

## HEALTH AND MEDICAL RESEARCH FUND

# Novel PCDH10-Wnt-MALAT1 regulatory axis in endometrioid endometrial adenocarcinoma

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## KEY MESSAGES

1. Protocadherin 10 (PCDH10) is silenced in endometrioid endometrial cancer through promoter hypermethylation.
2. Ectopic expression of PCDH10 inhibits tumour growth and induces cell apoptosis.
3. Transcriptomic analysis revealed that PCDH10 expression down-regulates metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). Further mechanistic studies uncovered that MALAT1 expression is transcriptionally induced by Wnt/ $\beta$ -catenin signalling.
4. We uncovered a novel PCDH10-Wnt/ $\beta$ -catenin-MALAT1 regulatory axis that contributes to development and progression of endometrioid

endometrial cancer.

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## Introduction

Endometrioid endometrial cancer (EEC) accounts for 80% to 90% of all endometrial cancers. Its key mutational events have been characterised, but the underlying molecular mechanisms remain poorly elucidated. We discovered a novel mir-193-YY1-APC regulatory axis that exerts functional roles in EEC development.<sup>1</sup> In this study, we investigated the tumour suppressive function of protocadherin 10 (PCDH10) in EEC. PCDH10 was proposed as a tumour suppressor, and its inactivation secondary to promoter hypermethylation has been detected in multiple cancers. Restoration of PCDH10 could inhibit cell growth, reduce clonogenicity, restrain cell invasion, and induce cell apoptosis.<sup>2</sup> The link between PCDH10 and EEC is unknown, and the molecular mechanisms await exploration. LncRNAs are RNA species over 200 nucleotides in length and play an important role in transcriptional regulation. LncRNAs are deregulated in different cancer contexts, including metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). Since its discovery as a prognostic factor for lung cancer metastasis, MALAT1 has been shown to be broadly up-regulated in a variety of cancer entities and to play critical roles in distinct cancer hallmark capabilities.<sup>3</sup> However, study of MALAT1 function in EEC is still lacking, and the transcriptional regulation of MALAT1 expression and the causes behind its deregulation are barely explored.

## Methods

A total of 76 cases of primary EEC and 45 cases of normal tissues were used in this study. All specimens, clinical information, and procedures were approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong.

In a xenograft mouse model,  $5 \times 10^6$  of control or PCDH10 stably expressing HEC-1-B cells were subcutaneously injected into the left and right flanks of the female athymic nude mice ( $n=5$  for each group). All animal experiments were approved by Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

Human EEC cell lines were obtained from American Tissue and Cell Culture and cultured as recommended.

A 516 bp fragment harbouring TCF4 binding site was amplified from genomic DNA and cloned into pGL3-basic vector. The mutant reporter was generated by mutating the TCF4 binding motif from CTTTGAA to CTTTGCG.

An RNA antisense probe was in vitro transcribed corresponding to 6871-7224 bp of MALAT1 (RefSeq accession, NR\_002819).

ChIP assays were carried out as described previously.<sup>4</sup> 5  $\mu$ g of antibodies against  $\beta$ -catenin or equal amount isotype IgG was used for each  $2 \times 10^7$  cell per ChIP. Immunoprecipitated genomic DNA was resuspended in 15  $\mu$ L of water. PCR was then performed with 1  $\mu$ L of DNA as a template on a 7900HT system.

The difference of PCDH10 mRNA expression between tumour and adjacent non-tumour tissues was analysed by the Mann-Whitney *U* test. For analysing the association of MALAT1 ISH scoring with clinical parameters, Pearson's Chi-square test was used. Statistical significance between two groups was assessed by Student's *t*-test. All tests were two sided, and a P value of <0.05 was considered statistically significant.

## Results

### PCDH10 is down-regulated in EEC through promoter hypermethylation

We examined PCDH10 mRNA expression in EEC cells lines and micro-dissected EEC tumour samples using normal endometrial tissue as controls. PCDH10 was significantly down-regulated in 76 tumour samples and all five cell lines, compared with 45 normal controls (Fig 1a). Aberrant hypermethylation in a CpG island (+8 ~ -328 bp upstream TSS) of PCDH10 promoter was detected in EEC tumours but not in normal endometrial tissues (Fig 1b). This finding was confirmed by analysing the genome-wide methylation data generated by The Cancer Genome Atlas project. Our analysis results from a cohort of 208 EEC patients and 34 normal controls showed a marked hypermethylation on the above region of PCDH10 promoter in EEC samples. Consistently, when treated with demethylation agent, 5-Aza, the promoter hypermethylation was markedly reduced and PCDH10 expression was restored (Fig 1c). Collectively, these results demonstrated that PCDH10 is down-regulated in EEC through its promoter hypermethylation.

### PCDH10 restoration inhibits proliferation and induces apoptosis in EEC cells

We performed gain-of-function study by overexpressing PCDH10 in EEC cells. Successful restoration of PCDH10 was found to inhibit cell proliferation as revealed by cell counting and MTS assay (Fig 1d). Furthermore, overexpression of PCDH10 impeded their abilities to grow in an attachment-independent manner (Fig 1e). In addition, a marked increase in the number of subG1 cells was detected in both AN3CA and HEC-1-B cells (Fig 1f), suggesting that PCDH10 may lead to cell apoptosis. Consistently, the Annexin V-PI double staining and TUNEL assays revealed that the number of apoptotic cells was significantly increased upon PCDH10 overexpression. Collectively, these results concluded that PCDH10 is a pro-apoptotic factor in EEC cells.

### RNA-sequencing reveals MALAT1 as a downstream factor of PCDH10

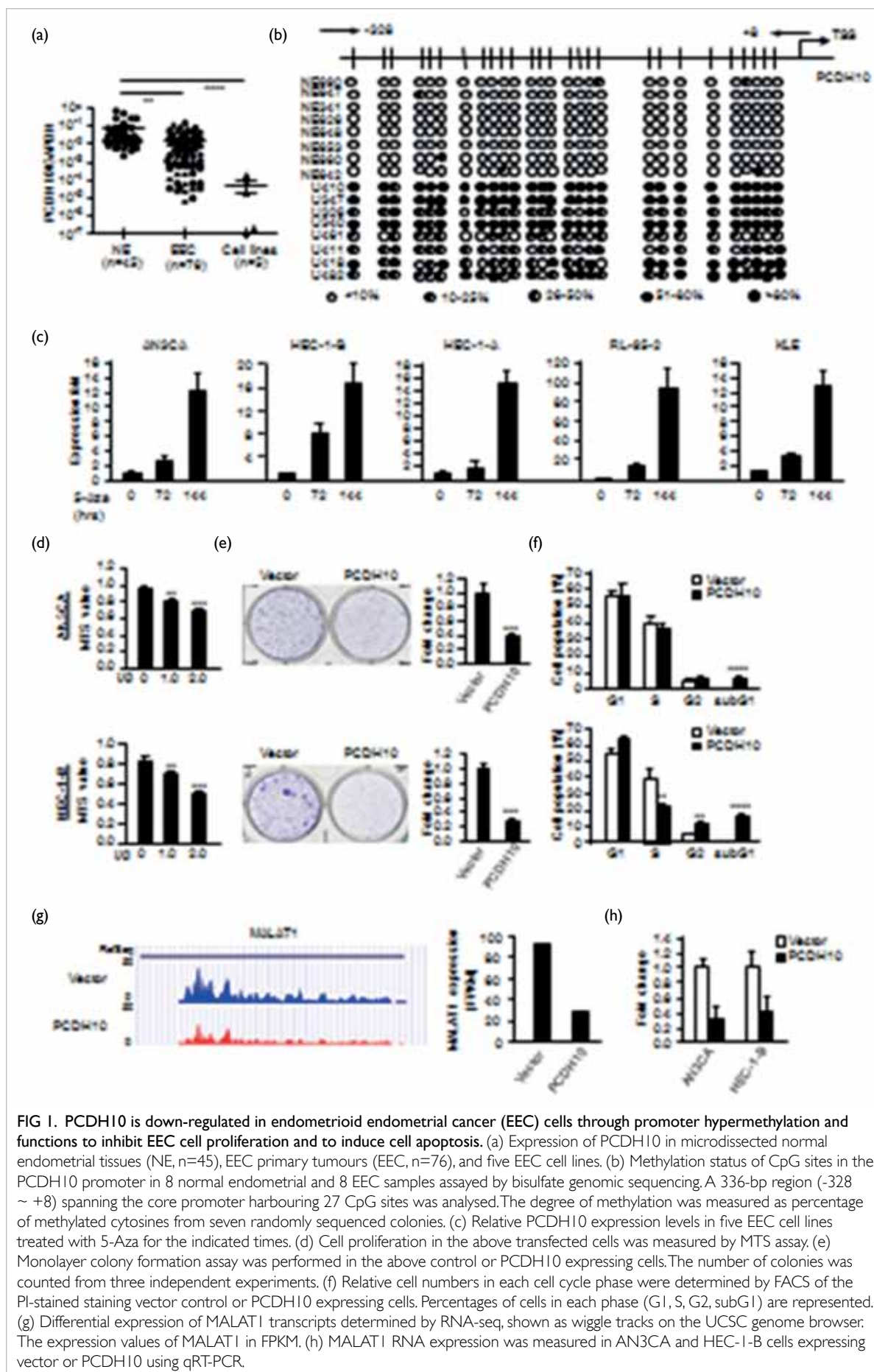
We performed a genome-wide analysis to globally

characterise PCDH10-affected transcriptomic changes. Among all potential targets, MALAT1 is one of the most significant targets downregulated upon overexpression of PCDH10. MALAT1 is well known to have an oncogenic role and involved in diverse processes of cancer development including cell proliferation, apoptosis, migration, and metastasis; nevertheless, its role in EEC has not been investigated. MALAT1 is abundantly expressed in EEC cells thus making the functional study relatively easy and feasible in a short period of time. MALAT1 is well studied in many systems and necessary reagents are relatively easy to obtain

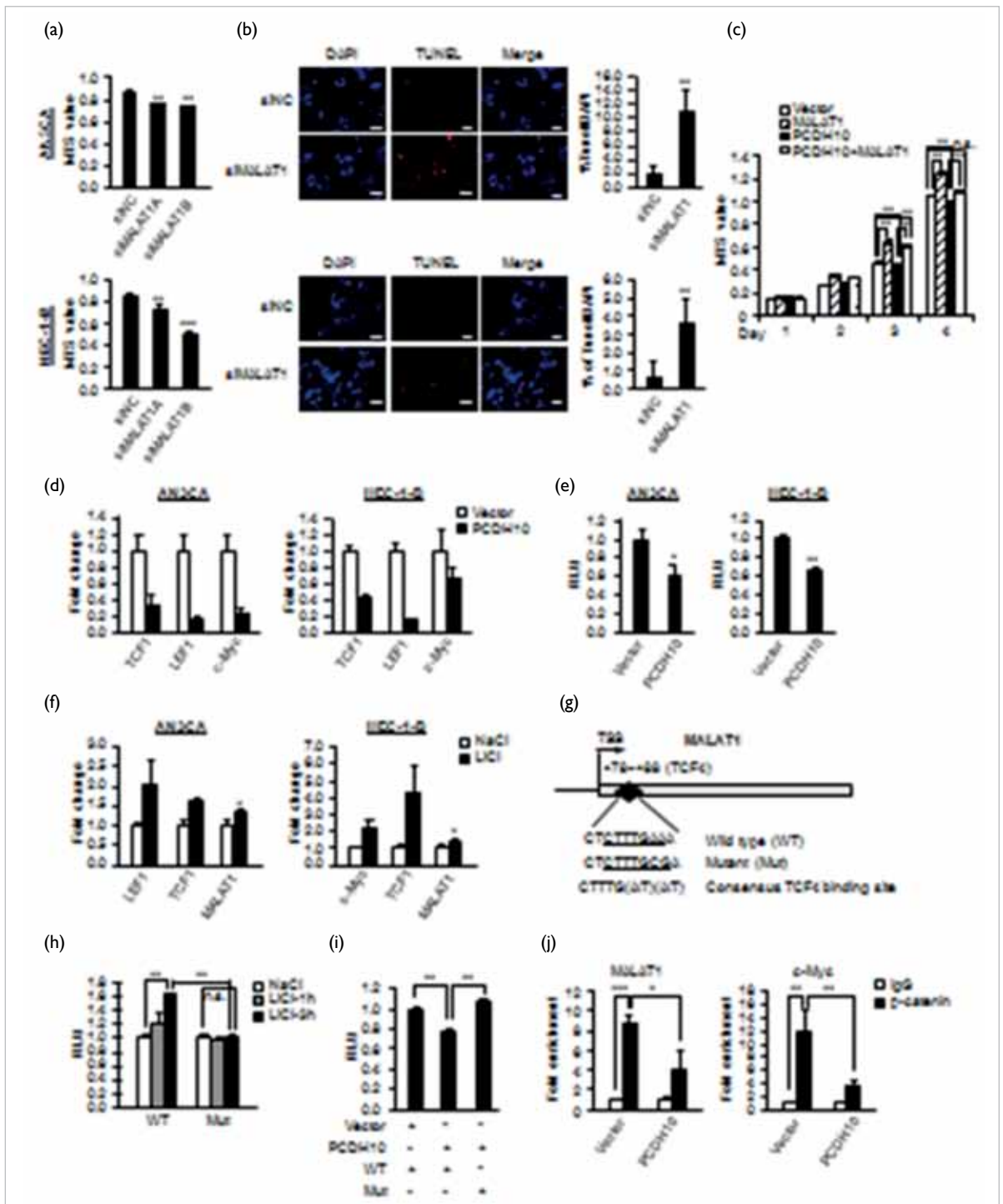
The RNA-seq data revealed that MALAT1 was decreased by approximately 70% in PCDH10 expressing cells (Fig 1g), which is further validated by independent qRT-PCR analyses (Fig 1h). To test whether MALAT1 is functionally downstream of PCDH10, we examined the effect of MALAT1 by using two siRNAs. Successful decrease of MALAT1 led to a significant delay in cell proliferation as revealed by cell counting assay and MTS assay (Fig 2a). Furthermore, siMALAT1 treatment induced a striking increase in apoptotic cell population (Fig 2b). Altogether the above findings suggested that MALAT1 knockdown phenocopied PCDH10 overexpression effect in EEC cells. Overexpression of MALAT1 reversed the inhibitory effect of PCDH10 on EEC cell growth (Fig 2c).

### PCDH10 suppresses MALAT1 transcription through inhibiting WNT/ $\beta$ -catenin signalling

PCDH-gamma was found to negatively regulate Wnt/ $\beta$ -catenin signalling.<sup>5</sup> We speculated that loss of PCDH10 may induce MALAT1 expression through activation of Wnt signalling. We examined the effect of PCDH10 restoration on Wnt signalling. PCDH10 overexpression caused a sharp decrease of several Wnt targets, including LEF1, TCF1, and c-MYC (Fig 2d) as well as Wnt signalling reporter activity (Fig 2e). Stimulation of Wnt signalling by lithium chloride led to a mild but significant increase of MALAT1 expression (Fig 2f). Moreover, we analysed publicly available TCF4 ChIP-seq data generated from various cell lines. In all the five cell lines, two TCF4 binding peaks proximal to the TSS of MALAT1 were detected and a consensus binding motif of TCF4 was found in the promoter region (+78 to +88 bp) [Fig 2g]. We cloned this region into a luciferase reporter (wild type) and found its activity was induced upon lithium chloride treatment; however, the response was lost when the TCF binding site is mutated (Mut) [Fig 2h]. Furthermore, PCDH10 over-expression could suppress the wild type but not the Mut activities (Fig 2i), suggesting PCDH10 regulates MALAT1 transcription through inhibiting Wnt signalling. We performed ChIP

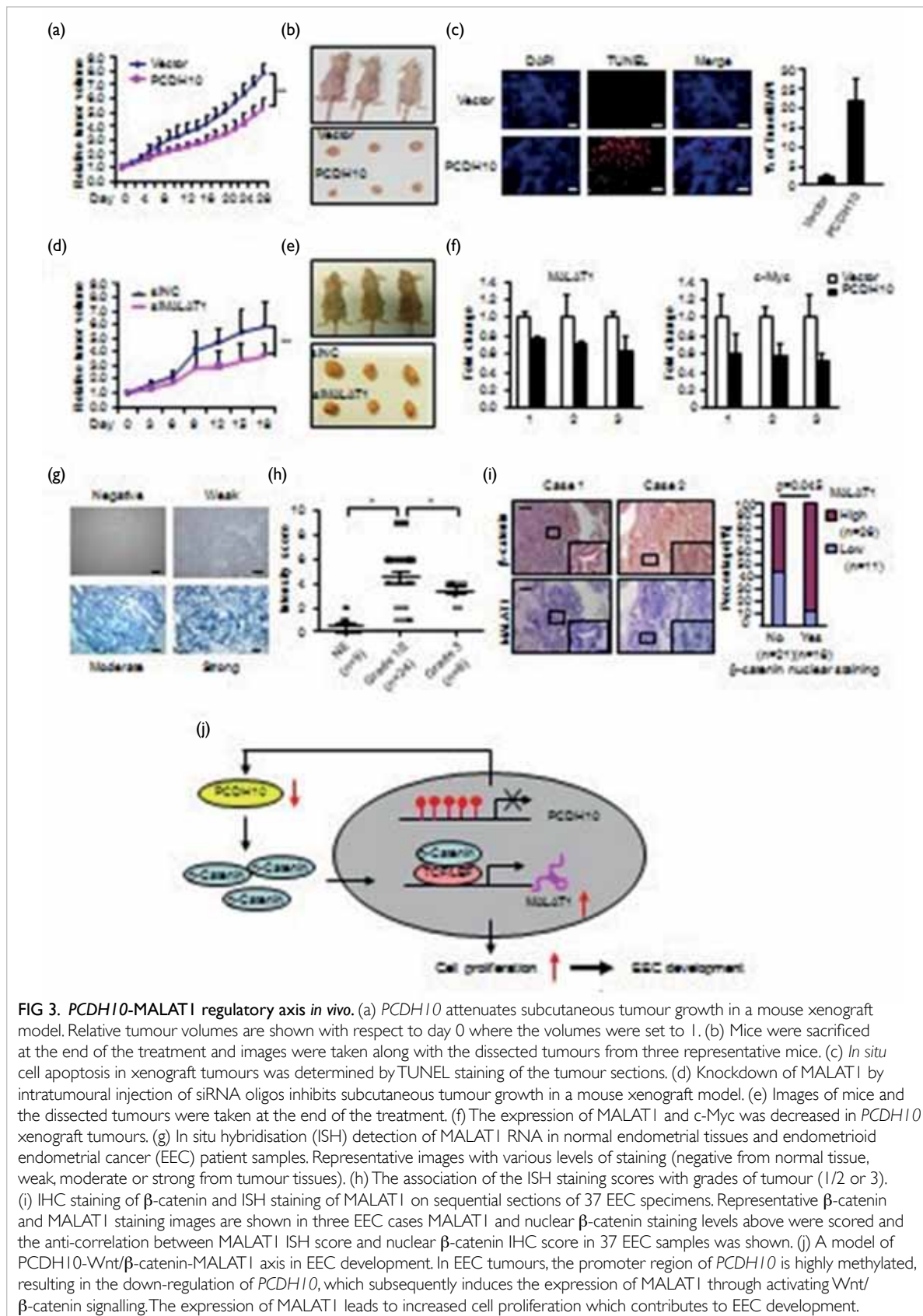


**FIG 1.** PCDH10 is down-regulated in endometrioid endometrial cancer (EEC) cells through promoter hypermethylation and functions to inhibit EEC cell proliferation and to induce cell apoptosis. (a) Expression of PCDH10 in microdissected normal endometrial tissues (NE, n=45), EEC primary tumours (EEC, n=76), and five EEC cell lines. (b) Methylation status of CpG sites in the PCDH10 promoter in 8 normal endometrial and 8 EEC samples assayed by bisulfate genomic sequencing. A 336-bp region (-328 ~ +8) spanning the core promoter harbouring 27 CpG sites was analysed. The degree of methylation was measured as percentage of methylated cytosines from seven randomly sequenced colonies. (c) Relative PCDH10 expression levels in five EEC cell lines treated with 5-Aza for the indicated times. (d) Cell proliferation in the above transfected cells was measured by MTS assay; (e) Monolayer colony formation assay was performed in the above control or PCDH10 expressing cells. The number of colonies was counted from three independent experiments. (f) Relative cell numbers in each cell cycle phase were determined by FACS of the PI-stained staining vector control or PCDH10 expressing cells. Percentages of cells in each phase (G1, S, G2, subG1) are represented. (g) Differential expression of MALAT1 transcripts determined by RNA-seq, shown as wiggle tracks on the UCSC genome browser: The expression values of MALAT1 in FPKM. (h) MALAT1 RNA expression was measured in AN3CA and HEC-1-B cells expressing vector or PCDH10 using qRT-PCR.



**FIG 2. MALAT1 is a functional downstream of PCDH10 and PCDH10 inhibits MALAT1 transcription through Wnt/ $\beta$ -catenin signalling pathway.** (a) Depletion of MALAT1 by siRNA oligos in AN3CA and HEC-I-B cells. Two siRNAs targeting MALAT1 (siMALAT1A and siMALAT1B) were used with a scramble sequence as control (siNC). Proliferation of the above transfected cells was determined by MTS assay. (b) Cell apoptosis was determined by TUNEL assay in AN3CA and HEC-I-B cells transfected with siMALAT1 or siNC. The index of TUNEL-positive cells is calculated. (c) HEC-I-B cells stably expressing PCDH10 or control vector were transiently transfected with MALAT1 expressing or a vector control plasmid, respectively. Cell proliferation was measured using MTS value at the indicated days after seeding. (d) PCDH10 decreases the mRNA expression levels of c-Myc, LEF1 and TCF1, in both AN3CA and HEC-I-B cells. (e) Transient expression of PCDH10 inhibits TOP-flash luciferase reporter activity in the above cells. (f) Expression of MALAT1 was increased by lithium chloride treatment; c-Myc, LEF1 or TCF1 expression was used as positive controls. (g) Schematic illustration of the promoter region of MALAT1 gene. The predicted TCF4 binding site with genomic location (+78 ~ +88) was displayed; wild type, mutant, and the consensus TCF binding sequences were indicated below. (h) Lithium chloride treatment increased the activity of the wild type but not the Mut reporter. Values were normalised by renilla levels. (i) PCDH10 inhibits the wild type but not the Mut reporter. Values were normalised by renilla levels. (j) ChIP-PCR detection of the  $\beta$ -catenin enrichment on the TCF binding site in HEC-I-B cells stably expressing PCDH10 or vector control. c-Myc genomic region harbouring a TCF binding site was used as a positive control. Enrichment values are relative to input.

to detect  $\beta$ -catenin enrichment on MALAT1 upon PCDH10 overexpression (Fig 2j). Together, these data demonstrated that PCDH10 suppresses MALAT1 expression through impairing  $\beta$ -catenin binding to its promoter. Expectedly, a robust enrichment of  $\beta$ -catenin was found on the identified TCF4 site and the enrichment was significantly diminished



**FIG 3. PCDH10-MALAT1 regulatory axis in vivo.** (a) PCDH10 attenuates subcutaneous tumour growth in a mouse xenograft model. Relative tumour volumes are shown with respect to day 0 where the volumes were set to 1. (b) Mice were sacrificed at the end of the treatment and images were taken along with the dissected tumours from three representative mice. (c) *In situ* cell apoptosis in xenograft tumours was determined by TUNEL staining of the tumour sections. (d) Knockdown of MALAT1 by intratumoural injection of siRNA oligos inhibits subcutaneous tumour growth in a mouse xenograft model. (e) Images of mice and the dissected tumours were taken at the end of the treatment. (f) The expression of MALAT1 and c-Myc was decreased in PCDH10 xenograft tumours. (g) In situ hybridisation (ISH) detection of MALAT1 RNA in normal endometrial tissues and endometrioid endometrial cancer (EEC) patient samples. Representative images with various levels of staining (negative from normal tissue, weak, moderate or strong from tumour tissues). (h) The association of the ISH staining scores with grades of tumour (1/2 or 3). (i) IHC staining of  $\beta$ -catenin and ISH staining of MALAT1 on sequential sections of 37 EEC specimens. Representative  $\beta$ -catenin and MALAT1 staining images are shown in three EEC cases MALAT1 and nuclear  $\beta$ -catenin staining levels above were scored and the anti-correlation between MALAT1 ISH score and nuclear  $\beta$ -catenin IHC score in 37 EEC samples was shown. (j) A model of PCDH10-Wnt/ $\beta$ -catenin-MALAT1 axis in EEC development. In EEC tumours, the promoter region of PCDH10 is highly methylated, resulting in the down-regulation of PCDH10, which subsequently induces the expression of MALAT1 through activating Wnt/ $\beta$ -catenin signalling. The expression of MALAT1 leads to increased cell proliferation which contributes to EEC development.

### PCDH10-MALAT1 regulatory axis in vivo

We evaluated the function of PCDH10-MALAT1 regulatory axis in vivo. We found PCDH10 overexpression markedly delayed tumour growth ( $n=5$ ,  $P<0.01$ , Fig 3a, b) and caused a severe cell apoptosis in vivo (Fig 3c), which was in agreement with its pro-apoptotic effect. To illustrate the effect of MALAT1, siRNA oligos were injected into the HEC-1-B xenograft tumour. A comparable phenotype as PCDH10 over-expression could be observed in siMALAT1 group (Fig 3d, e). We also detected reduced level of both MALAT1 and c-Myc in PCDH10 overexpressing tumours (Fig 3f), suggesting the existence of PCDH10-Wnt-MALAT1 axis in the xenografts.

To validate the above findings in EEC clinical samples, we examined the MALAT1 expression by in situ hybridisation on the paraffin sections. A much higher level was found in EEC samples as compared with normal tissues (Fig 3g). In addition, a strong MALAT1 signalling appeared to be associated with low histologic grade (grade 1/2 versus 3) [ $P=0.028$ , Fig 3h].

Analyses of tissue microarray data revealed that high expression of MALAT1 was linked with hyperplasia ( $P=0.017$ ), menopausal status ( $P=0.028$ ), no recurrence ( $P=0.032$ ), and low metastasis potential ( $P=0.041$ ). Further exploring The Cancer Genome Atlas data from a large cohort of EEC samples ( $n=253$ ), we detected a reverse association between PCDH10 and MALAT1 ( $P=0.0324$ ). Additionally, the expression level of MALAT1 by ISH staining is found strongly correlated with the total or nuclear level of  $\beta$ -catenin by IHC staining (Fig 3i). Altogether, these analyses confirmed the presence of PCDH10-Wnt/ $\beta$ -catenin-MALAT1 regulation in clinical samples.

### Discussion

We identified PCDH10 as a tumour suppressor in EEC. Our results showed that PCDH10 promoter is hypermethylated through analysis of locally collected EC samples and analysing The Cancer Genome Atlas data from worldwide EEC samples. Functional studies revealed that restoration of PCDH10 successfully reduced cell growth and induced cell apoptosis, in keeping with what was uncovered in other cancers. Notably, we could not recapitulate all functional features of PCDH10 demonstrated in other studies and the impact of PCDH10 on cell cycle also differs between two EEC cell lines, which

may reflect cell type and cancer type specific roles of PCDH10.

Our findings uncovered the transcriptomic influence exerted by PCDH10. Moreover, we identified MALAT1 as a functional downstream target of PCDH10 and showed that MALAT1 plays an oncogenic role in EEC. We demonstrated that MALAT1 is positively associated with hyperplasia, and negatively with metastasis, suggesting its predictive value as a molecular biomarker. The positive correlation of MALAT1 with hyperplasia and low grade EEC implies that dysregulation of MALAT1 is an early event in EEC development.

We provide novel insights into how PCDH10 acts on MALAT1 through modulating Wnt/ $\beta$ -catenin signalling. We identified MALAT1 as a direct transcriptional target of Wnt/ $\beta$ -catenin in EEC cells. According to ChIP-seq data from ENCODE, the TCF binding on this site is present in multiple cancer cell lines, thus Wnt regulation of MALAT1 likely occurs as a general pathway in various cancers.

Collectively, we identified a novel regulatory axis, PCDH10-Wnt-MALAT1 in the effort of elucidating the tumour suppressive function of PCDH10 (Fig 3j) and showed PCDH10 and lncRNA function in EEC and elucidated the transcriptional regulation of MALAT1.

### Acknowledgements

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### References

1. Ying J, Li H, Seng TJ, et al. Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation. *Oncogene* 2006;25:1070-80.
2. Gutschner T, Hammerle M, Diederichs S. MALAT1: a paradigm for long noncoding RNA function in cancer. *J Mol Med (Berl)* 2013;91:791-801.
3. Lu L, Sun K, Chen X, et al. Genome-wide survey by ChIP-seq reveals YY1 regulation of lincRNAs in skeletal myogenesis. *EMBO J* 2013;32:2575-88.
4. Dallosso AR, Hancock AL, Szemes M, et al. Frequent long-range epigenetic silencing of protocadherin gene clusters on chromosome 5q31 in Wilms' tumor. *PLoS Genet* 2009;5:e1000745.