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Identification of Achaete-scute complex-like 1 (ASCL1) target genes and evaluation of DKK1 and TPH1 expression in pancreatic endocrine tumours

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Abstract

Background: *ASCL1* role in pancreatic endocrine tumourigenesis has not been established. Recently it was suggested that ASCL1 negatively controls expression of the Wnt signalling antagonist *DKK1*. Notch signalling regulates expression of TPH1, the rate limiting enzyme in the biosyntesis of serotonin. Understanding the development and proliferation of pancreatic endocrine tumours (PETs) is essential for the development of new therapies.

Methods: *ASCL1* target genes in the pancreatic endocrine tumour cell line BON1 were identified by RNA interference and microarray expression analysis. Protein expressions of selected target genes in PETs were evaluated by immunohistochemistry.

Results: 158 annotated *ASCL1* target genes were identified in BON1 cells, among them DKK1 and TPH1 that were negatively regulated by ASCL1. An inverse relation of ASCL1 to DKK1 protein expression was observed for 15 out of 22 tumours (68%). Nine tumours displayed low ASCL1/high DKK1 and six tumours high ASCL1/low DKK1 expression. Remaining PETs showed high ASCL1/high DKK1 (n = 4) or low ASCL1/low DKK1 (n = 3) expression. Nine of twelve analysed PETs (75%) showed TPH1 expression with no relation to ASCL1.

Conclusion: A number of genes with potential importance for PET tumourigenesis have been identified. *ASCL1* negatively regulated the Wnt signalling antagonist *DKK1*, and *TPH1* expression in BON1 cells. In concordance with these findings DKK1 showed an inverse relation to ASCL1 expression in a subset of PETs, which may affect growth control by the Wnt signalling pathway.

Background

Pancreatic endocrine tumours (PETs) are derived from the embryologic endoderm and accounts for 1-2% of pancreatic cancer. The only currently curative therapy for patients with PETs is surgical resection. PETs occur sporadically or are familial in nature, caused by germ line mutations in the Multiple endocrine neoplasia 1 (*MEN1*) or von Hippel-Lindau (*VHL*) tumour suppressor genes. Understanding the molecular pathways that control PET development and proliferation are essential for possible development of novel therapies.

The basic helix loop helix (bHLH) transcription factor Achaete-scute complex homolog 1 (*Ascl1*) has been shown to play important regulatory roles in adrenal medullary chromaffin cells [1], thyroid parafollicular C-cells [2] and pulmonary endocrine cells [3]. *Ascl1* is tightly controlled by the Notch signalling pathway in the developing pancreas and governs the exocrine versus endocrine cell fate decision [4]. Forced Notch activation expands the pool of undifferentiated precursor cells and inhibits the initial emergence of endocrine cells and the following exocrine differentiation [5,6], whereas disruption of Notch signalling results in precocious endocrine differentiation [4]. The active form of Notch, NICD, induces the expression of Hairy and enhancer of split 1 (*HES1*) which in turn antagonises the expression of bHLH genes such as *ASCL1*, with subsequent inhibition of progenitor cell differentiation [7].

We have recently reported that ASCL1 is invariably expressed in PETs, and suggested that the observed lack of nuclear HES1 might contribute to the expression of ASCL1 in these tumours [8]. In lung cancer cells ASCL1 negatively regulates the expression of Dickkopf homologue 1 (*DKK1*) [9], an antagonist of the Wnt/β-catenin signalling pathway which is involved in the development of the exocrine pancreas [10] and in pancreatic beta cell proliferation [11]. Furthermore, overexpression of NOTCH1 in the human pancreatic endocrine tumour cell line BON1 leads to inhibition of ASCL1 expression, induction of HES1, reduced levels of endocrine markers such as synaptophysin, and also to major repression of TPH1 [12], the rate limiting enzyme in serotonin biosynthesis. Serotonin is together with other hormones implicated to cause the carcinoid syndrome.

Here we report on *ASCL1* target genes in BON1 cells transfected with *ASCL1* siRNA. In addition, the relation of DKK1 and TPH1 protein expression to ASCL1 expression is studied in sporadic and familial (MEN 1) PETs.

Methods

Cell culture

The polyclonal BON1 cell line (a kind gift from Dr. J. C. Thompson, Department of Surgery, University of Texas Medical Branch, USA) was grown in 1:1 mixture of F12K (Invitrogen, Life Technologies, Carlsbad, USA) and DMEM (SVA, Uppsala, Sweden) medium supplemented with 5% foetal bovine serum. The cells were grown at 37°C in a humidified 5.0% CO_2/air atmosphere. siRNA transfections were performed at 80% confluence. The BON1 cell line is one of few human pancreatic endocrine tumour cell lines available [13].

Immunofluorescent microscopy

BON1 cells were seeded on glass cover slips and fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) (Sigma Aldrich, St Louis, USA) for 30 min, and washed with PBS. The cells were permeabilised in 0.2% Triton X-100 (Sigma) in PBS for 5 min, washed again in PBS, and incubated in 5% foetal bovine serum in PBS for 60 min at room temperature. Primary as well as secondary antibodies were diluted in PBS containing 5% FBS. Cells were incubated with anti-ASCL1 mouse antibody (BD Biosciences, San Jose, USA) or anti-HES1 goat antibody (Santa Cruz Biotechnology, Santa Cruz, USA) followed by FITC-labelled secondary antibodies and TRITC-labelled phalloidin with a washing step in between. The cover slips were mounted on object slides by the use of Vectashield with DAPI (Vector laboratories, Burlingame, USA). Cells were photographed by an Axiocam HRm camera employing the Axiovision imaging software using a 63× plan-apochromat objective and a Zeiss Axioplan2 microscope (Carl Zeiss Inc., Oberkochen, Germany).

siRNA transfection

The two siRNAs were pre-designed (Ambion, USA, ID 114405 and AM4635). 5′-CGCGUUAUAGUAACUCC-CATT and 5′-UGGGAGUUACUAUAACGCGTG (siRNA/A) and 5′-AGUACUGCUUACGAUACGGTT and 5′-TTU-CAUGACGAAUGCUAUGCC (Control siRNA). Transfections were performed with 10-30 nmol siRNA in 12 well plates (80 0000 cells/well) using the jetSI-ENDO transfection reagent (Poly-Plus-Transfection SAS, Illkirch, France) according to the manufacturer's protocol. Samples were not pooled for downstream applications.

RNA isolation and cDNA synthesis

Cells were harvested 72 hours after transfection and total RNA was extracted using TriZol Reagent (Invitrogen) according to manufacturer's instructions. The RNA concentration and quality were assessed using the Agilent Bioanalyser (Agilent Technologies, Palo Alto, USA). The RNeasy Mini Kit (Qiagen, Holden, Germany) was used to further purify the RNA samples. cDNA was synthesised from 1 μg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR)

Relative mRNA expression was determined by qPCR, and compared to positive controls comprising lung carcinoid cell lines H727 and H720 (CRL-5815 and CRL-5838, LGC Promochem, Middlesex, UK. Data not shown). Commercially available primer and probe sets were used and measured against standard curves generated from dilution series of cDNA from cell lines H727, H720 and BON1. The following primers/probe mixes were used: *ASCL1*; Hs00269932_m1, *TCF3*; Hs01016249_m1, *DLL1*; Hs 00194509_m1, *SYP*; Hs00300531_m1, *TPH1*;

Hs00188220_m1, and *DKK1*; Hs00183740_m1 (Applied Biosystems). Reactions were performed and analysed using an Applied Biosystems PRISM 7700 Sequence Detector. Standard cycling conditions were used. Triplicate of each cDNA was used and each assay was performed twice. The gene-specific signals were normalised to expression of *ACTB* and *PPIA* endogenous control genes (primer/probe mix 4333762F and 4333763T).

Western blotting
Protein extracts for Western blotting were prepared by lysing the cells in RIPA buffer (Sigma-Aldrich) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Protein sample from each transfected well was separated in 12% SDS-polyacrylamide gradient gels (BioRad, Hercules, USA), transferred to PVDF membranes (GE Healthcare Europe GmbH, Uppsala, Sweden) and blocked with SuperBlock Blocking Buffer (Pierce Biotechnology, Rockford, USA) overnight at 4°C. The membranes were incubated with anti-ASCL1 monoclonal antibody (BD Biosciences) or anti-α- Tubulin monoclonal antibody (Santa Cruz Biotechnology) for 2 h. After briefly washing with PBS containing 0.1% Tween 20, the filters were incubated for 1 h with a secondary goat anti mouse antibody conjugated to horseradish peroxidase (1:5000 dilution). The filters were washed and developed using the Super Signal West femto kit (Pierce Biotechnology).

Microarray analysis
RNAs from successful siRNA transfection experiments where used for microarray expression analysis. The GeneChips, Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, USA) was used for the analysis. 100 nanograms of total RNA from each sample were used to prepare biotinylated fragmented cRNA using the two-cycle cDNA synthesis part. GeneChip were hybridised for 16 hours in a 45°C incubator, rotated at 60 rpm according to the GeneChip Expression Analysis Technical Manual (Rev. 5, Affymetrix). The arrays were washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip Scanner 3000 7 G.

Bioinformatics
Differentially regulated genes were determined by calculating the fold change between the nonspecific siRNA transfected cell samples and the siRNA-ASCL1 transfected samples. Subsequent analysis of the gene expression data was carried out in the freely available statistical computing language R http://www.r-project.org using packages available from the Bioconductor project http://www.bioconductor.org. The raw data was normalised using the robust multi-array average (RMA) [14] background-adjusted, normalized and log-transformed summarised values as first suggested by Li and Wong in 2001 [15]. In order to search for the differentially expressed genes between the samples from the different groups an empirical Bayes moderated *t* test was then applied [16], using the 'limma' package [17]. To address the problem with multiple testing, the *p*-values were adjusted according to Benjamini and Hochberg [18]. We selected as significant only probe sets with an adjusted *p*-value < 0.01 and an abs (log_2ratio) equal to or larger than1 (which corresponds to a two-fold change in expression) to investigate further.

Tissue specimens
Pancreatic endocrine tumour specimens were obtained from biobanks at the Department of Endocrine Oncology, the Department of Surgery, and the Department of Pathology at the Uppsala University Hospital. Frozen or paraffin embedded tissues were used. Tumours were initially frozen in liquid nitrogen and stored at –80°C until analysis. Inclusions were based on the availability of operative tissue specimens or biopsy material. Altogether two gastrinomas, two glucagonomas (one liver metastasis), five insulinomas and 14 non-functioning tumours were investigated. The mean age at diagnosis was 48 years (range 19-86). Seven tumours were from MEN 1 patients. The tumours were classified according to the WHO classification of endocrine neoplasms. For comparison, eight specimens of macroscopically determined non-tumourous pancreas adjacent to a pancreatic endocrine tumour were assessed by immunohistochemistry.

Immunohistochemistry
Twenty-two PETs were immunostained for DKK1. Frozen, acetone-fixed sections (6 μm) were incubated with an anti-DKK1 rabbit polyclonal antibody (SC-25516, Santa Cruz Biotechnology) diluted in PBS with 1% BSA. The reaction product was revealed using a biotinylated secondary antibody, Vectastain Elite ABC, (Vector) and the chromogen 3-amino-9-ethylcarbazol and 0.02% hydrogen peroxide as a substrate. Sections were counterstained with Mayer's haematoxylin and mounted. Twelve paraffin embedded PET specimens were immunostained for TPH1. The rehydrated sections were heat-retrieved and incubated with an anti-TPH1 mouse antibody (Sigma Aldrich). The reaction product was revealed using the EnVision system -HPR (DakoCytomation, Copenhagen, Denmark), and DAB as the chromogen. Sections were counterstained with Mayer's haematoxylin and mounted. Each PET specimen and non-tumourous pancreatic specimens were evaluated independently by the authors and graded as low, high or heterogeneous (*i.e.* areas of both low and high

expression present in the tumour). Immunostaining for ASCL1 has been published previously [8] and was graded as negative (–), weak (+), moderate (++), or strong (+++). In the present study we denoted strong (+++) staining in the cytoplasm as High and weak or moderate (+/++) as Low. Sections were photographed by an AxioCam MR camera employing the Axiovision imaging software using a LD A-plan 20×/40× 0.30 Ph1 objectives and a Zeiss Axiovert 40 microscope (Carl Zeiss Inc.).

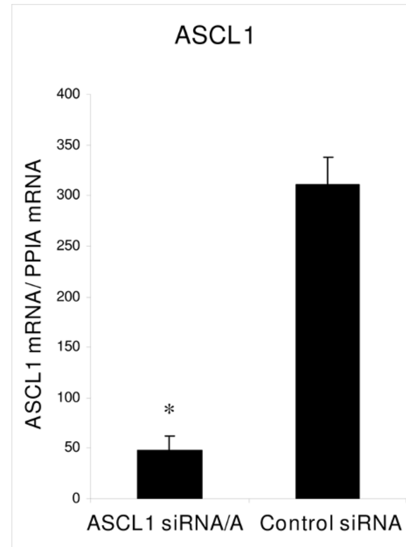
Statistical analysis
Unpaired *t* test was used for calculations regarding qPCR expression. A *p*-value below 0.05 was considered significant.

Ethical approval
Permission for this study was obtained from the Uppsala Ethical Committee, Sweden. Informed consent was gathered from all patients.

Results
Expression profiling in the pancreatic endocrine tumour cell line BON1
RNA interference and microarray expression analysis were employed in order to identify *ASCL1* target genes in BON1 cells. A specific siRNA to *ASCL1* (*ASCL1* siRNA/A) and one non-specific Control siRNA were transfected to BON1 cells. These cells are notoriously difficult to transfect and 30 nmol of siRNA was found to be optimal. *ASCL1* siRNA/A was found to significantly ($p < 0.0001$) reduce *ASCL1* mRNA expression compared to Control siRNA (Figure 1A). Importantly, ASCL1 protein expression was similarly reduced (Figure 1B). In order to further validate the experimental system for microarray expression analysis, the effects of reduced ASCL1 expression by RNAi was evaluated on the known or putative ASCL1 target genes Delta 1 (*DLL1*) and Synaptophysin (*SYP*). ASCL1 is known to bind to the DLL1 promoter and synergistically activate transcription together with Pou3f3 and Pou3f4 [19,20]. Reduced expression of ASCL1 negatively affects *SYP* expression in SCLC cells as well as in pulmonary endocrine cells of Ascl1 double null mice [3]. As a putative negative control we also assessed expression of the transcription factor *TCF3* (*E12*/*E47*); a recognised dimerisation partner of ASCL1 that is required for transcription activation of ASCL1 target genes [21,22]. The results showed that siRNA/A to *ASCL1* significantly reduced *DLL1* (p = 0.001) and *SYP* (p = 0.01) expression, while expression of *TCF3* was unaffected (Figures 2A-C).

Since we have observed lack of nuclear HES1 in PETs [8], protein expression in BON1 cells was investigated by fluorescent immunostaining. ASCL1 and HES1 were clearly expressed in BON1 cells, with prominent nuclear association (Figures 3 and 4).

(A)



(B)

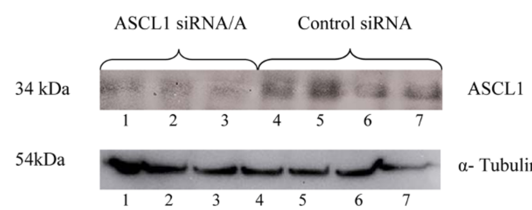


Figure 1
(A) Relative expression of *ASCL1* in siRNA-transfected BON1 cells as determined by qPCR. Expression levels were normalised to *ACTB* and *PPIA* with similar results. Data are presented as mean ± SEM of analysis in triplicates. * $p < 0.05$. (B) Western blotting analysis for ASCL1. Protein extracts from siRNA-transfected BON1 cells as indicated.

Six validated RNA samples from transfections with *ASCL1* siRNA/A and Control siRNA were selected for microarray expression analysis employing the Human Genome U133 Plus 2.0 GeneChip. A total of 433 transcripts showed at least a two-fold difference in expression in BON1 cells transfected with *ASCL1* siRNA compared to Control siRNA. Among annotated genes, 46 showed increased- and 112 reduced expression (Table 1; Table 2). As anticipated, *ASCL1* expression was decreased (Table 2). Expression of both *DKK1* and *TPH1* were found to be increased in *ASCL1* siRNA transfected cells (Table 1; Figure 5). Thus, *ASCL1* negatively regulates *DKK1* and *TPH1* in BON1 pancreatic endocrine tumour

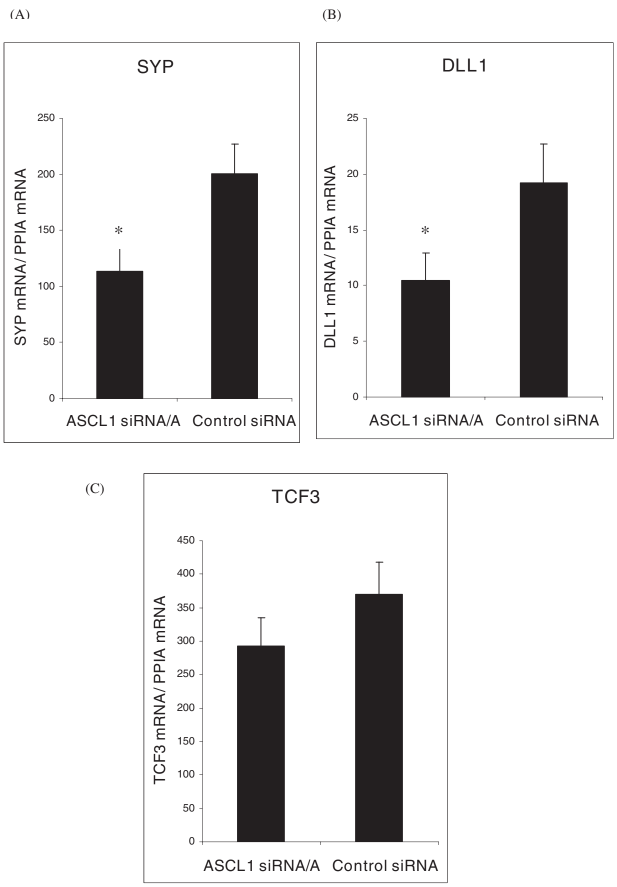


Figure 2
Relative expression of *SYP*, *DLL1*, and *TCF3* in siRNA-transfected BON1 cells as determined by qPCR. Expression levels were normalised to *ACTB* and *PPIA* with similar results. Data are presented as mean ± SEM of analysis in triplicates. * $p < 0.05$.

cells. Other *ASCL1* target genes included oncogenes (like *MYCN* and *RET*), those involved in the integrin system (*NRXN3*, *LAMA4* and *SMOCK2*), participating in apoptosis (*PDCD6*, *CFLAR* and *CCAR1*), as well as genes known to be involved in the Notch, Wnt, NFκβ, TGFβ and MAP kinas signalling pathways. Many of the *ASCL1* targets represent potential oncogenes and tumour suppressor genes.

Gene ontology (GO) were applied to identify the functional significance of all (n = 433) differentially expressed transcripts with known function(s) http://www.geneontology.org. Each differentially expressed transcript was placed in functional GO categories and over-represented categories are shown. The enrichment of the GO data was narrowed down to broad GO terms. The division was based on biological process, molecular function, and cellular components. The most over-represented GO biological process categories, according to number of involved transcripts, related to regulation of a biological or cellular processes, development, metabolic processes or transcription and regulation of transcription. For molecular function, most over-represented categories were binding activity (receptor, DNA or nucleic acid) and transcription regulation (cofactor or binding activity). For cellular components, the most over-represented category was transcripts involved in cellular junctions (Table 3).

Inverse expression of ASCL1 and DKK1 in the majority of investigated PETs

Expression of DKK1 was evaluated by immunohistochemistry in 22 out of the 23 analysed PETs (Figure 6; Table 4). Inverse relation of ASCL1 [8] to DKK1 expression was observed for 15 out of 22 tumours (68%). Of these, nine tumours displayed low ASCL1/high DKK1 and six tumours high ASCL1/low DKK1 expression. Thus, ASCL1 is likely to negatively regulate DKK1 transcription in these tumours, as has been shown to occur in A549 lung cancer cells [9]. The remaining PETs showed high ASCL1/high DKK1 (n = 4) or low ASCL1/low DKK1 (n = 3) expression. No relations of ASCL1/DKK1 expression to tumour syndrome, MEN 1, or WHO classification were observed.

TPH1 displays heterogeneous expression with no relation to ASCL1 in PETs

The amount of immunoreactivity varied for TPH1. Nine out of the twelve analysed PETs (75%) showed a heterogeneous expression pattern (Figure 6F, Table 4). High expression was seen in two tumours and low expression in one. Tumours with high or heterogeneous expression showed a somewhat lower TPH1 expression than control non-tumourous pancreatic tissue. No relations of ASCL1 to TPH1 expression or to clinical characteristics were observed.

Discussion

This study showed altogether 433 target transcripts (158 annotated genes) in the human pancreatic endocrine tumour cell line BON1 that directly or indirectly were regulated by *ASCL1*, among them several putative oncogenes and suppressor genes. *ASCL1* was found to negatively regulate *DKK1* and *TPH1* expression in BON1 cells. This may suggest that Notch1 signalling pathway regulatory factor(s) other than ASCL1 is involved in the reduced expression of *TPH1* observed in Notch1 over-expressing BON cells [12]. In order to investigate if this relation between *ASCL1*, *DKK1* and *TPH1 in vitro* might be of relevance *in vivo*, we analysed their protein expression in PETs. An inverse relation of ASCL1 to DKK1 expression was observed in 68% of the analysed

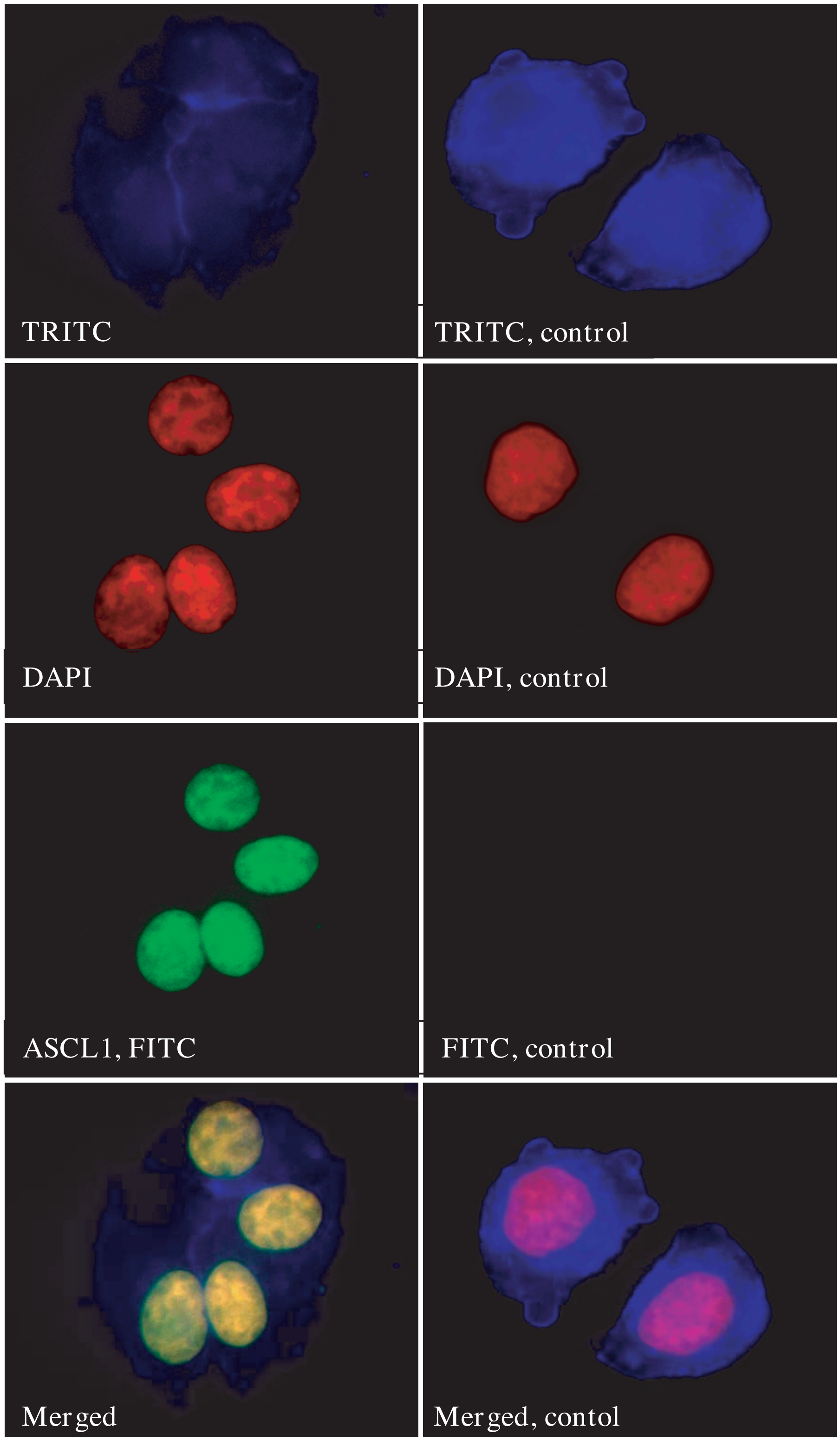


Figure 3
Fluorescent immunostaining of ASCL1 in BON1 cells. Cells were visualised by TRITC-labeled phalloidin (blue) and DAPI (red). Primary antibodies to ASCL1 were detected by FITC-labelled secondary antibodies (green). Yellow, indicates co-localisation (merged).

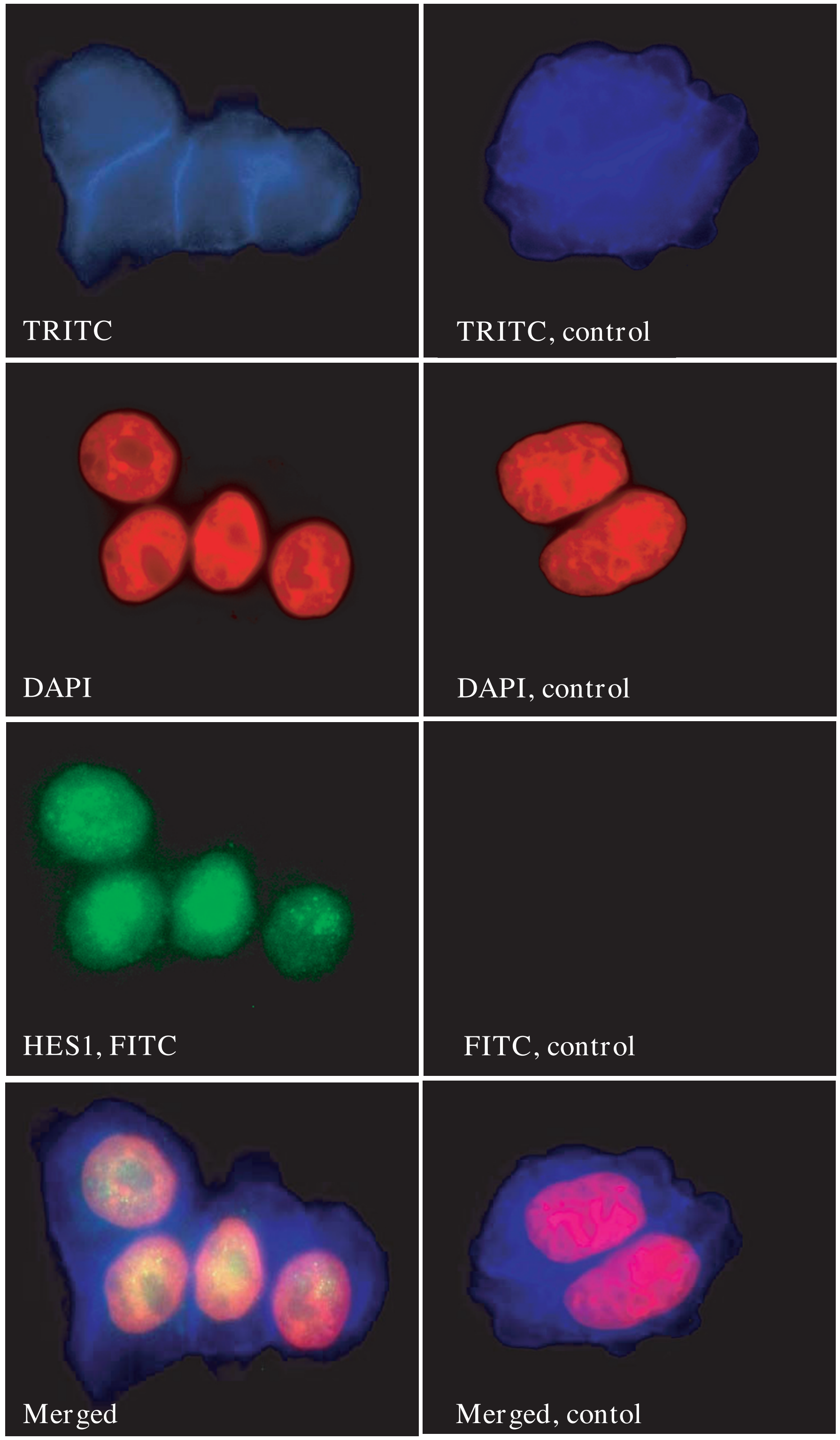


Figure 4
Fluorescent immunostaining of HES1 in BON1 cells. Cells were visualised by TRITC-labeled phalloidin (blue) and DAPI (red). Primary antibodies to HES1 were detected by FITC-labelled secondary antibodies (green). Yellow, indicates co-localisation (merged).

Table 1: Annotated genes with increased expression in BON1 cells transfected with siRNA to *ASCL1*

Gene Symbol	Gene Name	Location	Ratio	adj p Value
RBM24	RNA binding motif protein 24	6p22.3	4,708	0,0000391
HAS2	hyaluronan synthase 2	8q24.12	3,279	0,0002805
MYCN	v-myc myelocytomatosis viral related oncogene,	2p24.1	2,777	0,0000305
C13orf15	chromosome 13 open reading frame 15	13q14.11	2,594	0,0000701
APCDD1	adenomatosis polyposis coli down-regulated 1	18p11.22	2,569	0,0000305
LGR5	leucine-rich repeat-containing G protein-coupled receptor 5	12q22-q23	2,540	0,0003188
*DKK1**	dickkopf homolog 1 (Xenopus laevis)	10q11.2	2,349	0,0000155
*TPH1**	Tryptophan hydroxylase 1	11p15.3-p14	2,325	0,0002805
ID4	Inhibitor of DNA binding 4	6p22-p21	2,317	0,0001962
PVRL3	poliovirus receptor-related 3	3q13	2,295	0,0000949
TIMP2	TIMP metallopeptidase inhibitor 2	17q25	2,286	0,0000200
ASAM	adipocyte-specific adhesion molecule	11q24.1	2,254	0,0002763
SPOCK1	sparc/osteonectin, cwcv and kazal-like domains	5q31	2,251	0,0000796
NRXN3	neurexin 3	14q31	2,246	0,0002025
KL	klotho	13q12	2,240	0,0000150
GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	2q24.1	2,238	0,0000155
CLIC5	chloride intracellular channel 5	6p21.1-p12.1	2,235	0,0000313
EMG1	EMG1 nucleolar protein homolog (S. cerevisiae)	12p13	2,230	0,0000489
DRD1IP	dopamine receptor D1 interacting protein	10q26.3	2,219	0,0001121
CXCR7	chemokine (C-X-C motif) receptor 7	2q37.3	2,216	0,0000862
STOML3	stomatin (EPB72)-like 3	13q13.3	2,196	0,0003867
SI	sucrase-isomaltase (alpha-glucosidase)	3q25.2-q26.2	2,181	0,0000701
FGF13	fibroblast growth factor 13	Xq26.3	2,178	0,0000288
CHM	choroideremia (Rab escort protein 1)	Xq21.2	2,150	0,0000396
GLCE	glucuronic acid epimerase	15q23	2,144	0,0000561
DC2	DC2 protein	4q25	2,143	0,0000181
SMS	spermine synthase	Xp22.1	2,113	0,0003207
GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	2q24.1	2,095	0,0000586
LOC283454	hypothetical protein LOC283454	12q24.23	2,095	0,0000465
CXorf57	chromosome X open reading frame 57	Xq22.3	2,094	0,0003208
PTGES3	prostaglandin E synthase 3 (cytosolic)	12.	2,091	0,0000430
HS2ST1	heparan sulfate 2-O-sulfotransferase 1	1p31.1-p22.1	2,080	0,0001750
EMP1	epithelial membrane protein 1	12p12.3	2,066	0,0000357
EBAG9	estrogen receptor binding site associated, antigen, 9	8q23	2,058	0,0000919
VPS37B	vacuolar protein sorting 37 homolog B (S. cerevisiae)	12q24.31	2,049	0,0000640
NOV	nephroblastoma overexpressed gene	8q24.1	2,001	0,0000746

tumours (n = 22). No obvious relation between ASCL1 and TPH1 expression levels was found.

ASCL1 has been found to repress DKK1 transcription, a negative regulator of the Wnt signalling pathway in lung cancer cells, and is also the first transcriptional repressor identified for DKK1. The regulation is meditated by histone deacetylation and repressive lysine 27 trimetylation in the promoter region of DKK1 [9]. Moreover, downregulation of DKK1 has been associated with colorectal- and breast cancer (23, 24). On the other hand, DKK1 has also been identified as a potential prognostic and diagnostic marker for cohorts of breast cancer patients with poor prognosis [23] and increased circulating levels of DKK1 has been associated with the presence of bone metastases in patients with breast cancer [25] We note that 13 out of the 22 analysed PETs prominently expressed DKK1.

Wnt/β-catenin signalling is negatively regulated by DKK1 by inhibition of the complex formation between Wnts and its receptors, LRP5/6. It has been advocated that ASCL1 expression may favour cancer cell growth through repression of DKK1 with the consequential aberrant activation of the Wnt/β-catenin signalling pathway [9]. This may also apply to a subset of PETs as a total of 9 out of 22 PETs displayed low DKK1 immunoreactivity.

ASCL1 may have a coordinating role in production of serotonin by transcriptional regulation of TPH1 and could thereby be involved in causing the carcinoid syndrome in patients with PET [12]. Our results from the microarray expression analysis in BON1 cells suggested that TPH1 might constitute a ASCL1 target gene in BON1 cells. However, an obvious relation between ASCL1 and TPH1 protein expression levels were not found, and TPH1 showed a heterogeneous pattern of immunoreactivity in PETs.

Table 2: Annotated genes with decreased expression in BON1 cells transfected with siRNA to *ASCL1*

Gene Symbol	Gene Name	Location	Ratio	adj P Value
FAM87A	family with sequence similarity 87, member A	8p23.3	0,221	0,0000104
DKFZP761C1711	Hypothetical protein DKFZp761C1711		0,246	0,0000258
GABRA1	gamma-aminobutyric acid (GABA) A receptor, alpha 1	5q34-q35	0,260	0,0000172
GUSBP1	glucuronidase, beta pseudogene 1	7q21.11	0,277	0,0000104
TncRNA	trophoblast-derived noncoding RNA	11q13.1	0,284	0,0000104
LOC728411	Similar to Beta-glucuronidase precursor	5.	0,296	0,0000104
TTLL5	tubulin tyrosine ligase-like family, member 5	17q21.32	0,328	0,0001110
EBF1	Early B-cell factor 1	5q34	0,333	0,0000112
VPS13C	Vacuolar protein sorting 13 homolog C (S. cerevisiae)	15q21.3	0,341	0,0000150
RNF12	Ring finger protein 12	Xq13-q21	0,342	0,0000283
*ASCL1**	achaete-scute complex homolog 1 (Drosophila)	12q22-q23	0,351	0,0000162
SORBS2	sorbin and SH3 domain containing 2	4q35.1	0,353	0,0000161
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4	2q33.3-q34	0,359	0,0001092
FLJ38379	hypothetical protein FLJ38379	2q37.3	0,360	0,0001245
TCF12	Transcription factor 12	15q21	0,365	0,0000283
PGM5	phosphoglucomutase 5	9q13	0,365	0,0000499
RIT1	Ras-like without CAAX 1	1q22	0,366	0,0000150
ZCCHC7	Zinc finger, CCHC domain containing 7	9p13.2	0,371	0,0001730
LOC730168	hypothetical protein LOC730168///LOC732289	3q26.32	0,374	0,0000625
CFLAR	CASP8 and FADD-like apoptosis regulator	2q33-q34	0,374	0,0001083
C11orf80	chromosome 11 open reading frame 80	11q	0,375	0,0000579
CCAR1	Cell division cycle and apoptosis regulator 1	10q21.3	0,375	0,0000796
FAM81B	family with sequence similarity 81, member B	5q15	0,382	0,0000599
FLJ25770	hypothetical protein FLJ25770	4q21.1	0,383	0,0000181
LOC730390	SMA4///similar to SMA4	5q13	0,385	0,0000159
ZFAND6	Zinc finger, AN1-type domain 6	15q25.1	0,387	0,0000246
LOC728555	hypothetical protein LOC728555///LOC730391	5q13.2	0,388	0,0000151
GRAMD3	GRAM domain containing 3	5q23.2	0,394	0,0000136
CBFA2T2	core-binding factor, runt domain, alpha subunit 2	20q11	0,394	0,0000181
ZNF638	Zinc finger protein 638	2p13.2-p13.1	0,396	0,0019556
LOC728678	hypothetical protein LOC728678///LOC731914	3p22.3	0,397	0,0000150
CLCN5	chloride channel 5	Xp11.23-p11.22	0,398	0,0000170
ANXA13	annexin A13	8q24.13	0,412	0,0000181
RP11-506K6.3	Hypothetical LOC389362	6p25.2	0,415	0,0002632
FLJ23556	hypothetical protein FLJ23556	10q25.2	0,417	0,0000788
PFAAP5	Phosphonoformate immuno-associated protein 5	13q13.1	0,417	0,0000150
PCDHGA4	protocadherin gamma subfamily A, 4	5q31	0,419	0,0000274
LOC145474	hypothetical protein LOC145474	14q24.1	0,421	0,0003892
IFIT1	interferon-induced protein	10q25-q26	0,421	0,0006010
DKFZp547E087	hypothetical gene LOC283846	18p11.21	0,424	0,0000586
TXNIP	thioredoxin interacting protein	1q21.1	0,427	0,0000305
CDC14B	CDC14 cell division cycle 14 homolog B	9q22.33	0,432	0,0000885
RUFY2	RUN and FYVE domain containing 2	10q21.3	0,432	0,0001373
KLHL28	Kelch-like 28 (Drosophila)	14q21.3	0,435	0,0002095
MBNL2	Muscleblind-like 2 (Drosophila)	13q32.1	0,436	0,0000246
LOC730496	hypothetical protein LOC730496	1.	0,436	0,0000150
PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit	5p12	0,436	0,0002228
NR5A2	nuclear receptor subfamily 5, group A, member 2	1q32.1	0,438	0,0003777
LOC388743	similar to calpain 8	1q41	0,440	0,0000284
PTPN13	Protein tyrosine phosphatase, non-receptor type 13	4q21.3	0,441	0,0011559
RASAL2	RAS protein activator like 2	1q24	0,442	0,0003715
LOC730258	neuroblastoma breakpoint family, member 1, 3, 8, 10	1q21.1	0,443	0,0000150
ZNF518	Zinc finger protein 518	10q23.33	0,445	0,0002293
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	12p13	0,445	0,0000176
CTAGE5	CTAGE family, member 5	14q13.3	0,446	0,0000617
LOC440895	similar to LIM and senescent cell antigen-like domains 3	2q13	0,446	0,0003164
PELI1	Pellino homolog 1 (Drosophila)	2p13.3	0,449	0,0001121
FAM98A	Family with sequence similarity 98, member A	2p22.3	0,449	0,0000499
BRWD2	bromodomain and WD repeat domain containing 2	10q26	0,450	0,0000602
C20orf74	chromosome 20 open reading frame 74	20p11.22	0,450	0,0000926
MALAT1	metastasis associated lung adenocarcinoma transcript 1	2p16.3	0,452	0,0001060
RTN4	reticulon 4	2p16.3	0,453	0,0004446
LOC654342	Similar to lymphocyte-specific protein 1	2p11.1	0,454	0,0000284
CYorf15B	chromosome Y open reading frame 15B	Yq11.222	0,455	0,0001245

Table 2: Annotated genes with decreased expression in BON1 cells transfected with siRNA to *ASCL1* *(Continued)*

SMOC2	SPARC related modular calcium binding 2	6q27	0,456	0,0002821
CAPN2	calpain 2, (m/II) large subunit	1q41-q42	0,457	0,0000460
HEL308	DNA helicase HEL308	4q21.23	0,459	0,0006091
PDCD6	Programmed cell death 6	5pter-p15.2	0,459	0,0000722
LOC285147	hypothetical protein LOC285147	2p25.2	0,460	0,0001063
TFF3	trefoil factor 3 (intestinal)	21q22.3	0,460	0,0010306
RIMBP2	RIMS binding protein 2	12q24.33	0,461	0,0000520
C10orf93	chromosome 10 open reading frame 93	10q26.3	0,462	0,0000950
SUCNR1	succinate receptor 1	3q24-q25.1	0,462	0,0000603
LOC151878	hypothetical protein LOC151878	3p14.3	0,463	0,0003715
LRRFIP1	Leucine rich repeat (in FLII) interacting protein 1	2q37.3	0,465	0,0000339
ADAM12	ADAM metallopeptidase domain 12 (meltrin alpha)	10q26.3	0,465	0,0003076
GART	Phosphoribosylglycinamide, phosphoribosylaminoimidazole	21q22.11	0,466	0,0003142
PLGLB1/2	plasminogen-like B2///plasminogen-like B1	2p11-q11	0,467	0,0007445
RET	ret proto-oncogene	10q11.2	0,467	0,0000247
POLQ	polymerase (DNA directed), theta	3q13.33	0,467	0,0002632
KIAA1632	KIAA1632	18q12.3-q21.1	0,468	0,0004005
ADAM28	ADAM metallopeptidase domain 28	8p21.2	0,469	0,0000950
MSI2	Musashi homolog 2 (Drosophila)	17q22	0,469	0,0000344
JMJD1C	jumonji domain containing 1C	10q21.2-q21.3	0,470	0,0001013
DST	dystonin	6p12.1	0,473	0,0001509
NT5E	5'-nucleotidase, ecto (CD73)	6q14-q21	0,474	0,0000288
LYST	lysosomal trafficking regulator	1q42.1-q42.2	0,475	0,0001027
SYK	Spleen tyrosine kinase	9q22	0,476	0,0003933
DLG1	Discs, large homolog 1 (Drosophila)	3q29	0,476	0,0006683
RASSF6	Ras association (RalGDS/AF-6) domain family 6	4q13.3	0,476	0,0006133
TRA2A	transformer-2 alpha	1p36.11	0,478	0,0000701
UBE2D3	ubiquitin-conjugating enzyme E2D 3	4q24	0,479	0,0002430
TMEM46	transmembrane protein 46	13q12.13	0,480	0,0000248
INADL	InaD-like (Drosophila)	1p31.3	0,480	0,0002693
TTC30A	tetratricopeptide repeat domain 30A	2q31.2	0,484	0,0003577
SNAP25	Synaptosomal-associated protein, 25 kDa	20p12-p11.2	0,484	0,0000189
PRO2852	hypothetical protein PRO2852	9.	0,485	0,0000460
MLLT3	myeloid/lymphoid or mixed-lineage leukemia	9p22	0,486	0,0004334
RBM6	RNA binding motif protein 6	3p21.3	0,488	0,0002432
PPP2R5C	protein phosphatase 2, regulatory subunit B',	14q32	0,488	0,0000241
GOPC	Golgi associated PDZ and coiled-coil motif containing	6q21	0,489	0,0006453
LAMA4	laminin, alpha 4	6q21	0,489	0,0001402
SFRS15	splicing factor, arginine/serine-rich 15	21q22.1	0,490	0,0000344
KIF13A	kinesin family member 13A	6p23	0,491	0,0001644
CLASP2	cytoplasmic linker associated protein 2	3p22.3	0,493	0,0004942
MMAA	Methylmalonic aciduria (cobalamin deficiency) cblA type	4q31.22	0,493	0,0002644
C1orf192	chromosome 1 open reading frame 192	1q23.3	0,494	0,0006859
hCG_2003663	hCG2003663	9q22.32	0,494	0,0006724
SMOC1	SPARC related modular calcium binding 1	14q24.2	0,494	0,0001073
REV3L	REV3-like, catalytic subunit of DNA polymerase zeta	6q21	0,496	0,0001092
SMAD1	SMAD family member 1	4q31	0,496	0,0004345
TWF1	twinfilin, actin-binding protein, homolog 1 (Drosophila)	12q12	0,497	0,0001687
FBXO9	F-box protein 9	6p12.3-p11.2	0,497	0,0000391

Traditionally, much of the Notch signalling research has focused on the involvement of Notch signalling factors like ASCL1 in neural stem cell differentiation. Even though pancreatic endocrine cells have an endodermal origin they also share several molecular features with neurons. Like neurons in the central nervous system, differentiating endocrine cells in the pancreas appear in a scattered fashion within a field of progenitor cells. The different cell types are generated by lateral inhibition through Notch signalling [4]. With this in mind it is not surprising that the results from the GO analysis suggest that *ASCL1* target genes participate in cellular differentiation, migration and localisation of cells also in pancreatic endocrine cells.

Conclusion

The present findings support the notion that ASCL1 is involved in pancreatic endocrine tumourigenesis, where aberrant expression of DKK1 may play additional important roles. ASCL1 also directly or indirectly regulates expression of several putative oncogenes and

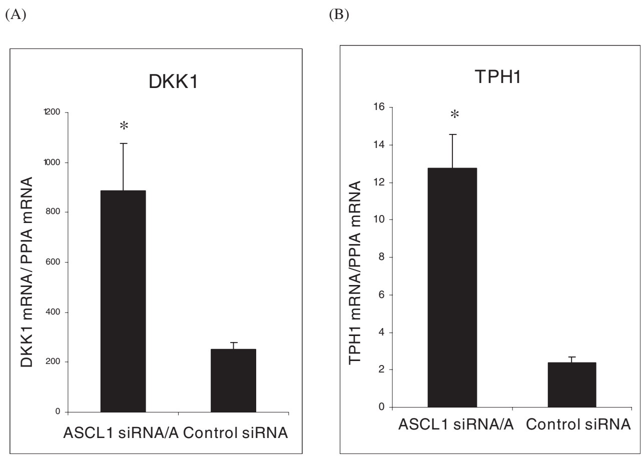


Figure 5
Relative expression of *DKK1* and *TPH1* in siRNA-transfected BON1 cells as determined by qPCR. Data are presented as mean ± SEM of analysis in triplicates. * $p < 0.05$.

Table 3: Functional GO categories

Systems	Categorys	No. of genes or transcripts
Biological processes		
	Regulation of biological or cellular process	342
	Development regulation/cellular	292
	Cellular metabolic process	189
	Transcription and regulation of transcription	144
	Biological/cellular adhesion	68
	Regulation of nucleo -base -side, -tide and nucleic acid metabolic process	62
	Regulation of gene expression	56
	Cell differentiation	44
	Locomotion, cellular or regulation of	29
	Cellular migration/localisation	26
	Phosphorylation	20
	Neurogenes	19
	Protein amino acid phosphorylation	17
	Carbohydrate biosynthetic process	5
	Integrin-mediated signalling pathway	5
Molecular functions		
	Binding activity, receptor, DNA, nucleic acid	305
	Transcription regulation/cofactor or binding activity	67
	Kinase activity	54
	Phosphotransferase activity	49
	Ligase activity ubiquitin/amino acid/small conjugating protein	24
	Ligase activity	20
	Enzyme activator activity	13
	Transmembrane receptor protein kinase activity	11
Cellular components		
	Cell junction	14
	Intercellular junction	8
	Basement membrane	5

Functional categories are based on GO annotation. Note that in GO function hierarchy, some genes/transcripts belong to multiple categories.

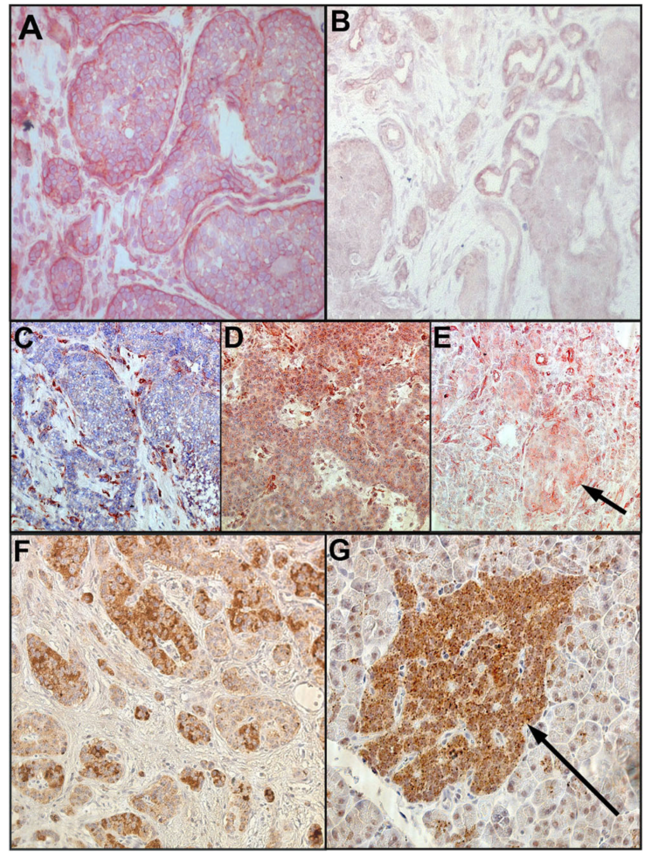


Figure 6
Immunohistochemical analysis of ASCL1, DKK1, and TPH1 in pancreatic endocrine tumours and non-tumourous pancreatic specimen. Pancreatic islets are indicated by arrows. Representative immunostainings are shown. (A) High ASCL1 expression in tumour no. 5 (×200), (B) Low ASCL1 expression in tumour no. 23 (×200), (C) Low DKK1 expression in tumour no. 5 (×200), (D) High DKK1 expression in tumour no. 10 (×200), (E) DKK1 expression in non-tumourous pancreas (×200), (F) heterogeneous TPH1 expression in tumour no. 10 (×200), (G) TPH1 expression in non-tumourous pancreas (×400).

tumours suppressor genes in pancreatic endocrine tumour cells that may contribute to the neoplastic process.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TAJ performed the experiments. TAJ, GW and BS participated in design of the study, interpreted the result and contributed to writing the paper. All authors read and approved the final version of the manuscript.

Table 4: Clinical characteristics and results of immunohistochemistry for ASCL1, DKK1 and TPH1

Tumour no	Gender	Age at diagnosis	WHO	Syndrome	ASCL1	DKK1	TPH1
1	M	53	2	NF	High	Low	Heterogeneous
2	F	47	1	NF/MEN 1	High	Low	Low
3	M	50	2	IN	High	Low	
4	F	86	1	IN	High	Low	
5	F	40	1	GA	High (p)	Low (p)	Heterogeneous
6	M	44	2	GL	High	Low	Heterogeneous
7	M	51	2	NF	Low	High	
8	F	46	2	NF	Low	High	
9	F	34	2	NF	Low	High	
10	M	62	1	NF/MEN 1	Low	High (p)	Heterogeneous (p)
11	M	53	2	NF/MEN 1	Low	High	
12	M	48	2	NF/MEN 1	Low	N/D	Heterogeneous
13	M	47	2	IN	Low	High	Heterogeneous
14	M	19	1	IN/MEN 1	Low	High	Heterogeneous
15*	M	53	2	GL	Low	High	High
16	M	72	2	GA	Low	High	
17	M	50	3	NF	High	High	
18	F	44	2	NF	High	High	
19	F	23	3	NF	High	High	
20	M	57	1	NF/MEN 1	High	High	High
21	F	44	1	NF	Low	Low	Heterogeneous
22	M	22	1	NF/MEN 1	Low	Low	High/Heterogeneous
23	M	64	1	IN	Low (p)	Low	

Immunoreactivity was graded as Low, High or Heterogeneous. ASCL1 immunoreactivity has been determined previously (8) were grading +, ++ is here denoted Low and +++ denoted High. N/D, not determined; *, liver metastasis; p, in Figure 6. WHO classifications: 1, well-differentiated endocrine tumour, 2, well-differentiated endocrine carcinoma, 3, poorly differentiated endocrine carcinoma. NF, non-functioning; MEN 1, Multiple Endocrine Neoplasia 1; IN, insulinoma; GA, gastrinoma; GL, glucagonoma

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