Research article

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

Térèse A Johansson1, Gunnar Westin2 and Britt Skogseid*1

Address: 1Department of Medical Sciences, Uppsala University, Uppsala University Hospital, SE-751 85 Uppsala, Sweden and 2Department of Surgical Sciences, Uppsala University, Uppsala University Hospital, SE-751 85 Uppsala, Sweden

E-mail: Térèse A Johansson - terese.johansson@medsci.uu.se; Gunnar Westin - gunnar.westin@surgsci.uu.se; Britt Skogseid* - britt.skogseid@medsci.uu.se

*Corresponding author

Published: 10 September 2009 Received: 1 March 2009

BMC Cancer 2009, 9:321 doi: 10.1186/1471-2407-9-321 Accepted: 10 September 2009

This article is available from: http://www.biomedcentral.com/1471-2407/9/321

© 2009 Johansson et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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 biosynthesis 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% CO2/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, Burlington, USA). Cells were photographed by an AxioCam HRm camera employing the Axiosvision 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’-CGCGUUAIAUGUAAGUCC-CATT and 5’-UGGGAGUUACUAACGCGTG (siRNA/A) and 5’-AGUACUGCUACGAUACGGIT and 5’-TTUAUAUCEGGAAGGCTACU (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, SYF; 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 (BD Biosciences) or anti-α Tubulin monoclonal antibody (BD Biosciences) 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 were used as 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 [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 (log2ratio) equal to or larger than 1 (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 (DakoCyto-mation, 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).

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
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, NF-kB, TGFβ and MAP kinases 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).
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 mediated by histone deacetylation and repressive lysine 27 trimethylation 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, LRPS/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 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  |
| CI3orf15    | 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       | 2.249  | 0.000155   |
| 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, cvc and kazal-like domains | 5q31        | 2.251  | 0.0000796  |
| NRXN3       | neurexin 3                        | 14q31          | 2.246  | 0.0002025  |
| KL          | klotho                            | 13q12          | 2.240  | 0.000150   |
| GP2D        | glycerol-3-phosphate dehydrogenase 2 (mitochondrial) | 2q24.1        | 2.238  | 0.0000155  |
| CLCS        | chloride intracellular channel 5   | 6p21.1-p12.1   | 2.235  | 0.0000313  |
| EMG1        | EMG1 nuclear 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  |
| FGFR13      | fibroblast growth factor 13        | Xq26.3         | 2.178  | 0.0000288  |
| CHM         | choroideremia (Rab escort protein 1) | Xq21.2        | 2.150  | 0.0000396  |
| GLCE        | glucronic acid epimerase            | 15q23          | 2.144  | 0.0000561  |
| DC2         | DC2 protein                        | 4q25           | 2.143  | 0.0001811  |
| SMS         | spermine synthase                  | Xp22.1         | 2.113  | 0.0003207  |
| GP2D        | glycerol-3-phosphate dehydrogenase 2 (mitochondrial) | 2q24.1        | 2.095  | 0.0000586  |
| LOC283454   | hypothetical protein LOC283454     | 12q44.23       | 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      | vacular protein sorting 37 homolog B (S. cerevisiae) | 12q44.31     | 2.049  | 0.0000640  |
| NOV         | nephroblastoma overexpressed gene  | 8q24.1         | 2.001  | 0.0000746  |
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 |
| DKKFZ761C1711 | Hypothetical protein DKKFZ761C1711 | | 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 | 0.296 | 0.0000110 |
| TTLS | tubulin tyrosine ligase-like family, member 5 | 17q21.32 | 0.328 | 0.0000110 |
| EBI1 | Early B-cell factor 1 | 5q34 | 0.333 | 0.0000112 |
| VPS13C | Vacular protein sorting 13 homolog C (S. cerevisiae) | 15q31.3 | 0.341 | 0.0000150 |
| 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 | 0.0000182 |
| VPS13 | Vacular protein sorting 13 homolog C (S. cerevisiae) | 15q31.3 | 0.365 | 0.0000182 |
| RIT1 | Ras-like without CAAX 1 | 1q22 | 0.366 | 0.0000150 |
| ZCCHC7 | Zinc finger, CCHC domain containing 7 | 9p13.2 | 0.371 | 0.00001730 |
| LOC730168 | hypothetical protein LOC730168///LOC732289 | 3q26.32 | 0.374 | 0.0000625 |
| CFLAR | CASP8 and FADD-like apoptosis regulator | 2q33-q34 | 0.374 | 0.0001083 |
| CI tar80 | chromosome 11 open reading frame 80 | 11q | 0.375 | 0.0000579 |
| CCAR1 | Cell division cycle and apoptosis regulator 1 | 10q23.1 | 0.375 | 0.0000796 |
| FAM81B | family with sequence similarity 81, member B | 5q15 | 0.382 | 0.0000599 |
| FLJ25770 | hypothetical protein FLJ25770 | 5q13 | 0.383 | 0.0000182 |
| LOC730390 | hypothetical protein LOC730390///similar to SMA4 | 5q13 | 0.385 | 0.0000150 |
| ZFAND6 | Zinc finger, AN1-type domain 6 | 15q21.1 | 0.387 | 0.0000246 |
| LOC7285S55 | hypothetical protein LOC728555///LOC73091 | 5q13.2 | 0.388 | 0.0000151 |
| GRAM3 | GRAM domain containing 3 | 5q32.2 | 0.394 | 0.0000136 |
| CBFA2T2 | core-binding factor, runt domain, alpha subunit 2 | 2q11.1 | 0.394 | 0.0000181 |
| ZNF638 | Zinc finger protein 638 | 2p13.2-p13.1 | 0.396 | 0.0001955 |
| 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 | 0.00002632 |
| FLJ33556 | hypothetical protein FLJ32556 | 10q25.2 | 0.417 | 0.0000788 |
| PFAAP5 | Phosphonofomate immuno-associated protein 5 | 1q31.3 | 0.417 | 0.0000150 |
| PCDHG4 | protocaderhin gamma subfamily A, 4 | 5q31 | 0.419 | 0.0000274 |
| LOC145474 | hypothetical protein LOC145474 | 1q24.1 | 0.421 | 0.00003892 |
| IFIT1 | interferon-induced protein | 10q25-q26 | 0.421 | 0.00006010 |
| DKKFZ547E087 | 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 | 0.00000885 |
| RUFY2 | RUN and FYVE domain containing 2 | 10q23.3 | 0.432 | 0.0001373 |
| KLHL28 | Kelch-like 28 (Drosophila) | 1q21.3 | 0.435 | 0.0002095 |
| MBNL2 | Muscleblind-like 2 (Drosophila) | 13q21.2 | 0.436 | 0.0000246 |
| LOC730496 | hypothetical protein LOC730496 | 1.0 | 0.436 | 0.0000150 |
| PRKAA1 | protein kinase, AMP-activated, alpha 1 catalytic subunit | 5p12 | 0.436 | 0.00002228 |
| NRS2A | nuclear receptor subfamily 5, group A, member 2 | 5q12.1 | 0.438 | 0.00003777 |
| LOC388743 | similar to calpain 8 | 1q21.1 | 0.440 | 0.0000284 |
| PTPN13 | Protein tyrosine phosphatase, non-receptor type 13 | 4q21.3 | 0.441 | 0.0001155 |
| RAM2 | RAS protein activator like 2 | 1q24 | 0.442 | 0.00003799 |
| 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 |
| SCN1A | sodium channel, nonvoltage-gated 1 alpha | 12p13 | 0.445 | 0.0000176 |
| CTAGE5 | CTAGE family, member 5 | 14q33.3 | 0.446 | 0.00000617 |
| LOC440895 | similar to LIM and senescent cell antigen-like domains 3 | 2q13 | 0.446 | 0.0003164 |
| PELI | Pellino homolog 1 (Drosophila) | 2p13.3 | 0.449 | 0.0001121 |
| FAM98A | Family with sequence similarity 98, member A | 2p22.3 | 0.449 | 0.00004999 |
| BRWD2 | bromodomain and WD repeat domain containing 2 | 10q26 | 0.450 | 0.0000602 |
| C2orf74 | chromosome 20 open reading frame 74 | 20p1.22 | 0.450 | 0.0000926 |
| MALAT1 | metastasis associated lung adenocarcinoma transcript 1 | 2p16.3 | 0.452 | 0.0001060 |
| RTN4 | reticulin 4 | 2p16.3 | 0.453 | 0.0004446 |
| LOC654342 | Similar to lymphocyte-specific protein 1 | 2p11.1 | 0.454 | 0.0002848 |
| Ctyf1 | chromosome Y open reading frame 15B | Yq11.222 | 0.455 | 0.0001245 |
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

---

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

| Gene Symbol | Gene Name | Chromosome | Adj. P Value | FDR |
|-------------|-----------|------------|--------------|-----|
| 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) | 2q22.3    | 0.460        | 0.0001306 |
| RIMBP2      | RIMS binding protein 2 | 12q24.33 | 0.461        | 0.0000520 |
| C10orf93    | chromosome 10 open reading frame 93 | 10q26.3   | 0.462        | 0.0000950 |
| SUCHR1      | succinate receptor 1 | 3q24-q25.1 | 0.462    | 0.0000603 |
| LOC151878   | hypothetical protein LOC151878 | 3p14.3  | 0.463        | 0.0003715 |
| LRRFPI      | 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    | 0.0000307 |
| GART        | Phosphoribosylglycinamide, phosphoribosylaminoimidazole | 2q22.11 | 0.466    | 0.0003142 |
| PGLB1/2     | 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 |
| JMD1C       | jumonji domain containing 1C | 10q21.2-q21.3 | 0.470 | 0.0001013 |
| DST         | dystin | 6p12.1   | 0.473        | 0.0001509 |
| NTSE        | 5’-nucleotidease, 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        | 0.000701 |
| UBED2D3     | ubiquitin-conjugating enzyme E2D 3 | 4q24  | 0.479        | 0.0002430 |
| TMEM46      | transmembrane protein 46 | 13q12.13 | 0.480    | 0.0002428 |
| INADL       | InaD-like (Drosophila) | 1p31.3 | 0.480        | 0.0002693 |
| TTC30A      | tetraspliceotide repeat domain 30A | 2q12.1  | 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 | 3p12.3 | 0.488    | 0.0002432 |
| PPP2R5C     | protein phosphatase 2, regulatory subunit B' | 1q432 | 0.488    | 0.0002421 |
| 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 |
| MMMAA       | Methylyinosinic aciduria (cubalin 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    | 0.0003435 |
| TWF1        | twinfilin, actin-binding protein, homolog 1 (Drosophila) | 12q12 | 0.497 | 0.0001687 |
| FBX09       | F-box protein 9 | 6p12.3-p11.2 | 0.497 | 0.000391 |
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 I | High  | Low  | Low  |
| 3         | M      | 50               | 2   | IN       | High  | Low  | Low  |
| 4         | F      | 86               | 1   | IN       | High  | Low  | Low  |
| 5         | F      | 40               | 1   | GA       | Low (p) | Low (p) | Heterogeneous |
| 6         | M      | 44               | 2   | GL       | High  | Low  | Low  |
| 7         | M      | 51               | 2   | NF       | Low   | High  | Heterogeneous |
| 8         | F      | 46               | 2   | NF       | Low   | High  | Low  |
| 9         | F      | 34               | 2   | NF       | Low   | High  | Low  |
| 10        | M      | 62               | 1   | NF/MEN I | Low (p) | Heterogeneous |
| 11        | M      | 53               | 2   | NF/MEN I | Low   | High  | Low  |
| 12        | M      | 48               | 2   | NF/MEN I | Low   | N/D  | Heterogeneous |
| 13        | M      | 47               | 2   | IN       | Low   | High  | Heterogeneous |
| 14        | M      | 19               | 1   | IN/MEN I | Low   | High  | Heterogeneous |
| 15        | M      | 53               | 2   | GL       | Low   | High  | High  |
| 16        | M      | 72               | 2   | GA       | Low   | High  | 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 I | High  | High  | High  |
| 21        | F      | 44               | 1   | NF       | Low   | Low   | Low   |
| 22        | M      | 22               | 1   | NF/MEN I | Low   | High  | 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 I; IN, insulinoma; GA, gastrinoma; GL, glucagonoma.

Acknowledgements
This work was supported by the Swedish Research Council, Swedish Cancer Society, and Lions Fund for Cancer Research. The authors are grateful to B. Bondeson, Dr. C. Martijn (Department of Surgical Sciences), A. von Malmborg, Dr J. Saras (Department of Medical Sciences) and H. Göransson (Uppsala Array Platform) for excellent experimental support and technical expertise. The BON1 cell line was kindly provided by Dr. J. C. Thompson (Department of Surgery, University of Texas Medical Branch, USA).

References
1. Huber K, Bruhl B, Guillemot F, Olson EN, Ernsberger U and Unsicker K: Development of chromaffin cells depends on MASH1 function. Development 2002, 129:4729–4738.
2. Lanigan TM, DeRaad SK and Russo AF: Requirement of the MASH-1 transcription factor for neuroendocrine differentiation of thyroid C cells. J Neurobiol 1998, 34:126–134.
3. Borges M, Linnoila RI, Velde van de Hj, Chen H, Nelkin BD, Mabry M, Baylin SB and Ball DW: An achaete-scute homologue essential for neuroendocrine differentiation in the lung. Nature 1997, 386:852–855.
4. Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendahl U and Edlund H: Notch signalling controls pancreatic cell differentiation. Nature 1999, 400:877–881.
5. Murtaugh LC, Stanger BZ, Kwan KM and Melton DA: Notch signalling controls multiple steps of pancreatic differentiation. Proc Natl Acad Sci USA 2003, 100:14920–14925.
6. Essi F, Ghosh B, Blankin AV, Lin JW, Albert MA, Yu X, MacDonald RJ, Civin CI, Real FX, Pack MA, Ball DW and Leach SD: Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas. Development 2004, 131:4213–4224.
7. Iso T, Kedes L and Hamamori Y: HES and HERP families: multiple effectors of the Notch signalling pathway. J Cell Physiol 2003, 194:237–255.
8. Johansson T, Lejonklou MH, Ekeblad S, Stålberg P and Skogseid B: Lack of Nuclear Expression of Hairy and Enhancer of Split-1 (HES1) in Pancreatic Endocrine Tumours. Horm Metab Res 2008, 40:354–359.
9. Osada H, Tomida S, Yatabe Y, Tateyama Y, Takeuchi T, Murakami H, Kondo Y, Sekido Y and Takahashi T: Roles of achaete-scute homologue 1 in DKK1 and E-cadherin repression and neuroendocrine differentiation in lung cancer. Cancer Res 2008, 68:1647–1655.
10. Wells JMT, Essi F, Boivin GP, Aronow BJ, Stuart W, Combs C, Sklenka A, Leach SD and Lowy AM: Wnt/beta-catenin signalling is required for development of the exocrine pancreas. BMC Dev Biol 2007, 7:4.
11. Rullifson JC, Karnik SK, Heiser PW, ten Berge D, Chen H, Gu X, Taketo MM, Nusse R, Heck AF, and Kim SK: Wnt signalling regulates pancreatic beta cell proliferation. Proc Natl Acad Sci USA 2007, 104:6247–6252.
12. Nakakura EK, Suriyanpong VR, Kunimalaiyaan M, Hsiao EC, Schuebel KE, Borges MW, Jin N, Collins BJ, Nelkin BD, Chen H and Ball DW: Regulation of neuroendocrine differentiation in gastrointestinal carcinoid tumour cells by notch signalling. J Clin Endocrinol Metab 2005, 90:4350–4356.
13. Parrish D, Ishizuka J, Townsend CM Jr, Haber B, Beauchamp RD, Karp G, Kim SW, Rajaraman S, Gleeley G Jr and Thompson JC: Characterization of a human pancreatic carcinoid in vitro: morphology, amine and peptide storage, and secretion. Pancreas 1994, 9:83–90.
14. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U and Speed TP: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003, 4:249–264.
15. Li C and Wong WH: Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci USA 2001, 98:31–36.
16. Smyth GK: Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004, 3:Article 3.
17. Wettenhall JM and Smyth GK: LimmaGUI: a graphical user interface for linear modelling of microarray data. Bioinformatics 2004, 20:3705–3706.

18. Hochberg Y and Ba Y: Controlling the false discovery rate: A practical and powerful approach to multiple testing. J Roy Stat Soc Ser B 1995, 57:289–300.

19. Kunisch M, Haenlin M and Campos-Ortega JA: Lateral inhibition mediated by the Drosophila neurogenic gene delta is enhanced by proneural proteins. Proc Natl Acad Sci USA 1994, 91:10139–10143.

20. Castro DS, Skowronska-Krawczyk D, Armant O, Donaldson JJ, Parras C, Hunt C, Critchley JA, Nguyen L, Gossler A, Götgens B, Matter JM and Guillenot F: Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev Cell 2006, 11:831–844.

21. Johnson JE, Birren SJ, Saito T and Anderson DJ: DNA binding and transcriptional regulatory activity of mammalian achaete-scute homologous (MASH) proteins revealed by interaction with a muscle-specific enhancer. Proc Natl Acad Sci USA 1992, 89:3596–3600.

22. Persson P, Jogi A, Grynfeld A, Pahlman S and Axelsson H: HASH-1 and E2-2 are expressed in human neuroblastoma cells and form a functional complex. Biochem Biophys Res Commun 2000, 274:22–31.

23. Forget MA, Turcotte S, Beauseigle D, Godin-Ethier J, Pelletier S, Martin J, Tanguay S and Lapointe R: The Wnt pathway regulator DKK1 is preferentially expressed in hormone-resistant breast tumours and in some common cancer types. Br J Cancer 2007, 96:646–653.

24. González-Sancho JM, Aguilera O, García JM, Pendás-Franco N, Peña C, Cal S, García de Herreros A, Bonilla F and Muñoz A: The Wnt antagonist DICKKOPF-1 gene is a downstream target of beta-catenin/TCF and is down regulated in human colon cancer. Oncogene 2005, 24:1098–1103.

25. Voorzanger-Rousselot N, Goehrig D, Journe F, Doriath V, Body JJ, Clézardin P and Garnero P: Increased Dickkopf-1 expression in breast cancer bone metastases. Br J Cancer 2007, 97:964–970.

Pre-publication history
The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/9/321/pre-pub