Transcriptome analysis of human cancer reveals a functional role of Heme Oxygenase-1 in tumor cell adhesion

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Abstract

Background: Heme Oxygenase-1 (HO-1) is expressed in many cancers and promotes growth and survival of neoplastic cells. Recently, HO-1 has been implicated in tumor cell invasion and metastasis. However, the molecular mechanisms underlying these biologic effects of HO-1 remain largely unknown. To identify a common mechanism of action of HO-1 in cancer, we determined the global effect of HO-1 on the transcriptome of multiple tumor entities and identified a universal HO-1-associated gene expression signature.

Results: Genome-wide expression profiling of Heme Oxygenase-1 expressing versus HO-1 silenced BeWo choriocarcinoma cells as well as a comparative meta-profiling of the preexisting expression database of 190 human tumors of 14 independent cancer types led to the identification of 14 genes, the expression of which correlated strongly and universally with that of HO-1 (P = 0.00002). These genes included regulators of cell plasticity and extracellular matrix (ECM) remodeling (MMP2, ADAM8, TGFB1, BGN, COL21A1, PXDN), signaling (CRIP2, MICB), amino acid transport and glycosylation (SLC7A1 and ST3GAL2), estrogen and phospholipid biosynthesis (AGPAT2 and HSD17B1), protein stabilization (IFI30), and phosphorylation (ALPPL2). We selected PXDN, an adhesion molecule involved in ECM formation, for further analysis and functional characterization. Immunofluorescence and Western blotting confirmed the positive correlation of expression of PXDN and HO-1 in BeWo cancer cells as well as co-localization of these two proteins in invasive extravillous trophoblast cells. Modulation of HO-1 expression in both loss-of and gain-of function cell models (BeWo and 607B melanoma cells, respectively) demonstrated a direct relationship of HO-1 expression with cell adhesion to Fibronectin and Laminin coated wells. The adhesion-promoting effects of HO-1 were dependent on PXDN expression, as loss of PXDN in HO-1 expressing BeWo and 607B cells led to reduced cell attachment to Laminin and Fibronectin coated wells.

Conclusions: Collectively, our results show that HO-1 expression determines a distinct ‘molecular signature’ in cancer cells, which is enriched in genes associated with tumorigenesis. The protein network downstream of HO-1 modulates adhesion, signaling, transport, and other critical cellular functions of neoplastic cells and thus promotes tumor cell growth and dissemination.

Background

Heme oxygenases are the rate-limiting enzymes in heme degradation that catalyze the conversion of heme into carbon monoxide, iron, and biliverdin. Heme oxygenase 1 (HO-1) has (cyto)protective properties and antiinflammatory, antiapoptotic, and antiproliferative capacities of HO-1 have been described in several cell types [1,2]. Under normal physiologic conditions HO-1 expression is low but can be upregulated in response to a wide range of stimuli and activated signaling molecules, including the HO-1 substrate heme, reactive oxygen species (ROS), nitric oxide species, prostaglandins, cytokines, growth factors such as insulin, and lipopolysaccharide [2]. Since heat shock (and other cellular stressors) lead to upregulation of HO-1, this molecule has also been termed heat-shock protein 32 (Hsp32).
A relation between malignant behavior and alterations in expression of HO-1 may exist. Elevated HO-1 has been detected in several cancer cell lines [3-6] and tumors (including lymphosarcoma, adenocarcinoma, hepatoma, glioblastoma, melanoma, prostate cancers, Kaposi sarcoma, squamous carcinoma, pancreatic cancer, brain tumors and myeloid leukemias; reviewed in [7]), thereby affecting tumor cell apoptosis, proliferation, invasion and metastasis [7]. Furthermore, HO-1 gene polymorphisms have been associated with increased cancer susceptibility [8,9].

Cell adhesion is an important determinant of organized growth and the maintenance of architectural integrity. Changes in cell-cell and cell-extracellular matrix (ECM) adhesion accompany the transition from benign tumours to invasive, malignant cancers and the subsequent metastatic dissemination of tumour cells [6,10,11]. Specifically, alterations in ECM remodeling have been shown to affect adhesion properties of neoplastic cells. Although several studies have linked expression of HO-1 with various stages of tumor progression [12-15], the molecular mechanisms underlying HO-1-mediated changes in adhesion of neoplastic cells remain elusive.

We used gene expression profiling as a global assay to identify a common gene set directly linked to HO-1 in 14 cancer types. One of the genes that emerged was PXDN, the human homologue of the *Drosophila* gene peroxidasein. PXDN is a cell surface peroxidase associated with the extracellular matrix [12] and was found to play a key role in HO-1-dependent cell adhesion of neoplastic cells in our investigations. Our results reflect, for the first time, that HO-1 mediates genome-wide effects on transcriptional regulation of genes potentially involved in tumorigenesis. Moreover, our findings provide insights into the mechanisms underlying HO-1-dependent tumor invasion and support the notion that HO-1 represents a molecular target in cancer.

**Materials and methods**

**Construction of transgenic cell lines**

Constitutive stable HO-1 knock-down in BeWo choriocarcinoma cells (European Collection of Cell Cultures (ECACC, Salisbury, UK)) was generated by transduction with a microRNA (miRNA) adapted retroviral vector. Briefly, an shRNAmir (microRNA-adapted short hairpin RNA) against human HO-1 in pSM2 vector (oligo ID: V2HS_133107; Open Biosystems, Huntsville, AL, USA) was subcloned into the LMP vector (Open Biosystems). Constitutive HO-1 overexpression in 607B melanoma cells [16] (kindly provided by Dr. Volker Wachek, was kindly provided by V. Wacheck; Department of Clinical Pharmacology, Medical University of Vienna, Austria) was generated by transduction with the retroviral vector MSCVpuro (Clontech, Mountain View, CA, USA) containing the human HO-1 cDNA [17]. For production of recombinant retroviruses, HEK293FT cells (Invitrogen, Carlsbad, CA, USA) were co-transfected with a vector containing the viral packaging proteins gag and pol, a vector containing env, and either LMP (ctrl), LMP-miHO1 (LMP containing miRNA against human HO-1), MSCV (ctrl) or MSCV-HO1 (MSCV containing the HO-1 cDNA). Vectors containing gag, env, and pol were kind gifts from Dr. Ewan Rosen (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA). Forty-eight hours after transfection, viral supernatants were collected, BeWo and 607B cells were transduced in the presence of polybrene (8 μg/ml). Stable integrants were selected with puromycin (5 μg/ml). Knock-down or overexpression of HO-1 was verified by Western blotting (Fig 1A and Fig Seven A).

**Transient Transfections**

Small interfering (si) RNA targeting human PXDN, negative control siRNA (oligo ID: HSS187890 or cat. no. 12935-200, respectively; Invitrogen), pCDNA 3.1 (Invitrogen), or a plasmid containing the full PXDN cDNA under control of the CMV promoter (clone ID: OCABo5050A058, ImaGenes, Berlin, Germany) were delivered into BeWo cells by nucleofection (Amaya, Lonza Bioscience) according to a previously optimized protocol [18]. Briefly, 1 × 10⁶ BeWo cells were nucleofected with siRNA (100 nmol/L) or 1 μg of control (pcDNA) or pPXDN plasmids following the manufacturers’ instructions (solution V, program X-005) (Amaxa Biosystems, Germany). Following transfection, cells were kept in culture for 48-72 hrs, followed by cell adhesion assays.

**Isolation of total RNA and DNA-Microarray expression profiling**

BeWo choriocarcinoma cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and were cultured in Ham F12 medium (Gibco Life Technologies, Paisley, UK) supplemented with 5% fetal bovine serum (FBS; Biochrom, AG, Berlin, Germany) and streptomycin/penicillin (Gibco) using standard culture conditions. Total RNA was extracted from subconfluent culture using an RNAeasy kit (Qiagen). Total RNA (200 ng) was then used for GeneChip analysis. Preparation of terminal-labeled cDNA, hybridization to genome-wide human gene Level 1.0 ST GeneChips (Affymetrix, Santa Clara, CA, USA) and scanning of the arrays were carried out according to manufacturer’s protocols https://www. affymetrix.com. RNA signal extraction, normalization and filtering was performed as described (http://www. bioconductor.org/; [19]). A variation filter was applied
for selecting informative (i.e., significantly varying) genes. The filtering criteria for the exemplary data sets required an interquartile range > 0.5 and at least one sample with expression intensity > 100. The full gene lists are now available at Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20404.

**Gene set enrichment analysis (GSEA)**

GSEA [20], is a computational method that determines whether a given set of genes (e.g. known pathways, specific areas of the genome or clusters from a cluster analysis) shows statistically significant differences between two phenotypic states (i.e. LMP vs. miHO-1). Briefly, the GSEA calculation involves 3 steps: calculation of an Enrichment Score (ES) followed by estimation of the significance level of ES and adjustment for Multiple Hypothesis Testing. We used a publicly available database of gene sets contained within the Molecular Signature Database (MSigDB; [20]) to test for enrichment upon HO-1 knockdown.

**Statistical Microarray Group Comparisons**

To calculate differential gene expression between individual sample groups, we performed a statistical comparison using the LIMMA package as described previously [19]. Briefly, LIMMA estimates the fold change between predefined sample groups by fitting a linear model and using an empirical Bayes method to moderate the standard errors of the estimated log-fold changes for each probe set [21]. A multiple testing correction based on the false discovery rate (FDR) was performed to produce adjusted p-values. All calculations were performed in “R.”
Human tumor gene expression databases

Human tumor gene expression data was used from the Global Cancer Map comprising 190 specimens of 14 different tumor types (breast, pancreas, lung, bladder, ovary, melanoma, uterus, renal, prostate, central nervous system, lymphoma, colorectal, mesothelioma, and leukemia) [22]. Gene expression data from the normal tissues were discarded. Only the data related to cancerous tissues were further analyzed. The GeneNeighbors module of the GenePattern software was used to identify genes, the expression of which was closely correlated with that of HO-1 [23]. Heatmap construction: We used the Pearson distance as a measure of similarity in the expression pattern. This algorithm produced a numerical score that represented the calculated Pearson distance for each gene relative to the HO-1 gene. The genes were then ranked so that the low score indicates the close similarity of the expression pattern of the particular gene with that of the HO-1 gene.

Kolmogorov-Smirnov statistics

To evaluate the significance of the coexpression pattern of genes, we used the Kolmogorov-Smirnov (KS) statistics. For our analysis, we selected the genes that are differentially expressed in LMP vs miHO1 cells with at least a 2-fold difference (i.e. out of 214 differentially expressed genes, 67 genes were coexpressed with HO1, leaving 45 input genes after mapping onto the respective arrays). We discarded the genes with either overly low or overly high expression levels (<50 and >15,000 relative units in more than half of the arrays, respectively). We also did not include genes that had either less than a 2-fold difference or less than a 50 relative unit difference across all tumor tissues. Finally, out of 16063 genes, 7978 remained. We then determined the positional distribution of the 45 genes within the list of 7978 genes ordered by the Pearson distance relative to HO-1 in the 190 tumor tissues and reported the 14 genes (out of the 45 input genes) being closest to HO-1. In other words, we selected the 14 genes displaying the smallest Pearson distance relative to HO-1. These genes are coexpressed with HO-1 in the tumor specimens and also induced by HO-1 in BeWo cells expressing HO-1 endogenously. We next calculated the KS score for these 14 genes using R. The higher the KS score, the more the expression pattern of the particular gene set is analogous across all tumors. We also performed the same KS analysis for 14 randomly selected genes using 100,000 permutations. The frequency of events when the KS score of the randomly chosen gene set was equal to or exceeded that of the target gene set was taken as a P value (P = 0.00002).

Real-time PCR

Total RNA (1 μg) was reverse transcribed into cDNA by MMLV enzyme (Promega, Mannheim, Germany) with random hexamers (1 μg/μg total RNA). All PCRs were performed using the SYBR Green kit (BioRad, Hercules, CA, USA). Primers for selected genes were designed using Primer3 software http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi with the following sequences:

- HO-1 (CAGGATTTTGTCAGGCGGCCCTGAAG, fwd; TGTGTTACAGGGAGGACCCTCACC, rev) ADAM8 (CCGCTACGTTGAGGCTGTATG, fwd; CCAGCATCTGGAACCTGCGAT, rev), COL21A1 (GAACCCCTGCTACCCTGGAC, fwd; GTGCCCTGCAATTCCTTG, rev), CRIP2 (CCTGTCAGCAAGAGGTG, fwd; 5’-CCCAAATCCCTGGACAGAG, rev), IFIG10 (CTTACGAAACGCACAGGA, fwd; GAACTCCCACCTGCACCTG, rev), MICB (ACCTCAGGAGGACCCTCTAC, fwd; GGAGGGAATGCAAGCCTC, rev), MMP2 (GACCCTGGAGAAGGGAACAG, fwd; CCATCGGCGTTCCTCCCATAC, rev), PXDN (GTCTGTCGCCCACTGACTG, fwd; GTTCGGCTGGGAATGCTG, rev), TGBF1 (TGGAGCTGGAGGACGCAGGA, fwd; GCCCGGGTTATGCTGGGTG, rev) and ARP (GCCAA TAAGGTCGCCGCTGTCTG, fwd; TCTTGCATCATCACACACAG, rev). Using the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Warrington, UK), PCR cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles at 94°C for 30 seconds, 60°C for 15 seconds and 72°C for 30 seconds and a 10 minutes terminal incubation at 72°C. Sequence Detector Software (SDS version 1.6.3, PE Applied Biosystems) was used to extract the PCR data, which were then exported to Excel (Microsoft, Redmond, WA) for further analyses. The RNA-amount of the human Arp gene was used as an internal control. Data were analyzed according to the 2-ΔΔCT method [24].

Western blot analyses

Western blot analyses were performed using standard protocols as recently done [25]. Equal amounts of protein lysates (35 μg) were separated on 10% SDS/polyacrylamide (PAA) gels and transferred onto Polyvinylidene fluoride (PVDF)-membranes (GE Healthcare, Amersham, Buckinghamshire, UK). After blocking filters were incubated overnight (4°C) with monoclonal mouse antibodies against human HO-1 (clone OSA110; 1:1000; Stressgen, Ann Arbor, MI, USA), PXDN (clone clone A01; 1:1000; Abnova, Taipei City, Taiwan), β-actin (1:5000; Abcam, Cambridge, MA, USA). After 1 h of treatment (room temperature) with secondary antibodies (anti-mouse Ig horseradish peroxidase linked, Amersham; 1:20,000) signals were...
developed by using ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech, Piscatway, NJ, USA).

**Immunofluorescence**

Cells grown on coverslips were washed, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X 100 and blocked with goat serum. Expression of HO-1 and PXDN were detected with the antibodies described above. Cells were incubated with secondary antibodies conjugated to Alexa594 or Alexa488 (1:500; Molecular Probes, Eugene, OR, USA) and visualized using a Zeiss Axioskop 2 microscope, Zeiss Axiocam and Photoshop. As negative control coverslips were incubated with the respective isotype control IgG and secondary antibodies (data not shown). First trimester placental tissue were dehydrated and embedded in paraffin (Merck) as described elsewhere [26]. Serial sections (2-3 μm) were prepared, deparaffinized and finally heated in a microwave oven (×2 5 min, 850 W). After incubation in blocking solution (NEN, Boston, MA, USA), slides were incubated overnight with primary antibodies, washed 3 times in PBS (each 5 min) and followed by incubation with secondary antibodies conjugated to Alexa594 or Alexa488 (1 hour, Molecular Probes, Eugene, OR). The following primary antibodies/dilutions were utilized: PXDN (Sigma, 1:50) cytookeratin 7 (clone OV-TL, 8.3 μg/ml, DAKO, Glostrup, Denmark), HO-1 (clone OSA-110, 1:1000, Stressgen, Ann Arbor, MI), Ki67 (clone KiS5, 10 μg/ml, Chemicon), Kip2/p57 (C-20, rabbit, 2 μg/ml, Santa Cruz Biotechnolgy, Santa Cruz, CA) and Vimentin (clone Vim 3B4, Dako). As a negative control, the primary antibody was replaced by buffer or isotype IgG. Finally, all sections were counterstained with 1 μg/ml DAPI (Roche) and covered with Fluoromount-G (Soutech, Birmingham, AL).

**Cell Adhesion Assay**

Adhesion assays were performed as described by [27], with minor modifications. 96 well plates were coated overnight at 4°C with Fibronectin (10 μg/ml; SIGMA), rat tail Collagen , or with Laminin (10 μg/ml; SIGMA) in PBS. Wells were rinsed and blocked for 1 h with 1% BSA in PBS. Logarithmic phase cells were harvested with trypsin and plated at 40,000 cells per well. After 30 min of incubation at 37°C, wells were rinsed to remove non-adherent cells. Adhered cells were fixed in 10% formalin for 5 min and stained with 0.1% crystal violet (in 20% MeOH) for 5 min. Excess dye was washed off with water and absorbance was measured at 595 nm. Bars represent mean absorbance +/- SEM of each condition tested in triplicates. All values have had background subtracted that represents cell adhesion to wells blocked with 1% BSA in PBS.

**Cell Invasion assay**

The invasion of BeWo cells was measured by using the Transwell chambers (Chemicon, Millipore, CA) according to the manufacturer’s protocol. Briefly, the BeWo cells were electroporated with 20 μM of a control siRNA or siRNA targeting human PXDN with the Amaxa method as described elsewhere [18]. 24 hours later, the cells were seeded onto the membrane of the upper chamber of the transwell at a concentration of 2×10⁷/ml in 500 μl of DMEM/F12 medium. The medium in the upper chamber was serum-free. The medium at the lower chamber contained 10% Foetal Calf serum as a source of chemoattractants. Cells that passed through the Matrigel coated membrane were stained with Cell Stain Solution containing crystal violet supplied in the Transwell Invasion assay (Chemicon, Millipore, CA) and photographed after 20 hours of incubation.

**Cell Proliferation Assay**

The effect of CO on proliferation of RAECs was determined with a nonradioactive bromodeoxyuridine (BrdU)-based cell-proliferation assay [28] (per the manufacturer’s guidelines; Roche, Basel, Switzerland). Following electroporation of 1×10⁶ BeWo cells with 20 μM control or PXDN siRNA according to a previously optimized protocol [18], 2500 cells were seeded into 96 well plates and left for 24 hrs to recover. The cells were stimulated to proliferate with 10% FBS and BrdU incorporation was measured at indicated time points.

**Statistical analysis**

Student’s t test was used for comparison between the groups. P value < 0.05 was considered significant.

**Results**

**Gene expression profiling**

We used gene expression profiling to determine the genome-wide effect of HO-1 on the transcriptome of BeWo choriocarcinoma cells. BeWo cells were used in these experiments because these cells show relatively high levels of endogenous HO-1 expression. Expression of HO-1 was silenced in BeWo cells by a micro-RNA adapted retroviral vector targeting human HO-1. Western blotting demonstrated an efficient knockdown of HO-1 expression in BeWo cells stably expressing miHO-1 (henceforth referred to as ‘miHO-1’) as compared to BeWo cells stably expressing the LMP control sequence (referred to as ‘LMP’) (Fig. 1). RNA isolated from control (LMP) or miHO1 infected (miHO-1) cells was labeled and hybridized to human genome-wide gene level 1.0 ST arrays. Among 214 differentially expressed genes with statistical significance (adjusted p-value < 0.05), 67 genes were expressed at higher levels in HO-1...
| Probe Set ID   | Mean Fold Change (LMP vs miHO-1) | Adj. P-Value | Gene Symbol | Gene Name                           |
|---------------|----------------------------------|--------------|-------------|-------------------------------------|
| NM_005330_at  | 7.82                             | 0.0073       | HBE1        | hemoglobin, epsilon 1               |
| NM_024913_at  | 7.74                             | 0.0067       | C7orf58     | chromosome 7 open reading frame 58  |
| NM_00110533_at| 5.56                             | 0.0073       | C7orf58     | chromosome 7 open reading frame 58  |
| NM_206828_at  | 5.10                             | 0.0077       | NLRP7       | NLR family, pyrin domain containing 7|
| NM_002133_at  | 5.09                             | 0.0262       | HMOX1       | heme oxygenase (decycling) 1        |
| NM_139176_at  | 4.90                             | 0.0081       | NLRP7       | NLR family, pyrin domain containing 7|
| NM_004530_at  | 4.83                             | 0.0098       | MMP2        | matrix metallopeptidase 2 (gelatinase A) |
| XM_001720850_at| 4.48                             | 0.0092       | LOC100134134| similar to peroxidasin homolog      |
| NM_030820_at  | 4.34                             | 0.0223       | COL21A1     | collagen, type XXI, alpha 1         |
| NM_012293_at  | 4.27                             | 0.0092       | PXDN        | peroxidasin homolog (Drosophila)    |
| XM_00171515_at| 3.86                             | 0.0106       | LOC100131375| similar to peroxidasin homolog      |
| NM_000660_at  | 3.60                             | 0.0306       | TGFBI       | transforming growth factor, beta 1  |
| NM_001312_at  | 3.39                             | 0.0146       | CRIP2       | cysteine-rich protein 2             |
| NM_004425_at  | 2.99                             | 0.0262       | ECM1        | extracellular matrix protein 1      |
| NM_006227_at  | 2.91                             | 0.0306       | PLTP        | phospholipid transfer protein       |
| NM_182676_at  | 2.91                             | 0.0306       | PLTP        | phospholipid transfer protein       |
| XM_001718318_at| 2.91                             | 0.0318       | LOC100131307| hypothetical protein LOC100131307  |
| NM_000413_at  | 2.88                             | 0.0262       | HS17B1      | hydroxy steroid (17-beta) dehydrogenase 1 |
| NM_178422_at  | 2.87                             | 0.0301       | PAQR7       | progestin and adipQ receptor family member VII |
| NM_022664_at  | 2.86                             | 0.0262       | ECM1        | extracellular matrix protein 1      |
| NM_001109_at  | 2.76                             | 0.0284       | ADAM8       | ADAM metallopeptidase domain 8      |
| NM_000391_at  | 2.64                             | 0.0306       | TPP1        | tripeptidyl peptidase 1             |
| XM_001726123_at| 2.64                             | 0.0262       | LOC100133842| similar to lectin, galactoside-binding, soluble, 3 binding protein |
| NM_003045_at  | 2.63                             | 0.0262       | SLC7A1      | solute carrier family 7, member 1   |
| NM_005931_at  | 2.63                             | 0.0231       | MICB        | MHC class I polypeptide-related sequence B |
| NM_000414_at  | 2.61                             | 0.0262       | APOE        | apolipoprotein E                    |
| NM_006927_at  | 2.60                             | 0.0262       | ST3GAL2     | ST3 beta-galactoside alpha-2,3-sialyltransferase 2 |
| NM_001042423_at| 2.60                             | 0.0334       | SLC16A3     | solute carrier family 16, member 3 (monocarboxylic acid transporter 4) |
| NM_004207_at  | 2.59                             | 0.0332       | SLC16A3     | solute carrier family 16, member 3 (monocarboxylic acid transporter 4) |

**Upregulated in HO-1 expressing control (LMP) cells:**

**Downregulated in HO-1 expressing control (LMP) cells:**
expressing control (LMP) cells and 147 genes in cells deficient in HO-1 (miHO-1, see Additional file 1). Top 30 differentially expressed genes are shown in Table 1. An obvious feature of HO-1 was its effect on the expression of genes which are either directly or indirectly linked to cell adhesion and the integrity or remodelling of extracellular matrix (CD24, HOOK1, LAYN, HEY1, MME, RRAS2, FZD3, KIF14, KIF18A, DAAM1, BCL6, PLS1, ERBB2IP, BGN, FSD1, IFI30, LGALS3BP, FMNL1, TMSL3, CORO1A, TFF1, CLEC11A, ADAM8, ECM1, PLTP, TGFB1, PXDN, COL21A1 and MMP2; Table 1, and Additional Files 1 &2).

Pathway prediction analyses

To further explore the dataset, GSEA [20] was used to identify groups of functionally related genes with expression patterns that correlate with HO-1 expression. GSEA is a method for interpreting gene expression data that focus on groups of genes sharing common biological function, chromosomal location or regulation. This approach can show important effects on pathways, which might be missed in single-gene analyses [20]. Fig. 1C displays the top 10 pathways regulated by HO-1 expression in BeWo cells. Amongst others, HO-1 expressing BeWo cells were significantly enriched in pathways regulating extracellular matrix orchestration and signal transduction. Plotting of the enrichment score vs the rank-ordered gene list for the top-scoring gene set ‘extracellular region’ illustrates increased expression of ECM molecules and their remodeling enzymes in HO-1 expressing BeWo LMP cells (Fig. 1D). A more detailed analysis of this pathway revealed enhanced expression of several ECM molecules including extracellular matrix-1 (ECM1), collagen type IX, α3 (COL9A3, sargycloccans β and -ε (SGCβ and SBCε), and the matrix remodeling factors MMP2 and TGFβ1 (Fig. 1E). The significant gene sets ‘receptor binding’, integral to plasma membrane’ and ‘system development’ contained further genes related to cell plasticity and ECM organization, including IGF2, placental growth factor (PGF), collagen type I α1 (COL1A1), fibroblast growth factor receptors 3 and 4 (FGFR-3,-4), ADAM8, (see Additional File 3). These observations suggest that HO-1 expressing cells produce factors relevant to cell-matrix adhesion as well as their degrading enzymes.

**HO-1 gene signature in 190 human tumors**

We next determined whether expression of the putative HO-1 target genes identified in BeWo cells (Additional File 1) correlates with HO-1 expression levels in human tumors. For these purpose, we performed data mining using the GCM database. This database includes the expression profiling data of 16,063 genes of 190 individual tumors of the 14 human cancer types. Using R/Bioconductor, we ranked 7978 genes (filtering described in methods) according to their level of coexpression with HO-1. The Pearson distance was used as an unbiased measure of the expression pattern similarity of the target gene with the expression pattern of HO-1. The Pearson distance was used as an unbiased measure of the expression pattern similarity of the target gene with the expression pattern of HO-1. Using the data of Additional File 1, we then selected the top 14 individual genes, expression of which most uniformly correlated with that of HO-1 both in BeWo LMP cells and in 190 human tumors (Fig. 2A and Table 2). To confirm that the coexpression of these 14 genes with HO-1 is statistically significant we applied KS statistics. One hundred thousand trials with a randomly selected set of 14 genes undermined the high statistical significance of the 14 identified genes (P = 0.00002). Fig. 2B

### Table 1: Top 30 genes up- or downregulated statistically significant more than 2-fold in BeWo control (LMP) cells compared with cells deficient in HO-1 (miHO-1) (Continued)

| Gene ID       | Mean Fold Change | P Value | Description                                      |
|--------------|------------------|---------|-------------------------------------------------|
| XM_001127597_at | -2.98            | 0.0178  | FLJ32810 hypothetical protein FLJ32810           |
| NM_0309052_at  | -2.95            | 0.0178  | CAB39L calcium binding protein 39-like           |
| NM_001079670_at| -2.92            | 0.0178  | CAB39L calcium binding protein 39-like           |
| NM_001726_at   | -2.88            | 0.0297  | BRDT bromodomain, testis-specific                |
| NM_179397_at   | -2.87            | 0.0178  | GOLM1 golgi membrane protein 1                   |
| NM_014827_at   | -2.81            | 0.0321  | ZC3H11A zinc finger CCCH-type containing 11A     |
| NM_001042482_at| -2.78            | 0.0306  | TPKN thiamin pyrophosphokinase 1                 |
| NM_004052_at   | -2.77            | 0.0306  | BNP3 BCL2/adenovirus E1B 19 kDa interacting protein 3 |
| NM_006424_at   | -2.75            | 0.0262  | SLC34A2 solute carrier family 34 (sodium phosphate), member 2 |
| NM_005900_at   | -2.73            | 0.0262  | SMAD1 SMAD family member 1                       |
| NM_001008397_at| -2.72            | 0.0262  | GPX8 glutathione peroxidase 8                    |
| NM_018169_at   | -2.71            | 0.0284  | C1orf35 chromosome 12 open reading frame 35     |
| NR_003942_at   | -2.68            | 0.0318  | SNORD76 small nuclear RNA, C/D box 76           |
| XM_001726844_at| -2.66            | 0.0375  | LOC100130123 PRO2870                           |
| NM_207189_at   | -2.65            | 0.0276  | BRDT bromodomain, testis-specific                |

Mean fold change values of n = 2 experiments.

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shows the expression pattern of the 14 highly significant HO-1 target genes in the 190 tumor samples, which include ADAM8, AGPAT2, MICB, ST3GAL2, SLC7A1, HSD17B1, MMP2, IFI30, COL21A1, ALPPL2, CRIP2, BGN, TGFB1 and PXDN. To corroborate our results, we used qRT-PCR to determine the mRNA levels of 8 HO-1 target genes in BeWo LMP and miHO-1 cells (Fig. 2C). These 8 HO-1 target genes were selected based on their putative role in regulation of cell plasticity/motility based on Gene Ontology classification and PubMed searches. According to our results, the expression levels of these genes were the lowest in miHO-1 cells. Western blotting of LMP and miHO-1 BeWo cell extracts confirmed the increased levels of PXDN in LMP cells (Fig. 3A). Immunofluorescence analysis of routinely cultured, subconfluent LMP and miHO-1 BeWo cells further corroborated western blotting data, showing increased (mostly perinuclear) PXDN staining in LMP cells (Fig. 3B).

**HO-1 and PXDN colocalize in invasive trophoblast**

To confirm a link of HO-1 with PXDN, we determined the expression of HO-1 and PXDN in first trimester placenta tissues. Among the Cytokeratin-positive (=villous, extravillous as well as syncytiotrophoblast) cells, Ki67- or p57-staining indicated proliferating (non-invasive) or invasive, differentiated extravillous trophoblast cells, respectively (Fig. 4). Immunofluorescence analysis of

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**Figure 2 HO-1 target gene expression in 190 human tumors**

(A) The heatmap shows the expression pattern of 14 HO-1 target genes including HO-1 itself in the 190 human tumors of the 14 most common cancer types included in the GCM database. Red and blue squares denote high versus low levels of expression of each individual gene normalized to its mean expression across all tumor samples, respectively. (B) Relative gene expression of 8 selected HO-1 target genes. The Y-axis displays the normalized DNA Microarray signal across 190 tumor samples (comprising the X-axis). Cancer types are shown above the plot. (C) mRNA levels were measured by qRT-PCR using the RNA samples isolated from HO-1 expressing (LMP) and HO-1 silenced (miHO-1) BeWo cells. The expression values were normalized relative to Arp. The levels of mRNA in LMP and miHO-1 cells are shown in percentage relative to LMP cells (set to 100%). Bars represent mean (+/- SEM) of three independent experiments.
serial sections revealed pan-trophoblastic HO-1 and PXDN staining (Fig. 4), however, proximal extravillous trophoblasts in the cell column stained strongest for HO-1 and PXDN. Based on our immunostaining data, we concluded that the expression of HO-1 is coupled to an up-regulation of PXDN in first trimester placenta.

HO-1 affects cell adhesion to extracellular matrix molecules via PXDN

We examined the effect of HO-1 knockdown on the attachment of BeWo LMP and miHO-1 cells to fibronectin, laminin and collagen type I using cell adhesion assays. In this assay, nonadherent cells were removed gently and the remaining adherent cells were fixed, stained and analysed by light microscopy. As shown in Fig. 5A, HO-1 expressing cells (LMP) became much more adherent compared with HO-1 deficient (miHO-1) cells. The adherent cells were measured at 550 nm following staining with crystal violet. As shown in Fig. 5B, the absorbance of LMP cells was significantly higher than that of miHO-1 cells (P > 0.05). This effect was more pronounced in the order Laminin > Fibronectin > Collagen type I. It is noteworthy that very few cells adhered to control wells (termed 'Ctrl'). To examine if the HO-1 target gene PXDN is accountable for the increased adhesiveness of HO-1 expressing BeWo cells, we repeated adhesion assays with BeWo cells silenced for PXDN expression. We observed diminished PXDN mRNA and protein levels two days after transfection of LMP and miHO-1 BeWo cells with a PXDN-specific siRNA, but not with a negative control siRNA, (Fig. 5C and 5D, respectively). We evaluated effects of PXDN knockdown on cell adhesion to Fibronectin and Laminin, as BeWo cells most efficiently adhere to these matrix proteins. Transfection with a control siRNA did not alter the inhibitory effect of reduced HO-1 levels on adhesion of BeWo cells to Fibronectin or Laminin (Fig. 5E and 5F). While PXDN-knockdown did not alter cell adhesion properties of HO-1 deficient BeWo cells (miHO-1), siRNA-mediated PXDN-knockdown abolished the stimulatory effect of HO-1 on cell adhesion observed in LMP cells (Fig. 5E and 5F). To minimize the risk of off-target effects, we repeated the cell adhesion experiments with an alternative siRNA against PXDN with similar results (Additional File 4). To undermine a role of PXDN in cell adhesion, we transiently overexpressed PXDN in BeWo miHO1 cells. Ectopic expression of PXDN (pPXDN) resulted in enhanced adhesion to Laminin and Fibronectin, as compare to cells transfected with a control pasmid (Fig. 5G).

To verify that the effects of HO-1 on cell adhesion and PXDN expression are truly related to HO-1, we generated a HO-1 gain-of-function cell model using 607B melanoma cells, which have no detectable endogenous HO-1 expression. As shown by western blotting (Fig. 6A), retroviral HO-1 gene transfer into 607B cells resulted in stable HO-1 overexpression (MSCV-HO1) as compared to cells transduced with a virus containing empty retroviral backbone (MSCV). Adhesion to Fibronectin and Laminin was more pronounced in 607B cells overexpressing HO-1 (MSCV-HO1) as compared to control infected cells (MSCV; Fig. 6B). Furthermore, MSCV-HO1 cells expressed higher levels of PXDN, compared with MSCV control cells (Fig. 6C). To investigate if PXDN has pro-adhesive properties in 607B cells, similar to BeWo cells, adhesion assays were repeated using PXDN-silenced MSCV-HO1 cells. siPXDN, but not siCtrl-treatment of 607B MSCV-HO1 cells

| Gene Accession | Gene Symbol | Distance from HO-1 | Fold Change (LMP/miHO-1) |
|----------------|-------------|--------------------|-------------------------|
| NM_001109      | ADAM8       | 0.5570885          | 2.76                    |
| NM_0010172727  | AGBP1       | 0.6074327          | 2.05                    |
| NM_009391      | MICB        | 0.66872585         | 2.63                    |
| NM_009179      | ST3GAL2     | 0.680627           | 2.60                    |
| NM_003045      | SLC7A1      | 0.70778143         | 2.63                    |
| NM_000413      | HSD17B1     | 0.7225144          | 2.88                    |
| NM_008610      | MMP2        | 0.7414118          | 4.83                    |
| NM_006332      | IF30        | 0.7581253          | 2.24                    |
| NM_030820      | COL21A1     | 0.7659843          | 4.34                    |
| NM_031313      | ALPPL2      | 0.77093726         | 2.10                    |
| NM_001312      | CRIPI       | 0.78032595         | 3.39                    |
| NM_001711      | BGN         | 0.8124546          | 2.01                    |
| NM_000660      | TGFB1       | 0.82223743         | 3.60                    |
| NM_012293      | PXDN        | 0.82782316         | 4.27                    |

NOTE: Distance from HO-1 corresponds to ranking derived from the Pearson distance from HO-1. Fold change (LMP/miHO-1), the ratio of gene expression levels in HO-1 expressing cells versus HO-1 silenced cells.
Figure 3 Knock-down of HO-1 in BeWo cells diminishes PXDN protein expression. (A) PXDN detection by western blotting of LMP and miHO-1 BeWo cells. β-actin indicates equal loading. A representative example is shown. (B) Immunofluorescent detection of PXDN (=red) and nuclei (DAPI = blue) in cultured LMP and miHO-1 BeWo cells. Fields shown are representative of each population.
efficiently knocked-down PXDN mRNA levels (~10-fold reduction; Fig. 7D). Furthermore, PXDN-knockdown in 607B MSCV-HO1 cells resulted in a significant reduction in cell adhesion to Fibronectin and Laminin, as compared to siCtrl-treated cells (Fig. 6E). Subsequently, we examined the effect of silenced PXDN expression on BeWo cell growth and invasion. Compared to siCtrl transfected cells, PXDN-silenced cells showed significant decreased cell growth over 96 hrs (Fig. 7A). Of note, knockdown of HO-1 in BeWo cells did not affect cell proliferation (data not shown). When testing the abilities of BeWo cells to invade through the 8-μm pores on the polycarbonate membrane coated with matrigel, we found that the knock-down of endogenous PXDN

Figure 4 PXDN and HO-1 co-localize in first trimester placenta. Immunohistochemical analyses of serial sections of placental tissues (between 7 and 14th weeks of gestation) stained with different antibodies, counterstained with DAPI and analysed by fluorescence microscopy as described in Materials and Methods. Trophoblast and stromal cells are marked by Cytokeratin 7 (CK7, green) and Vimentin (VIM, red) staining, respectively. Ki67 (red) staining indicates poorly invasive but proliferative EVT, whereas p57 (green) positive cells account for invasive, differentiated EVT. HO-1 and PXDN were stained on separate serial sections. Photographs were taken at 400-fold magnification. VC, villous core; EVT, extravillous trophoblast; CC, cell column; ST, syncytiotrophoblast. Note the relative higher abundance of PXDN and HO-1 in CTB as compared with EVT. Magnification: 200x.
Figure 5 HO-1 mediates cell adhesion via PXDN in BeWo choriocarcinoma cells via PXDN. (A) Control-infected (LMP) or miHO-1 (miHO-1) infected BeWo cells were seeded on fibronectin (FN), collagen type I (Col I), laminin (Ln) or tissue culture plastic (TC) and assayed for adhesion as described in Materials and Methods. Photographs were taken at a 40x magnification. (B) The absorbance of fixed and crystal-violet stained cells at 550 nm of ctrl (LMP) infected cells were arbitrarily set to 100% in each experiment. Note that HO-1 deficient BeWo cells (miHO-1) became less adherent than LMP cells expressing HO-1 endogenously (*P < 0.05 vs LMP cells). PXDN knocked down in BeWo LMP and miHO-1 cells after transient transfection with a control (siCtrl) or PXDN-specific (siPXDN) siRNA, as determined by real-time PCR (C) and western blotting (D). β-actin indicates equal loading. Effect of PXDN-knockdown on cell adhesion to fibronectin (E) or laminin (F) in control-infected (LMP) or miHO-1 (miHO-1) infected BeWo cells. For comparison, OD-values of ctrl (LMP) infected cells were arbitrarily set to 100% in each experiment. Note that HO-1 expressing cells (LMP), but not HO-1 deficient cells (miHO-1) became less adherent following PXDN-knockdown (*P < 0.05 vs siCtrl-treated LMP cells). (G) Effect of ectopic PXDN expression on cell adhesion of miHO1 BeWo cells. For comparison, OD-values of cells transfected with an empty control plasmid (pcDNA) were arbitrarily set to 100% in each experiment. Note that PXDN overexpressing cells (pPXDN), but not mock-transfected cells (pcDNA) became more adherent (*P < 0.05 vs pcDNA treatment).
expression significantly reduced cell invasion as compared to siCtrl treated BeWo LMP cells (P < 0.05; Fig. 7B)

Discussion
In cancer, HO-1 influences tumor cell survival, apoptosis, invasion and metastasis as well as resistance of certain tumors to chemotherapeutic agents [7,17]. These changes suggest alterations of signal transduction and transcription pathways, which HO-1 affects either directly or indirectly. To identify these regulatory mechanisms and to determine the identity of the universal genes, expression of which is affected by HO-1, we silenced HO-1 expression in BeWo choriocarcinoma cells (miHO-1) and performed gene expression profiling of these cells relative to BeWo cells which express
HO-1 endogenously ('LMP'). An interesting aspect of the 214 identified genes whose expression was affected by HO-1, was the regulation of multiple genes linked to cell plasticity/motility and ECM maintenance. In the course of invasion tumor cells leave normal structures by passing through basal membrane and migrate into the surrounding stroma. These events include significant changes in cell morphology as well as close interaction of cells with extracellular matrix (ECM) and structural rearrangement of the latter. Further evidence for a role of HO-1 in modulating cell plasticity was revealed by pathway prediction analysis, which demonstrated modulation of genes of the extracellular region as well as underlying signal transduction pathways (GSEA; Fig 1). Consistent with our data, TGFβ1 was identified as a HO-1 target gene in a microarray comparison of prostate cancer cells with varying HO-1 protein levels [12]). Several potential mechanisms underlying gene regulation by HO-1 can be envisioned that also emphasize a potential role of the enzymatic products of HO-1: regulation by HO-1 can be envisioned that also emphasize a potential transcription factor or coregulator [29,36] Of note, we detected a fraction of total cellular HO-1 protein in the nucleus of BeWo cells (data not shown). Further studies are warranted to investigate potential signaling pathways triggered by HO-1, (including the role of nuclear HO-1) in gene regulation.

To provide unbiased proof for the role of HO-1 in genome-wide transcriptional regulation, irrespective of the cancer tissue type, we performed a metaprofiling analysis using the GCM database of 190 human tumors of 14 different types. The motivation of this data mining strategy was to identify which genes from the 214 putative HO-1 target genes, determined in BeWo cells, most closely correlated with the expression of HO-1 in 190 tumor samples. This unbiased comparative analysis revealed 14 HO-1 universal target genes: proteolytic ADAM8 and MMP2, acyltransferase AGPAT2, cell surface protein MICB, extracellular glycosylase ST3GAL2, amino acid transporter SLC7A1, steroid dehydrogenase HSD17B1, thiol reductase IFI30, alkaline phosphatase ALPPL2, intracellular adapter protein CRIP2, extracellular matrix constituents BGN and COL21A1, multifunctional cytokine TGFβ1, and peroxidase PXDN. The expression of these genes is strongly correlated with that of HO-1 (P = 0.00002). The results of our data mining and our subsequent statistical analyses were validated by using qRT-PCR, Western blotting, and immunostaining of LMP and miHO1 cells. Immunofluorescence staining of first trimester placenta specimens confirmed that HO-1 immunoreactivity is coupled to that of PXDN in trophoblast cells (Fig. 4), which share the capacity to migrate and invade surrounding tissues similar to malignant cells [37]. Based on these results, we suggest that HO-1 stimulates multiple transcriptional changes and affects several cellular pathways, including extracellular matrix organization (MMP2, ADAM8, TGFβ1, BGN, COL21A1, PXDN), signaling (CRIP2, MICB), amino acid transport and glycosylation (SLC7A1 and ST3GAL2), estrogen and phospholipid biosynthesis (AGPAT2 and HSD17B1), protein stabilization (IFI30) and phosphorylation (ALPPL2). Many of these genes are directly associated with cancer; further studies are warranted to identify the role of the HO-1 associated genes in the tumorigenic proteries of HO-1.

Given that cell adhesion is intrically linked to tumor progression/invasion, and that the HO-1 gene signature features many regulators of cell adhesion, we investigated potential effects of HO-1 on cell adhesion in HO-1 silenced BeWo cells and HO-1 overexpressing 607B melanoma cells. Knockdown of HO-1 in BeWo cells reduced adhesion to various ECM molecules, having strongest effect on Laminin (Fig. 5). Stronger adhesion of 607B cells overexpressing HO-1 confirmed a positive role of HO-1 in cell adhesion (Fig. 7). Previously, we
have shown that loss of HO-1 expression in BeWo cells resulted in increased cell motility, based on boyden chamber assays [25]. Thus, at least in BeWo cells, knockdown of HO-1 decreases cell adhesion with a concomitant increase in cell motility. A reduction in cell adhesion with a concomitant increase in cell motility is one hallmark of mesenchymal-amoeboid transition (MAT), a process describing a change in (cancer cell) movement from mesenchymal to amoeboid mode. Such type of movement was shown to be characteristic of certain malignancies, including prostate cancer [10,11].

We hypothesized that one of the HO-1 signature genes, many of which represent potential regulators of cell plasticity, mediates the adhesion-promoting effect of HO-1. One promising and novel candidate was PXDN, which could alter cell-ECM interaction either by stabilization of the ECM through protein-protein interactions via leucine-rich repeats and immunoglobulin loops, as well as by enzymatically formed tyrosine-tyrosine cross-links [38]. PXDN, also known as MG50, is a peroxidasin associated with the endoplasmatic reticulum, and expressed in melanoma, breast cancer, colon cancer, ovarian cancer, renal carcinoma as well as metastatic gliomas [4,38-40]. Silencing of PXDN abolished the adhesion-promoting effect of endogenous HO-1 in BeWo (LMP) and 607B (MSCV-HO1) cells (Fig. 5 and Fig. 6), while PXDN knockdown did not affect cell adhesion in HO-1 deficient cells. We hypothesize that the PXDN dosage may be very critical for the adhesive response, as PXDN levels in miHO-1 cells treated with a PXDN specific siRNA were ~50 times lower compared to LMP cells (Fig. 5): If inhibition of BeWo cell adhesion correlates with PXDN - levels, maybe there exists a threshold level for PXDN. However, the phenotype of miHO1 cells could be rescued by PXDN overexpression (Fig. 6G). The reduced (~50%) matrigel invasion of PXDN-silenced BeWo cells is most likely due to proproliferative properties of PXDN (Fig 7). However, additional mechanisms must prevail as cell growth in PXDN silenced cells was inhibited by approximately 30% after 24 hrs, the duration of the cell invasion assay. Importantly, to our knowledge, this is the first time showing functional effects of PXDN expression levels on cell adhesion and invasion. Further extensive experiments are needed to determine the molecular mechanism by which PXDN modulates cell adhesion and invasion, and how it is linked to the adhesion-promoting properties of HO-1.

To conclude, our unbiased large scale genome-wide studies clarified, for the first time, the molecular signature of HO-1 in cancer and identified the genes which are functionally, universally, and most consistently linked with HO-1 expression among multiple tumor types. The identification of the HO-1 target genes will undoubtedly help to understand the complex network of cellular and molecular events, which are linked to the role of HO-1 in cancer. Ongoing studies will shed light on the functional significance of these individual genes.

Additional material

Additional file 1: Transcriptional signature of HO-1 in BeWo choriocarcinoma cells. The data provided represent the 214 differentially expressed genes with statistical significance (adjusted p-value < 0.05) between HO-1 expressing control (LMP) cells and cells deficient in HO-1 (miHO-1).

Additional file 2: quantitative real-time PCR validation of 5 genes regulated upon HO-1 knockdown. Graphical presentation for five differentially expressed genes selected for qRT-PCR validation. mRNA levels were measured by qRT-PCR using the RNA samples isolated from HO-1 expressing (LMP) and HO-1 silenced (miHO-1) BeWo cells. The expression values were normalized relative to Arp. The levels of mRNA in LMP and miHO-1 cells are shown in percentage relative to LMP cells (set to 100%). Bars represent mean (+/- SEM) of three independent experiments. Real-time PCR verification of genes statistically significant overexpressed in cells deficient of HO-1. Primers for selected genes were designed using Primer3 software http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi with the following sequences: TNFSF10 (CTGGGACCAGGAAAGAAGC, fwd; GCTCAGGAATGATGCGCAC, rev), PEG3 (TTCCTACCAAGCTACTGCT, fwd; GGTGTCGCGTCCTAGT, rev), GOLM1 (Agctttgcacctgcagac, fwd; CCTGCAGCCTCGTCTCC, rev), C2B38L1 (Ccaaacaagagagctgctca, fwd; GCTGAGCTAATGACAGTG, rev), CD24 (CCAACTAATGGCCACCAAG, , fwd; TTGTACCTAGGAGCCAACC, rev). The RNA amount of the human Arp gene was used as an internal control. Data were analyzed according to the 2 ΔΔCt method [24].

Additional file 3: Pathway analysis in HO-1 deficient BeWo cells. Graphical presentation of 9 gene sets as heatmaps that correlate with HO-1 expression in BeWo cells, identified by GSEA. Downregulated genes are shown in blue and upregulated genes in red.

Additional file 4: PXDN-knockdown in BeWo cells using an alternative siRNA oligo targeting human PXDN. Upper panel: PXDN knocked down in BeWo LMP cells after transient transfection with a control (sICtr) or PXDN-specific (sIPXDN#2) siRNA (Invitrogen, oligo ID: H55187891), as determined by real-time PCR. Lower panel: Effect of PXDN-knockdown using sIPXDN#2 on cell adhesion to fibronectin or laminin in control-infected (LMP) BeWo cells. For comparison, OD-values of LMP cells treated with a control siRNA (sICtr) were arbitrarily set to 100% in each experiment. Note that HO-1 expressing cells (LMP) became less adherent following PXDN-knockdown.

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Authors’ contributions
ST carried out the GeneChip and bioinformatic as well as statistical analysis and drafted the manuscript. AJ carried out adhesion assays and western blotting. SH and MK carried out the immunostaining. MM designed primers and performed real-time PCR measurements. JH generated retroviral constructs, conducted retroviral gene transductions and cell proliferation experiments.
assays. JL performed cell invasion assays and transient transfections. HP and OW participated in the design and coordination of the study. MB conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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