Tumorigenesis and Neoplastic Progression

Expression of Cyclophilin B is Associated with Malignant Progression and Regulation of Genes Implicated in the Pathogenesis of Breast Cancer

Feng Fang,* Ayanna J. Flegler,* Pan Du,[†]
Simon Lin,[†] and Charles V. Clevenger*

From the Breast Cancer Program,* Robert H. Lurie
Comprehensive Cancer Center & Department of Pathology; and
the Bioinformatics Core,[†] Robert H. Lurie Comprehensive Cancer
Center, Northwestern University, Chicago, Illinois

Cyclophilin B (CypB) is a 21-kDa protein with peptidyl-prolyl *cis*-trans isomerase activity that functions as a transcriptional inducer for Stat5 and as a ligand for CD147. To better understand the global function of CypB in breast cancer, T47D cells with a small interfering RNA-mediated knockdown of CypB were generated. Subsequent expression profiling analysis showed that 663 transcripts were regulated by CypB knockdown, and that many of these gene products contributed to cell proliferation, cell motility, and tumorigenesis. Real-time PCR confirmed that STMN3, S100A4, S100A6, c-Myb, estrogen receptor α , growth hormone receptor, and progesterone receptor were all down-regulated in si-CypB cells. A linkage analysis of these array data to protein networks resulted in the identification of 27 different protein networks that were impacted by CypB knockdown. Functional assays demonstrated that CypB knockdown also decreased cell growth, proliferation, and motility. Immunohistochemical and immunofluorescent analyses of a matched breast cancer progression tissue microarray that was labeled with an anti-CypB antibody demonstrated a highly significant increase in CypB protein levels as a function of breast cancer progression. Taken together, these results suggest that the enhanced expression of CypB in malignant breast epithelium may contribute to the pathogenesis of this disease through its regulation of the expression of hormone receptors and gene products that are involved in cell proliferation and motility. (*Am J Pathol* 2009, 174:297–308; DOI: 10.2353/ajpath.2009.080753)

Cyclophilin B (CypB) is a 21-kDa protein belonging to the cyclophilin family of peptidyl-prolyl *cis*-trans isomerase.¹

In this role, cyclophilins promote alterations in protein conformation by binding to substrates in a proline-specific manner and enabling conversion of the proline imide bond through 180° of rotation.² Cyclophilins are widely expressed in the human body and highly conserved throughout the evolution. Cyclophilins act as molecular chaperones that fold, translocate, and process newly synthesized proteins.³ They are also binding targets of cyclosporine A, a complex that inhibits the activation of the calcineurin/nuclear factor of activated T-cells signaling pathway, which contributes to the immunosuppressive effects of this drug.^{2,4} By the reversible modification of their protein structure, cyclophilins also serve as signaling switches,⁵ regulating the activity of transforming growth factor β^6 receptor; epidermal growth factor receptor,⁷ tyrosine kinases,⁸ and transcription factors such as c-Myb⁹ and interferon regulatory factor 4.¹⁰

Structurally, CypB contains a high degree of homology with other members of the cyclophilin family in its core β -barrel/isomerase region, which contains a surface hydrophobic pocket that constitutes the proline binding motif. Both the N- and C-termini of CypB differ significantly from other cyclophilin family members. Within the N-terminus, CypB contains a nuclear translocation motif (DEKKKGPKV), while an endoplasmic reticulum (ER) retention sequence (AIAKE) resides in its C-terminus. This ER-retention motif is proteolytically clipped in the ER, enabling secretion of CypB into the extracellular milieu.¹ CypB is present in serum and breast milk in concentrations of up to 150 ng/ml.^{11,12} Interestingly, when examined biochemically, the majority of CypB can be found to reside in the cell nucleus,¹³ with lower quantities in the ER compartment.¹⁴ Within the ER, CypB could be found in association with nascent precursor proteins complexed

Supported by the National Institutes of Health (RO1 CA102682), the Avon and Lynn Sage Foundations, and the Zell Scholar's Fund.

Accepted for publication October 9, 2008.

Address reprint requests to Charles V. Clevenger, MD, PhD, Department of Pathology, Northwestern University, Lurie 4-107, 303 East Superior Street, Chicago, IL 60611. E-mail: clevenger@northwestern.edu.

with protein components of the ER protein translocation apparatus.¹⁵

The larger biological function of CypB has remained enigmatic. Studies have indicated that CD147 may serve as a cell surface receptor for CypB, as activation of this receptor by CypB results in calcium transport and mitogen-activated protein kinase activation^{16–18}; as such CypB has been considered by some to be a cytokine. CypB is also required during the replication of the hepatitis C virus genome, providing a new therapeutic opportunity in the treatment of this disease.^{19,20} However, in addition to these actions, CypB also functions within the nucleus at the level of transcriptional regulation. CypB facilitates the transcriptional activity of Stat5 by inducing the release of the repressor PIAS3,^{21,22} resulting in significantly enhanced Stat5-mediated gene expression. Given the association of CypB with other intranuclear transcription factors (Clevenger lab, unpublished data), these findings suggest that CypB could serve to coordinate global networks of gene expression.

To examine the effects of CypB on gene expression and global cellular function in human breast cancer, microarray analysis was performed on T47D breast cancer stable transduced cells that overexpressed CypB small interfering siRNA, resulting in an effective knockdown of CypB (si-CypB) or non-specific control of siRNA for luciferase knockdown (si-Luc). These data, validated by subsequent real-time PCR analysis, revealed significant alterations in the expression of genes related to cell proliferation, cell migration, and hormone response in the si-CypB cells. When coupled with data obtained from *in vitro* functional analysis of these cells, as well as the measurement of CypB levels in matched normal, malignant, and metastatic human breast tissues, our findings indicate that CypB action may significantly contribute to the pathogenesis of human breast cancer.

Materials and Methods

Cell Lines, Vectors, and Reagents

The estrogen receptor α (ER α) positive human breast cancer cell line T47D (ATCC, Manassas, VA) and HEK293FT (Invitrogen, Carlsbad, CA) were maintained in Dulbecco's Modified Eagle Medium (Hyclone) supplemented with 10% fetal bovine serum, 50 μ g/ml penicillin, and 50 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. The vectors used here include: pGL410-SV40 (The promoterless pGL4.10 vector was digested with BglII/HindIII, and inserted with SV40 minimal promoter digested from pGL2-promoter vector with BglII/HindIII), pGL2-promoter vector, pGL4.10 and renilla luciferase reporter pGL4.73 (The latter three vectors from Promega, Madison WI). Antibodies used in this study are: anti- α -Tubulin (Invitrogen, 32-2500), anti-CypB (Invitrogen, 37-0600), and Alexa 488 fluor-conjugated second antibody (Invitrogen, A11029).

Establishment of Stable or Transient CypB siRNA Cells

SiRNA targeted to the 3'-end region of the CypB coding sequence (termed si-CypB) and the luciferase gene (termed si-Luc) in the pHIV-7/Puro vectors were used to generate stable pools of transduced cells.²³ These vectors were generously provided by Dr. Hengli Tang (Florida State University). HEK293FT cells were transfected with 3 μ g pHIV-7/Puro vector and 9 μ g of Viral Power Packaging (Invitrogen) using Lipofectamine 2000 (Invitrogen). Virus was harvested post-transfection, and filtered through a 0.45 μ m filter to remove cellular debris. Two ml of the lentiviral supernatants were used to transduce T47D cells (50% confluency in T75 flask) in the presence of 7 μ g/ml of polybrene (Invitrogen). After 24 hours following transduction, the transduced cells were selected with 1 μ g/ml puromycin for 10 days. The resultant stable pools maintained in 1 μ g/ml puromycin were then used for the outlined studies. For independent validation of the effects of siRNA knockdown of CypB, a siRNA against the central region of the CypB coding sequence (cat# D-001136-01-05, Dharmacon, Lafayette, CO) was transfected in T47D cells in parallel with the controls si-glyceraldehyde-3-phosphate dehydrogenase (cat# AM4623 from Ambion), and si-control RNA (cat# D-001210-01 from Dharmacon) using RNAiMAX (Invitrogen). After 48 hours, cells were harvested for Western blot or RT-PCR (Reverse transcription polymerase chain reaction).

Microarray and Data Analysis

Microarray analysis was conducted with an Illumina Human Ref-6 version 2 Expression Chip (Illumina, San Diego, CA). Three independent T47D cultures were used for RNA isolation with RNeasy plus mini kit (Qiagen, Valencia, CA). RNA quality was checked by Agilent Bioanalyzer (Santa Clara, CA). An Ambion labeling kit was used for labeling cDNA followed by hybridization to Illumina chips. Scan data were extracted by Illumina Beadstudio and subsequently analyzed using Bioconductor lumi package.²⁴ The data were first variance stabilization transformed²⁵ and then normalized by a quantile normalization method. To reduce false positives, probes with measurement value below the background level (detection *P* value <0.01) in all hybridizations were filtered out; 17,901 probes were kept for subsequent statistical analysis. Genes with significant differential expression levels were identified using analysis of variance with Bayes adjustment of the variance implemented in Bioconductor limma package.²⁶ To control the effects of multiple testing and reduce false positives, the gene selection of differentially expressed genes is based on: the false discovery rate (FDR) adjusted *P* value <0.05, fold-change ≥ 1.5 (up or down). The identified genes were used for the outlined studies (see Supplemental Table S1 available at <http://ajp.amjpathol.org>). Microarray data were deposited in the Gene Expression Omnibus database with

accession number GSM324466 (GEO database, <http://www.ncbi.nlm.nih.gov/geo/>).

Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) was used to identify gene networks according to biological functions and/or diseases in the Ingenuity Pathways Knowledge Base (Ingenuity Systems, Redwood City, CA). The transcripts with known gene identifiers (HUGO gene symbols) and expression levels (fold change ≥ 1.5 up or down, $P < 0.01$, FDR < 0.05) were filtered and then entered into the Ingenuity Pathways Knowledge Base IPA 4.0 (see Supplemental Table S1 available at <http://ajp.amjpathol.org> for gene list). Each gene identifier mapped in the Ingenuity Pathways Knowledge Base was termed as a focus gene, which was overlaid into a global molecular network established from the information in the Ingenuity Pathways Knowledge Base. Each network contained a maximum of 35 focus genes. Genes were represented as nodes with different shapes and colors (see figure legends), and biological relationships were represented by edges (different lines). All edges were supported by at least one reference as interaction or action.²⁷ The nodes (genes) and the edges were described in the figures and figure legends.

Reverse Transcription and Real-Time PCR Validation

Two micrograms of RNA was used for cDNA synthesis in 20 μ l reaction volume using Superscript III first strand synthesis kit (Invitrogen). Four μ l of cDNA (2.5 ng/ μ l related to RNA concentration), 1 μ l primers (2 μ mol/L each), and 5 μ l of 2 $\times$ Power SYBR Mastermix (Applied Biosystems, Foster city, CA) were used for real-time PCR in 10 μ l reaction volume performed in 384-well plate. Real-time PCR was conducted on ABI 7900HT Thermocycler (Applied Biosystems). All real-time PCR re-

actions were run in triplicate. Data were normalized to 18S rRNA, and fold change was represented as $2^{-\Delta\Delta Ct}$ [$2^{-(Ct \text{ target-Ct } 18S)_{\text{siCypB}} - (Ct \text{ target-Ct } 18S)_{\text{siLuc}}}$]. Primers for real-time PCR are listed in Table 1.

Luciferase Assay

A dual luciferase assay was conducted according to Fang et al.²⁸ The firefly luciferase reporter pGL410-SV40 (500 ng/well) and renilla luciferase control vector pGL4.73 (5 ng/well) were transiently transfected into 2×10^5 cells in 24-well plate using Lipofectamine LTX (Invitrogen). Following transfection, cells were cultured in growth medium for 24 hours before luminescence assay. Data are represented as RLU (relative light unit, ratio of luciferase/renilla).

Western Blot

Fifty microgram of lysates from T47D cells were subjected to 10% SDS-polyacrylamide electrophoresis gel, transferred onto polyvinylidene difluoride membrane and immunoblotted with the antibodies (1 μ g/ml) described above. Images were captured using a Fujifilm LAS-3000 image analyzer (Fuji, Japan).²⁸

Cell Motility Assay

Cell motility was assayed using Boyden chambers (12 μ mol/L, 12-well polycarbonate membrane transwell, Corning), as previously described.²⁹ To enhance motility non-occlusively, the lower surface of Boyden chamber inserts were coated with 200 μ l Collagen I (25 ng/ μ l, 5 μ g total in 1 $\times$ PBS). T47D cells were arrested in defined (fetal bovine serum-free) medium for 24 hours, then detached with Versene (Invitrogen); 2×10^5 detached cells were resuspended in the 500 μ l of defined medium and added into the upper Boyden chamber. One ml of defined medium with 10% fetal bovine serum, or 17- β -estradiol (E2,

Table 1. Primers Used for Real-Time PCR

Gene name	Primer sequences	Start	End	RNA accession number
18S rRNA	olg200 5'-CCCCATGAACGAGGGAATT-3' olg201 5'-GGGACTTAATCAACGCAAGCTT-3'	1626	1686	NR_003286
c-Myb	olg80 5'-GCCAATTATCTCCGAATCGAA-3' olg81 5'-ACATTGTTTTCCAATTCTCCCCT-3'	337	507	NM_005375
ER α	olg331 5'-GGAAGCTACTGTTTGCTCCTAACTTG-3' olg332 5'-AGATCTCCACCATGCCCTCTAC-3'	1561	1635	NM_000125
GHr	olg337 5'-CCAGGCCCTAAAGACAAATCTTCT-3' olg338 5'-AAAAAGTCTCTCGCTCAGGTGAA-3'	164	236	NM_000163
PR	olg345 5'-TCAGTGGGCAGATGCTGTATTT-3' olg346 5'-GCCACATGGTAAGGCATAATGA-3'	3766	3862	NM_000926
S100A4	olg323 5'-CAAGCTCAACAAGTCAGAACTAAAGG-3' olg324 5'-GCTTCTGGAAGCAGCTTCATC-3'	150	241	NM_002961
S100A6	olg347 5'-CTCACCATTGGCTCGAAGCT-3' olg348 5'-AGTCTTCCATCAGCCTTGCAA-3'	438	492	NM_014624
STMN3	olg349 5'-TGCTGTCGCTCATCTGCTCC-3' olg350 5'-TCACCTCCATGTCCCCGTACT-3'	132	209	NM_015894

Primers were obtained using Primer Express 3.1 software, from Primerbank or from published sequences (STMN3⁶¹, S100A6⁶²). Most primer pairs span two exons, except the intronless 18S rRNA. Primers were tested by dissociation curve analysis to assure only a single amplicon. The base pairs that the forward primer starts and reverse primer ends are based on mRNA sequence. All primers are located inside the coding sequence.

$10^{-7}\text{M})^{30}$ was placed in the lower chambers. Cells were cultured for 20 hours and the migrated cells were counted under microscope for five fields per insert with triplicate inserts in each experiment.

Cell Proliferation Assay

T47D cells (4×10^4 cells) were plated in 96-well plate and cultured in the growth medium for 24 hours. Each well was pulsed with $0.5 \mu\text{Ci}$ of [^3H] thymidine ($48 \text{ Ci}/\text{mmol}$; Amersham Pharmacia Biotech) for 4 hours. Cells were harvested onto the membrane with Filtermate Harvester (Perkin Elmer, Waltham, MA) before analysis with a MicroBeta TriLux Scintillation Counter (Perkin Elmer).

Breast Tissue Microarray

Breast tissues were obtained from the Tissue Core of the Northwestern University Breast Cancer Program in the form of a tissue microarray (TMA). All tissues were stripped of all patient identifiers before use, and thus were anonymous to the investigators. The TMA examined included matched normal adjacent, invasive ductal carcinoma, and lymph node metastasis from 11 patients. In addition, unmatched ductal carcinoma *in situ* (DCIS) specimens from nine patients were also present on the TMA. The presence of tumor and integrity of each tissue was confirmed by H&E staining. Before analysis by both immunofluorescent (IF) and immunohistochemical (IHC) approaches, the TMA was incubated at 65°C for 10 minutes, before deparaffinization in xylene and rehydration through graded ethanol, followed by antigen retrieval in citrate buffer (Zymed 00-5001, $\text{pH} = 6.0$) at 95°C for 20 minutes. The slides were blocked in the blocking buffer (2.5% bovine serum albumin and 0.1% Triton X-100) for 10 minutes and incubated with CypB antibody (1:10 dilution for IF; 1:20 dilution for IHC) overnight at 4°C . Antigen-antibody complexes were detected for IF by incubation with an Alexa 488 fluor-conjugated second antibody (1:100 dilution) for one hour. For IHC, detection of antibody-antigen complex used an HRP-conjugated secondary antibody, followed by diaminobenzidine labeling, as previously described.³¹ Visual scoring of the IF-labeled images was performed as described previously³¹ with modification. Given that virtually 100% of the breast epithelial demonstrated some level of anti-CypB IF labeling, scoring was restricted to assessment of label intensity on a 0 to 3 scale (0 = absent, 1 = dim, 2 = bright, 3 = very bright). Comparable semiquantitative results were noted with specimens labeled by anti-CypB IHC labeling (not shown).

Statistical Analysis

All experiments described here were performed at least three times. Statistical analysis was performed on GraphPad Prism 4 (GraphPad Software, La Jolla, CA), and specified in the figure legends. The results are shown as the means with error bars depicting $\pm$ SEM.

Results

The Effects of CypB Knockdown on Global Gene Expression

To assess the effects of CypB expression on global gene expression in breast cancer, two T47D stable cell pools (transfected with the si-Luc siRNA expression vector targeting the luciferase gene as a non-human target control, and the si-CypB siRNA expression vector targeting the CypB gene) were generated. Western blot results showed that the parental T47D cells (also called wild-type, WT) and si-Luc cells had a similar level of CypB expression, while the CypB protein level was barely detected in the si-CypB cells (Figure 1A). The stable knockdown of CypB mRNA was also confirmed by real-time PCR (data not shown) and microarray analysis (see Supplemental Table S1 available at <http://ajp.amjpathol.org>).

Figure 1. Characterization of CypB knockdown in T47D cells. **A:** Western blot analysis using anti-CypB and control antibodies (with anti- α -tubulin as loading control) reveals decreased expression of CypB in the various siRNA transfectants. Upper panel, expression of CypB is reduced in the si-CypB stable transfectant pool as opposed to the wild-type (WT) or the si-Luc control pool. Lower panel, T47D cells transiently transfected with siRNA against si-glyceraldehyde-3-phosphate dehydrogenase, siControl, or siCypB again demonstrate the specific knockdown of CypB. **B:** Luciferase siRNA control, but not CypB siRNA, effectively targets plasmid-based luciferase expression. T47D cells were transfected with firefly luciferase reporter (500 ng of pGL410-SV40) and renilla luciferase control (5 ng of pGL4.73). The transfected cells were cultured in growth medium for 24 hours before luminescence assay. The results are shown as the means $\pm$ SEM. Statistical analysis was performed using one-way analysis of variance. *** $P < 0.001$. **C:** Heat map analysis reveals a global view of genes up- and down-regulated in si-CypB cells compared with si-Luc control cells. **D:** Volcano map demonstrates the relationship between the observed fold change in gene expression and the P value significance of such changes in si-CypB cells compared with si-Luc cells. The dotted lines represent the P value and fold-change cut-offs, the red dots represent the selected genes filtered by criteria (fold change ≥ 1.5 up or down, $P < 0.01$, FDR < 0.05).

Figure 2. si-RNA-mediated knockdown of CypB decreases the expression of two motility-relevant gene products and the motility of T47D cells. **A** and **B**: Real-time PCR validated that STMN3 (**A**) and S100A4 (**B**) expression was down-regulated in T47D pools stably or transiently transfected with one of two non-homologous siRNA. The y axis label "Fold change" is defined in the materials and methods section; "Stable" refers to the cell pools stably transfected with the si-CypB-pHIV-7/puro construct; "Transient" refers to cells transfected directly with a CypB siRNA targeting a differing coding sequence than that used in the stable transfectants. **C**: Cell motility assay using Boyden chamber assay revealed a significant reduction in T47D motility in the si-CypB cells in both resting and fetal bovine serum-stimulated conditions. Cellular migration was quantitated by phase-contrast microscopy. Magnification = original $\times 100$. Statistical analysis was performed using analysis of variance (ANOVA).

A luciferase assay confirmed that firefly luciferase expression was decreased in the control si-Luc T47D cells when co-transfected with a luciferase expression construct (Figure 1B). To corroborate the findings obtained with the siRNA knockdown pools, a CypB siRNA targeting a different region of CypB was transiently transfected with parallel controls into T47D cells (Figure 1A); these cells (demonstrating a 70% reduction in CypB levels) were used to independently validate the effects of the CypB knockdown in stable pools to exclude off-target effects (see Figure 2).

To explore the effect of CypB knockdown on global gene expression, cDNA microarray analysis was conducted with si-CypB and the control si-Luc stable transduced cell pools. To assess the statistical significance of these data, a Bayesian-moderated *t*-test was used to take advantage of the large number of genes being analyzed simultaneously to yield more reliable variance estimates. To reduce false positives, genes above-background in at least one experiment were used for subsequent analysis. Stringent criteria (fold change ≥ 1.5 up or down, $P < 0.01$, FDR < 0.05) were used to filter differentially expressed genes. Two-dimensional hierarchical clustering was applied to these filtered probes to generate a global overview of gene expression map (heat map). The heat map showed a remarkable difference in gene expression pattern between si-Luc and si-CypB groups. It also indicated, in a global view, highly consistent results between the triplicates in each group (Figure 1C). To simultaneously correlate the size of effects on gene expression (log₂-fold change as y axis) and the statistical significance ($-\log_{10} P$ value as x axis) at the global level, a volcano plot was used to compare the difference of gene expression between si-CypB and si-Luc groups (Figure 1D). The red dots represent the selected differentially expressed genes (FDR < 0.05 , fold change ≥ 1.5), significantly regulated by CypB knockdown. Of 663 differentially expressed genes, 297 (45%) were up-regulated and 366 (55%) were down-regulated (see Supplemental Table S1 available at <http://ajp.amjpathol.org>). Tables 2 and 3 list the top 30 transcripts up-regulated (Table 2) or down-regulated (Table 3) by CypB knockdown.

CypB Knockdown Is Associated with Decreased Cell Motility

CypB has been shown to induce cell migration in peripheral blood T lymphocytes.¹⁸ Microarray analysis also revealed that many genes implicated in cell motility were regulated in si-CypB cells, (see Supplemental Table S1 available at <http://ajp.amjpathol.org>); STMN3 (stathmin-like 3) has the second highest-fold down-regulation in the si-CypB cells (see Table 3). STMN3 is involved in signal transduction, regulation of microtubule dynamics, and tumor cell invasion.³² S100A4 (also known as CAPL or metastasin) is also down-regulated, and this protein is widely studied in the breast cancer (Table 3). S100A4 is a calcium-binding, metastasis-associated protein, and plays a role in motility and invasion during metastasis.³³ These two genes were chosen for real-time PCR validation due to their importance in breast cancer and their significant reductions in mRNA expression observed in the si-CypB cells. Real-time PCR validated that STMN3 and S100A4 were significantly down-regulated in si-CypB stable cells (Figure 2, A and B). To confirm that these results were not the consequence of an off-target effect associated with the siRNA used in the stable transfectant cell pools, T47D cells were transiently transfected with a siRNA targeting an independent sequence in CypB (Figure 2, A and B). These studies revealed that this transient knockdown of CypB using a different siRNA still resulted in a significant reduction in the level of expression of STMN3 and S100A4, albeit at a level somewhat lower than that observed in the si-CypB stable pools. We believe that the decreased level of down-regulation of STMN3 and S100A4 noted in the transiently transfected cells receiving si-CypB is a direct reflection of the reduced efficiency of this construct in reducing CypB levels in these transfectants (ie, 70% vs. 95%; Figure 1) as opposed to the si-CypB stable pools.

Given that the expression of multiple genes were altered by CypB knockdown (Figure 1C; see Supplemental Table S1 available at <http://ajp.amjpathol.org>), the effect

Table 2. The List of Top 30 Transcripts Up-Regulated by CypB Knockdown

Symbol	Description	Fold change	EntrezID
SNAR	small NF90-associated RNA A53129250	8.7	100126799
BCYRN1	brain cytoplasmic RNA 1, Bc1 analog (mouse)	8.4	618
SPINK4	serine peptidase inhibitor, Kazal type 4	5.7	27290
VCX	variable charge, X-linked	4.9	26609
VCX2	variable charge, X-linked 2	3.7	51480
VCX3B	variable charge, X-linked 3B	3.5	425054
PNPO	Pyridoxamine 5'-phosphate oxidase	3.4	55163
MMD	monocyte to macrophage differentiation-associated	3.1	23531
MT2A	metallothionein 2A	2.9	4502
ANKRD43	ankyrin repeat domain 43	2.8	134548
MT1A	metallothionein 1A	2.8	4489
UCP2	uncoupling protein 2 (mitochondrial, proton carrier)	2.7	7351
CLIC6	chloride intracellular channel 6	2.7	54102
SAR1B	SAR1 gene homolog B (<i>S. cerevisiae</i>)	2.6	51128
S100P	S100 calcium binding protein P	2.6	6286
RPIA	ribose 5-phosphate isomerase A	2.6	22934
APOD	apolipoprotein D	2.5	347
ALDH3B2	aldehyde dehydrogenase 3 family, member B2	2.4	222
ATP6V0B	ATPase, H ⁺ transporting, lysosomal 21kDa, V0 subunit b	2.4	533
PIP	prolactin-induced protein	2.4	5304
HSPA8	heat shock 70kDa protein 8	2.3	3312
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	2.3	10049
C3orf52	chromosome 3 open reading frame 52	2.3	79669
SLC5A6	solute carrier family 5, member 6	2.3	8884
DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	2.3	55466
NIPSNAP1	nipsnap homolog 1 (<i>C. elegans</i>)	2.3	8508
EPB41L3	erythrocyte membrane protein band 4.1-like 3	2.3	23136
CKS1B	CDC28 protein kinase regulatory subunit 1B	2.2	1163
NR2F1	nuclear receptor subfamily 2, group F, member 1	2.2	7025
FAM89B	family with sequence similarity 89, member B	2.2	23625

of CypB knockdown on cell motility was analyzed by Boyden chamber migration assay. As anticipated, these results showed that cell motility was significantly decreased in cells with siRNA-mediated knockdown of CypB as compared with controls (Figure 2C).

The Expression of Genes Implicated in the Regulation of Cell Proliferation Is Altered by CypB Knockdown

Following the establishment of the si-CypB T47D knockdown pools, it was initially noted that the proliferation of these cells (compared with the si-Luc cells or the parental T47D line) was appreciably slower, with no evidence of excess cell death (data not shown). The Gene Ontology database (www.geneontology.org) was searched using the AmiGo browsing tool³⁴ to generate heat maps of genes related to cell proliferation. Heat map analysis showed that many genes implicated in the regulation of cell proliferation, including the S100A6 gene,³⁵ were down-regulated in si-CypB cells (Figure 3A). The transcription factor c-Myb that contributes to both proliferation, differentiation, and breast cancer pathogenesis,³⁶ was also significantly down-regulated in si-CypB cells. Validation of these results using real-time PCR confirmed that S100A6 and c-Myb gene expression was decreased in si-CypB cells (Figure 3, B and C). The si-CypB cell morphology was similar to wild-type and si-Luc cells, but with a slower growth rate compared with wild-type and si-Luc cells (Figure 3D). Cell proliferation assay using

H³-thymidine incorporation confirmed that cell proliferation was reduced in si-CypB cells (Figure 3E). Taken together these studies indicate that CypB levels can significantly modulate breast cancer cell proliferation and the genes involved in this process.

The Genes Regulated by CypB Knockdown Contribute to Essential Cell Functions

IPA is widely used for the meta-analysis of gene expression, proteomics and metabolic data to elucidate tumor progression, biomarker discovery and drug discovery.²⁷ To assess the global effects of CypB on gene expression, IPA was used to systematically visualize the relationships of genes regulated by CypB knockdown. The transcripts from microarray results were filtered (see Material and methods) and 633 transcripts were input into the IPA Base. IPA was used to overlay the CypB-regulated genes onto global networks developed from the information contained in the Ingenuity Pathways Knowledge Base. Of the 27 function networks (score >3 is considered as significant according to IPA) significantly impacted by CypB knockdown, the network of genes associated with function in cancer biology were most profoundly impacted. The identities of the top six other networks significantly impacted by CypB knockdown in T47D cells are graphed in Figure 4B. The genes within the cancer function network were then used to generate an interaction network by the IPA. In this interaction network, the estrogen receptor α (ESR1, hereafter referred to as ER α) was

Table 3. The List of Top 30 Transcripts Down-Regulated by CypB Knockdown

Symbol	Description	Fold change	EntrezID
PPIB	Peptidylprolyl isomerase B (cyclophilin B)	-13.1	5479
STMN3	Stathmin-like 3	-5.4	50861
TXNIP	Thioredoxin interacting protein	-4.4	10628
CADM1	Cell adhesion molecule 1	-4.2	23705
ASS1	Argininosuccinate synthetase 1	-4.1	445
GPC6	Glypican 6	-3.9	10082
PTRF	Polymerase I and transcript release factor	-3.6	284119
C22orf16	Chromosome 22 open reading frame 16	-3.5	400916
PAPSS2	3[prime]-phosphoadenosine 5[prime]-phosphosulfate synthase 2	-3.4	9060
SULF1	Sulfatase 1	-3.2	23213
TFF3	Trefoil factor 3 (intestinal)	-3.2	7033
TUBB2B	Tubulin, beta 2B	-3.0	347733
SQSTM1	Sequestosome 1	-2.9	8878
S100A4	S100 calcium binding protein A4	-2.9	6275
TNNC1	Troponin C type 1 (slow)	-2.9	7134
PTGER3	Prostaglandin E receptor 3 (subtype EP3)	-2.8	5733
ATP1B1	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	-2.8	481
LAMP3	Lysosomal-associated membrane protein 3	-2.7	27074
CYBRD1	Cytochrome b reductase 1	-2.7	79901
OLR1	Oxidized low density lipoprotein (lectin-like) receptor 1	-2.6	4973
SLC35A1	Solute carrier family 35, member A1	-2.6	10559
FAM129A	Family with sequence similarity 129, member A	-2.6	116496
GPD1L	Glycerol-3-phosphate dehydrogenase 1-like	-2.6	23171
FBXO32	F-box protein 32	-2.5	114907
RAP2C	RAP2C, member of RAS oncogene family	-2.4	57826
CDH3	Cadherin 3, type 1, P-cadherin (placental)	-2.4	1001
CA12	Carbonic anhydrase XII	-2.4	771
MATN2	Matrilin 2	-2.4	4147
FKBP5	FK506 binding protein 5	-2.4	2289
DKFZP586H2123	Regeneration associated muscle protease	-2.4	25891

prominently situated (Figure 4A). Real-time PCR confirmed that ER α was down-regulated in si-CypB cells (Figure 4C). As widely recognized, the expression of ER α is used to biologically stratify breast cancer phenotype, and the determination of its expression has significant prognostic and therapeutic implications. Estrogen has multiple effects on breast cancer cells, including an increase in motility and expression of the c-Myc oncogene.³⁰ To test if the CypB siRNA-mediated decrease in ER α expression resulted in decreased ER-enhanced gene expression and cancer motility, RT-PCR analysis of the c-Myc oncogene and Boyden chamber migration assays in the presence or absence of estradiol were performed. These analyses revealed that the estrogen-induced increase in c-Myc expression was reduced by 78% in si-CypB cells (as compared with the si-Luc control pools; data not shown) and that the estrogen-induced cell motility was also significantly decreased in si-CypB cells (Figure 4D). These findings further suggest that the levels of CypB within a cell may serve to regulate breast cancer biology in part through its regulation of ER α expression and function.

CypB Knockdown Decreases Receptor Expression

As the pathogenesis and progression of breast cancer is intimately associated with the expression of receptors for hormones and growth factors, heat map analysis focusing on the effects of CypB knockdown on receptor expression was performed (Figure 5A), with the effect of this

knockdown on selected receptor expression of relevance to breast cancer biology shown in the Figure 5B. Real-time PCR confirmed that growth hormone receptor (GHR) and progesterone receptor (PR) were down-regulated in si-CypB cells (Figure 5, C and D). The prolactin receptor (PRLr) was also confirmed to be down-regulated in si-CypB cells, and the larger effect of CypB knockdown on PRL-induced signaling will be the focus of a separate manuscript (Fang et al manuscript in preparation). Interestingly, si-CypB cells demonstrated no alteration in their mRNA expression of either the epidermal growth factor receptor or erbB2 receptor (not shown). In addition, no alteration in the expression of either CD147, or its downstream regulated pro-apoptotic gene product Bim, was observed (data not shown). Taken together these results suggest that the function of CypB may be of particular relevance to breast cancer cells of the luminal phenotype, given its significant effects on the expression of the ER, PR, PRLr, and GHR.

Analysis of CypB Expression in Normal and Malignant Breast Tissues

The above data suggest that the regulation of CypB levels may significantly impact the expression of gene networks relevant to the biology of breast cancer. If so, one could hypothesize that CypB expression could be elevated as a function of neoplastic progression within breast tissues. To test this hypothesis, the levels of CypB protein were evaluated by microscopic evaluation of both semiquantitative anti-CypB IF and IHC on a progression

Figure 3. si-RNA mediated knockdown of CypB decreases the expression of two proliferation-relevant genes and cell proliferation of T47D cells. **A:** Heat map analysis (GO:0008283, cell proliferation from AmiGO) reveals that genes implicated in cell proliferation are altered by CypB knockdown. c-Myb was manually added in this cell proliferation category (GO:0008283) due to its widely reported role for cell proliferation. **B** and **C:** Real-time PCR validated that the expression of S100A6 (**B**) and c-Myb (**C**) was down-regulated in the si-CypB cells. **D:** Cell growth (as measured by viable cell number) was reduced in si-CypB cells. Cells were cultured in the growth medium for 3 days before trypan blue exclusion staining to measure the number of viable cells; the number of non-viable cells in each of the cell pools is less than 2%. **E:** cell proliferation was decreased in si-CypB cells. Growth was measured by ³H-thymidine incorporation. Statistical analysis was performed using Student's *t*-test (**B** and **C**) or one-way analysis of variance (**D** and **E**). **P* < 0.05, ***P* < 0.01.

breast TMA. The TMA used consisted of normal adjacent, primary malignant, and lymph node metastasis from 11 patient-matched specimens. Within this cohort there were 8 ER+/PR+ patients, 2 ER-/PR- patients, and one Her2+ patient, with an equal distribution of grade. In addition, the TMA included nine non-matched DCIS specimens. As seen in the IHC photomicrograph presented in Figure 6A and B, CypB levels were increased in malignant breast epithelium, with a significant concentration of CypB within the nucleus. Semiquantitative analysis of the anti-CypB IF present on the TMA demonstrated a significant increase in CypB levels in DCIS/primary malignant/metastasis versus normal tissues (Figure 6C). Comparable semiquantitative results were obtained with anti-CypB IHC (data not shown). Anecdotally, the mag-

Figure 4. CypB knockdown results in a significant alteration in the expression of genes implicated in cancer as assessed by interaction network analysis. **A:** The top interaction network generated using IPA analysis included genes associated with cancer. The color indicates the upregulation of gene expression induced by CypB knockdown (red), down-regulation of gene expression (green) and protein complexes (gray). **B:** Functional categories identified by IPA for the 663 CypB-regulated genes. The categories were obtained from the Molecular and Cellular Function analytic tool in the IPA database. **C:** Real-time PCR validation of down-regulated expression of ERα in si-CypB cells. **D:** A cell motility assay showed decreased motility of si-CypB cells. The defined medium in the bottom Boyden chambers were supplemented with 17β-estradiol (E2, 10⁻⁷ M). Statistical analysis was performed using a student *t*-test (**C**) or two-way analysis of variance (**D**). ****P* < 0.001.

nitude of CypB increase seen in malignant versus adjacent normal tissues was comparable in ER+, ER-, and Her2+ tumors. Although an increase in CypB levels were noted in primary malignant versus DCIS tissues, suggesting discrete increases in CypB as a function of malignant progression, caution should be used in overinterpreting these results, as the DCIS tissues were nonmatched. Regardless, given the regulation of hormone receptors and genes implicated in proliferation and motility by CypB, the up-regulation of this prolyl isomerase in breast cancer could significantly modulate the biology of this disease.

Discussion

Within the nucleus, ongoing research has demonstrated that CypB functions as a transcriptional inducer

Figure 5. Expression of the GHR and PR was down-regulated in si-CypB cells. **A** and **B**: Heat map analysis (GO:0004872, receptor activity from AmiGO) revealed that the expression of many receptors was altered by CypB knockdown. **C** and **D**: Real-time PCR validated the down-regulation of growth hormone receptor (GHR) and progesterone receptor (PR) in si-CypB cells. Statistical analysis was performed using student *t*-test (**B** and **D**). **P* < 0.05, ***P* < 0.01.

of Stat5-mediated gene expression.^{21,22} At the cell surface, CypB also serves as a ligand for CD147 receptor (also known as the extracellular metalloproteinase inducer (EMMPRIN)), that in turn regulates mitogen-activated protein kinase activation, motility, calcium transport,^{16–18} and the expression of the pro-apoptotic protein Bim.³⁷ Given these important functions, it was hypothesized that CypB could serve as ligand for CD147 and/or as a transcription inducer in the regulation of breast cancer biology. To test this hypothesis, the combined use of validated gene expression profiling with relevant biological functional assays of CypB-siRNA transfected T47D breast cancer cells and anti-CypB TMA analysis was used. These studies in the ER⁺ T47D line have revealed that down-regulation of CypB profoundly altered the gene expression of hormone receptors, and function networks relevant to the biology of breast cancer. Ongoing work in the lab seeks to expand the expression profiling of the effects of CypB reduction in breast cancer cells with differing phenotypes, such as ER[–]/PR[–] and Her2⁺ cells.

With large data sets retrieved by microarray, it is frequently difficult to determine whether the genes are direct or indirect targets of the protein of interest by hierarchical gene cluster analysis alone.²⁷ In this work, based on genes regulated by CypB knockdown, IPA was used to

Figure 6. Anti-CypB IHC and IF analysis of a breast progression TMA revealed that CypB levels increased in malignant breast tissues. **A** and **B**: Representative anti-CypB IHC of matched normal (**A**) vs. malignant (**B**) primary breast tissues. The photomicrograph demonstrates the observed increase in CypB expression in malignant tissues, with accumulation of the anti-CypB label over the cell nucleus. Magnification = original $\times 400$. **C**: Quantification of anti-CypB IF labeling in malignant tissues. Semiquantitative analysis of the intensity IF label is described in Materials and Methods. The results are shown as the means with error bars depicting $\pm$ SEM. Statistical analysis was performed using one-way analysis of variance. ***P* < 0.01 and ****P* < 0.001 as compared with normal tissues.

systematically analyze gene expression patterns to identify those protein networks most significantly altered by CypB knockdown in the T47D breast cancer cell line. As seen in Figure 4B, protein networks implicated in cancer, cell growth (cell proliferation, cell cycle, cell death), and cell movement were significantly affected by reduction in CypB levels. Interestingly, hormone receptors for both sex steroid receptors (ER α and PR) and peptide hormone receptors (GHR and PRLr) were significantly decreased by CypB knockdown. Each of these hormone receptors has been implicated in various aspects of breast cancer biology, with ER α obviously having the most established prognostic and therapeutic role.^{38,39} IPA analysis revealed (Figure 4A) that many gene products functionally implicated in the actions of ER α are modulated by CypB knockdown, including those involved in cell proliferation⁴⁰ and migration.³⁰ The reduction in both PRLr and GHR expression by CypB knockdown is also notable in that both of these receptors are frequently associated with the Jak2/Stat5 signaling pathway.⁴¹ Thus a reduction in CypB would appear to down-regulate PRL and GH-triggered signaling at two separate levels—by reducing PRLr and GHR expression and by decreasing Stat5 activation. Further substantiating this, reports from several

labs have noted correlations between ER α , PR, and PRLr expression.^{42–45} In mammary epithelial cells, PRL has been reported to increase cytosolic ER α and PR concentrations.⁴⁶ The activity of ER α has been long recognized to up-regulate the expression of PR.⁴⁷ In turn, recent studies using MCF7 cells with a tetracycline-inducible PRL expression construct resulted in increased levels of ER α , PRLr, and PR levels and increased estrogen responsiveness. Indeed, the PRL-induced upregulation of ER α by PRL is believed to be in part due to PRL-induced Jak2/Stat5 signaling.⁴⁸ Taken together, these findings suggest that by regulating ER α , PR, and PRLr expression at multiple levels, the expression of CypB may have significant effects on hormone responsiveness in breast cancer.

In addition to the down-regulation of hormone receptors noted following CypB knockdown, gene products implicated in cell motility and growth were significantly altered. In terms of motility, two validated genes identified by expression profiling were S100A4 and STMN3. S100A4 overexpression in breast cancer patients has been associated with reduced survival,⁴⁹ while others have found correlations between S100A4, osteopontin expression, and breast cancer angiogenesis and metastasis.⁵⁰ A member of the stathmin family, STMN3 is involved in microtubule dynamics. STMN3 is associated with ovarian cancer progression,³² but little is known of its association with the biology of breast cancer. However, other members of the stathmin family have been found to correlate with outcomes in human breast cancer.⁵¹ In terms of proliferation, two genes, namely c-Myb and S100A6, were also validated to be significantly down-regulated following CypB knockdown. Both of these gene products are relevant to the biology of breast cancer. While c-Myb is classically associated with cell differentiation and proliferation³⁶ in the hematopoietic system, recent studies have identified both expression and function for this protein in breast cancer. Immunohistochemistry using human breast tissues revealed increased levels of c-Myb protein in *in situ* and invasive breast cancers compared with normal tissues.³¹ c-Myb was found to correlate with ER status,⁵² and studies have also found that c-Myb is regulated by estrogen and may contribute to the biology of ER+ breast cancer cells.^{52–54} Our lab has previously demonstrated a functional relationship between CypB and Stat5^{21,22} and more recently has found that c-Myb can positively regulate Stat5 activity Fang F, Ryszczyn MA, Clevenger CV: Role of c-Myb during PRL-induced-signaling in breast cancer. *Endocrinology*, 2008, In press. Thus, CypB may regulate both c-Myb and Stat5 at multiple levels, both directly, through its regulation of c-Myb expression, and indirectly, by altering the levels of c-Myb available to interact with Stat5. Indeed, both CEBP β and c-Myc, transcription factors whose expression is regulated by Stat5 and c-Myb, were down-regulated in the si-CypB T47D cells (data not shown). Less is known of the functional relationships of S100A6, also known as calyculin, however this calcium-binding protein is clearly up-regulated in breast cancer cells and tissues⁵⁵ and knockdown of this gene appears to decrease both cell proliferation and motility.³⁵ Interestingly, S100A6 has been observed in prolactin re-

ceptor immunoprecipitates,⁵⁶ providing yet another mechanism through which CypB knockdown could modulate breast cancer-relevant, hormone receptor-mediated signaling.

With respect to its global cellular function, the relative contributions of CypB action at the cell surface via its CD147 receptor versus its function in the nucleus as a transcriptional inducer in breast cancer cells remains to be fully investigated. However, the current study has provided some intriguing observations to that end. First, while significant accumulations of CypB were noted within the nucleus of breast epithelium in the TMA, little was noted in the extracellular milieu or at the cell surface. Second, CypB siRNA-mediated knockdown had little effect on the expression of either CD147 or the pro-apoptotic protein Bim, whose expression is down-regulated by CD147 activity (increased levels of Bim were thus anticipated in the si-CypB cells, but this was not observed). Third, several genes directly regulated by the activity of Stat5, namely CEBP β , and c-Myc (not shown) were expressed at decreased levels in the si-CypB T47D cells. While these observations certainly do not exclude a role for CypB acting through CD147 in this setting, they are supportive of a nuclear function for this prolyl isomerase in breast cancer cells and tissues.

The role of Stat5 in breast cancer remains enigmatic. On one hand, when mice heterozygous for Stat5a were crossed with the SV40-T-antigen mouse model of mammary cancer, significant reductions in the numbers, size, and progression of resultant tumors were noted.⁵⁷ On the other hand, the presence of phosphorylated Stat5a has been associated with a well-differentiated phenotype in breast cancer both *in vitro*⁵⁸ and *in vivo*.⁵⁹ How CypB as a transcriptional inducer of Stat5 may alter its larger biological function at this time is uncertain and is most likely multifactorial (given the large number of genes affected by CypB knockdown), however, it is clear from the data presented here that the levels of CypB are directly associated with enhanced proliferation and motility.

The relevance of these observations are further substantiated by the anti-CypB immunohistochemistry and immunofluorescence analysis shown here that reveal an up-regulation of CypB in malignant versus normal breast tissues. Future tissue-based studies regarding the expression of CypB will focus on both breast cancer phenotype and patient outcome. In addition our lab has recently demonstrated that the inhibitor of cyclophilin PPI activity, cyclosporine A, has profound effects on the growth, motility, invasion, and metastasis of breast cancer both *in vitro* and *in vivo*.⁶⁰ These studies have raised the interesting question of whether the effects of cyclosporine A in breast cancer are due to its inhibition of the PPI activity of CypA or CypB, or the downstream inhibition of calcineurin/ nuclear factor of activated T-cells by the cyclosporine A/CypA complex (or some combination of the above). Regardless, our studies presented here demonstrate that a decrease in CypB levels (and therefore an overall decrease in CypB PPI activity) can profoundly alter the expression of genes and cellular functions relevant to the pathogenesis and progression of

breast cancer. In this regards, the development of additional pharmacological agents that specifically target each of the cyclophilins may have significant utility in the treatment of this disease.

Acknowledgments

Yvonne Feeney and Jennifer Whitehead are respectively thanked for their assistance with immunofluorescence and tissue microarray support.

References

- Price ER, Zydowsky LD, Jin MJ, Baker CH, McKeon FD, Walsh CT: Human cyclophilin B: a second cyclophilin gene encodes a peptidyl-prolyl isomerase with a signal sequence. *Proc Natl Acad Sci USA* 1991, 88:1903–1907
- Fischer G, Tradler T, Zarnt T: The mode of action of peptidyl prolyl cis/trans isomerases in vivo: binding vs. catalysis. *FEBS Lett* 1998, 426:17–20
- Bhattacharyya T, Karnezis AN, Murphy SP, Hoang T, Freeman BC, Phillips B, Morimoto RI: Cloning and subcellular localization of human mitochondrial hsp70. *J Biol Chem* 1995, 270:1705–1710
- Kofron JL, Kuzmic P, Kishore V, Colon-Bonilla E, Rich DH: Determination of kinetic constants for peptidyl prolyl cis-trans isomerases by an improved spectrophotometric assay. *Biochemistry* 1991, 30: 6127–6134
- Hunter T: Prolyl isomerases and nuclear function. *Cell* 1998, 92:141–143
- Huse M, Chen YG, Massague J, Kuriyan J: Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. *Cell* 1999, 96:425–436
- Lopez-Illasaca M, Schiene C, Kullertz G, Tradler T, Fischer G, Wetzker R: Effects of FK506-binding protein 12 and FK506 on autophosphorylation of epidermal growth factor receptor. *J Biol Chem* 1998, 273:9430–9434
- Brazin KN, Mallis RJ, Fulton DB, Andreotti AH: Regulation of the tyrosine kinase Itk by the peptidyl-prolyl isomerase cyclophilin A. *Proc Natl Acad Sci USA* 2002, 99:1899–1904
- Levenson JD, Ness SA: Point mutations in v-Myb disrupt a cyclophilin-catalyzed negative regulatory mechanism. *Mol Cell* 1998, 1:203–211
- Mamane Y, Sharma S, Petropoulos L, Lin R, Hiscott J: Posttranslational regulation of IRF-4 activity by the immunophilin FKBP52. *Immunity* 2000, 12:129–140
- Allain F, Boutillon C, Mariller C, Spik G: Selective assay for CyPA and CyPB in human blood using highly specific anti-peptide antibodies. *J Immunol Methods* 1995, 178:113–120
- Mariller C, Allain F, Kouach M, Spik G: Evidence that human milk isolated cyclophilin B corresponds to a truncated form. *Biochim Biophys Acta* 1996, 1293:31–38
- Le Hir M, Su Q, Weber L, Woerly G, Granelli-Piperno A, Ryffel B: In situ detection of cyclosporin A: evidence for nuclear localization of cyclosporine and cyclophilins. *Lab Invest* 1995, 73:727–733
- Price ER, Jin M, Lim D, Pati S, Walsh CT, McKeon FD: Cyclophilin B trafficking through the secretory pathway is altered by binding of cyclosporin A. *Proc Natl Acad Sci USA* 1994, 91:3931–3935
- Klappa P, Freedman RB, Zimmermann R: Protein disulphide isomerase and a luminal cyclophilin-type peptidyl prolyl cis-trans isomerase are in transient contact with secretory proteins during late stages of translocation. *Eur J Biochem* 1995, 232:755–764
- Yurchenko V, O'Connor M, Dai WW, Guo H, Toole B, Sherry B, Bukrinsky M: CD147 is a signaling receptor for cyclophilin B. *Biochem Biophys Res Commun* 2001, 288:786–788
- Melchior A, Denys A, Deligny A, Mazurier J, Allain F: Cyclophilin B induces integrin-mediated cell adhesion by a mechanism involving CD98-dependent activation of protein kinase C-delta and p44/42 mitogen-activated protein kinases. *Exp Cell Res* 2008, 314:616–628
- Pakula R, Melchior A, Denys A, Vanpouille C, Mazurier J, Allain F: Syndecan-1/CD147 association is essential for cyclophilin B-induced activation of p44/42 mitogen-activated protein kinases and promotion of cell adhesion and chemotaxis. *Glycobiology* 2007, 17:492–503
- Fernandes F, Poole DS, Hoover S, Middleton R, Andrei AC, Gerstner J, Striker R: Sensitivity of hepatitis C virus to cyclosporine A depends on nonstructural proteins NS5A and NS5B. *Hepatology* 2007, 46:1026–1033
- Heitman J, Cullen BR: Cyclophilin B escorts the hepatitis C virus RNA polymerase: a viral achilles heel? *Mol Cell* 2005, 19:145–146
- Ryczyn MA, Reilly SC, O'Malley K, Clevenger CV: Role of cyclophilin B in prolactin signal transduction and nuclear retrotranslocation. *Mol Endocrinol* 2000, 14:1175–1186
- Ryczyn MA, Clevenger CV: The intranuclear prolactin/cyclophilin B complex as a transcriptional inducer. *Proc Natl Acad Sci USA* 2002, 99:6790–6795
- Robida JM, Nelson HB, Liu Z, Tang H: Characterization of hepatitis C virus subgenomic replicon resistance to cyclosporine in vitro. *J Virol* 2007, 81:5829–5840
- Du P, Kibbe WA, Lin SM: lumi: a pipeline for processing Illumina microarray. *Bioinformatics* 2008, 24:1547–1548
- Lin SM, Du P, Huber W, Kibbe WA: Model-based variance-stabilizing transformation for Illumina microarray data. *Nucleic Acids Res* 2008, 36:e11
- Smyth GK: Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004, 3:Article3
- Ganter B, Giroux CN: Emerging applications of network and pathway analysis in drug discovery and development. *Curr Opin Drug Discov Devel* 2008, 11:86–94
- Fang F, Antico G, Zheng J, Clevenger CV: Quantification of PRL/Stat5 Signaling with a Novel pGL4-CISH Reporter. *BMC Biotechnol* 2008, 8:11
- Miller SL, Antico G, Raghunath PN, Tomaszewski JE, Clevenger CV: Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells. *Oncogene* 2007, 26:4668–4678
- Malek D, Gust R, Kleuser B: 17-Beta-estradiol inhibits transforming-growth-factor-beta-induced MCF-7 cell migration by Smad3-repression. *Eur J Pharmacol* 2006, 534:39–47
- McHale K, Tomaszewski JE, Puthiyaveetil R, Livolsi VA, Clevenger CV: Altered expression of prolactin receptor-associated signaling proteins in human breast carcinoma. *Mod Pathol* 2008, 21:565–571
- Walter-Yohrling J, Cao X, Callahan M, Weber W, Morgenbesser S, Madden SL, Wang C, Teicher BA: Identification of genes expressed in malignant cells that promote invasion. *Cancer Res* 2003, 63:8939–8947
- Davies BR, O'Donnell M, Durkan GC, Rudland PS, Barraclough R, Neal DE, Mellon JK: Expression of S100A4 protein is associated with metastasis and reduced survival in human bladder cancer. *J Pathol* 2002, 196:292–299
- Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G: Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000, 25:25–29
- Breen EC, Tang K: Calcylin (S100A6) regulates pulmonary fibroblast proliferation, morphology, and cytoskeletal organization in vitro. *J Cell Biochem* 2003, 88:848–854
- Weston K: Reassessing the role of C-MYB in tumorigenesis. *Oncogene* 1999, 18:3034–3038
- Yang JM, O'Neill P, Jin W, Foty R, Medina DJ, Xu Z, Lomas M, Arndt GM, Tang Y, Nakada M, Yan L, Hait WN: Extracellular matrix metalloproteinase inducer (CD147) confers resistance of breast cancer cells to Anoikis through inhibition of Bim. *J Biol Chem* 2006, 281: 9719–9727
- Katzenellenbogen BS, Katzenellenbogen JA: Estrogen receptor transcription and transactivation: estrogen receptor alpha and estrogen receptor beta: regulation by selective estrogen receptor modulators and importance in breast cancer. *Breast Cancer Res* 2000, 2:335–344
- Katzenellenbogen BS, Montano MM, Le Goff P, Schodin DJ, Kraus WL, Bhardwaj B, Fujimoto N: Antiestrogens: mechanisms and actions in target cells. *J Steroid Biochem Mol Biol* 1995, 53:387–393
- Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tjujague M, Strom A, Treuter E, Warner M, Gustafsson JA: Estrogen

- receptors: how do they signal and what are their targets. *Physiol Rev* 2007, 87:905–931
41. Clevenger CV, Furth PA, Hankinson SE, Schuler LA: The role of prolactin in mammary carcinoma. *Endocr Rev* 2003, 24:1–27
42. Bonneterre J, Peyrat JP, Beuscart R, Demaille A: Biological and clinical aspects of prolactin receptors (PRL-R) in human breast cancer. *J Steroid Biochem Mol Biol* 1990, 37:977–981
43. Murphy LJ, Murphy LC, Vrhovsek E, Sutherland RL, Lazarus L: Correlation of lactogenic receptor concentration in human breast cancer with estrogen receptor concentration. *Cancer Res* 1984, 44:1963–1968
44. Ormandy CJ, Hall RE, Manning DL, Robertson JF, Blamey RW, Kelly PA, Nicholson RI, Sutherland RL: Coexpression and cross-regulation of the prolactin receptor and sex steroid hormone receptors in breast cancer. *J Clin Endocrinol Metab* 1997, 82:3692–3699
45. Clevenger CV, Chang WP, Ngo W, Pasha TL, Montone KT, Tomaszewski JE: Expression of prolactin and prolactin receptor in human breast carcinoma. Evidence for an autocrine/paracrine loop. *Am J Pathol* 1995, 146:695–705
46. Ederly M, Imagawa W, Larson L, Nandi S: Regulation of estrogen and progesterone receptor levels in mouse mammary epithelial cells grown in serum-free collagen gel cultures. *Endocrinology* 1985, 116:105–112
47. Schultz JR, Petz LN, Nardulli AM: Estrogen receptor alpha and Sp1 regulate progesterone receptor gene expression. *Mol Cell Endocrinol* 2003, 201:165–175
48. Frasor J, Gibori G: Prolactin regulation of estrogen receptor expression. *Trends Endocrinol Metab* 2003, 14:118–123
49. Jenkinson SR, Barraclough R, West CR, Rudland PS: S100A4 regulates cell motility and invasion in an in vitro model for breast cancer metastasis. *Br J Cancer* 2004, 90:253–262
50. de Silva Rudland S, Martin L, Roshanlall C, Winstanley J, Leinster S, Platt-Higgins A, Carroll J, West C, Barraclough R, Rudland P: Association of S100A4 and osteopontin with specific prognostic factors and survival of patients with minimally invasive breast cancer. *Clin Cancer Res* 2006, 12:1192–1200
51. Brattsand G: Correlation of oncoprotein 18/stathmin expression in human breast cancer with established prognostic factors. *Br J Cancer* 2000, 83:311–318
52. Guerin M, Sheng ZM, Andrieu N, Riou G: Strong association between c-myc and oestrogen-receptor expression in human breast cancer. *Oncogene* 1990, 5:131–135
53. Gudas JM, Klein RC, Oka M, Cowan KH: Posttranscriptional regulation of the c-myc proto-oncogene in estrogen receptor-positive breast cancer cells. *Clin Cancer Res* 1995, 1:235–243
54. Drabsch Y, Hugo H, Zhang R, Dowhan DH, Miao YR, Gewirtz AM, Barry SC, Ramsay RG, Gonda TJ: Mechanism of and requirement for estrogen-regulated MYB expression in estrogen-receptor-positive breast cancer cells. *Proc Natl Acad Sci USA* 2007, 104:13762–13767
55. Maelandsmo GM, Florenes VA, Mellingsaeter T, Hovig E, Kerbel RS, Fodstad O: Differential expression patterns of S100A2, S100A4 and S100A6 during progression of human malignant melanoma. *Int J Cancer* 1997, 74:464–469
56. Murphy LC, Murphy LJ, Tsuyuki D, Duckworth ML, Shiu RP: Cloning and characterization of a cDNA encoding a highly conserved, putative calcium binding protein, identified by an anti-prolactin receptor antiserum. *J Biol Chem* 1988, 263:2397–2401
57. Ren S, Cai HR, Li M, Furth PA: Loss of Stat5a delays mammary cancer progression in a mouse model. *Oncogene* 2002, 21:4335–4339
58. Sultan AS, Xie J, LeBaron MJ, Ealley EL, Nevalainen MT, Rui H: Stat5 promotes homotypic adhesion and inhibits invasive characteristics of human breast cancer cells. *Oncogene* 2005, 24:746–760
59. Nevalainen MT, Xie J, Torhorst J, Bubendorf L, Haas P, Kononen J, Sauter G, Rui H: Signal transducer and activator of transcription-5 activation and breast cancer prognosis. *J Clin Oncol* 2004, 22:2053–2060
60. Zheng J, Koblinski JE, Dutson LV, Feeney YB, Clevenger CV: Prolyl isomerase cyclophilin A regulation of Jak2 and the progression of human breast cancer. *Cancer Res* 2008, 68:7769–7778
61. Bieche I, Maucuer A, Laurendeau I, Lachkar S, Spano AJ, Frankfurter A, Levy P, Manceau V, Sobel A, Vidaud M, Curmi PA: Expression of stathmin family genes in human tissues: non-neural-restricted expression for SCLIP. *Genomics* 2003, 81:400–410
62. Ebihara T, Endo R, Kikuta H, Ishiguro N, Ma X, Shimazu M, Otoguro T, Kobayashi K: Differential gene expression of S100 protein family in leukocytes from patients with Kawasaki disease. *Eur J Pediatr* 2005, 164:427–431