Identification of protein targets for microRNAs (miRNAs) is a significant challenge due to the complexity of miRNA-mediated regulation. We have previously demonstrated that miR-193b targets estrogen receptor-α (ERα) and inhibits estrogen-induced growth of breast cancer cells. Here, we applied a high-throughput strategy using quantitative iTRAQ (isobaric tag for relative and absolute quantitation) reagents to identify other target proteins regulated by miR-193b in breast cancer cells.

iTRAQ analysis of pre-miR-193b transfected MCF-7 cells resulted in identification of 743 unique proteins, of which 39 were down-regulated and 44 up-regulated as compared with negative control transfected cells. Computationally predicted targets of miR-193b were highly enriched (sevenfold) among the proteins whose level of expression decreased after miR-193b transfection. Only a minority of these (13%) showed similar effect at the mRNA level illustrating the importance of post-transcriptional regulation. The most significantly repressed proteins were selected for validation experiments. These data confirmed 14–3-3ζ (YWHAZ), serine hydroxyl transferase (SHMT2), and aldo-keto reductase family 1, member C2 (AKR1C2) as direct, previously uncharacterized, targets of miR-193b. Functional RNAi assays demonstrated that specific combinations of knockdowns of these target genes by siRNAs inhibited growth of MCF-7 cells, mimicking the effects of miR-193b overexpression. Interestingly, the data imply that besides targeting ERα, the miR-193b effects include suppression of the local production of estrogens and other steroid hormones mediated by the AKR1C2 gene, thus provoking two separate molecular mechanisms inhibiting steroid-dependent growth of breast cancer cells.

In conclusion, we present here a proteomic screen to identify targets of miR-193b, and a systems biological approach to mimic its effects at the level of cellular phenotypes. This led to the identification of multiple genes whose combinatorial knock-down likely mediates the strong anti-cancer effects observed for miR-193b in breast cancer cells. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.005322, 1–9, 2011.

MicroRNAs (miRNAs) regulate gene expression post-translationally by binding primarily to the 3’ untranslated region (3’UTR) of their target mRNAs, resulting in mRNA destabilization or translational repression (1, 2). Genes encoding 1048 human miRNAs have so far been identified (miRBase v.16.0) (3), and miRNAs are predicted to regulate the expression of up to 60% of all human protein-encoding genes (4). A large number of bioinformatic methods have been developed for miRNA target prediction (1), but these are still highly unspecific and inaccurate because miRNAs typically contain an imperfect match to their target sequences. In addition, a single miRNA can target hundreds of proteins and a single protein can be influenced by multiple miRNAs (1, 5, 6). Hence, comprehensive understanding of the phenotypic effects of miRNAs at the level of the entire cell is currently difficult.

mRNA profiling by microarrays has been widely used for miRNA target identification, but microarrays only detect the effects of miRNAs at the transcriptional level, and will miss targets repressed solely at the translational level. However, compared with mRNA profiling techniques changes in protein levels are difficult to assess in a high-throughput fashion. Recently, we introduced a protein lysate microarray technique for high-throughput identification of miRNAs targeting estrogen receptor-α (ERα) protein (7). In the present study, we have carried out a systematic analysis of target protein and mRNA levels in living cells after transfection with a pre-miRNA construct.
struct leading to forced up-regulation of the miRNA molecule. We compared quantitative proteomics data obtained by using iTRAQ (isobaric tag for relative quantitation) reagents with mRNA profiling data obtained from microarrays to characterize miRNA targets in breast cancer cells. We focused on dissecting the effects of miR-193b, which is known to directly target ERα, and to inhibit growth of breast cancer cells by inducing a cell cycle arrest in G₁ phase (7). Using iTRAQ based quantitative mass spectrometry we found that the expression of 39 out of 743 investigated proteins was repressed by miR-193b, and that the predicted miR-193b targets were highly enriched among the down-regulated proteins. The impact of miR-193b on the protein levels of key targets were validated, and individual siRNA mediated knock-downs were performed to demonstrate that specific combinations of these could mimic the phenotypic effects observed after miR-193b transfection.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—MCF-7 cells were obtained from Interlab Cell Line Collection (ICLC, Italy), and cultured in Dulbecco’s modified Eagles medium (DMEM) (1 g/l glucose) supplemented with 10% fetal bovine serum (FBS), 2 ml L-glutamine and 1% penicillin/ streptomycin. Cells were regularly screened for Mycoplasma with PCR using Mycoplasma Plus™ PCR Primer Set from Agilent Technologies Inc. (Santa Clara, CA).

Human Pre-miR™ miRNA Precursors, Anti-miR™ inhibitors, and an siRNA for human estrogen receptor-α (GGCCGAAUUC-GAUAUCGGTT) were purchased from Ambion (Austin, TX). miRCURY LNA™ inhibitor for miR-193b was obtained from Exiqon (Vedbaek, Denmark). siRNAs for YWHAZ (SI00764813), SHMT2 (SI03155985), PTPLB (SI02653084), KIF11 (SI02653693), and AllStars Negative Control were purchased from Qiagen Inc. (Valencia, CA).

For iTRAQ labeling, 100 µg of proteins were precipitated with six volumes of cold acetone at −20 °C for 2 h. Protein digestion and labeling were done according to ITRAQ 4-plex Reagent Kit (Applied Biosystems, Foster City, CA). Protein pellets were suspended in dissolution buffer (0.5 M triethylammonium bicarbonate), and denatured and disulfide bonds were cleaved with 50 mM Tris-(2-carboxyethyl)phosphine. Each sample was then digested by 10 µg of sequence grade modified trypsin (Promega Corp, Madison, WI) in the lysis buffer before use. Unsolubilized material was removed by centrifugation at +4 °C at 15,000 × g for 20 min. Protein concentration was measured by DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

For iTRAQ labeling, 100 µg of proteins were precipitated with six volumes of cold acetone at −20 °C for 2 h. Protein digestion and labeling were done according to iTRAQ 4-plex Reagent Kit (Applied Biosystems, Foster City, CA). Protein pellets were suspended in dissolution buffer (0.5 M triethylammonium bicarbonate), and denatured and disulfide bonds were cleaved with 50 mM Tris-(2-carboxyethyl)phosphine. Each sample was then digested by 10 µg of sequence grade modified trypsin (Promega) at +37 °C overnight, and labeled with the iTRAQ reagents.

Before pl-based peptide prefraccionation, the samples were desalted by C18 Empore disks (3 µ) and dried peptides were dissolved in IPG strip rehydration solution (4 µl urea, 2% (v/v) IPG buffer pH 3–10). Peptides were loaded on a 13-cm IPG strip with a pH gradient of 3–10. Electrofocusing of peptides was performed with IPGphor (Amersham Biosciences, GE Healthcare, Piscataway, NJ) until 20 kVh. After focusing, the strip was cut into 12 fractions, peptides were extracted as described previously (8) and desalted again to remove urea.

**LC-ESI/MS/MS and Data Analysis**—Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) analyses were performed on a nanoflow HPLC system (Famos, Switchos and Ultimate, LC Packings/Dionex, Sunnyvale, CA) coupled to a QSTAR Pulsar i mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada). Peptides were first loaded on a precolumn (0.3 × 5 mm PepMap C18, LC Packings) and subsequently separated on an in-line 15 cm C18 column (75 µm × 15 cm, Magic µm 100 Å C18, Michrom BioResources Inc., Sacramento, CA). A linear 120 min gradient (from 2% to 34% acetonitrile) was used to elute peptides. Data acquisition parameters suggested by Applied Biosystems were used.

Protein identification and quantitation were carried out using the ProteinPilot software (version 2.0.1, Applied Biosystems, MDS-Sciex) with the Paragon™ search algorithm (9). The software compares relative intensity of proteins present in samples based on the intensity of reporter ions released from each labeled peptide and automatically calculates protein ratios and p values for each protein. The data from 12 liquid chromatography tandem MS (LC-MS/MS) analyses were merged and searched against combined human Swiss-Prot (version 54.6) - TrEMBL (version 37.6) protein sequence database containing 76,122 protein entries. The following search parameters were used: iTRAQ 4-plex as the sample type, digestion with trypsin, and cysteine alkylation with methyl methanethiosulfate. The precursor and fragment ion mass accuracy, number of missed cleavages permitted, fixed and variable modifications as well the peak list generating parameters are built-in functions of ProteinPilot. For protein identification, 95% confidence was used. Only those proteins with p < 0.05 were accepted for quantitation and further validation experiments.

**Western Blot Validation of iTRAQ Data**—Aliquots of total cell lysates were fractionated on SDS-polyacrylamide gels and transferred to Whatman Protran nitrocellulose membrane (Schleicher & Schuell, Whatman Inc, Florham Park, NJ). The membranes were blocked against nonspecific binding using 5% skim milk. Membranes were probed with specific antibodies for YWHAZ (Santa Cruz Biotechnology Inc., Santa Cruz, CA), AKR1C2 (Abnova, Taipei, Taiwan), MCM7 (Cell Signaling Technology, Danvers, MA), SHMT2 (Abcam, Cambridge, UK), HSP90α (Cell Signalning Technology) and PTPLB (Abnova). Equal loading was confirmed by probing the same membrane with a specific antibody for human β-actin (Sigma-Aldrich). The blots were visualized by enhanced chemiluminescence (ECL) detection system (Pierce, Thermo Scientific, Rockford, IL).

**Real-time Quantitative PCR Analysis**—Total cellular RNAs were isolated with miRNA™ Total RNA isolation kit (Ambion Inc.). For cDNA synthesis, 200 ng of total RNA was reverse transcribed with High Capacity cDNA Reverse Transcription kit (Bio-Rad Laboratories, Hercules, CA). Thereafter, the cDNAs were diluted 1/10 and the Taqman quantitative real-time PCR (qRT-PCR) analysis performed with Applied Biosystems 7900HT instrument using specific primers and probes for YWHAZ, SHMT2, AKR1C2, PTPLB, MCM7, HSP90αB1, and glyceroldehyde 3-phosphate-dehydrogenase (GAPDH) designed by the Universal Probe Library Assay Design Center (Roche Applied Biosciences, Basel, Switzerland) (supplementary Table S1). The results were analyzed with SDS 2.3 and RQ manager softwares (Applied Biosystems), and the expression of the miRNAs determined by relative quantitation method using GAPDH as an endogenous control. The data were collected from three separate biological experiments, which were each run with triplicates.

**UTR Reporter Constructs and Luciferase Assays**—Fragments covering the wild type and mutated miR-193b target sites in the YWHAZ, SHMT2, PTPLB, and MTHFD1 3’ untranslated region (UTR) regions, and AKR1C2 5’UTR region (supplementary Table S2) were cloned into the Spe/MluI sites of a pMiR-REPORT Luciferase vector (Am-
bion Inc.) downstream of a luciferase gene. For luciferase assays, MCF-7 cells (15,000 per well) were plated 24 h before transfection onto white, clear-bottom 96-well plates in normal culture medium without antibiotics. The cells were cotransfected with 50 ng of the 3’UTR reported plasmids, 50 ng Renilla luciferase plasmid, and with 50 nM pre-miR construct with Lipofectamine 2000 (Invitrogen Inc.) as previously described (7). The luciferase activity was assayed 24 h after transfection with Dual-Glo Luciferase Assay System (Promega Corp, Madison, WI) and measured with Envision Plate-reader (Perkin Elmer Inc, Wellesley, MA).

**Proliferation Assays**—For assaying the cell growth, MCF-7 cells (8000/well) were reverse transfected with 5 nM, 10 nM, and 20 nM miRNAs or siRNAs in 96-well plates using SiLentFect (Bio-Rad). The growth curves were generated during 88 h period of time by using the INCUCYTE™ Live-Cell Imaging System (Essen BioSciences, Ann Arbor, MI), which provides a time-lapse method for quantifying cell growth inside the cell culture incubator. Cell viability was measured with CellTiter-Glo Cell Viability Assay (Promega), as previously described (7). The lists of predicted targets from each prediction program were compared with the lists of miR-193b regulated genes separately. Enrichment of the predictions was calculated by comparing the proportion of predicted targets among the miR-193b down-regulated transcripts to predicted targets among all proteins detected in the control sample, or by comparing the proportion of predicted targets among the miR-193b down-regulated transcripts to predicted targets among all genes on the array.

**RESULTS**

**Identification of miR-193b Targets by iTRAQ Proteomics**—We have previously shown, that miR-193b directly targets ERα, induces cell cycle arrest, and inhibits growth when overexpressed in breast cancer cells (7) (supplementary Fig. S1). To study the effects of miR-193b in MCF-7 cells in more detail and to identify additional protein targets for miR-193b, we used a proteomics approach. For that purpose, MCF-7 cells were transfected with a pre-miR construct for miR-193b and with a negative control scrambled pre-miR, and incubated for 48 h. Thereafter, the cells were harvested, lyzed, and subjected to iTRAQ labeling. Relative protein expression differences between negative control pre-miR and pre-miR-193b-transfected MCF-7 cells were then measured by mass spectrometry (Fig. 1A). Protein identification and quantitation were done using the ProteinPilot software (version 2.0.1) with the Paragon™ search algorithm (9). The software compares relative intensity of proteins present in samples based on the intensity of reporter ions released from each labeled peptide and automatically calculates protein ratios and p values for each protein. For protein identification, 95% confidence was used. Only those proteins with p < 0.05 were accepted for quantitation and further validation experiments.

With iTRAQ, a total of ~1000 proteins were identified, and 743 of those were unique, common proteins in two biological replicate analyses. Of those, 39 were down-regulated and 44 up-regulated 48 h post miR-193b transfection (Fig. 1B and supplementary Tables S3 and S4). We next compared the protein data with gene expression microarray data obtained from our previous study, where miR-193b targets were analyzed at the mRNA level 24 h post-transfection (7). For the microarray experiment, 24 h time point was selected in order to detect the more rapidly occurring changes at the mRNA level and to avoid indirect changes occurring at later time points. Most of the identified proteins (96%) were detected also at the mRNA level (Fig. 1B). However, only five (13%) of the candidate protein targets for miR-193b (SHMT2, YWHAZ, HN1L, PIP4K2C, and PTPL) were significantly (>twofold) inhibited at the mRNA level according to the microarray profiling data from Leivonen et al. 2009 (7), and target predictions from TargetScan, PicTar, and miRBase. IEF, isoelectric focusing; LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry.

**Fig. 1. Identification of novel targets for miR-193b by proteomic expression profiling with iTRAQ tagging.** A. Workflow for identification of differentially expressed proteins between pre-miR-193b transfected and pre-miR negative control transfected MCF-7 cells (48 h post-transfection). B. Overview of the iTRAQ data obtained after miR-193b overexpression, compared with microarray profiling data from Leivonen et al. 2009 (7), and target predictions from TargetScan, PicTar, and miRBase. IEF, isoelectric focusing; LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry.
yses for the proteomics data using the Molecular Signatures Database (MSigDB), and the results indicated that, in addition to the ERα signaling, cell cycle related pathways and genes are significantly enriched among miR-193b target proteins (supplementary Table S5).

Next, we tested how miRNA target predictions correlate with our data. miR-193b target predictions were obtained with three different algorithms: TargetScan (10), PicTar (11), and miRBase (3). As shown in Fig. 2A, the predicted targets for miR-193b were highly enriched (sevenfold) among the down-regulated proteins, as compared with all the proteins identified in a control MCF-7 sample. Similarly, in the microarray data, the predicted targets were highly enriched (5.5-fold) among the transcripts down-regulated by miR-193b, when compared with all genes on the array (Fig. 2B).

Validation of Putative miR-193b Targets by Western Blot and qRT-PCR Analyses—For validation experiments, we selected proteins that were identified in two replicate iTRAQ assays with a 95% confidence for protein identification (p <0.05 in both replicates), as calculated by the ProteinPilot software (Table I). Four of the most significantly down-regulated proteins (YWHAZ, SHMT2, PTPLB, and MTHFD1) were predicted to be targeted by miR-193b by TargetScan or miRBase, and three of those (YWHAZ, SHMT2, and PTPLB) were also among the most prominently down-regulated genes at the mRNA level (Table I). Western blot analyses of the selected proteins corroborated the potent down-regulation of AKR1C2, YWHAZ, PTPLB, SHMT2, MCM7, and HSP90AB1 (HSP90β) proteins by miR-193b 48 h after transfection (Fig. 3A). qRT-PCR analyses showed that miR-193b down-regulated AKR1C2, YWHAZ, PTPLB, SHMT2, MCM7, and HSP90AB1 also at the mRNA level (Fig. 3B). These data were mainly in line with the microarray data, except for HSP90AB1, whose transcript was not down-regulated in the microarray analysis (Table I).

YWHAZ, SHMT2, and AKR1C2 are Direct Targets of miR-193b—YWHAZ, SHMT2, PTPLB, and MTHFD1 contain predicted miR-193b target sites at their 3’UTR region, whereas AKR1C2 contains a putative miR-193b site in its 5’UTR region (Fig. 4A). To analyze whether miR-193b directly interacts with these predicted sites, we subcloned fragments covering the miR-193b binding sites into a luciferase reporter vector. In addition, we designed constructs with mutated miR-193b target sites. The resulting 3’UTR-luciferase reporter plasmids were transfected in MCF-7 cells together with pre-miR-193b and with a Renilla luciferase control plasmid. Transfection of miR-193b significantly inhibited the reporter activity of SHMT2 and YWHAZ 3’UTR and AKR1C2 5’UTR wild-type constructs, as compared with pre-miR negative controls (Fig. 4B). In contrast, miR-193b had no effect on the luciferase activity of the mutated constructs, indicating that miR-193b directly targets YWHAZ and SHMT2 by interacting with their 3’UTR, and AKR1C2 by interacting its 5’UTR. The luciferase activities of the PTPLB or MTHFD1 3’UTR constructs were not affected by miR-193b, indicating that miR-193b does not target its predicted sites in the PTPLB or MTHFD1 3’UTR.

To further study the importance of miR-193b in regulating SHMT2, AKR1C2, and YWHAZ, we inhibited miR-193b with specific anti-miRNAs and analyzed the effect on the protein level 96 h after transfection. As shown in Fig. 4C, miR-193b inhibition by 5 nM anti-miR-193b or LNA-anti-miR-193b resulted in up-regulation of SHMT2 and AKR1C2 protein levels (by 1.6- to 1.8-fold). With YWHAZ, the change was modest (1.4-fold), but consistently observed. Conceivably, there are also other factors regulating YWHAZ and inhibition of miR-193b is not enough to up-regulate YWHAZ.

siRNA-mediated Knockdown of miR-193b Regulated Genes Inhibits Breast Cancer Cell Growth—We have previously shown that miR-193b potently suppresses the growth and viability of MCF-7 cells in vitro ((7) and supplementary Fig. S1). To study
TABLE I

Most significant, replicating downregulated proteins identified in iTRAQ. As a selection criteria, protein confidence p/H11021 > 0.05 (analyzed by Protein Pilot software v.2.0.1) was used. The table includes gene symbol, accession number, protein name, ratios, number of peptides used for quantitation, and p values of expression between scrambled negative control miRNA (Scr) and miR-193b transfected samples, percent coverage (95%) (%Cov(95)), mRNA expression (fc, fold change scrambled vs. miR-193b), and miR-193b target predictions for the proteins. Target predictions were obtained with TargetScan, PicTar, and miRBase, and mRNA data from Leivonen et al. 2009 (7).

| Gene Symbol | Accession | Protein name | Replicate I | Replicate II |
|-------------|-----------|--------------|-------------|--------------|
|            |           | miRNA fc     | mRNA fc     |
|            |           | (mean)       | (mean)      |
|            |           | p-val         | p-val       |
|            |           | %Cov(95)      | %Cov(95)    |
| miR-193b:Scr | miR-193b:Scr |              |             |
| AKR1C2     | Q5SR16    | Aldo-keto reductase family 1, member C2 | 0.699 | 0.708 |
| MCM7       | P33993    | DNA replication licensing factor MCM7 | 0.709 | 0.708 |
| Q9Y2Z6     | SHMT2     | CGI-07 protein | 0.759 | 0.761 |
| NMD3       |           |              |             |             |
| PTPLB      | P63104    | 14–3-3 protein zeta/delta | 0.791 | 0.784 |
| YWHAZ      | P68363    | Heat shock protein HSP90 | 0.810 | 0.813 |
| SHMT2      | Q6NSA3    | DUTP pyrophosphatase-like member A | 0.832 | 0.832 |
| HSP90AB1   | P08238    | Heat shock protein HSP90 | 0.892 | 0.892 |
| MTHFD1     | P11586    | C-1-tetrahydrofolate synthase, cytoplasmic | 0.892 | 0.892 |
| TUBB3      | Q13509    | Tubulin beta-3 chain | 0.892 | 0.892 |
| TUBA1B     | P28328    | Tubulin alpha-1B chain | 0.892 | 0.892 |
| HSP90AB1   | P11586    | C-1-tetrahydrofolate synthase, cytoplasmic | 0.892 | 0.892 |
| TUBB3      | Q13509    | Tubulin beta-3 chain | 0.892 | 0.892 |
| TUBA1B     | P28328    | Tubulin alpha-1B chain | 0.892 | 0.892 |
| HSP90AB1   | P11586    | C-1-tetrahydrofolate synthase, cytoplasmic | 0.892 | 0.892 |


discussion

Identification of miRNA targets is central to understanding the biologic roles of miRNAs. Computational and experimental approaches to identify miRNA targets have proven remarkably difficult because of the complexity of the miRNA-mediated regulation. Proteomic profiling of human cells with SILAC following miRNA transfections revealed how individual miRNAs modulated the levels of hundreds of proteins (5, 6). Therefore, high-throughput methods for miRNA target identification are required.

In the present study, we have performed quantitative iTRAQ experiments in combination with gene expression microarray profiling, bioinformatic predictions, and functional assays to identify protein targets for miR-193b in breast cancer cells. miR-193b directly targets ERFα, inhibits breast cancer cell proliferation and induces cell cycle arrest in G1 (7). With the iTRAQ analysis, we identified 39 proteins to be down-regulated by miR-193b at 48 h time point. Of these, only 13% were significantly repressed at the mRNA level according to miR-193b transfection, emphasizing the importance of protein...
level detection of miRNA targets. A recent study by Guo et al. (2010) demonstrated that miRNAs predominantly act by decreasing mRNA levels as a consequence of mRNA destabilization. The discrepancies here could partly be due to different time-points used in the microarray and proteomics analyses, 24 h and 48 h, respectively. Furthermore, the steady-state levels of mRNA and protein rarely correlate directly because of complicated post-transcriptional control mechanisms (12, 13). Although proteomic strategies are currently limited to the identification of only a fraction of the cell’s proteome, they complement the microarray experiments as they offer a read-out of the functional component of the transcript activity. The exact mechanism of miRNA-mediated repression is still unclear, and a combination of both mRNA and corresponding protein data are expected to reveal deeper insights into this mode of biological regulation. Furthermore, all methods for identification of miRNA target genes are susceptible to detection of indirect targets that are repressed through secondary effects, and experimental validation of the targets is required.

The prediction algorithms mainly use 3’UTR databases as the source of predictions. Therefore, they will miss putative miRNA targeting beyond the 3’UTR regions. For instance, AKR1C2 was not predicted to be targeted by miR-193b by TargetScan, PicTar, or miRBase. Li et al. (21) have shown that during breast cancer cell metastasis, the expression of miR-193b is down-regulated, which in turn up-regulate urokinase-type plasminogen activa-

![Fig. 3. Validation of the iTRAQ data. A, Western blot showing miR-193b-mediated down-regulation of AKR1C2, MCM7, PTPLB, SHMT2, YWHAZ, and HSP90β (HSP90AB1). MCF-7 cells were transfected with miR-193b or negative control miRNA (20 nM), and incubated for 48 h. Thereafter, cell lysates were analyzed with Western blotting. Equal loading was confirmed by probing the same filters with β-actin. B, MCF-7 breast cancer cells were transfected with miR-193b or negative control miRNA and incubated for 24 h or 48 h. Thereafter, the total cellular RNAs were isolated and analyzed with Taqman qRT-PCR for the expression of AKR1C2, MCM7, PTPLB, SHMT2, YWHAZ, and HSP90AB1 mRNAs. The results normalized for GAPDH are shown as relative expression using negative control miRNA as a reference. The data were collected from three independent experiments, which were run with triplicates (Mean ± S.D.).]
YWHAZ, SHMT2, and AKR1C2 are direct targets for miR-193b. A. The complementarity of miR-193b sequence to the five down-regulated genes. B. Activity of luciferase reporters containing wild type (wt) or mutated (mut) putative miR-193b target sites in the YWHAZ, SHMT2, PTPLB, and MTHFD1 3′ UTR, or AKR1C2 5′ UTR. The reporters (50 ng) were co-transfected with Renilla luciferase control vector (50 ng) and with pre-miR-193b (50 nM), and the luciferase activities measured after 24 h incubation. Firefly luciferase activity was normalized to that of Renilla luciferase. The data shown are mean ± S.E. of at least four independent experiments, each performed with five replicates. **p < 0.01. C. The effect of miR-193b inhibition on the expression of SHMT2, AKR1C2 and YWHAZ. MCF-7 cells were transfected with negative control anti-miRNA (anti-Scr) or with miR-193b inhibitors (anti-193b or LNA-193b) at 5 nM concentrations. After 96 h incubation, cells were harvested and the cell lysates were analyzed with Western blotting. The numbers below each panel show quantitations relative to anti-Scr, which was set to 1.0.
Proteomic Identification of miR-193b Targets

Inhibition of miR-193b targets suppresses MCF-7 cell growth. A, MCF-7 cells were transfected with miR-193b or with siRNAs for YWHAZ, SHMT2, PTPLB, MCM7, AKR1C2, AKR1C1, and HSP90AB1 at 20 nM concentration alone or in different combinations, as indicated. Cell growth was monitored over 88 h period of time in Incucyte Live-Cell Imaging System, which provides a time-lapse method for quantifying cell growth inside the cell culture incubator. As a negative control, Allstars negative control siRNA (Qiagen) was used, and as a positive control, an siRNA for KIF11 was used. The data represent average of two independent assays, which were both done with three technical replicates (mean ± S.D.). B, The levels of SHMT2, YWHAZ, PTPLB, AKR1C2, and MCM7 proteins after siRNA transfections (72 h) were analyzed by Western blotting.

Our functional assays demonstrated that knocking down miR-193b targets by siRNAs phenocopied the anti-proliferative effects of miR-193b. However, with siRNA-mediated silencing potent growth inhibitory effects were mostly evident only after synergistic inhibition of multiple miR-193b target genes. This highlights the fact that miRNAs regulate the expression of several genes and their cell phenotypic effects are often not mediated by a single target.

To conclude, we present here that proteomic expression profiling is an efficient strategy to functionally identify protein expression and contribute to the development of breast cancer. In addition, miR-193b expression was related to tumor stage and tumor grade, and correlated with the metastasis-free survival of breast cancer patients. Here, the identified miR-193b targets included other known breast cancer-associated genes, such as AKR1C2 and YWHAZ. YWHAZ belongs to the 14–3-3 family of proteins, and it has been shown to be overexpressed in >40% of advanced stage breast cancers, confer cancer cell apoptosis resistance, and correlate with poor survival in breast cancer patients (22, 23). AKR1C2 belongs to the aldo-keto reductase (AKR) superfamily of enzymes which participate in the local production of steroid hormones, such as estrogens, progesterones, and androgens (24). AKRs have been suggested as potential therapeutic targets in breast cancer due to their positive effects on proliferative signaling, which stimulates the growth of hormone-dependent and -independent breast cancer. Silencing AKR1C2 and another member of the same family, AKR1C1, very efficiently inhibited MCF-7 cell growth, suggesting that these genes are essential for MCF-7 cell survival. Recently, we demonstrated that ERα signaling is directly regulated by miR-193b in breast cancer cells (7). Together, these findings suggest that miR-193b regulates estrogen signaling by multiple mechanisms: by directly inhibiting the estrogen receptor as well as regulating the production of estrogen. This results in suppression of cell growth.
targets and mechanisms of action for miRNAs that have anti-cancer effects when transfected into breast cancer cells. In addition, we provide functional data on novel targets for a breast cancer associated miRNA, miR-193b, whose effects likely involve several distinct steps of ERα signaling, such as steroid synthesis and down-regulation of the ERα receptor.

Acknowledgment—We thank Pauliina Toivonen for technical assistance.

This study was financially supported by the Academy of Finland Translational Genome-Scale Biology Center of Excellence, the Finnish Cancer Society, Sigrid Juselius Foundation, and by Academy of Finland postdoctoral researcher’s grants to SKL and PO.

To whom correspondence should be addressed: VTT Technical Research Centre of Finland, Medical Biotechnology, Itäinen Pitkäkatu 4C, P.O. Box 106, FI-20521 Turku, Finland. Tel.: +358 40 0386 010; Fax: +358 20 7222 840; E-mail: suvi-katri.leivonen@vtt.fi.

REFERENCES

1. Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions. Cell 136, 215–233
2. Guo, H., Ingolia, N. T., Weissman, J. S., and Bartel, D. P. (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 463, 835–840
3. Griffiths-Jones, S., Saini, H. K., van Dongen, S., and Enright, A. J. (2008) miRBase: tools for microRNA genomics. Nucleic Acids Res. 36, D154–D154.
4. Friedman, R. C., Farh, K. K., Burge, C. B., and Bartel, D. P. (2009) Most mammalian microRNAs are conserved targets of microRNAs. Genome Res. 19, 92–105
5. Baek, D., Villen, J., Shin, C., Camargo, F. D., Gygi, S. P., and Bartel, D. P. (2008) The impact of microRNAs on protein output. Nature 455, 64–71
6. Selbach, M., Swansonhammer, B., Thierfelder, N., Fang, Z., Kuhn, R., and Rajewsky, N. (2008) Widespread changes in protein synthesis induced by microRNAs. Nature 455, 56–63
7. Leivonen, S.-K., Mäkelä, R., Östling, P., Kohonen, P., Haapa-Paananen, S., Kivelä, K., Enery, E., Askila, A., Heliöstam, K., Sahilberg, N., Kristensen, V. N., Barresen-Dale, A. L., Saviranta, P., Perälä, M., and Kallioniemi, O. (2008) Protein lysate microarray analysis to identify microRNAs regulating estrogen receptor signaling in breast cancer cell lines. Oncogene 28, 3926–3936
8. Cargile, B. J., Talley, D. L., and Stephenson, J. L. J., Jr. (2004) Immobilized pH gradients as a first dimension in shotgun proteomics and analysis of the accuracy of pI predictability of peptides. Electrophoresis 25, 938–945
9. Shilov, I. V., Seymour, S. L., Patel, A. A., Loboda, A., Tang, W. H., Keating, S. P., Hunter, C. L., Nuwaysir, L. M., and Schaeffer, D. A. (2007) The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol. Cell Proteomics 6, 1638–1655
10. Grimmson, A., Farh, K. K., Johnston, W. K., Garrett-Engele, P., Lim, L. P., and Bartel, D. P. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol. Cell 27, 91–105
11. Krek, A., Grün, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., MacMenamin, P., da Piedade, I., Gunalas, K. C., Stoffel, M., and Rajewsky, N. (2005) Combinatorial microRNA target predictions. Nat. Genet. 37, 495–500
12. Chen, J., Colangelo, C., Williams, K., and Gerstein, M. (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol. 4, 117
13. Calaluce, R., Kubin, M. M., Davis, J. W., Magee, J. D., Chen, J., Kuwano, Y., Gorospe, M., and Atasoy, U. (2010) The RNA binding protein HuR differentially regulates unique subsets of microRNAs in estrogen receptor negative and estrogen receptor positive breast cancer. BMC Cancer 10, 26
14. Ow, S. Y., Salim, M., Noirel, J., Evans, C., Rehman, I., and Wright, P. C. (2009) ITRAQ underestimation in simple and complex mixtures: “the good, the bad and the ugly”. J Proteome Res 8, 5347–5355
15. Karp, N. A., Huber, W., Sadowski, P. G., Charles, P. D., Hester, S. V., and Lilley, K. S. (2010) Addressing accuracy and precision issues in ITRAQ quantitation. Mol. Cell Proteomics 9, 1885–1897
16. Chen, J., Feliotter, H. E., Paré, G. C., Zhang, X., Pemberton, J. G., Garady, C., Lai, D., Yang, X., and Tron, V. A. (2010) MicroRNA-193b represses cell proliferation and regulates cyclin D1 in melanoma. Am J Pathol 176, 2520–2529
17. Grey, F., Tirabassi, R., Meyers, H., Wu, G., McWeeney, S., Hook, L., and Nelson, J. A. (2010) A viral microRNA down-regulates multiple cell cycle genes through mRNA 5’UTRs. PLoS Pathog 6, e1000867
18. Lee, I., Ajay, S. S., Yook, J. I., Kim, H. S., Hong, S. H., Kim, N. H., Dhanaum, D. M., Chinnayay, A. M., and Athey, B. D. (2009) New class of microRNA targets containing simultaneous 5’-UTR and 3’-UTR interaction sites. Genome Res. 19, 1175–1183
19. Orom, U. A., Nielsen, F. C., and Lund, A. H. (2008) MicroRNA-10a binds the 5’UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell 30, 460–471
20. Lytie, J. R., Yario, T. A., and Stetiz, J. A. (2007) Target miRNAs are repressed as efficiently by microRNA-binding sites in the 5’ UTR as in the 3’ UTR. Proc. Natl. Acad. Sci. U.S.A. 104, 9667–9672
21. Li, X. F., Yan, P. J., and Shao, Z. M. (2009) Downregulation of miR-193b contributes to enhance urokinase-type plasminogen activator (uPA) expression and tumor progression and invasion in human breast cancer. Oncogene 28, 3937–3948
22. Lu, J., Guo, H., Treerakkitkornmongkol, W., Li, P., Zhang, J., Shi, B., Ling, C., Zhou, X., Chen, T., Chao, P. J., Feng, X., Seewaldt, V. L., Muller, W. J., Sahin, A., Hung, M. C., and Yu, D. (2009) 14–3–3zeta Cooperates with ErbB2 to promote ductal carcinoma in situ progression to invasive breast cancer by inducing epithelial-mesenchymal transition. Cancer Cell 16, 195–207
23. Neal, C. L., Yao, J., Yang, W., Zhou, X., Nguyen, N. T., Lu, J., Danes, C. G., Guo, H., Lan, K. H., Ensor, J., Hittleman, W., Hung, M. C., and Yu, D. (2009) 14–3–3zeta overexpression defines high risk for breast cancer recurrence and promotes cancer cell survival. Cancer Res. 69, 3425–3432
24. Penning, T. M., and Byrns, M. C. (2009) Steroid hormone transforming aldo-keto reductases and cancer. Ann. N.Y. Acad. Sci. 1155, 33–42