

# PDGF induced microRNA alterations in cancer cells

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## ABSTRACT

**Platelet derived growth factor (PDGF) regulates gene transcription by binding to specific receptors. PDGF plays a critical role in oncogenesis in brain and other tumors, regulates angiogenesis, and remodels the stroma in physiologic conditions. Here, we show by using microRNA (miR) arrays that PDGFs regulate the expression and function of miRs in glioblastoma and ovarian cancer cells. The two PDGF ligands AA and BB affect expression of several miRs in ligand-specific manner; the most robust changes consisting of *let-7d* repression by PDGF-AA and *miR-146b* induction by PDGF-BB. Induction of *miR-146b* by PDGF-BB is modulated via MAPK-dependent induction of *c-fos*. We demonstrate that PDGF regulates expression of some of its known targets (e.g. cyclin D1) through miR alterations and identify the epidermal growth factor receptor (EGFR) as a new PDGF-BB target. We show that its expression and function are repressed by PDGF-induced *miR-146b* and that *mir-146b* and EGFR correlate inversely in human glioblastomas. We propose that PDGF-regulated gene transcription involves alterations in non-coding RNAs and provide evidence for a miR-dependent feedback mechanism balancing growth factor receptor signaling in cancer cells.**

## INTRODUCTION

Two structurally similar receptor tyrosine kinases are activated by the platelet derived growth factor, PDGF (1,2). PDGF induces cell growth and survival (3), transformation (4), migration, vascular permeability and

wound healing (5). PDGF is a dimeric molecule composed of two disulfide-bound chains (A and B) (6). It binds selectively to receptor subunits, based on specific epitopes (7), PDGF-AA binding PDGFR- $\alpha$ , PDGF-AB and -BB recognizing both PDGFR- $\alpha$  and PDGFR- $\beta$  subunits (2). Two recently cloned PDGF chains (C and D) (8,9) bind the PDGFR- $\alpha$  and PDGFR- $\beta$ , respectively. PDGFR activation occurs through dimerization and autophosphorylation of tyrosine residues in the intracellular domain (10). The two PDGFRs mediate similar, but not identical processes, PDGFR- $\beta$  being more efficient in mediating chemotactic effects (11), transformation (12) and intracellular Ca<sup>++</sup> traffic (13).

Experimental studies have implicated aberrant PDGFR signaling in oncogenesis, particularly in glioblastomas (GBM) (14), sarcomas (15) and selected epithelial cancers [breast and ovarian cancer (16,17)]. PDGFR activation in cancer occurs as a consequence of gene amplification [GBMs (14)], chromosomal rearrangements (18) or activating mutations (19). Activation of PDGFR in tumors can also occur through autocrine or paracrine stimulation (20–23) as both tumor and normal cells in the stroma secrete PDGF. Work in our laboratory demonstrated that PDGFR and its ligands are expressed in ovarian tumors, PDGF is detectable in malignant ascites, and the PDGFR is activated by tumor cell-secreted ligand (24). Autocrine PDGFR activation was also described in brain tumors as an important step to tumor progression (21,25). PDGF signaling in neural stem cells was linked to tumor initiation (26) and autocrine PDGF stimulation of de-differentiated astrocytes induced gliomagenesis (21,23). Autocrine activation of the receptor may contribute to adverse clinical outcome in ovarian (16) and other tumors (22,27,28). PDGFR stimulation leads to activation of intracellular signaling, particularly of Akt, Src and ras/MAPK1/2, with

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significant cross-talk documented between these pathways (17,24,29). The mechanisms accounting for differences between effects elicited by distinct PDGF ligands are not well understood, having been previously attributed to cell-specific contexts.

MicroRNAs (miR) are single stranded 16–24 nt regulatory RNAs that repress target gene expression by inhibiting translation or by promoting target mRNA degradation. Emerging evidence implicates miRs in human cancer, where they act either as oncogenic factors (through repression of tumor suppressor genes) or as tumor suppressors (30,31). As each miR targets multiple genes, it is predicted that ~30–40% of genes deregulated in human cancer are under the control of cancer-associated microRNAs (32). Through their effects on gene expression in cancer cells, miRs regulate key cellular mechanisms such as cell differentiation, survival, proliferation and metabolism (33). miR dysregulation in tumor cells may occur constitutively (through deletion, amplification of chromosomal fragments or mutations) (34) or as consequence of micro-environmental factors (e.g. hypoxia, cytokines, estrogens) (35–37). The goal of this study was to identify miRs regulated by PDGFs and their functions in cancer cells.

Here we identify non-coding RNAs (ncRNAs) specifically regulated by each of the two PDGF ligands, AA and BB. We show that PDGF-BB, but not AA, induces *miR-146b* expression through a Fos-dependent mechanism and that in turn, this miR regulates the expression of the epidermal growth factor receptor, the EGFR. We provide evidence that a ncRNA modulates the balance between two growth factor receptor pathways in cancer cells (PDGFR and EGFR), suggesting the existence of a feedback loop that allows cancer cells to use one pathway selectively over the other. We propose that alterations in miR expression represent one potential mechanism by which PDGFs regulate target gene expression (e.g. *cyclin D1* and *EGFR*). Other miRs induced or repressed by PDGFs and their effects on gene transcription are identified and discussed.

## MATERIALS AND METHODS

### Materials and cell lines

PDGF BB, PD98059 and LY294002 were from Sigma (St Louis, MO, USA), PDGF AA from PeproTech (Rocky Hill, NJ, USA) and EGF and VEGF from R&D (Minneapolis, MN). All cells were treated with PDGF BB and AA at 25 ng/ml unless otherwise specified and with EGF at 20 and 150 ng/ml. U118-MG, U87-MG glioblastoma cells, and human WI-38 fibroblasts (ATCC, Manassas, VA, USA) were grown according to ATCC instructions. The immortalized ovarian cells C272/hTert/E7 were previously described (38) and grown in a 1:1 mixture of MCDB 105 (Sigma) and M199 media (Cellgro, Herndon, VA, USA) supplemented with FBS. Neuroblastoma cells SHSY-5Y were from Dr Carol Thiele of the National Cancer Institute (39). Nineteen de-identified snap-frozen human glioblastoma specimens were acquired from the Indiana University Cancer Center

Tissue Bank and from the Brain Tumor Tissue Bank (BTTB) of London Health Sciences Centre (London, ON, Canada). Their use was approved by the Institutional Review Board.

### MicroRNA microarrays and statistical analysis

RNA extracted from cells stimulated with PDGF or control was biotin labeled and hybridized to custom made microRNA chips containing 900 probes for human mature and precursor miRs; 700 probes for mouse miRs, 900 probes for ultraconserved sequences and controls (array express accession, A-MEXP-1246). All probes on the array were duplicated, and average values were used for the analysis. Two biological replicates for each time-point (0, 2, 6, 12 and 24 h) were analyzed. Data were normalized using quantiles algorithm ([www.bioconductor.org](http://www.bioconductor.org)). Absent calls were assigned at a threshold of 4.5 (log<sub>2</sub>) before statistical analysis. *miR* probes were excluded from analysis if <20% of expression data values had at least a 1.5-fold change in either direction from the miR median value. Two-sample *t*-test (with random variance model) utilizing the Biometric Research Branch (BRB) array tool version 3.6.0 (<https://linus.nci.nih.gov/BRB-ArrayTools.html>) was used to perform the class comparison between unstimulated and PDGF-stimulated samples or between PDGF-AA and PDGF-BB treated samples. miRs significant at the 0.01 level (two-sided) of the univariate test are reported. Hierarchical clustering was performed by using the expression of the differentially regulated miRs across experiments. The mean was computed over all samples from the same class. MiRs and arrays were mean-centered and normalized by using GENE CLUSTER 3.0. Average linkage clustering was performed by using Euclidean distance and displayed using TreeView program (<http://rana.lbl.gov/EisenSoftware.htm>). Clustering trees display average expression values after log<sub>2</sub> transformation. Principal component analysis (PCA) used unsupervised and supervised classification algorithms from Partek Genomics Suite (<http://www.partek.com/>) of normalized and filtered data.

### Immunoblotting

Actively growing cells were lysed into RIPA buffer containing leupeptin (1 µg/ml), aprotinin (1 µg/ml), PMSF (400 µM) and Na<sub>3</sub>VO<sub>4</sub> (1 mM). Cell lysates were sonicated and incubated on ice for 30 min. Cellular debris was removed by centrifugation for 15 min. Equal amounts of protein were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane and treated with primary antibodies against phospho-PDGFRα (Santa Cruz, sc-12911-R), PDGFRα (Cell Signaling, #3164), phospho-PDGFRβ (Cell Signaling, #3166S), PDGFRβ (Santa Cruz, sc-339), phospho-AKT (Cell Signaling, #4060X), phospho-MAPK-P42/44 (Cell Signaling, #9101S), phospho-EGFR (Cell Signaling, #2236S), EGFR (Santa Cruz, sc-03), c-fos (Santa Cruz, sc-52), cyclin D1 (Cell Signaling, #2922) and GAPDH (Santa Cruz) during an overnight incubation at 4°C. After incubation with corresponding HRP-labeled secondary antibodies, enhanced chemiluminescence was used

for protein visualization. Western blots were repeated in independent conditions at least twice.

### Quantitative real-time reverse transcription-PCR

Mature miR expression was quantified by qPCR from total RNA extracted from cell cultures or tumors with RNA STAT-60 reagent (Tel-Test Inc.). Total RNA (200–500 ng) was reverse transcribed with TaqMan<sup>®</sup> MicroRNA Reverse Transcription Kit (Applied Biosystems). miR expression levels were quantified by using probes from TaqMan<sup>®</sup> MicroRNA Assays (Applied Biosystems) on an ABI Prism 7900 platform (Applied Biosystems) according to the manufacturer's protocol. At the end of the PCR reaction a melting curve was generated and the cycle threshold ( $C_t$ ) was recorded. Relative expression of microRNA of interest was calculated as  $\Delta C_t$ , measured by subtracting the  $C_t$  of the reference microRNA from that of the RNU49 control (for cancer cell lines) and snRNA U6 (for human glioblastoma samples). qPCR for EGFR used the FastStart Taqman Probe Master (Roche) and results were normalized to *GAPDH*. Primers are included in Supplementary Table S1. Results are presented as means  $\pm$  standard deviation (SD) of replicates. Each measurement was performed in duplicate and repeated. Student's *t*-test compared mean miR expression levels between groups and Pearson coefficient measured the correlation between *EGFR* and *miR-146b*. A  $P < 0.05$  was deemed significant.

### Transfection

To knockdown *let-7d* and *miR-146b*, we used locked nucleic acids (LNA) MiRCURY LNA<sup>TM</sup> and scrambled LNA from Exiqon (Denmark). To overexpress *let-7d* and *miR-146b*, we used pre-miR<sup>TM</sup> miRNA Precursor and negative control #1 from Ambion (Austin, TX, USA). Transfection was facilitated by Dreamfect (Oz Biosciences) according to manufacturer's instructions. siRNA against *c-fos* and scrambled siRNA (control) were purchased from Dharmacon (Lafayette, CO, USA). Typically miR or gene knock-down or overexpression were achieved within 24–48 h of transfection. After transfection and/or treatments, cells were harvested for RNA or protein analyses at specified time-points.

### Reporter assay

A Dual-Luciferase Assay (Promega) was performed to quantify EGFR 3'-UTR activities. EGFR 3'-UTR reporter clone pMirTarget-EGFR was from Origene (SC216236). The predicted miR-146b target site between 1064 and 1085 bp was deleted by using the QuickChange II Site-directed Mutagenesis Kit (Agilent Technologies #200523-5) to generate the pMirTarget-Mut clone and verified by sequencing. U118 cells were co-transfected with pMirTarget-EGFR or pMirTarget-Mut and control reporter plasmid (renilla) at a ratio of 10:1 by using FuGENE HD transfection Reagent (Roche). Luminescence was measured by using a TD-20/20 luminometer (Turner Biosystems). Experiments were performed in triplicate and repeated in independent

conditions two times. To control for transfection efficiency, values for luminescence were normalized to renilla activity.

### BrdU assay

An ELISA based assay (Roche Applied Science) measured cell proliferation of C272-hTert/E7 cells transfected with *let-7d* precursor and control and stimulated with PDGF-AA, according to manufacturer's instructions.

### Chromatin immunoprecipitation

To detect the interaction between *c-fos* and the *miR-146b* promoter, we used chromatin immunoprecipitation (ChIP). In brief, C272-hTert/E7 cells were treated with PDGF-BB for 3 h, then fixed in 1% formaldehyde, lysed into 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl and sonicated. The soluble chromatin was pre-cleared by incubation with sheared salmon sperm DNA-protein. One portion of the supernatant was used as a DNA input control, and the remainder was incubated with rabbit IgG or a Fos antibody (overnight, 4°C). Immunoprecipitated complexes were incubated with protein A slurry, washed successively with low-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], high-salt buffer (500 mM NaCl), LiCl buffer [0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)] and Tris-EDTA (pH 8.0), and then eluted with 1% SDS, 100 mM NaHCO<sub>3</sub> buffer. Cross-linking of protein-DNA complexes was reversed by incubation with 5 M NaCl at 65°C for 4 h, and DNA was digested with 10 mg/ml of proteinase K (1 h, 45°C). The DNA was extracted with phenol-chloroform and subjected to PCR amplification using primers designed for the predicted *c-fos* binding domain in the *miR-146b* promoter: CAGTGGCTCACAC CTGTAATTC (f) and GTAGCACAATCACGGCTT ACTG (r). The 242-bp PCR products were resolved by 2% agarose-ethidium bromide gel electrophoresis, visualized by UV and analyzed with the Kodak gel analysis system. As another negative control, DNA immunoprecipitated with *c-fos* antibody was amplified with primers designed for a fragment located 1000 bp downstream of *miR-146b* (F: 5'-GCCTACCAAAGTGC TGGGATTAC-3' and R: 5'-ATATGCTGGACGCTGG ACTAGAC-3').

### Statistical analysis

The Student's *t*-test compared quantitative data between groups;  $P < 0.05$  being significant.

## RESULTS

### MiRNAs regulated by PDGF

We hypothesized that the response to PDGF involves alterations in miR expression and employed miR microarrays to identify such changes. U118 glioblastoma cells expressing both subunits of the PDGFR,  $\alpha$  and  $\beta$ , were treated with PDGF-AA or PDGF-BB over a 24 h



**Table 1.** Specific PDGF AA inducible or repressible microRNAs

miR	Chromosome	Fold-changes	P-value	Expression in cancer	Function
mir-126-5p	9q24	-19.1	4.54E-05	Lost in breast cancer	Suppresses cell proliferation
miR-149	2q37	4.8	0.0003	Down in glioblastoma, melanoma	Not known
miR-185	22q11.21	3.9	0.0089	Up in hepatocellular and bladder cancer	Not known
miR-663	20p11.21	5.5	0.0011	Up in ovarian, lost in breast cancer	Epigenetic silencing in breast cancer
miR-769-5p	19q13.33	5.2	0.0087	Not known	Not known
Let-7d	9q22	-9.5	0.0048	Down in hepatocellular, ovarian, lung cancer	Suppresses cell proliferation
miR-770P	14q42	-6.0	0.002	Not known	Not known

**Table 2.** Specific PDGF BB inducible or repressible microRNAs

miR	Chromosome	Fold-changes	P-value	Expression in cancer	Function
miR-106b	7q22.1	4.6	0.0433	Up in prostate cancer	Suppresses E2F1 and p21
miR-30b	8q24	-9.2	0.005	Down in cancer stem cells	Self renewal and survival of cancer stem cells
miR-30c	1p34	-5.6	1.16E-05	Down in cancer stem cells	Self renewal and survival of cancer stem cells
miR-146b	10q24.32	9.3	1.96E-05	Up in thyroid, breast, pancreatic cancer	Suppresses KIT Inducible by TNF
miR-198	3q13	3.2	0.003	Down in hepatocellular carcinoma up in prostate cancer	Not known
miR-181a1	1q31.32	6.2	0.006	Up in pancreatic, thyroid and colon cancer	Target of p53
miR-631	15.q24	4.7	0.0006	Not known	Not known
miR-1	18q11	3.1	0.004	Up in prostate cancer, down-regulated in other cancers	Suppresses E2F1 Synaptic signaling
miR-613	12p13	3.4	0.004	Not known	Not known
miR-129-1	7q32.1	5.3	0.039	Up in retinoblastoma, down-regulated in other cancers	Not known
miR-216	2p18.1	2.9	0.0017	Down-regulated in pancreatic cancer	Not known
miR-499	20q11.22	3.7	0.0062	Not know	Expressed in myocytes, involved in cell senescence
miR-564	3p21.31	9.4	1.84E-05	Not known	Not known

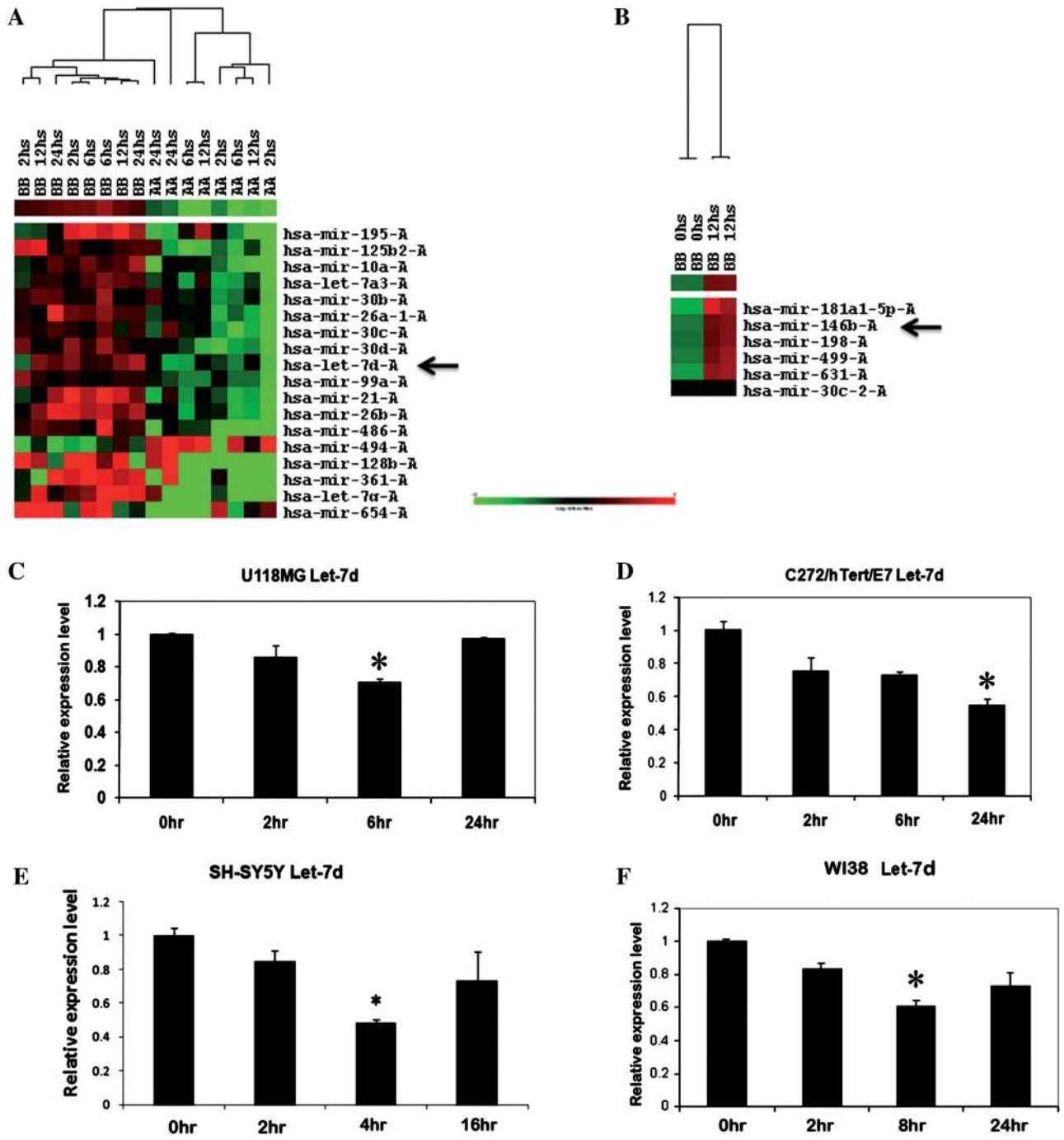
time-course. RNA extracted at 0, 2, 6, 16 and 24 h was used for miR expression profiling. A two-sample *t*-test with random variance was used to perform the class comparison between un-stimulated and PDGF-stimulated samples and miRs significant at the  $P < 0.01$  level of the univariate test are reported. Twenty miRs were induced or repressed by PDGF, with 7 being altered by PDGFAA (four up-regulated and three down-regulated) and 13 affected by PDGF BB (11 up-regulated and two down-regulated). Tables 1 and 2 summarize miRs whose expression is altered by PDGFs compared to un-stimulated cells. Surprisingly no miR was altered in a similar manner by both PDGF-AA and PDGF-BB at  $P < 0.01$ . Comparison of miR profiles combining all time-points after PDGF-AA or PDGF-BB stimulation revealed two miRs (*let-7g* and *miR-556*) most differentially altered by the two PDGFs ( $P < 0.01$ , Supplementary Table S1).

Cluster analysis shows global differences between miRs altered by PDGF-AA and PDGF-BB, *let-7d* being one of the miRs repressed by PDGF-AA (Figure 1A) and *mir-146b* being one of the miRs induced by PDGF-BB (Figure 1B). These two miRs were studied in further detail in subsequent analyses. Supervised and

unsupervised PCA miR classification was performed for samples stimulated with PDGF-AA or PDGF-BB at different timepoints versus un-stimulated samples. Analyses summarized in Supplementary Figure S1A and B demonstrate significant differences in miR expression patterns induced by the two PDGFs versus un-stimulated cells. Likewise PCA was used to classify miRs altered by PDGF-AA versus PDGF-BB (Supplementary Figure S1C-F) demonstrating discriminatory miR profiles elicited by the two PDGF ligands. Selected miRs emerging from the microarray analysis were confirmed by qPCR. The U118MG, C272/hTert/E7 and WI38 cells express both PDGF receptor subunits, while U87MG cells express predominantly PDGFR- $\beta$  (Supplementary Figure S2A). Supplementary Figure S2 includes selected validation studies confirming by qPCR miR alterations induced by PDGF, such as *miR-106b* up-regulation by PDGF-BB.

#### PDGF-AA represses *let-7d* in cancer cells

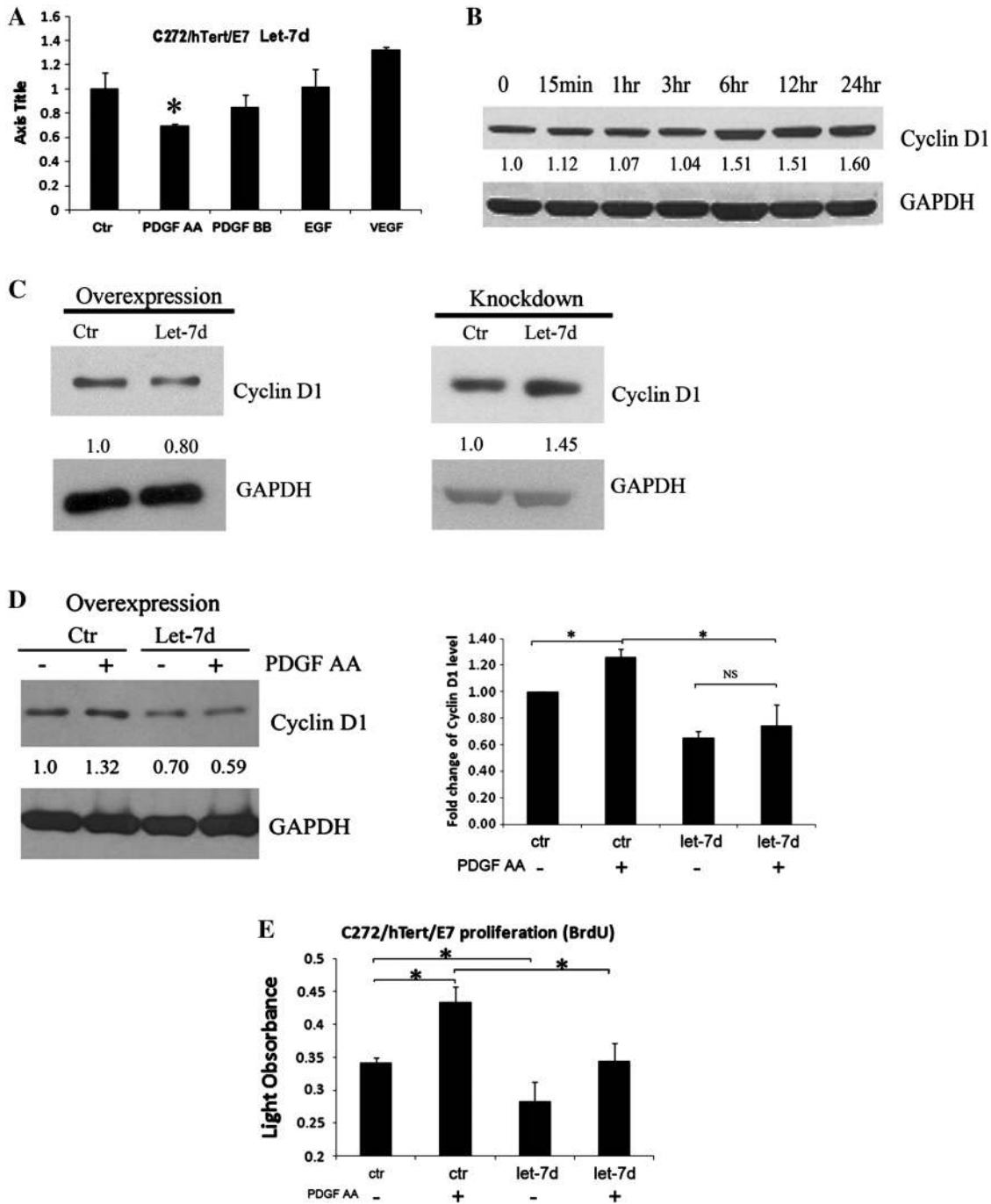
Further confirmation of the microarray generated data focused on *let-7d* as a specific target of PDGF-AA and on *miR-146b* as a PDGF-BB inducible miR. Up to 50% *let-7d* repression by PDGF-AA was evident U118MG,



**Figure 1.** Hierarchical clustering demonstrates specific effects of PDGF-AA and PDGF-BB on miR expression in U118 cells. (A) Differential expression of miRs is induced by PDGF-AA and PDGF-BB at different timepoints. Among other miRs, *let-7d* is suppressed by PDGF-AA (see arrow). (B) Effects of PDGF-BB on miR expression (0 versus 12h). Among other miRs, *mir-146b* is induced by PDGF-BB (see arrow). qPCR quantifies *let-7d* expression levels in U118 MG (C), C272/hTert/E7 (D), SH-SY5Y (E) and WI38 cells (F) after PDGF-AA treatment. Statistical significance is marked by asterisks.

C272hTert/E7, SH-SY5Y and WI38 cells within 6h of PDGF-AA stimulation. The effect of PDGF-AA on *let-7d* occurred rapidly (2–6h, Figure 1C–F), although some variability in the kinetic response of *let-7d* to PDGF-AA was evident in the different cell lines tested. For instance in C272/hTert/E7 cells the repression of *let-7d* persisted for 24h, whereas in U118MG cells the

level of *let-7d* returned to normal 24h after PDGF-AA stimulation. While some of the PDGFR-expressing cancer cells tested here secrete PDGF, the neuroblastoma cells SH-SY5Y cells express both PDGF receptors but do not secrete PDGFs (40). Thus the effects of PDGF-AA on *let-7d* expression are independent of autocrine secretion of this growth factor (Figure 1E).



**Figure 2.** PDGF-AA down-regulates *let-7d* expression. (A) qPCR quantifies *let-7d* expression in C272/hTert/E7 cells in response to PDGF-AA, PDGF-BB, VEGF and EGF. *RNU49* was used as control. (B) Western blotting for cyclin-D1 in C272/hTert/E7 cells treated with PDGF-AA. (C) Western blotting for cyclin D1 in U118MG cells transfected with *let-7d* precursor or control (left panel). Western blotting for cyclin D1 in U118MG cells transfected with LNA targeting *let-7d* or scrambled LNA (right panel). (D) Western blotting assessed cyclin D1 in C272/hTert/E7 cells treated with PDGF-AA for 6h after transfection of *let-7d* precursor or control (left panel). Densitometry quantifies cyclin D1 expression levels relative to GAPDH in independent experiments. Statistical significance is indicated by asterisks (NS, not significant). (E) BRDU assay quantifies cell proliferation of C272/hTert/E7 cells transfected with *let-7d* precursor and control and stimulated with PDGF-AA or vehicle. Statistical significance is marked by asterisks.

To test the specificity of the miR response to individual growth factors, *let-7d* expression was quantified in C272hTert/E7 cells treated with PDGF-AA, PDGF-BB, EGF and VEGF. A ~40% decrease in *Let-7d* level was observed in response to PDGF-AA, with no measurable

changes observed in PDGF-BB, EGF or VEGF-treated cells (Figure 2A).

To test the functional significance of *let-7d* repression by PDGF-AA, we measured its effects on one of its best documented targets, cyclin D1 (33). Cyclin D1 is induced

by PDGF-AA (Figure 2B) and its up-regulation is known to be implicated in PDGF-stimulated cell proliferation (41). To test its role in the context of PDGF-regulated miR alterations, *Let-7d* was overexpressed and knocked down and cyclin D1 expression levels were measured. Overexpression in U118MG cells was achieved by transfection of the *let-7d* pre-miR and confirmed by qPCR (not shown). Overexpression of *let-7d* repressed cyclin D1 expression (Figure 2C, left panel). Likewise *let-7d* knockdown was achieved by transient transfection of an LNA targeting this miR and confirmed by qPCR (not shown). *Let-7d* knockdown induced cyclin D1 (Figure 2C, left panel). To show that repression of *let-7d* by PDGF-AA was significant to the regulation of cyclin D1 by PDGF, this miR was overexpressed. Subsequent treatment with PDGF-AA in cells overexpressing *let-7d* did not significantly affect cyclin D1 expression ( $P = 0.3$ ) as compared to the effect of PDGF-AA on cyclin-D1 in cells transfected with a control sequence ( $P = 0.02$ , Figure 2D). Further, this effect was implicated in PDGF-AA-induced cell proliferation, as *let-7d* overexpression inhibited PDGF-AA induced BRDU incorporation in proliferating cells (Figure 2E,  $P = 0.03$ ). Collectively, these data indicate that PDGF-AA regulates critical cell cycle components and S-phase entry in part via down-regulation of *let-7d*.

#### PDGF-BB induces *miR-146b* in cancer cells

Next, we focused on the effects of PDGF-BB and showed by qPCR that *miR-146b* was induced by up to 2-fold in U118MG, U87MG, C272hTert/E7 and SH-SY5Y cells within 6–16h (Figure 3A). The induction in *miR-146b* expression was sustained (up to 24h) in the cancer cells tested here. Lesser induction of *miR-146b* (~30%) was observed in non-transformed WI38 fibroblasts stimulated with PDGF-BB (Figure 3B). To test the specificity of this miR's response to individual growth factors, C272/hTert/E7 cells were treated with PDGF-AA, PDGF-BB, EGF and VEGF and *miR-146b* was quantified. A 2-fold increase in *miR-146b* expression level was noted in response to PDGF-BB, while PDGF-AA, EGF and VEGF did not induce significant variation of *miR-146b* levels (Figure 3C).

Akt and MAPK (42) are the two major oncogenic pathways activated by PDGF stimulation, and we sought to determine their contribution to *miR-146b* up-regulation. Therefore we measured the effects of a MEK (PD98059), PI3K (LY294002) and PDGFR (imatinib mesylate) inhibitors on *miR-146b*. Pre-treatment with these inhibitors prevented *miR-146b* induction by PDGF-BB (Figure 3D), suggesting that both the ERK (inhibited by PD98059) and the AKT (inhibited by LY294002) signaling pathways are involved in *miR-146b* induction.

To generate mechanistic insight into PDGF chains specific effects on miR expression, we assessed the differential impact of PDGF-AA versus PDGF-BB on the major downstream signaling pathways. In C272/hTert/E7 cells, PDGF-AA induced phosphorylation of PDGFR $\alpha$ , but not of PDGFR $\beta$  within 30 min

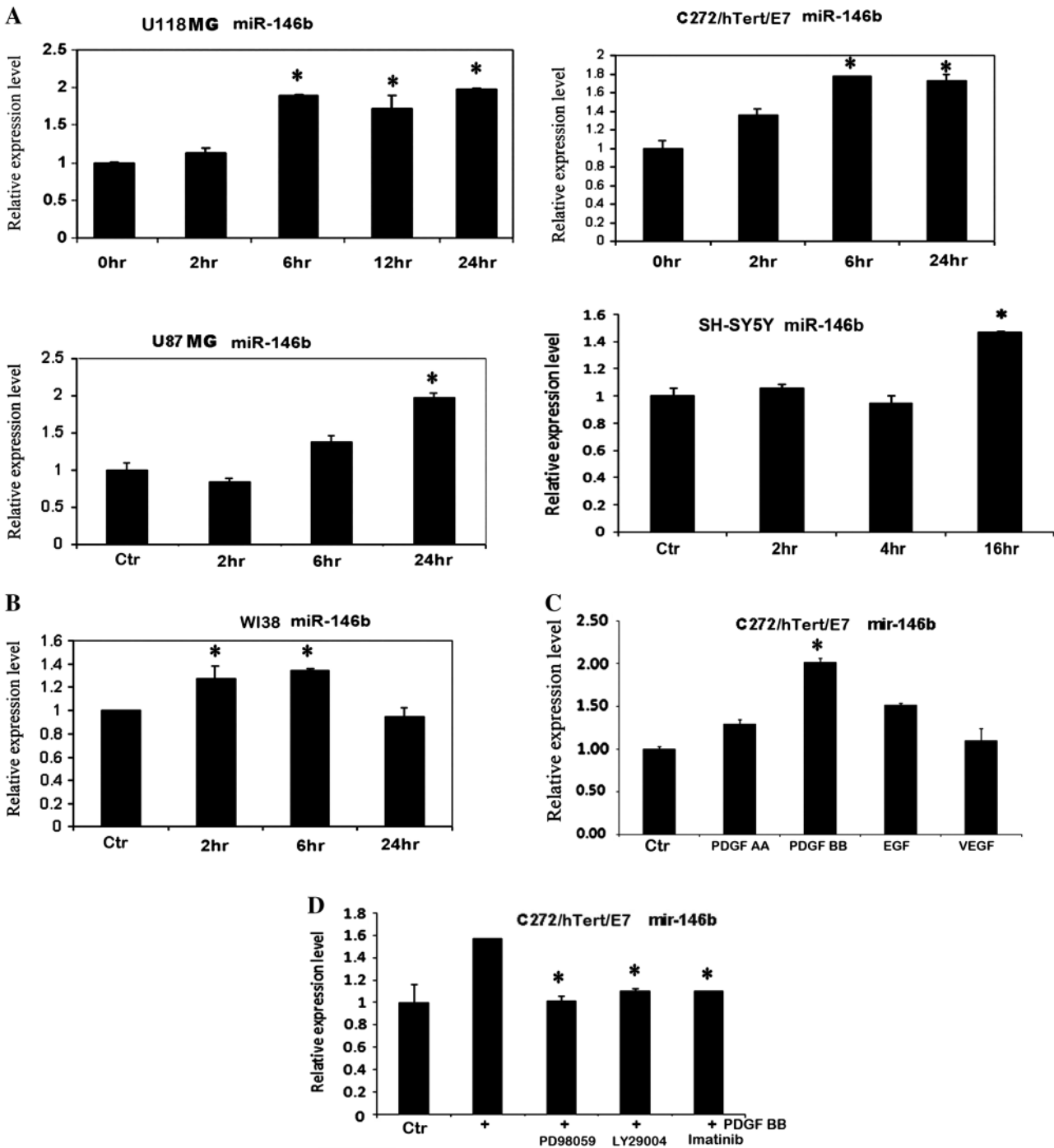
(Figure 4A). In contrast, PDGF-BB led to phosphorylation of both receptor subunits and its effects were more prominent than those of PDGF-AA. Akt was rapidly phosphorylated after stimulation with each of the two PDGFs. At equivalent doses of PDGF (25 ng/ml), PDGF-BB induced Akt phosphorylation more prominently compared to PDGF-AA. Likewise, MAPK p42/p44 was and more rapidly and robustly activated by PDGF-BB compared to PDGF-AA (Figure 4A), suggesting that the former is a more potent stimulator of this pathway. An important transcription factor induced by PDGF is *c-Fos* (43). In C272HTERT/E7 cells, *c-fos* expression was markedly induced by PDGF-BB within 1–3h, but not measurably by PDGF-AA (Figure 4B, upper and middle panels). As the main mechanism implicated in *c-fos* engagement by PDGF is the activation of MAPK pathway (44), we measured the effects of its inhibition. The MAPK inhibitor, PD98059, blocked *c-fos* induction by PDGF-BB, while LY294002 did not affect it (Figure 4B, lower panel), indicating that PDGF-BB engages *c-fos* more effectively than PDGF-AA, and does so by activating the MAPK pathway.

To further understand the relevance of PDGF to the induction of *miR-146b*, we performed an *in silico* search upstream of the *miR-146b* sequence for known transcription factors activated by PDGF (<http://microna.sanger.ac.uk/sequences/ftp/genomes/hsa.gff>). This led to the identification of a putative binding site for *c-fos* at position –2300 upstream of *miR-146b* (Figure 4D). *c-fos* knockdown by siRNA repressed the expression level of *miR-146b* induced by PDGF-BB (Figure 4C,  $P = 0.007$ ). Binding of *c-fos* to the *miR-146b* promoter was shown by CHIP utilizing primers encompassing from –2447 to –2205 region upstream of the transcription starting site of *miR-146b*. *c-fos* interaction with this *miR-146b* promoter region was enhanced by PDGF-BB (Figure 4D, lower panel), further supporting the significance of *c-fos* to PDGF-BB induced up-regulation of *miR-146b*.

#### *MiR-146b* modulates the balance between PDGF and EGF signaling

We next searched for possible targets of *miR-146b* by using bioinformatic predictive tools (<http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi>). Having learned that in other systems EGFR is a *miR-146b* target (45), we tested whether changes in *miR-146b* expression alter EGFR expression. Transfection of a commercial *miR-146b* mimic led to repression of EGFR protein expression level (Figure 5A, left panel). Conversely, transfection of a LNA targeting *miR-146b* caused its knock down (not shown) and subsequent up-regulation of EGFR protein expression level (Figure 5A, right panel). However, as expected, the variation in *miR-146b* expression levels resulting from ectopic overexpression or LNA-mediated knock down of *miR-146b* achieved in these experiments exceeded the range of PDGF-BB induced changes in cancer cells. Therefore, to test whether by altering *miR-146b*'s expression, PDGF-BB



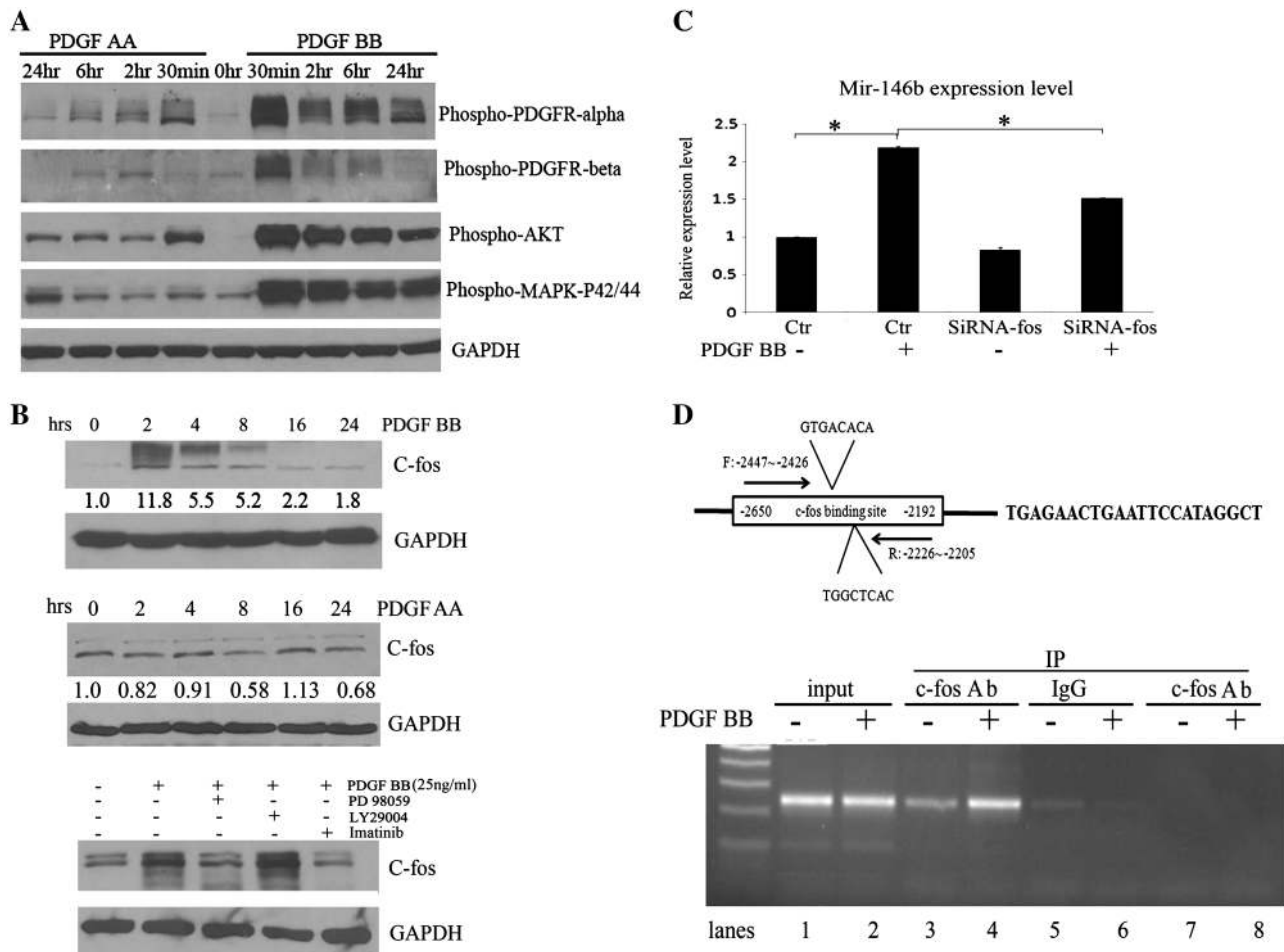


**Figure 3.** PDGF BB up-regulates miR-146b. (A) qPCR quantifies *miR-146b* in U118, U87-MG, C272/hTert/E7 and SH-SY5Y after PDGF-BB treatment. (B) qPCR quantifies *miR-146b* in WI38 fibroblasts after PDGF-BB treatment. (C) qPCR quantifies *miR-146b* in C272/hTert/E7 cells in response to PDGF-AA, PDGF-BB, VEGF and EGF. (D) qPCR quantifies *miR-146b* in C272hTert/E7 cells treated with PDGF-BB and PD98059, LY294002 and imatinib mesylate. *RNU49* was used as control for all qPCR reactions.

regulates the expression level of EGFR; we measured the receptor's expression after PDGF-BB treatment and noted down-regulation of EGFR protein level 6–24h after PDGF-BB stimulation (Figure 5B, upper panel). In contrast, PDGF-AA did not decrease measurably EGFR expression, in concordance with the known lack of induction of *miR-146b* by PDGF-AA (Figure 5B, lower panel).

To investigate whether *miR-146b* has a significant contribution to the effect of PDGF-BB on EGFR, this miR was overexpressed and cells were treated with vehicle or PDGF-BB. Overexpression of *miR-146b* in C272hTERT/E7 cells down-regulated EGFR expression as compared to cells transfected with control miR. EGFR expression was further suppressed in cells



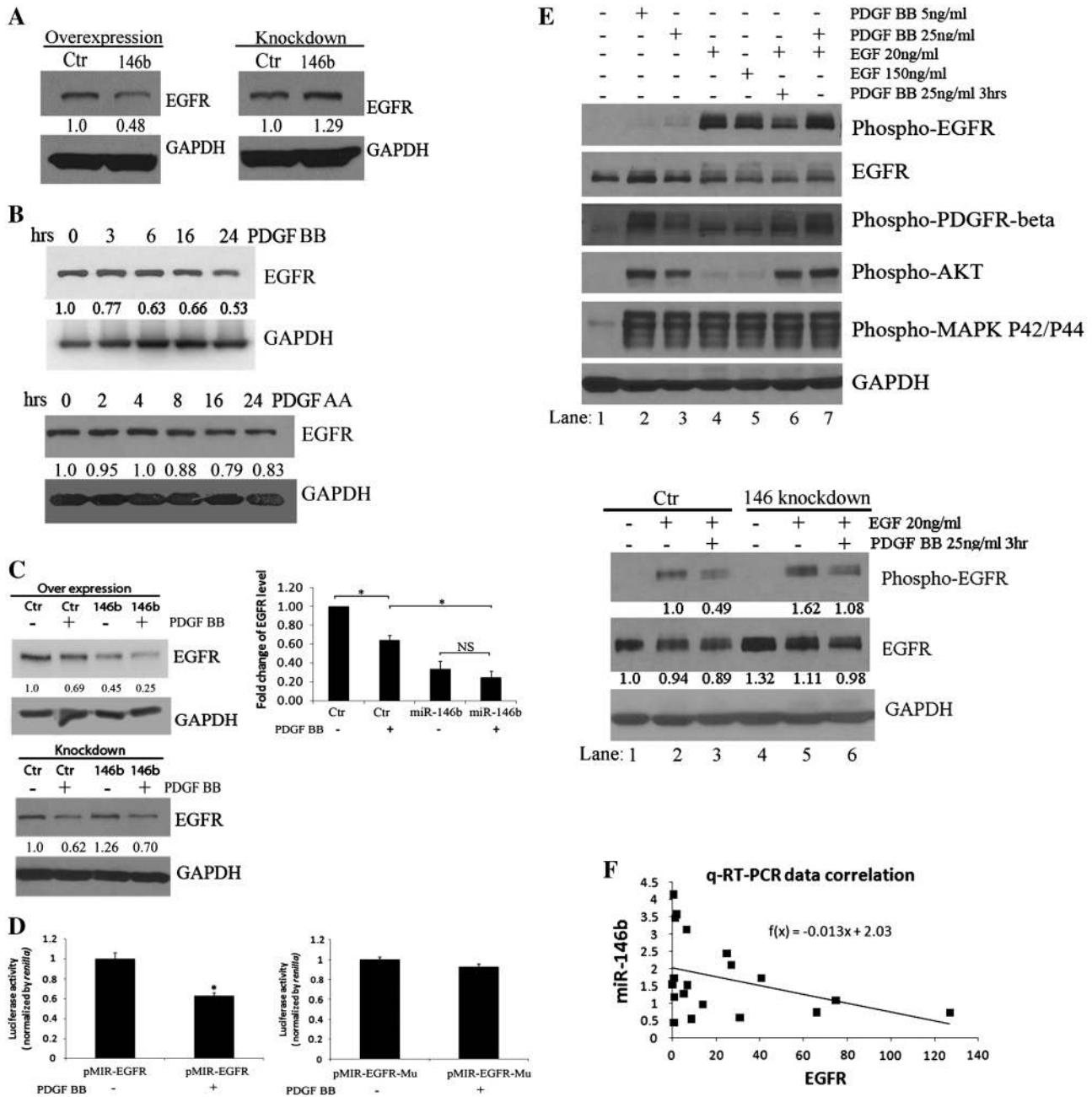


**Figure 4.** Mechanism of miR-146b regulation by PDGF BB. (A) Phosphorylation of PDGFR $\alpha$  and  $\beta$ , Akt and MAPKp42/p44 was measured by western blotting in C272hTert/E7 cells stimulated with PDGF-AA (25 ng/ml) or PDGF-BB (25 ng/ml) for 30 min to 24 h. (B) Western blotting for c-Fos in C272/hTert/E7 cells treated with PDGF-BB for 2–24 h (upper panel). Western blotting for c-Fos in C272/hTert/E7 cells treated with PDGF-AA for 2–24 h (middle panel). Western blotting quantifies c-fos in C272hTert/E7 cells stimulated with PDGF-BB in the presence or not of PD98059 and LY294002 inhibitors (lower panel). (C) C272hTert/E7 cells were transfected with *c-fos* targeted siRNA or scrambled siRNA (control) and treated with PDGF BB. *Mir-146b* was quantified by qPCR ( $P = 0.007$ ). Statistical significance is marked by asterisks. (D) Schematic representation of the *c-fos* binding site at position  $-2300$  upstream of the *mir-146b* and the position of primers chosen for amplification in the ChIP assay. ChIP assay tests binding of *c-Fos* to the *mir-146b* promoter region after PDGF-BB stimulation. A 242-bp PCR product was detected by gel agarose electrophoresis from chromatin immunoprecipitated with an antibody against *c-fos* (lanes 3 and 4) using primers flanking the *c-fos* binding region of the *mir-146b* promoter ( $-2447$  to  $-2205$  bp). Positive controls are amplicons from input chromatin (lanes 1 and 2). Negative controls consist of chromatin immunoprecipitated with non-specific IgG and amplified with primers specific to the *mir-146b* promoter (lanes 5 and 6) and chromatin immunoprecipitated with *c-fos* antibody and amplified with primers away from the predicted *c-fos* binding site (lanes 7 and 8).

overexpressing *miR-146b* after treatment with PDGF-BB (Figure 5C, upper panels). To further validate that the regulation of EGFR is mediated by *miR-146b* through binding to its 3'-UTR region, we measured the effects of PDGF-BB on the activity of an EGFR's 3'-UTR-luciferase reporter. PDGF-BB repressed the reporter activity of the wild-type 3'-UTR fragment ( $P = 0.04$ ; Figure 5C, lower left panel), but not that of a 3'-UTR fragment lacking the *miR-146b* seed sequence ( $P = 0.56$ , Figure 5C, lower right panel). These data suggest that one possible mechanism through which PDGF-BB regulates EGFR expression occurs via modulation of *miR-146b* expression levels.

To gain further insight into the functional significance of the effects of PDGF on EGF-EGFR signaling, C272/hTert/E7 cells that express both PDGFRs and the EGFR,

were stimulated with PDGF-BB and EGF, either alone, in combination, or in succession. Two doses of PDGF (5 and 25 ng/ml) and of EGF (20 and 150 ng/ml) were used (Figure 5D, upper panel). As anticipated, PDGF-BB phosphorylated the PDGFR- $\beta$ , but not the EGFR; while EGF potently phosphorylated the EGFR. MAPK42/44 activation was induced by PDGF-BB as well as by EGF at comparable levels, but Akt phosphorylation was more potently induced by PDGF-BB compared to EGF (Figure 5C, top panel). Interestingly, pre-stimulation with PDGF-BB for 3 h prior to EGF, attenuated EGFR phosphorylation as compared to EGF-induced receptor activation (Figure 5D, top panel, lane 6 versus 4 and 5). This suggested that PDGF-BB may induce a factor which inhibits the effects of EGF. This effect was not observed when the two growth factors



**Figure 5.** MiR-146b targets the EGFR. (A) Western blotting for EGFR in U118MG cells transfected with *miR-146b* precursor or control (left) or with LNA targeting *miR-146b* or scrambled LNA (right). (B) Western blotting for EGFR in C272/hTert/E7 cells treated with PDGF-BB for 3–24 h (upper panel). Western blotting for EGFR in C272/hTert/E7 cells treated with PDGF-AA for 2–24 h (lower panel). (C) Western blotting assessed EGFR expression in C272/hTERT/E7 cells treated with PDGF-BB for 6 h after transfection of *miR-146b* precursor or control leading to *miR-146b* over-expression. Densitometry quantifies EGFR expression levels relative to GAPDH in independent experiments. Statistical significance is indicated by asterisks (upper panels, NS, not significant). Reporter assay measured the activity of an EGFR 3'-UTR-luciferase construct before and after stimulation with 50 ng/ml of PDGF-BB (lower panels). Reporter activity of wild-type EGFR 3'-UTR (left) and mutated 3'-UTR (right) were measured and normalized to renilla activity. Statistical significance is marked by asterisks; NS, non-significant. (D) Phosphorylation of PDGFR $\beta$ , EGFR, Akt and MAPK p42/p44 were measured by western blotting (upper panel) in C272hTert/E7 cells stimulated for 30 min with PDGF BB (5 ng/ml, lane 2 and 25 ng/ml, lane 3), EGF (20 ng/ml, lane 4 and 150 ng/ml, lane 5) or a combination of PDGF and EGF (pre-treatment with PDGF for 3 h, followed by EGF; lane 6; or concomitant stimulation with PDGF and EGF for 30 min; lane 7). Phosphorylation of EGFR and total expression of EGFR were measured by Western blotting in C272hTert/E7 cells (lower panel) transfected with LNA targeting *miR-146b* or control. Cells were then treated with EGF (20 ng/ml, lanes 2 and 5) or with PDGF for 3 h prior to stimulation with EGF (lanes 3 and 6). Densitometry quantifies EGFR phosphorylation and total expression relative to GAPDH. (E) EGFR and *miR-146b* relative expression plot obtained by using relative expression data after standardization of EGFR relative expression values and *miR-146b*.  $F(x)$  is the estimated linear trend function.

were administered simultaneously (Figure 5D, top panel, lane 7 versus 6). Activation of Akt and MAPK42/44 downstream of the EGFR was not affected by the sequence of growth factor administration, likely because of the overlapping effects of PDGF and EGF on these pathways.

To demonstrate that PDGF-induced inhibition of EGFR phosphorylation occurred at least in part via induction of *miR-146b*, we knocked-down *miR-146b* prior to stimulation with EGF or with PDGF followed by EGF. *miR-146b* knockdown accentuated EGFR activation in response to EGF when compared to control-LNA transfected cells (Figure 5D, lower panel, lanes 2 and 5) and this occurred in parallel with an increase in the expression level of EGFR. Further, *miR-146b* knockdown prevented partially the attenuation of EGFR phosphorylation induced by pre-treatment with PDGF prior to EGF (Figure 5D, lower panel, lanes 3 and 6). These data demonstrate how a miR fine tunes the cancer cells' response to different growth signals.

Lastly, to illustrate the functional significance of *miR-146b* expression to EGFR's expression *in vivo*, we measured by qPCR the expression levels of EGFR and *miR-146b* in 19 human glioblastoma specimens. *miR-146b* and *EGFR* expression levels were inversely correlated (Pearson coefficient  $-0.38$ ,  $P = 0.05$ , one tail, Figure 5E), demonstrating that this miR-receptor interaction can still be detected in tumors despite the complexity of *in vivo* systems.

## DISCUSSION

Significant literature, including our previous studies, demonstrates that autocrine PDGFR activation plays a critical role in cancer progression via regulation of specific molecular networks (21,22,24). In the current study, we provide new evidence that PDGF-initiated signaling alters the human miRnome, with significant repercussions on target gene expression and function. This report implicates alterations of ncRNAs in the effects of PDGF signaling on gene regulation in cancer cells and demonstrates that distinct PDGF ligands have specific effects on miR expression.

The original observations that a significant proportion of miRs exhibit altered expression patterns in tumors, with or without corresponding genomic alterations, established the foundation for the ongoing 'ncRNAs revolution' in oncology (34,46). Mechanistically, cancer-associated miRs directly or indirectly regulate genes with critical roles in tumor formation, including oncogenes or tumor suppressors (46). Additionally, miRs respond to cytokines, estrogen, and a variety of stresses, which are relevant to our understanding of the cancer microenvironment (47), as well as of other non-malignant disorders or of normal physiological responses.

The impact of our studies is at least 3-fold. First, the two PDGF ligands (AA and BB) exhibit compelling differences in their impact on miRs expression. Repression of *let-7d* is specifically induced by PDGF-AA, whereas induction of *miR-146b* is specific to PDGF-BB. To our

knowledge, this represents the first evidence for PDGF ligand-specific miRs response, and may help explain some of the observed different effects elicited by the two forms (12,48). PDGFs' effects on miR expression have been described in non-transformed mesenchymal cells in rapport to cell proliferation and migration induced by these growth factors (49–51). However, miRs previously described to be inducible by PDGF in smooth muscle cells (e.g. *miR-24*, *miR-221*) were not identified by the global approach (miR arrays) utilized in our study. These differences may be attributable to the cancer phenotype studied here or to the threshold for detection of differences in gene expression levels imposed by the use of miR arrays.

Second, transcription factors regulate miR expression in a fashion similar to the regulation of classic genes, well documented examples including *HIF-miR-210* (47) and *p53-miR-34a* (52). Here we show that PDGF induces *miR-146b* through a *c-fos* dependent mechanism. These findings expand the accepted classical pathway of gene expression modulated by PDGF through recruitment of factors that activate gene transcription to include a new mechanism dependent on ncRNAs. This model may help to improve our understanding of how different downstream responses are derived from relatively similar upstream growth factors.

Third, EGFR expression is regulated by *miR-146b* in response to PDGF-BB. While *miR-146b* has been shown to target EGFR (45), we provide evidence for a feedback mechanism stemming from PDGFR signaling, potentially providing proof of principle for a shift between growth factor signaling in cancer cells. We also demonstrate that the EGFR and *miR-146b* expression are inversely correlated *in vivo*. These findings suggest that ncRNAs participate in the fine tuning of cellular responses to external stimuli, balancing one growth factor pathway versus another. Given that the two growth factors' effects on cell proliferation and survival are to some extent redundant, this mechanism may explain how cells adapt to stimuli from the micro-environment by turning off un-necessary growth or survival pathways. It is also possible that the ncRNA-modulated shift between response to PDGF (a mesenchymal factor) and EGF (an epithelial factor) may have implications for maintaining the balance between epithelial and mesenchymal features of cancer cells, and perhaps influence the transition between the two states. For this study, we dissected this mechanism in the context of cancer cells, however we do not exclude the possibility that a similar feedback loop may operate in non-transformed cells during physiologic responses.

The above findings also point to potential clinical applications of this regulatory mechanism. When PDGF signaling is turned off by inhibitors, PDGF inducible miRs may be silenced and subsequently their targets, including the alternative growth pathway (e.g. EGF-EGFR), may become de-repressed. If operative in tumors *in vivo*, this link may contribute to resistance to PDGFR inhibitors. Understanding this mechanism may help to develop strategies that take into account the interconnected arrays of growth factors in the tumor environment.



In summary, our findings expand the classical view of PDGF-initiated signaling and provide insight into the regulation of known and/or new targets of PDGF. The involvement of PDGF-inducible ncRNAs may explain differences between biological effects of specific PDGF ligands and feedback regulatory mechanisms between growth pathways in cancer cells. These findings have implications for development of therapies targeting the PDGFR in cancer.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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