

MicroRNA profile indicates downregulation of the TGF β pathway in sporadic non-functioning pituitary adenomas

Henriett Butz · István Likó · Sándor Czirják · Péter Igaz · Márta Korbonits · Károly Rác · Attila Patócs

Published online: 10 November 2010
© Springer Science+Business Media, LLC 2010

Abstract MicroRNAs (miRs) are small, 16–29 nucleotide long, non-coding RNA molecules which regulate the stability or translational efficiency of targeted mRNAs via RNA interference. MiRs participate in the control of cell proliferation, cell differentiation, signal transduction, cell death, and they play a role in carcinogenesis. The aims of our study were to analyse the expression profile of miRs in sporadic clinically non-functioning pituitary adenomas (NFPA) and in normal pituitary tissues, and to identify biological pathways altered in these pituitary tumors. MiR expression profiles of 12 pituitary tissue specimens (8 NFPA and 4 normal pituitary tissues) were determined using miR array based on quantitative real-time PCR with 678 different primers. Five overexpressed miRs and mRNA expression of Smads (Smad1-9), *MEG* and *DLK1*

genes were evaluated with individual Taqman assays in 10 NFPA and 10 normal pituitary tissues. Pathway analysis was performed by the DIANA-mirPath tool. Complex bioinformatical analysis by multiple algorithms and association studies between miRs, Smad3 and tumor size was performed. Of the 457 miRs expressed in both NFPA and normal tissues, 162 were significantly under- or overexpressed in NFPA compared to normal pituitary tissues. Expression of *Smad3*, *Smad6*, *Smad9*, *MEG* and *DLK1* was significantly lower in NFPA than in normal tissues. Pathway analysis together with in silico target prediction analysis indicated possible downregulation of the TGF β signaling pathway in NFPA by a specific subset of miRs. Five miRs predicted to target *Smad3* (miR-135a, miR-140-5p, miR-582-3p, miR-582-5p and miR-938) were overexpressed. Correlation was observed between the expression of seven overexpressed miRs and tumor size. Downregulation of the TGF β signaling through Smad3 via miRs may have a possible role in the complex regulation of signaling pathways involved in the tumorigenesis process of NFPA.

Electronic supplementary material The online version of this article (doi:10.1007/s11102-010-0268-x) contains supplementary material, which is available to authorized users.

H. Butz · P. Igaz · K. Rác · A. Patócs (✉)
2nd Department of Medicine, Faculty of Medicine, Semmelweis University, 46 Szentkirályi str., Budapest 1088, Hungary
e-mail: patatt@bel2.sote.hu

I. Likó
Gedeon Richter Plc, Budapest, Hungary

S. Czirják
National Institute of Neurosurgery, Budapest, Hungary

M. Korbonits
Department of Endocrinology, Barts and the London School of Medicine, Queen Mary University of London, London EC1M 6BQ, UK

A. Patócs
Molecular Medicine Research Group, Hungarian Academy of Sciences, Budapest, Hungary

Keywords Pituitary adenoma · miRNA · Expression profile · Pathway analysis · TGF β · Smad3

Introduction

MicroRNAs (miRs) are short, non-coding RNA molecules that posttranscriptionally regulate gene expression via RNA interference [1]. The mature miR molecules are approximately 16–29 nucleotides long and bind to the 3'UTR of mRNAs as antisense regulators to prevent translation or to cause mRNA degradation [2–4]. Until April 2010, 14,197 entries have been submitted to miR-Base Release 15.0 representing hairpin precursor miRs

giving rise to 15,632 mature miRs in 133 species. In *Homo sapiens* 940 miR sequences have been recorded. Their roles have been considered in development [5], cell proliferation [6], differentiation [7], apoptosis [8] and tumorigenesis (reviewed in Ref. 9) including leukaemia [10], lung [11], breast cancer [12], and various endocrine tumors e.g. thyroid carcinoma [13], endocrine pancreatic tumor [14], ovarian cancer [15] and pituitary tumor [16–18].

Pituitary adenomas account for at least 10% of intracranial tumors with a prevalence of 1 in 1,000 people in the general population [19]. Although these tumors are mostly benign, they may cause significant morbidity due to hormonal dysfunction and mass effect. Genetic background of sporadic pituitary adenomas, especial of NFPA is poorly characterized.

The activin/transforming growth factor- β (TGF- β) family consists of evolutionary conserved polypeptides which play a prominent role in regulating pituitary tumor growth and prolactin secretion from pituitary lactotrope cells. Members of this family, activin, TGF β and bone morphogenetic proteins (BMPs) are dimeric molecules composed of two subunits linked by a disulfide bridge. The signal transduction begins with ligand binding to a single transmembrane serine/threonine kinase type II receptor, followed by transphosphorylation and activation of the type I receptor. The activated type I receptor phosphorylates intracellular mediators known as receptor-regulated Smads (R-Smads: Smad2 and Smad3). Two phosphorylated R-Smad subunits form a heterotrimer with Smad4 subunit that translocates to the nucleus, where it associates with various binding partners including menin, and binds to DNA [reviewed in Ref. 20, 21]. The tumor suppressor effect of the TGF β pathway includes interference with cell cycle by inducing expression of p15^{Ink4b} and p21^{Waf1}, inhibition of cyclin D-CDK4/6 and cyclin E/A-CDK2 complexes [22, 23], and induction of apoptosis. Also, the TGF β pathway represses expression of c-myc, a transcription factor that promotes cell proliferation, and Id protein nuclear factors which inhibit cell differentiation [24, 25].

Somatic mutations of genes encoding TGF β receptors are rare in human pituitary tumors [26] and no difference in TGF β RII expression has been found between NFPA and normal pituitary samples [27]. However, microarray studies have indicated that FSH, LH and TSH β -subunit, which are under TGF β regulation, are underexpressed in NFPA samples suggesting that TGF β signaling is repressed in NFPA [28, 29]. In addition, TGF β administration decreased proliferation and increased apoptosis of HP75 cells, a cell line derived from a clinically non-functioning pituitary tumor [29, 30].

The aim of this study was to examine the miR expression profile of NFPA and normal pituitary samples and to identify alterations in biological pathways of NFPA. In addition, we studied the mRNA expression of *Smads*

(*Smad1-9*) in the same samples in order to detect those miRs which may inhibit the TGF β pathway through downregulation of Smad3 and may, therefore play a role in NFPA tumorigenesis.

Materials and methods

Patients

Ten NFPA and 10 normal pituitary tissue specimens were examined. Adenoma tissues were removed by transsphenoidal surgery at the Hungarian National Institute of Neurosurgery. All adenoma samples were gathered with the permission of the Local Committee on Human Research, after obtaining informed consent from each patient. The diagnosis of NFPA was based on clinical findings, hormone levels of patients and immunohistochemistry. Routine immunohistochemical examination included immunostaining for anterior pituitary hormones (GH, PRL, ACTH, LH, FSH and TSH) and staining for the MIB1 proliferation marker. All routine immunohistochemical studies were performed at the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary. Normal pituitary samples were obtained by autopsy within 6 h of death from patients with no evidence of any endocrine disease (University Clinical Centre, Belgrade, Serbia). Demographic data of the patients are summarized in Table 1.

RNA isolation

Tissue specimens were immediately frozen in liquid nitrogen after the adenoma removal, and stored at -80°C until use. Total RNA was extracted with miRNeasy Mini Kit (Qiagen Inc., Chatsworth, CA). RNA integrity and concentration was measured using Agilent Bioanalyzer 2100 System (Agilent Tech Inc., Santa Clara, USA).

miR profiling in pituitary tissues using TLDA cards

miR expression profile was analyzed in 8 NFPA and 4 normal pituitary samples using TaqMan Low Density Array (TLDA) Human MicroRNA Panel v.2 (Applied Biosystems, Foster City, CA). All procedures were performed following the manufacturer's instructions. Briefly, 300 ng total RNA per sample was reverse transcribed using Megaplex RT primer Pool A and B up to 380 stem-looped primers per pool and TaqMan MicroRNA Reverse Transcription Kit (P/N: 4366596). Pre-amplification was carried out by Megaplex Preamp Primers and TaqMan Preamp Master Mix. Real-time PCR amplifications were run in TaqMan Human MicroRNA Array A and B (PN: 4398965,

Table 1 Clinical and pathological findings in patients with clinically non-functioning pituitary adenomas

	Age	Sex	Tumor size before operation (mm ³)	Immunohistochemistry							Molecular biological procedure
				PRL	GH	ACTH	FSH	LH	TSH	MIB1 (%)	
1	29	F	6000	Neg	Neg	Neg	++	Neg	Neg	0	miR-TLDA Smads qRT-PCR
2	34	M	26250	Neg	Neg	Neg	++	Neg	Neg	0	miR-TLDA Smads qRT-PCR
3	75	F	8400	Neg	Neg	Neg	+	Neg	Neg	0	miR-TLDA Smads qRT-PCR
4	36	M	1750	Neg	Neg	++	Neg	Neg	Neg	0	miR-TLDA Smads qRT-PCR
5	28	F	24937	+	+	Neg	Neg	Neg	Neg	0	miR-TLDA Smads qRT-PCR
6	58	M	5130	+	Neg	Neg	Neg	Neg	Neg	0	miR-TLDA Smads qRT-PCR
7	70	F	15000	Neg	Neg	Neg	Neg	Neg	Neg	0	miR-TLDA Smads qRT-PCR
8	79	M	7200	Neg	Neg	Neg	Neg	Neg	Neg	0	miR-TLDA Smads qRT-PCR
9	52	F	8800	Neg	Neg	NA	NA	NA	NA	NA	Smads qRT-PCR
10	69	M	11700	Neg	Neg	NA	NA	NA	NA	NA	Smads qRT-PCR

M male, F female, GH growth hormone, PRL prolactin, ACTH adrenocorticotrop hormone, FSH follicle stimulating hormone, LH luteinizing hormone, TSH thyroid stimulating hormone, MIB1 proliferation marker, NA not available, + positive immunostaining

4398966) using Megaplex Primer Pools, Human Pools Set v3.0 (PN: 4444750) on 7900HT Real Time PCR System (Applied Biosystems). Real-time RT-PCR data analysis was performed using Real-Time StatMinerTM software (Integromics, Granada, Spain). Expression level was calculated by the ddCt method, and fold changes were obtained using the formula 2^{-ddCt} .

Real-time quantification of individual miRs revealed by TLDA miR array

Expression level of hsa-miR-135a (PN: 4427975, Assay ID: 000460), hsa-miR-140-5p (PN: 4427975, Assay ID: 001187), hsa-miR-582-3p (PN: 4427975, Assay ID: 002399), hsa-miR-582-5p (PN: 4427975, Assay ID: 001983), hsa-miR-938 (PN: 4427975, Assay ID: 002181), RNU44 (PN: 4427975, Assay ID: 001094), RNU48 (PN: 4427975, Assay ID: 001006), U6 snRNA (MammU6, PN: 4427975, Assay ID: 001973) were determined in pituitary samples using individual TaqMan MicroRNA Assays (Applied Biosystems) according to the protocol provided by the supplier. Briefly, reverse transcriptions were performed with miRNA specific stem-loop RT primer using TaqMan MicroRNA Reverse Transcription Kit (P/N: 4366596) followed by qRT-PCR using TaqMan assays and TaqMan Universal PCR Master Mix. Reactions were run in triplicate in 384-well plates on 7900 HT RealTime PCR System (Applied Biosystems). Expression level was calculated by ddCt method, and fold changes were obtained using the formula 2^{-ddCt} .

Pathway analysis

Significantly differentially expressed miRs were uploaded into the DIANA-mirPath tool. In silico target prediction by TargetScan v5. followed by enrichment analysis of

multiple miR target genes by Pearson's Chi-squared test comparing each set of miR targets to all known KEGG pathways was performed. KEGG [31] is a database resource that provides knowledge on several genomes as well as their relationships to biological systems. It has been utilized as a systematic knowledge base for molecular and network biology [32]. For further investigation we excluded miRs with $-\ln(P\text{-value}) < 3$ ($P < 0.05$).

Gene expression

mRNA expression of genes involved in identified pathways was evaluated in 10 NFPA and 10 normal pituitary tissues including each of the 12 samples used for the miR expression experiment. Reverse transcription of 1 µg of total RNA was performed using the Superscript III First Strand Synthesis Kit (Invitrogen, USA). The mRNA expression was determined with qRT-PCR using the 7500 Fast Real-Time PCR System (Applied Biosystems). Quantitative RT-PCR was carried out in triplicate in 20 µl reaction volume containing 10 µl TaqMan Fast Universal PCR Master Mix, 250 pM TM probe, and 10-fold diluted cDNA in RNase-free water. The used inventoried probes were synthesised by Applied Biosystems (P/N: 4331182, SMAD1: Hs00195432_m1, SMAD2: Hs00183425_m1, SMAD3: Hs00232219_m1, SMAD4: Hs00929647_m1, SMAD5: Hs00195437_m1, SMAD6: Hs00178579_m1, SMAD7: Hs00178696_m1, SMAD9: Hs00195441_m1, DLK1: Hs00171584_m1, MEG3: Hs01087966_m1, and endogenous controls: 18S: Hs99999901_s1, ACTB: Hs99999903_m1, GAPDH: Hs99999905_m1, B2M: Hs99999907_m1, HPRT1: Hs99999909_m1). The most appropriate housekeeping genes were determined by Normfinder software. Based on these results, the geometric mean of GAPDH and ACTB was used as a computed endogenous control.

Expression level was calculated by the ddCt method, and fold changes were obtained using the formula $2^{-\text{ddCt}}$.

In silico target prediction for *Smad3*

In addition to the DIANA-mirPath tool for target prediction, we used three computational target prediction algorithms: microrna.org (<http://www.microrna.org/microrna/home.do>), Targetscan 4.0 (<http://www.targetscan.org>) and MicroCosm (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>).

Statistical analysis

MiR expression data obtained by TLDA were normalized using three housekeeping genes, RNU44, RNU48 and MammalianU6 selected by Normfinder integrated in StatMiner. Normfinder calculates the overall stability for all candidate housekeeping genes tested on a sample set indifferent from its composition. It generates an overall stability rank from distinct intragroup and intergroup measures of variability. By grouping the samples into tumoral and normal tissues, the best combination of MammU6, RNU44 and RNU48 has been identified, and an endogenous control has been computed by their geometric mean. The same computed endogenous control was used in single miR expression experiments. After data normalisation, miR expression levels in NFPA were compared to those found in normal pituitary tissues using *T*-test or Mann–Whitney *U* test depending on the results of Shapiro–Wilks normality test. Statistical analysis was performed using Integromics RealTime StatMiner and Statistica 7.0 software (StatSoft Inc., Tulsa, USA). Hierarchical cluster analysis was performed by UPGMA clustering method using Euclidean distance for similarity measuring. Results were visualized as heatmaps. For correlation analysis between miR expression, *Smad3* expression and tumor size Pearson's or Spearman rank correlation tests were used depending on the data distribution evaluated by normality test. A value of $P < 0.05$ was considered to be significant.

Results

MiR expression profile of non-functioning pituitary adenomas

miRs with threshold cycle (CT) ≥ 35 were removed from the analysis and were considered as unexpressed. Of the 670 miRs studied, 205 were unexpressed in any pituitary tissues while 3 miRs (miR-124*, miR-515-5p and miR-872) were expressed only in NFPA tissues and 5 miRs (miR-198, miR-299-5p, miR-497*, miR-548c-3p and

miR-622) only in normal pituitary tissues. Of the 457 miRs expressed in both normal and tumor tissues, 92 were significantly underexpressed and 70 significantly overexpressed in NFPA compared to normal pituitary tissues (Suppl. Table 1). Unsupervised hierarchical cluster analysis using significantly altered miRs correctly classified each of the 12 pituitary samples and it separated 3 groups among the NFPA samples (Fig. 1).

Validation of the TLDA miR array

Five overexpressed miRs predicted to target *Smad3* (miR-135a, miR-140-5p, miR-582-3p, miR-582-5p and miR-938) were analysed by individual miR assays. All these miRs were significantly overexpressed in NFPA samples in agreement with findings obtained from the TLDA miR array (Fig. 2).

Chromosomal localization of the differentially expressed miRs

Chromosomal localization of differentially expressed miRs showed that the 162 under- or overexpressed miRs map to 35 chromosomal regions. In the underexpressed group, 4 hotspot regions with 43 miRs, while in the overexpressed group one hotspot region with 8 miRs were identified (Fig. 3, Suppl. Table 1). mRNA expression of two genes (*MEG3* and *DLK1*) mapped to chromosome region 14q32 was measured by qRT-PCR. Both genes were significantly underexpressed in NFPA samples (Fig. 4).

Correlation between miR expression and clinicopathological findings

There was a significant negative correlation between tumor size and the expression level of 18 miRs (Suppl. Table 2). Of these, 6 miRs (miR-450b-5p, miR-424, miR-503, miR-542-3p, miR-629 and miR-214) were significantly underexpressed (Fig. 5), one miR (miR-592) was significantly overexpressed in NFPA compared to normal pituitary tissues and 11 miRs (miR-224, miR-581, miR-221*, miR-504, miR-215, miR-143, miR-103, miR-510, miR-181a, miR186, miR-708) did not differ significantly in tumor samples compared to normal. Four underexpressed miRs (miR-450b-5p, miR-424, miR-503, miR-542-3p) clustered together in chromosomal region Xq26.3.

In silico pathway analysis of differentially expressed miRs

To highlight the potential pathogenetic role of differentially expressed miRs in NFPA and to identify potentially altered biological processes via miRs, an in silico pathway

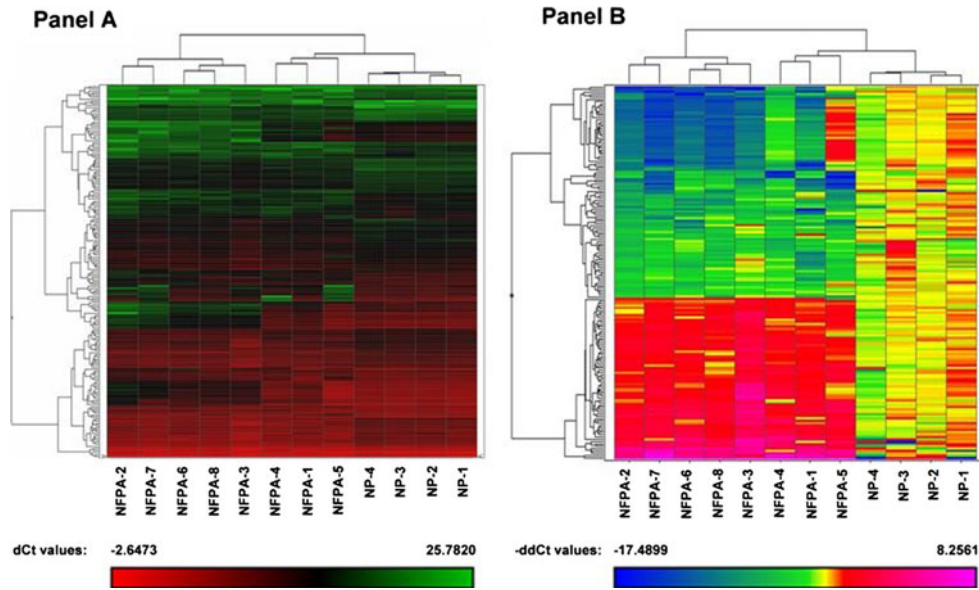
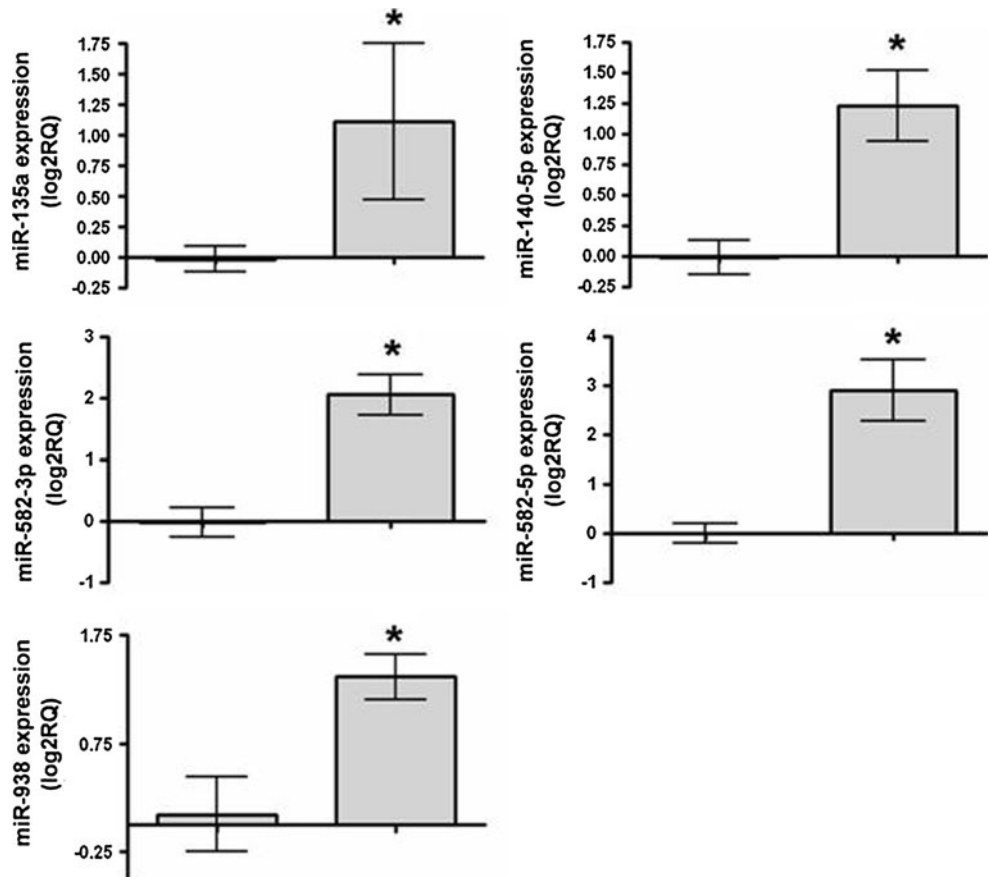


Fig. 1 Hierarchical clustering of the 12 different pituitary samples based on their miR expression patterns. 162 miRs with significant differences in expression were subjected to hierarchical clustering by UPGMA clustering method using Euclidean distance for similarity measuring. Data were normalized using 3 housekeeping genes:

RNU44, RNU48 and MammalianU6. **a** cluster analysis of dCt values, **b** cluster analysis of -ddCt values. Overexpression is indicated with red/pink, whereas underexpression is coded with green/blue. *NFPA* clinically non-functioning pituitary adenoma sample, *NP* normal pituitary sample (Color figure online)

Fig. 2 Expression of miRs by individual Taqman miR assays. *Left column* NP, *right column* NFPA samples. Columns are means \pm SE. * $P < 0.05$



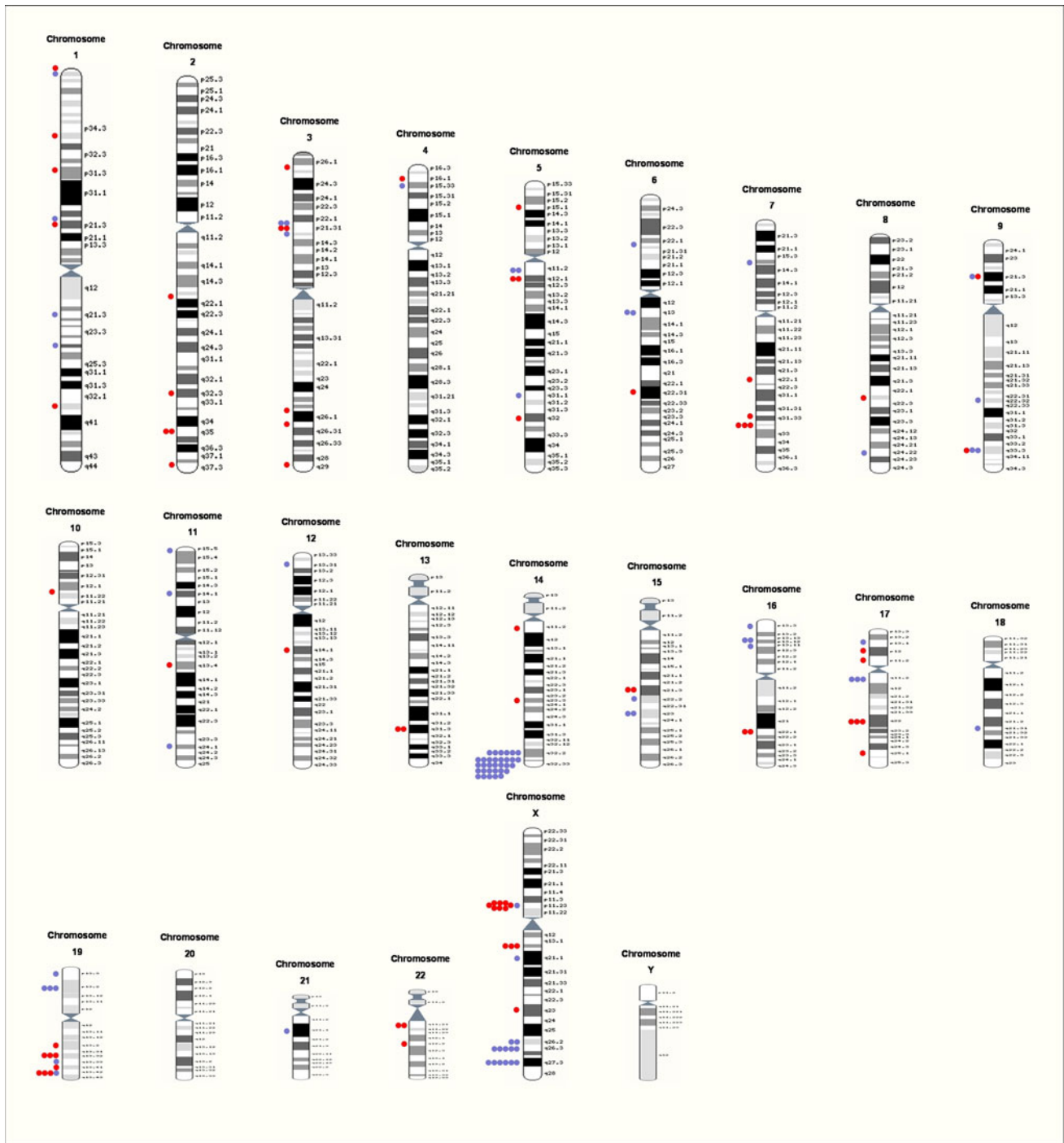


Fig. 3 Chromosomal localization of significantly differentially expressed miRNAs in clinically non-functioning pituitary tumors (each spot represents one miR; red spot overexpressed miR, blue spot underexpressed miR) (Color figure online)

analysis was performed. Using the DIANA-mirPath tool several possible pathways modified by differentially expressed miRNAs were identified (Suppl. Tables 3, 4). Most importantly, comparison of overexpressed miRNAs indicated the possible involvement of the TGFβ signaling pathway with 205 miR-mRNA interactions involving 51 genes and 39 miRNAs in NFPA tissues.

mRNA expression of *Smads* and in silico analysis of miRNAs potentially targeting *Smad3* mRNA

To further explore the possible involvement of the TGFβ signaling pathway in the pathogenesis of NFPA, mRNA expression of *Smads* (*Smad1-9*) were analyzed in pituitary tissues. As shown in Fig. 6., mRNAs of *Smad3*, *Smad6* and

Fig. 4 Chromosomal region of 14q32.2–32.31, harboring *MEG*, *DLK1* and miRs (a). 51 miRs and 53 small nucleolar RNA (snoRNA) encoded here. We measured 46 of the 51 miRs, of those 26 miRs showed underexpression in pituitary tumors compared to normal tissue. Underexpression of *MEG* and *DLK1* in pituitary tumors (right columns) compared to normal tissues (left columns). Columns are means \pm SE. * $P < 0.05$ (b)

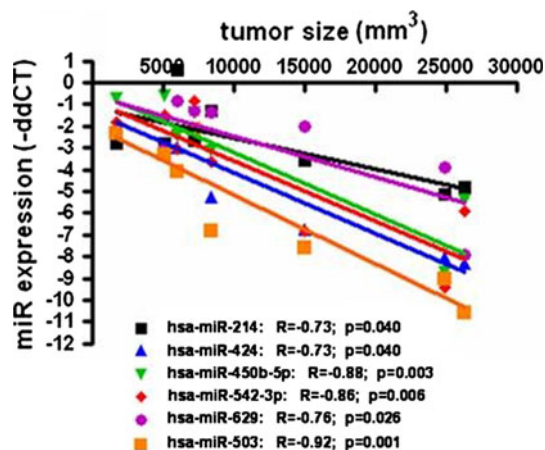
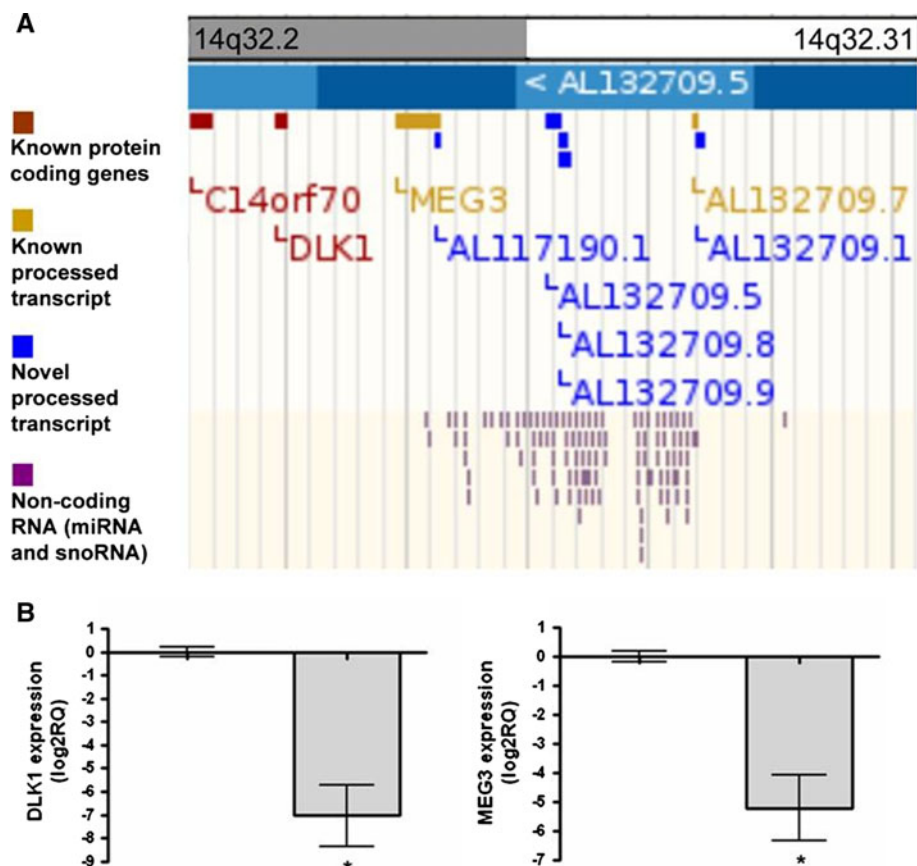


Fig. 5 Significant correlations between expression of underexpressed miRs and tumor size

Smad9 (= *Smad8*) were significantly ($P < 0.001$) underexpressed in NFPA compared to normal pituitary tissues. Expression of *Smad3* but not *Smad6* and *Smad9* showed a tendency to correlate with tumor size ($P = 0.08$, $P = 0.89$ and $P = 0.33$).

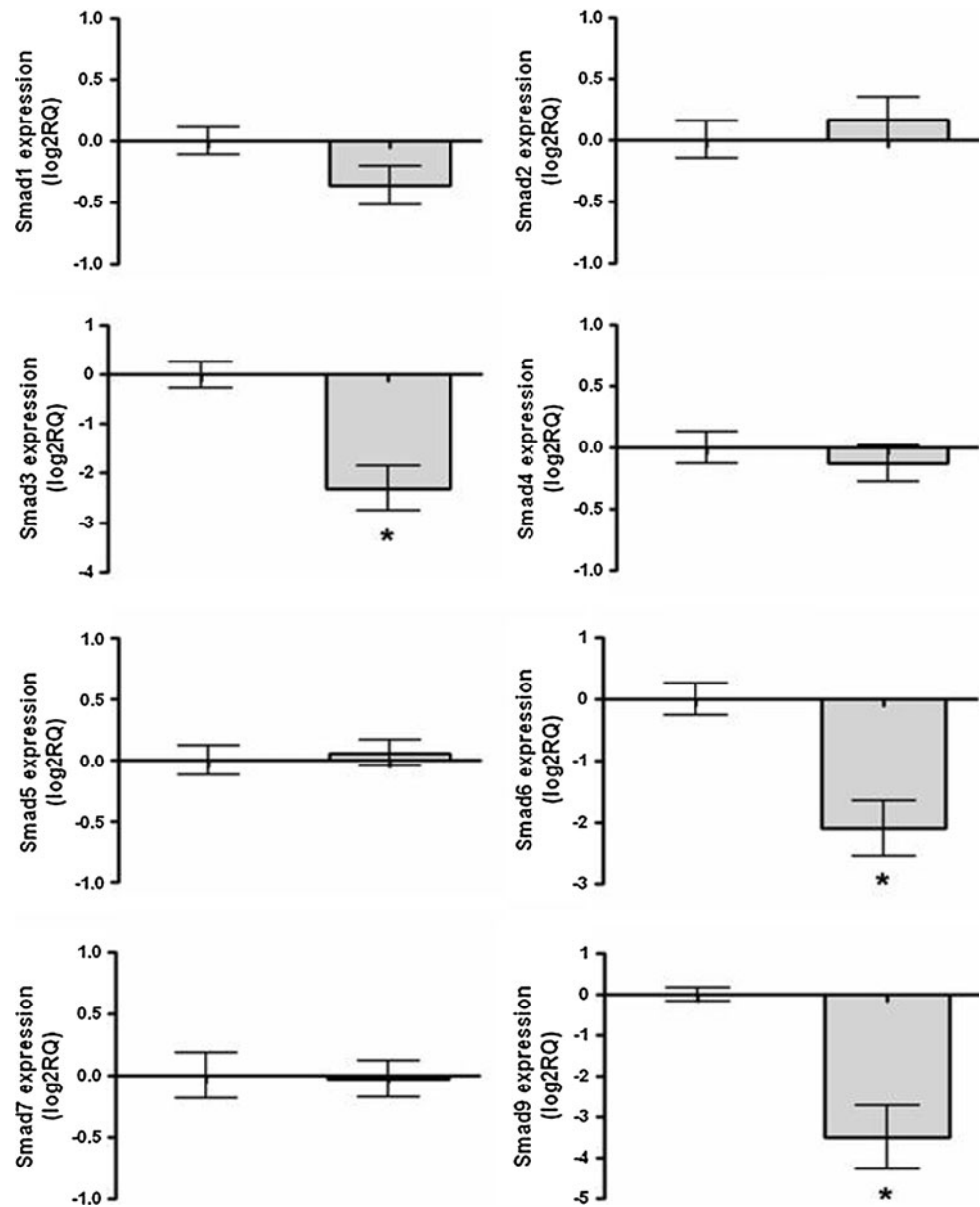
In order to reveal a possible link between *Smad3* mRNA expression and differentially expressed miRs in NFPA, we performed correlation analysis. This analysis revealed that the expression of 80 miRs negatively correlated with

Smad3 expression. Using 4 different algorithms for in silico target prediction, we were able to narrow this list to 19 overexpressed miRs potentially targeting *Smad3* in NFPA (Table 2).

Discussion

In this study, we have assessed the miR expression profiles of NFPA and normal pituitary tissues using TLDA. This method has been successfully applied in earlier studies including ours for the identification of significantly altered miRs in different tumor types such as adrenal and pituitary tumors [33–35]. Because appropriate normalisation of data is critical to ensure accurate and reliable results, miR expression data were normalised to a computed house-keeping gene. Using this approach, we have identified several miRs with significantly different expression between NFPA and normal pituitary. Of the significantly altered miRs, 4 overexpressed (miR-128a, miR-93, miR-378 and miR-429) and one underexpressed miR (miR-543) have been validated previously by qRT-PCR in 18 NFPA and 10 normal pituitary samples (data not shown) [34, 36, 37], ensuring that the expression profile determined by TLDA was accurate. In addition, we measured 5 of 19

Fig. 6 Expression of *Smads* in clinically non-functioning pituitary tumors. Y axis represents each of *Smads* expression (log₂RQ). Columns are means \pm SE. **P* < 0.05



miRs potentially targeting *Smad3* (miR-135a, miR-140-5p, miR-582-3p, miR-582-5p and miR-938) by individual miR assays. All of them and the previously measured miR-429 showed significant overexpression in NFPA samples compared to normal pituitary.

We found that unsupervised hierarchical clustering of miR array results rendered all normal tissues into one group, while NFPA samples were separated into three groups. Three of the 8 NFPA tissues belonged to a group clustered closer to normal pituitary tissues than the other 5 NFPA tissues. However, we were unable to reveal differences in clinical, hormonal or immunohistochemical findings between these three groups of NFPA. This finding may support previous proteomic and mRNA expression

data showing that NFPA tissues obtained from different patients are not homogeneous [38–40].

Chromosomal localization of differentially expressed miRs indicated several regions, including 14q32.2–14q32.31 for 32 underexpressed miRs (Suppl. Table 1, Fig. 3), suggesting that this chromosomal region may contain tumor suppressor gene(s). Indeed, *MEG3*, a tumor suppressor gene maps to this region and it has been found to be underexpressed by promoter hypermethylation in NFPA tumors [41, 42]. Beside *MEG3*, *DLK1* is also mapped to 14q32.31 and in our experiment both genes were underexpressed in NFPA samples (Fig. 4), supporting that expression of those miRs is regulated along with their host genes or their environment [2, 43].

Table 2 List of the 19 overexpressed miRs targeting *Smad3* in clinically non-functioning pituitary adenomas identified by multiple in silico target prediction algorithms, expression level of the identified miRs and their correlations with *Smad3* expression

miR name	Correlation between <i>Smad3</i> –miR		miR expression level		miR targeting <i>SMAD3</i>			
	r(X,Y)	P-value	Fold change	P-value	DIANA-mirPath	TargetScan	microRNA.org	MicroCosm
hsa-miR-93*	−0.811	0.001	1.5	0.048	–	–	–	Yes
hsa-miR-135a	−0.747	0.005	1.8	0.025	–	Yes	–	–
hsa-miR-139-5p	−0.579	0.049	5.2	0.013	–	Yes	–	–
hsa-miR-140-5p	−0.608	0.036	2.8	0.011	–	–	Yes*	–
hsa-miR-153	−0.700	0.011	5.0	0.000	–	Yes	–	–
hsa-miR-200b	−0.657	0.020	1.8	0.045	–	Yes	–	–
hsa-miR-362-5p	−0.609	0.035	3.4	0.036	–	Yes	–	–
hsa-miR-429	−0.623	0.030	3.1	0.001	Yes	Yes	–	–
hsa-miR-491-3p	−0.808	0.001	2.1	0.005	Yes	–	–	–
hsa-miR-500	−0.706	0.010	3.2	0.016	–	Yes	–	–
hsa-miR-501-3p	−0.684	0.014	3.8	0.038	–	Yes	–	–
hsa-miR-501-5p	−0.671	0.017	4.3	0.004	–	Yes	–	–
hsa-miR-545	−0.728	0.007	2.6	0.035	Yes	Yes	–	–
hsa-miR-570	−0.676	0.016	3.3	0.000	Yes	Yes	Yes	–
hsa-miR-579	−0.631	0.028	1.9	0.018	–	Yes	–	–
hsa-miR-582-3p	−0.671	0.017	31.3	0.004	Yes	–	–	–
hsa-miR-582-5p	−0.720	0.008	160.4	0.004	–	–	Yes	–
hsa-miR-641	−0.636	0.026	4.9	0.004	–	Yes	–	–
hsa-miR-938	−0.609	0.036	7.4	0.000	–	Yes	Yes	–

Yes, positive prediction; –, not predicted; *, experimentally validated in earlier studies

Another interesting locus, Xq26.3 was identified in our study with highly underexpressed miRs (miR-424, miR-450b-5p, miR-542-3p, miR-542-5p and miR-503). Three of these miRs, miR-424, miR-542-3p and miR-503 have been predicted to target cyclin D1 and cyclin D2 which are frequently overexpressed in NFPA [44–47]. In addition, miR-503 has been validated to target directly cyclin D1 and it has been suggested to be a putative tumor suppressor [48]. Also, a potentially important target of miR-503 revealed by in silico prediction is the cell cycle regulator CDC25. CDC25 and Wee1 kinase have opposite effects in cell cycle regulation. In our previous study, we found that Wee1 kinase was downregulated by a specific subset of overexpressed miRs in pituitary tumors including NFPA [37]. These findings support the view that cell cycle alteration affecting the regulatory loop between Wee1 and CDC25 is influenced by miRs and that loss of expression of these miRs may result in tumor growth [37].

We have found 19 miRs correlating with tumor size (Suppl. Table 2). Of these miRs, miR-629 and miR-214 potentially target Bcl2, an antiapoptotic molecule and therefore, they may contribute to tumorigenesis through improper regulation of apoptosis. MiR-592 has been reported to be underexpressed in colon tumors deficient in mismatch repair compared to those with proficient

mismatch repair [49]. We observed the upregulation of this miR in NFPA compared to normal pituitary tissues, which supports the findings of Zhu et al. [50] showing that genomic instability frequently found in colon cancer is very unusual in pituitary tumors.

In order to predict the function of miRs in biological processes and to identify pathogenetic pathways potentially modified by posttranscriptional regulation, we performed an in silico pathway analysis for significantly differentially expressed miRs using DIANA-mirPath tool. Several pathways have been identified in NFPA including the TGF β signaling pathway with 205 miR-mRNA interactions involving 51 genes and 39 overexpressed miRs suggesting the complexity of the regulatory network altered in pituitary tumors. For analyzing TGF β pathway in our samples, we measured the expression profile of Smads. These molecules are central regulators of the TGF β and BMP pathways in the pituitary; and their alterations are critical steps towards tumor formation and progression [51]. In previous studies no differences have been found at the level of TGF β receptors between NFPA and normal pituitary tissues, but FSH, LH and TSH β -subunit which are under TGF β regulation [28] were underexpressed in NFPA [27]. Among Smads, we found that *Smad3*, *Smad6* and *Smad9* (=Smad8) were significantly underexpressed. Smad3 is a

downstream molecule of the TGF β signaling, as it forms a complex with Smad4 which translocates to the nucleus. Smad6 and Smad7 can inhibit this process as well as BMP signaling transduction. We found that Smad6 was significantly underexpressed in NFPA samples, however, Smad7 expression did not change. In addition, BMP signaling downstream molecules (Smad1, Smad5 and Smad8) were not altered in these NFPA samples.

Based on these results, we have focused on Smad3, the main TGF β downstream molecule, and based on our in silico pathway analysis we performed a correlation analysis between Smad3 and miRs expression followed by complex bioinformatical target prediction. We identified 19 miRs overexpressed and potentially targeting *Smad3*, of which miR-140-5p has already been experimentally validated to target *Smad3* directly [52]. Interestingly, it has been shown that not only miRs are able to influence Smad expression but Smads are also capable to enhance miRs maturation by binding p68, a component of Drosha microprocessor complex [53]. TGF β /BMP regulated miRs (T/B miRs) contain in their stem region of primary transcripts a conserved sequence similar to Smad binding element (CAGAC or CAGGG). BMP specific Smad1 and Smad5, as well as TGF β specific Smad3 interact with the p68 and enhance T/B miRs' expression posttranscriptionally [54].

Previous studies showed that 44 T/B miRs exist in the human genome [54]. Our finding of 5 underexpressed miRs (miR-199a-5p, miR-214, miR-509-5p, miR-487b and miR-877) may indicate that downregulation of these miRs may be the result of a global inhibition of the TGF β /BMP pathway and may suggest that a negative feed-back regulation loop, similar to that reported for miR-20a and E2F [55], may exist between some miRs and the TGF β /BMP downstream molecule Smads. However, the T/B miR miR-140-3p (the antisense strand of miR-140-5p) showed overexpression in tumors, perhaps indicating that overexpression of this miR may involve other mechanism(s).

Posttranscriptional regulation of the TGF β pathway by miRs has been implicated in both development [56, 57] and tumorigenesis [58, 59]. The TGF β tumor suppressor pathways are affected by miR-106b-25 and the miR-17-92 cluster cooperatively. MiR-106b, miR-93, miR-17-5p and miR-20a suppress p21^{Waf1} expression, which is required for TGF β -induced cell cycle arrest, and earlier studies showed that p21^{Waf1} expression can be detected only in less than 5% of cells of NFPA [60]. MiR-25 and miR-92 inhibit BIM expression, which is essential for TGF β -dependent apoptosis [58, 59]. In our previous study, we found that miR-93, -20a and -17-5p were significantly overexpressed in NFPA compared to normal pituitary tissues.

Another important link between Smad3 and pituitary tumorigenesis arises from the direct interaction between

Smad3 and the tumor suppressor menin. Inactivation of menin blocks TGF β and activin signaling, antagonizing their growth-inhibitory properties in anterior pituitary cells [61]. It is well established that *MEN1* gene mutations play a role in MEN1-related pituitary tumorigenesis, but MEN1 gene mutations seem to be extremely rare in sporadic pituitary adenomas [62, 63]. However, tumor transformation is associated with a significant reduction in menin protein levels in a high percentage of pituitary adenomas [64] and studies by several groups using RT-PCR [65–67] showed no differences in MEN1 mRNA levels between neoplastic and normal pituitary tissues. These data together may raise the possibility that posttranscriptional mechanisms, at least partly determined by MEN1 targeting miRs, may play an important role in the regulation of menin expression. Indeed, 4 miRs (miR-149, miR-570, miR-592, miR-769-5p) in our study potentially targeting the *MEN1* transcript showed significant overexpression but the direct regulation of menin expression by these miRs needs further studies.

The complexity of the regulatory network altered in pituitary tumors is underlined by our pathway analysis as well. In addition to the TGF β pathway, we found the potential involvement of other known pathways, such as MAPK (over-activated in GH producing and NFPA as shown in B-Raf overexpression [68] and proteomics experiments [69]), Akt/mTOR [70], ERK1/2 [71] and Wnt and Notch pathways (Suppl. Tables 3, 4).

Related to pituitary, it has been found in earlier studies that Wnt inhibitors are down-regulated in NFPA [45]. The Wnt downstream effector β -catenin has been shown to exhibit an increased immunostaining in 57% of pituitary adenomas. The Wnt- β -catenin pathway stabilizes PITX2 mRNA, and PITX2 directly activates cyclin D. The changes in WIF1, SFRP1, PITX2, and cyclin D1 expression support the possibility that elevated Wnt- β -catenin signaling is important for pituitary tumorigenesis [45]. In our pathway analysis, Wnt pathway are concerned by miRs with 249 interactions between 72 genes and 44 miRs including overexpressed miRs potentially targeting Wnt inhibitors; WIF1 (miR-128, miR-137, miR-140-3p, miR-200b, miR-330-5p, miR-373, miR-429), SFRP2 (miR-128, miR-340, miR-373) and SFRP4 (miR135a, miR-153, miR-182, miR-183, miR-26a, miR-340, miR-342-3p, miR-500, miR-501-3p, miR-519c-3p, miR-532-5p) Both Wnt and Notch signaling has been described to be modulated by miRs. MiR-17-5p, an overexpressed miR in pituitary, has been found to result in Wnt signaling activation through targeting *HBPI* [72]. Mir-148a has been shown to repress Wnt signaling, and we found it underexpressed in pituitary tissues, suggesting its role in the activation of this pathway [73]. Recently, miR-449 has been described as a modifier of the Wnt pathway in primary pigmented nodular

adrenocortical disease (PPNAD) through targeting *WISP2* [74] and, therefore, its underexpression in NFPA may promote activation of the pathway. We found *Mir-199b* to be underexpressed in pituitary tissues and in vitro experiments has confirmed its inhibitory effect on cell proliferation rate [75].

Recent studies using microarrays and proteomics have emphasized the importance of Notch signaling in pituitary tumorigenesis. Notch3 signaling has been already connected to lung cancer and aggressive T-cell neoplasias. Notch has been shown to be strongly overexpressed and *DLK1*, a potential ligand of Notch3, has been found to be one of the most markedly down-regulated genes in NFPA [39]. *ASCL1*, another component of the Notch pathway, has been also strongly down-regulated in NFPA. It has been suggested that high levels of Notch3 could repress *ASCL1* leading to the loss of *DLK1* expression in these tumors. In our experiments, *miR-197* and *-33b*, which are predicted to target *DLK1*, were significantly overexpressed in NFPA tissues and, therefore, they could result in a loss of *DLK1* expression.

In conclusion, our findings support the possibility that the *TGF β* signaling is repressed and modulated by miRs in NFPA via *Smad3* whose expression can be directly regulated by several overexpressed miRs including the experimentally validated *miR-140*. In addition, the complexity of the regulatory network identified by miR profile may suggest that the decrease of *TGF β* signaling via

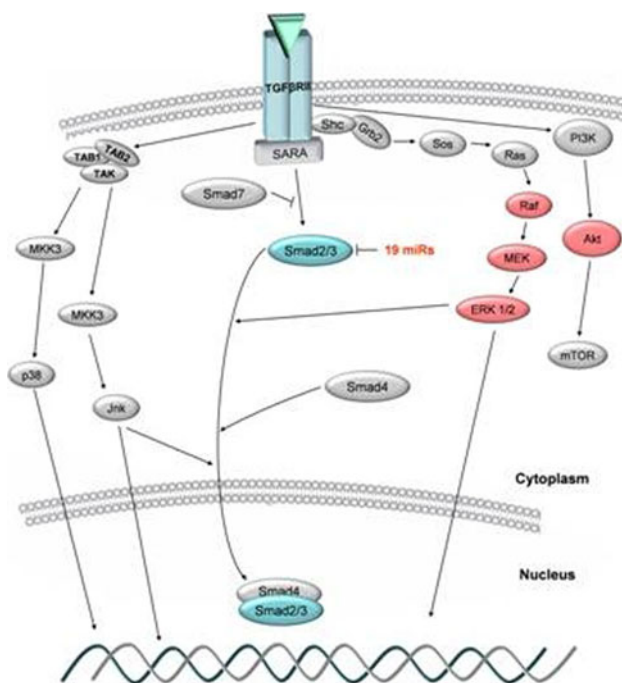


Fig. 7 The *TGF β* pathway influenced by miRs. *Blue* codes represent underexpressed, and *red* codes illustrate overexpressed component of the *TGF β* pathway in clinically non-functioning pituitary adenomas (Color figure online)

Smad3 may result in a shift toward alternative, non-*Smad* pathways, including Ras-MAPK, p38, c-Jun, and PI3 K-Akt (Fig. 7). These alternative pathways have been already considered as contributing factors in pituitary tumorigenesis.

Acknowledgments We are very grateful to Dr V. Zivkovic, Belgrade, Serbia for the provision of the control samples. This work was supported by grant ETT 040-09. A.P. is a recipient of János Bólyai Research Fellowship.

Conflict of interest None.

References

- Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. *Science* 294:853–858
- Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281–297
- Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N (2008) Widespread changes in protein synthesis induced by microRNAs. *Nature* 455:58–63
- Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP (2008) The impact of microRNAs on protein output. *Nature* 455:64–71
- Lee RC, Ambros V (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* 294:862–864
- O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 435:839–843
- Chen CZ, Li L, Lodish HF, Bartel DP (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science* 303:83–86
- Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM (2005) *miR-15* and *miR-16* induce apoptosis by targeting *BCL2*. *Proc Natl Acad Sci USA* 102:13944–13949
- Deng S, Calin GA, Croce CM, Coukos G, Zhang L (2008) Mechanisms of microRNA deregulation in cancer. *Cell Cycle* 7:3729–3741
- Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM (2002) Frequent deletions and down-regulation of micro-RNA genes *miR15* and *miR16* at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 99:15524–15529
- Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T, Takahashi T (2004) Reduced expression of the *let-7* microRNAs in human lung cancers in association with shortened post-operative survival. *Cancer Res* 64:3753–3756
- Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Camoiglio M, Ménard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M, Croce CM (2005) MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 65:7065–7070
- He H, Jazdzewski K, Li W, Liyanarachchi S, Nagy R, Volinia S, Calin GA, Liu CG, Franssila K, Suster S, Kloos RT, Croce CM, de la Chapelle A (2005) The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci USA* 102:19075–19080

14. Roldo C, Missiaglia E, Hagan JP, Falconi M, Capelli P, Bersani S, Calin GA, Volinia S, Liu CG, Scarpa A, Croce CM (2006) MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. *J Clin Oncol* 24:4677–4684
15. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Ménard S, Croce CM (2007) MicroRNA signatures in human ovarian cancer. *Cancer Res* 67:8699–8707
16. Bottoni A, Piccin D, Tagliati F, Luchin A, Zatelli MC, Degli Uberti EC (2005) miR-15a and miR-16-1 down-regulation in pituitary adenomas. *J Cell Physiol* 204:280–285
17. Bottoni A, Zatelli MC, Ferracin M, Tagliati F, Piccin D, Vignali C, Calin GA, Negrini M, Croce CM, Degli Uberti EC (2007) Identification of differentially expressed microRNAs by microarray: a possible role for microRNA genes in pituitary adenomas. *J Cell Physiol* 210:370–373
18. Amaral FC, Torres N, Saggiaro F, Neder L, Machado HR, Silva WA Jr, Moreira AC, Castro M (2009) MicroRNAs differentially expressed in ACTH-secreting pituitary tumors. *J Clin Endocrinol Metab* 94:320–323
19. Daly AF, Burlacu MC, Livadariu E, Beckers A (2007) The epidemiology and management of pituitary incidentalomas. *Horm Res* 68:195–198
20. Derynck R, Zhang YE (2003) Smad-dependent and Smad-independent pathways in TGF- β family signalling. *Nature* 425:577–584
21. Ikushima H, Miyazono K (2010) Cellular context-dependent “colors” of transforming growth factor- β signaling. *Cancer Sci* 101:306–312
22. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF (1995) Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through p53-independent mechanism. *Proc Natl Acad Sci USA* 92:5545–5549
23. Hannon GJ, Beach D (1994) p15INK4B is a potential effector of TGF- β -induced cell cycle arrest. *Nature* 371:257–261
24. Yagi K, Furuhashi M, Aoki H, Goto D, Kuwano H, Sugamura K, Miyazono K, Kato M (2002) c-myc is a downstream target of the Smad pathway. *J Biol Chem* 277:854–861
25. Miyazono K, Miyazawa K (2002) Id: a target of BMP signaling. *Sci STKE* 151:pe40
26. D’Abronzio FH, Swearingen B, Klibanski A, Alexander JM (1999) Mutational analysis of activin/transforming growth factor- β type I and type II receptor kinases in human pituitary tumors. *J Clin Endocrinol Metab* 84:1716–1721
27. Evans CO, Young AN, Brown MR, Brat DJ, Parks JS, Neish AS, Oyesiku NM (2001) Novel patterns of gene expression in pituitary adenomas identified by complementary deoxyribonucleic acid microarrays and quantitative reverse transcription-polymerase chain reaction. *J Clin Endocrinol Metab* 86:3097–3107
28. Wang Y, Fortin J, Lamba P, Bonomi M, Persani L, Roberson MS, Bernard DJ (2008) Activator protein-1 and smad proteins synergistically regulate human follicle-stimulating hormone beta-promoter activity. *Endocrinology* 149:5577–5591
29. Kulig E, Jin L, Qian X, Horvath E, Kovacs K, Stefaneanu L, Scheithauer BW, Lloyd RV (1999) Apoptosis in nontumorous and neoplastic human pituitaries: expression of the Bcl-2 family of proteins. *Am J Pathol* 154:767–774
30. Danila DC, Zhang X, Zhou Y, Haidar JN, Klibanski A (2002) Overexpression of wild-type activin receptor alk4-1 restores activin antiproliferative effects in human pituitary tumor cells. *J Clin Endocrinol Metab* 87:4741–4746
31. Kanehisa M, Goto S (2000) KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28:27–30
32. Aoki KF, Kanehisa M (2005) Using the KEGG database resource. *Curr Protoc Bioinformatics Chapter 1:Unit 1.12*
33. Hui AB, Shi W, Boutros PC, Miller N, Pintilie M, Fyles T, McCready D, Wong D, Gerster K, Waldron L, Jurisica I, Penn LZ, Liu FF (2009) Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. *Lab Invest* 89:597–606
34. Butz H, Liko I, Czirjak S, Igaz P, Zivkovic V, Korbonits M, Racz K, Patocs A (2009) Screening for over-expressed microRNAs in human sporadic pituitary adenomas. The Endocrine Society’s Annual Meeting (ENDO), Washington, DC, Abstract OR39-3, pp 158
35. Tömböl Z, Szabó PM, Molnár V, Wiener Z, Tölgyesi G, Horányi J, Riesz P, Reismann P, Patócs A, Likó I, Gaillard RC, Falus A, Rácz K, Igaz P (2009) Integrative molecular bioinformatics study of human adrenocortical tumors: microRNA, tissue-specific target prediction, and pathway analysis. *Endocr Relat Cancer* 16:895–906
36. Butz H, Likó I, Boyle B, Lendvai N, Igaz P, Czirjak S, Korbonits M, Rácz K, Patócs A (2009) Methods of microRNA research and their use in pituitary adenomas. *J Hung Soc Intern Med* 62:355–362 (Hungarian, abstract in English)
37. Butz H, Likó I, Czirjak S, Igaz P, Munayem Khan M, Zivkovic V, Bálint K, Korbonits M, Rácz K, Patócs A (2010) Down-regulation of Wee1 kinase by a specific subset of microRNA in human sporadic pituitary adenomas. *J Clin Endocrinol Metab* 95:E181–E191
38. Zhan X, Evans CO, Oyesiku NM, Desiderio DM (2003) Proteomics and transcriptomics analyses of secretagogin down-regulation in human non-functional pituitary adenomas. *Pituitary* 6:189–202
39. Moreno CS, Evans CO, Zhan X, Okor M, Desiderio DM, Oyesiku NM (2005) Novel molecular signaling and classification of human clinically nonfunctional pituitary adenomas identified by gene expression profiling and proteomic analyses. *Cancer Res* 65:10214–10222
40. Farrell WE (2006) Pituitary tumours: findings from whole genome analyses. *Endocr Relat Cancer* 13:707–716
41. Gejman R, Batista DL, Zhong Y, Zhou Y, Zhang X, Swearingen B, Stratakis CA, Hedley-Whyte ET, Klibanski A (2008) Selective loss of MEG3 expression and intergenic differentially methylated region hypermethylation in the MEG3/DLK1 locus in human clinically nonfunctioning pituitary adenomas. *J Clin Endocrinol Metab* 93:4119–4125
42. Zhang X, Zhou Y, Mehta KR, Danila DC, Scolavino S, Johnson SR, Klibanski A (2003) A pituitary-derived MEG3 isoform functions as a growth suppressor in tumor cells. *J Clin Endocrinol Metab* 88:5119–5126
43. Baskerville S, Bartel DP (2005) Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA* 11:241–247
44. Hibberts NA, Simpson DJ, Bicknell JE, Broome JC, Hoban PR, Clayton RN, Farrell WE (1999) Analysis of cyclin D1 (CCND1) allelic imbalance and overexpression in sporadic human pituitary tumors. *Clin Cancer Res* 5:2133–2139
45. Elston MS, Gill AJ, Conaglen JV, Clarkson A, Shaw JM, Law AJ, Cook RJ, Little NS, Clifton-Bligh RJ, Robinson BG, McDonald KL (2008) Wnt pathway inhibitors are strongly down-regulated in pituitary tumors. *Endocrinology* 149:1235–1242
46. Simpson DJ, Frost SJ, Bicknell JE, Broome JC, McNicol AM, Clayton RN, Farrell WE (2001) Aberrant expression of G(1)/S regulators is a frequent event in sporadic pituitary adenomas. *Carcinogenesis* 22:1149–1154
47. Jordan S, Lidhar K, Korbonits M, Lowe DG, Grossman AB (2000) Cyclin D and cyclin E expression in normal and adenomatous pituitary. *Eur J Endocrinol* 143:R1–R6
48. Jiang Q, Feng MG, Mo YY (2009) Systematic validation of predicted microRNAs for cyclin D1. *BMC Cancer* 9:194

49. Sarver AL, French AJ, Borralho PM, Thayaniy V, Oberg AL, Silverstein KA, Morlan BW, Riska SM, Boardman LA, Cunningham JM, Subramanian S, Wang L, Smyrk TC, Rodrigues CM, Thibodeau SN, Steer CJ (2009) Human colon cancer profiles show differential microRNA expression depending on mismatch repair status and are characteristic of undifferentiated proliferative states. *BMC Cancer* 9:401
50. Zhu J, Guo SZ, Beggs AH, Maruyama T, Santarius T, Dashner K, Olsen N, Wu JK, Black P (1996) Microsatellite instability analysis of primary human brain tumors. *Oncogene* 12:1417–1423
51. Lebrun JJ (2009) Activin, TGF-beta and menin in pituitary tumorigenesis. *Adv Exp Med Biol* 668:69–78
52. Pais H, Nicolas FE, Soond SM, Swinger TE, Clark IM, Chantry A, Moulton V, Dalmay T (2010) Analyzing mRNA expression identifies Smad3 as a microRNA-140 target regulated only at protein level. *RNA* 16:489–494
53. Davis BN, Hilyard AC, Lagna G, Hata A (2008) SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* 454:56–61
54. Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A (2010) Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Droscha. *Mol Cell* 39:373–384
55. Sylvestre Y, De Guire V, Querido E, Mukhopadhyay UK, Bourdeau V, Major F, Ferbeyre G, Chartrand P (2007) An E2F/miR-20a autoregulatory feedback loop. *J Biol Chem* 282:2135–2143
56. Choi SJ, Moon JH, Ahn YW, Ahn JH, Kim DU, Han TH (2005) Tsc-22 enhances TGF-beta signaling by associating with Smad4 and induces erythroid cell differentiation. *Mol Cell Biochem* 271:23–28
57. Martello G, Zacchigna L, Inui M, Montagner M, Adorno M, Mamidi A, Morsut L, Soligo S, Tran U, Dupont S, Cordenonsi M, Wessely O, Piccolo S (2007) MicroRNA control of Nodal signalling. *Nature* 449:183–188
58. Mishra L, Shetty K, Tang Y, Stuart A, Byers SW (2005) The role of TGF-beta and Wnt signaling in gastrointestinal stem cells and cancer. *Oncogene* 24:5775–5789
59. Petrocca F, Vecchione A, Croce CM (2008) Emerging role of miR-106b-25/miR-17-92 clusters in the control of transforming growth factor beta signaling. *Cancer Res* 68:8191–8194
60. Neto AG, McCutcheon IE, Vang R, Spencer ML, Zhang W, Fuller GN (2005) Elevated expression of p21 (WAF1/Cip1) in hormonally active pituitary adenomas. *Ann Diagn Pathol* 9:6–10
61. Hendy GN, Kaji H, Sowa H, Lebrun JJ, Canaff L (2005) Menin and TGF-beta superfamily member signaling via the Smad pathway in pituitary, parathyroid and osteoblast. *Horm Metab Res* 37:375–379
62. Prezant TR, Levine J, Melmed S (1998) Molecular characterization of the men1 tumor suppressor gene in sporadic pituitary tumors. *J Clin Endocrinol Metab* 83:1388–1391
63. Wenbin C, Asai A, Teramoto A, Sanno N, Kirino T (1999) Mutations of the MEN1 tumor suppressor gene in sporadic pituitary tumors. *Cancer Lett* 142:43–47
64. Theodoropoulou M, Cavallari I, Barzon L, D'Agostino DM, Ferro T, Arzberger T, Grüber Y, Schaaf L, Losa M, Fallo F, Ciminale V, Stalla GK, Pagotto U (2004) Differential expression of menin in sporadic pituitary adenomas. *Endocr Relat Cancer* 11:333–344
65. Asa SL, Somers K, Ezzat S (1998) The MEN-1 gene is rarely down-regulated in pituitary adenomas. *J Clin Endocrinol Metab* 83:3210–3212
66. Farrell WE, Simpson DJ, Bicknell J, Magnay JL, Kyrodimou E, Thakker RV, Clayton RN (1999) Sequence analysis and transcript expression of the MEN1 gene in sporadic pituitary tumours. *Br J Cancer* 80:44–50
67. Satta MA, Korbonits M, Jacobs RA, Bolden-Dwinfour DA, Kaltsas GA, Vangeli V, Adams E, Fahlbusch R, Grossman AB (1999) Expression of menin gene mRNA in pituitary tumours. *Eur J Endocrinol* 140:358–361
68. Ewing I, Pedder-Smith S, Franchi G, Ruscica M, Emery M, Vax V, Garcia E, Czirják S, Hanzély Z, Kola B, Korbonits M, Grossman AB (2007) A mutation and expression analysis of the oncogene BRAF in pituitary adenomas. *Clin Endocrinol (Oxf)* 66:348–352
69. Zhan X, Desiderio DM (2010) Signaling pathway networks mined from human pituitary adenoma proteomics data. *BMC Med Genomics* 3:13
70. Musat M, Korbonits M, Kola B, Borboli N, Hanson MR, Nanzer AM, Grigson J, Jordan S, Morris DG, Gueorguiev M, Coculescu M, Basu S, Grossman AB (2005) Enhanced protein kinase B/Akt signalling in pituitary tumours. *Endocr Relat Cancer* 12:423–433
71. Dworakowska D, Wlodek E, Leontiou CA, Igreja S, Cakir M, Teng M, Prodromou N, Góth MI, Grozinsky-Glasberg S, Gueorguiev M, Kola B, Korbonits M, Grossman AB (2009) Activation of RAF/MEK/ERK and PI3K/AKT/mTOR pathways in pituitary adenomas and their effects on downstream effectors. *Endocr Relat Cancer* 16:1329–1338
72. Li H, Bian C, Liao L, Li J, Zhao RC (2010) miR-17-5p promotes human breast cancer cell migration and invasion through suppression of HBP1. *Breast Cancer Res Treat BMC Genomics* 11:320
73. Qin L, Chen Y, Niu Y, Chen W, Wang Q, Xiao S, Li A, Xie Y, Li J, Zhao X, He Z, Mo D (2010) A deep investigation into the adipogenesis mechanism: profile of microRNAs regulating adipogenesis by modulating the canonical Wnt/beta-catenin signaling pathway. *BMC Genomics* 11:320
74. Iliopoulos D, Bimpaki EI, Nesterova M, Stratakis CA (2009) MicroRNA signature of primary pigmented nodular adrenocortical disease: clinical correlations and regulation of Wnt signaling. *Cancer Res* 69:3278–3282
75. Garzia L, Andolfo I, Cusanelli E, Marino N, Petrosino G, De Martino D, Esposito V, Galeone A, Navas L, Esposito S, Gargiulo S, Fattet S, Donofrio V, Cinalli G, Brunetti A, Vecchio LD, Northcott PA, Delattre O, Taylor MD, Iolascon A, Zollo M (2009) MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma. *PLoS One* 4:e4998