Integrative analysis of miRNA and mRNA expression profiles in pheochromocytoma and paraganglioma identifies genotype-specific markers and potentially regulated pathways

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Abstract

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are rare neuroendocrine neoplasias of neural crest origin that can be part of several inherited syndromes. Although their mRNA profiles are known to depend on genetic background, a number of questions related to tumor biology and clinical behavior remain unanswered. As microRNAs (miRNAs) are key players in the modulation of gene expression, their comprehensive analysis could resolve some of these issues. Through characterization of miRNA profiles in 69 frozen tumors with germline mutations in the genes SDHD, SDHB, VHL, RET, NF1, TMEM127, and MAX, we identified miRNA signatures specific to, as well as common among, the genetic groups of PCCs/PGLs. miRNA expression profiles were validated in an independent series of 30 composed of VHL-, SDHB-, SDHD-, and RET-related formalin-fixed paraffin-embedded PCC/PGL samples using quantitative real-time PCR. Upregulation of miR-210 in VHL- and SDHB-related PCCs/PGLs was verified, while miR-137 and miR-382 were confirmed as generally upregulated in PCCs/PGLs (except in MAX-related tumors). Also, we confirmed overexpression of miR-133B as VHL-specific miRNAs, miR-488 and miR-885-5p as RET-specific miRNAs, and miR-183 and miR-96 as SDHB-specific miRNAs. To determine the potential roles miRNAs play in PCC/PGL pathogenesis, we performed bioinformatic integration and pathway analysis using matched mRNA profiling data that indicated a common enrichment

Key Words
- pheochromocytoma
- paraganglioma
- microRNA
- differentiation

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of pathways associated with neuronal and neuroendocrine-like differentiation. We demonstrated that miR-183 and/or miR-96 impede NGF-induced differentiation in PC12 cells. Finally, global proteomic analysis in SDHB and MAX tumors allowed us to determine that miRNA regulation occurs primarily through mRNA degradation in PCCs/PGLs, which partially confirmed our miRNA–mRNA integration results.

Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are rare neuroendocrine tumors of neural crest origin. Up to an estimated 60% of PCCs/PGLs are associated with germline or somatic mutations in one many susceptibility genes (RET; VHL; NF1; SDH subunits A, B, C, D, and AF2; TMEM127; MAX; and HIF2A (EPAS1); Baysal et al. 2000, Cascon et al. 2009, Mannelli et al. 2009, Bayley et al. 2010, Burnichon et al. 2010, 2012a,b, Qin et al. 2010, Comino-Mendez et al. 2011, 2013, Zhuang et al. 2012, Lorenzo et al. 2013, Pacak et al. 2013, Taieb et al. 2013, Toledo et al. 2013, Yang et al. 2013). Although two decades of comprehensive study of the clinical features associated with the known PCC/PGL genes has improved patient care and genetic counseling, the highly variable behavior of these tumors continues to complicate disease management (reviewed by Raimundo et al. (2011)). Other approaches, such as high-throughput OMIC technologies or deep sequencing, have demonstrated their robustness to gain further insight into the molecular mechanisms behind PCCs/PGLs. In fact, several mRNA expression profiling studies have demonstrated that unsupervised analysis of PCCs/PGLs show different gene signatures depending on the primary mutation (Eisenhofer et al. 2004, Dahia 2006, Favier et al. 2009, Lopez-Jimenez et al. 2010, Comino-Mendez et al. 2011, 2013, Zhuang et al. 2012, Lorenzo et al. 2013, Pacak et al. 2013, Taieb et al. 2013, Toledo et al. 2013, Yang et al. 2013). More specifically, ‘cluster 1’, containing VHL- and SDHx-related tumors, is associated with a pseudohypoxic gene signature, while RET-, NF1-, TMEM127-, and MAX-related PCCs/PGLs allocate to ‘cluster 2’, enriched in the PI3K/AKT and RAS signaling pathways (Maher & Eng 2002, Eisenhofer et al. 2004, Dahia 2006, Favier et al. 2009, Lopez-Jimenez et al. 2010, Qin et al. 2010, Comino-Mendez et al. 2011, Jiang & Dahia 2011). Although promising mutation-specific markers have been identified through this extensive characterization (Eisenhofer et al. 2004, Dahia 2006, Favier et al. 2009, Lopez-Jimenez et al. 2010), a more complete picture of the gene expression regulation is required to better understand the mechanisms involved in PCC/PGL development.

Small non-coding microRNAs (miRNAs), capable of posttranscriptionally regulating the expression of an estimated one third of all metazoan protein-coding genes (Bartel 2004), have not been fully characterized in PCCs/PGLs. miRNAs bind to semi-complimentary sites at the 3′-UTR of targeted mRNA, which can result in mRNA degradation and/or translational truncation (Bartel 2004, Lim et al. 2005), and thus can affect gene expression.

To date, three miRNA expression profiling studies have been performed on PCCs/PGLs (Meyer-Rochow et al. 2010, Tombol et al. 2010, Patterson et al. 2012). This study identifies for the first time miRNA expression levels in seven genetic classes of PCCs/PGLs (VHL, SDHB, SDHD, RET, NF1, TMEM127, and MAX mutants), which were validated in an independent series. Integration of matched miRNA and mRNA expression profiles identified miRNA-regulated gene expression networks that may contribute to PCC/PGL pathogenesis. Functional studies in PC12 cells showed that miR-183 and/or miR-96 impede NGF-induced differentiation. Furthermore, global proteomic analysis of SDHB- and MAX-related tumors indicated that miRNA regulation occurs primarily through mRNA degradation in PCCs/PGLs and suggested that miR-183/96 may contribute to SDHB-related PCC/PGL development by targeting ezrin (EZR) to interfere with differentiation. Our study not only identifies genotype-specific miRNAs in PCCs/PGLs but also provides new possible insights into PCC/PGL development.

Materials and methods

Tumor and normal tissues

Fresh frozen (n = 69) and formalin-fixed paraffin-embedded (FFPE; n = 30) specimens, including PCCs/PGLs and normal adrenal medullas (nAM), were collected by hospitals through the Spanish National Tumor Bank...
Network (CNIO) and by the Instituto Oncologico Veneto in Italy. Written informed consent to collect phenotypic and genotypic data was obtained from all participants in accordance with Institutional Review Board (IRB)-approved protocols of each center. Fresh frozen and FFPE tissues were prepared as described previously (Lopez-Jimenez et al. 2010).

Hematoxylin and eosin staining from all samples were evaluated by two pathologists and selected only those contained at least 85% tumor cells. The material derived from normal adrenal gland available was obtained from multiorgan donations and selected by pathologists. All frozen and FFPE tissues were previously genetically characterized (Lopez-Jimenez et al. 2010, Comino-Mendez et al. 2011) and classified accordingly. Frozen specimens used for miRNA expression profiling contained germline mutations in the following genes: VHL (n=13), SDHB (n=9), SDHD (n=4), all parasympathetic PGLs, RET (n=14), NF1 (n=4), TMEM127 (n=3), and MAX (n=3); the series also included 14 sporadic PCCs/PGLs (with no mutations in the known susceptibility genes) and six nAM tissue samples. Of 63 tumors, only five were malignant (SDHB_4, SDHB_5, MAX_1, MAX_2, and Sporadic_14).

Owing to limited availability of material, an independent collection of 30 PCC/PGL FFPE samples was used for validation, with germline mutations in the following genes: VHL (n=6), SDHB (n=6), SDHD (n=7), all parasympathetic PGLs, RET (n=8), and two nAM tissues. The clinical features and the genetic characteristics of frozen and FFPE samples are detailed in Table 1.

**RNA extraction**

Total RNA was isolated from frozen tissues using the TRI Reagent Kit (MRC, Cincinnati, OH, USA). RNA purity and integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All samples used for hybridization had an RNA integrity number of at least 7. For FFPE samples, total RNA isolation was done using the RNeasy FFPE Kit (Qiagen). RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNase treatment was performed using the DNA-free Kit (Ambion, Austin, TX, USA) following the manufacturer’s instructions.

**miRNA expression profiling and data processing**

miRNA expression profiling was performed using the Agilent Human miRNA Microarray Kit version 2.0 (G4470B, Agilent Technologies). For each tissue sample, 100 ng total RNA was hybridized and processed in accordance with the manufacturer’s instructions. The arrays were scanned with a G2565C DNA microarray scanner (Agilent Technologies), images were processed using the Agilent Feature Extraction (AFE) Software package version 10.1.1 (Agilent Technologies), and data were exported as text files. The exported data files were read into R using the Limma package (Gentleman et al. 2004), and the processed miRNA signal was obtained with the AgiMicroRNA package applying the RMA algorithm to obtain the normalized dataset (Lopez-Romero et al. 2010).

miRNAs not expressed in more than 75% of the samples in each class were filtered. miRNA data were further processed by filtering flat patterns, and all subsequent analyses were performed using only miRNAs that passed both filters.

**Unsupervised analysis**

In order to increase specificity, unsupervised hierarchical cluster analysis was performed with only miRNAs with significant expression (one-way ANOVA, false discovery rate (FDR) <0.002). Samples were grouped according to their expression profiles using GeneCluster 3.0 (de Hoon et al. 2004) and viewed in a visualizer that displays cluster profiles and relevant cluster member information.

**Supervised analysis and miRNA marker selection**

Differential expression of miRNAs was computed vs nAM samples independently for each experimental groups by non-permutation t-test analysis with limma Pomelo II web tool (Morrissy & Diaz-Uriarte 2009). This way, we were able to identify the miRNA signature associated with PCC/PGL experimental groups. As nAM was included as a calibrator, its effect cancels out when comparing between experimental groups. To facilitate the identification of specific miRNAs related to the genetic background, sporadic tumors were initially considered to identify miRNAs common among all PCCs/PGLs, but not taken into account thereafter as they are genetically undefined. For each comparison (genetic group vs nAM), only miRNAs with an FDR <0.05 and a fold change (log2) ≥ ±1.3 were considered significantly differentially expressed. By submitting these lists of miRNAs to Venn diagram analysis (VENNY, http://bioinfogp.cnb.csic.es/tools/venny/index.html), we identified miRNAs specific to, as well as those common among all, genetic groups of PCCs/PGLs. In addition to the above criteria, we...
<table>
<thead>
<tr>
<th>Tumor ID*</th>
<th>Frozen/FFPE</th>
<th>Germline mutation</th>
<th>Protein</th>
<th>Sex</th>
<th>Age</th>
<th>Location</th>
<th>PCC/PGL</th>
<th>M/B</th>
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<td>p.?</td>
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<td>p.?</td>
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MB, malignant/benign. Malignancy was defined as the presence of metastasis where chromaffin cells are normally absent. NA, not available.

aTumor ID indicates the primary germline mutation followed by tumor identification number (i.e. germline mutation_ID#).
bPublication submitted.
cNF1 mutation determined based on clinical criteria. Mutations were annotated according to the corresponding transcript ID for each gene analyzed: SDHB (ENST00000375499), SDHD (ENST00000375549), VHL (ENST00000256474), RET (ENST00000355710), TMEM127 (ENST00000258439), NFI (ENST00000358273), and MAX (ENST00000358664).

considered reported biological functions, when available, in the final selection of candidate miRNAs for validation.

RT and quantitative real-time PCR

First-strand cDNA synthesis by RT of total RNA was performed using the miRCURY LNA Universal RT miR PCR system (Exiqon, Vedbaek, Denmark) according to the manufacturer’s recommendations. Quantitative real-time PCR (qRT-PCR) were performed on an ABI PRISM 7900HT analyzer (Applied Biosystems) using the LNA miR-PCR primer/SYBR Green mix (Exiqon) in accordance with the manufacturer’s recommendations. Reactions were performed in triplicate, and negative controls were included in all series of qRT-PCRs. The qRT-PCR data were imported into qBase (Hellemans et al. 2007), and after assessing the
stability of five endogenous reference RNAs, we selected the most stable ones, SS-rRNA, SNORD48, and SNORD66, for normalization. Relative miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed using StatPlus version 2009 (AnalystSoft, http://www.analystsoft.com/en/). Statistical differences between the four genetic classes of PCCs/PGLs (SDHB, SDHD, VHL, and RET mutants), as well as nAM, were assessed using Kruskal–Wallis one-way ANOVA ($P<0.05$).

### Integration of mRNA and miRNA expression data

Using matched transcriptomic (mRNA) data available from the same tumors (Lopez-Jimenez et al. 2010), integration with miRNA profiles was performed using the MiRNA And Genes Integrative Analysis (MAGIA) web tool (Sales et al. 2010), for each sample in a given experimental group. First, this integration analysis was applied to all PCCs/PGLs regardless of genetic background and nAMs and then to each genetic group, filtering those miRNAs common among all PCCs/PGLs (FDR $<0.05$). The log$_2$-transformed mRNA data contained 19,620 genes. The tool (Sales the MiRNA And Genes Integrative Analysis (MAGIA) web integration with miRNA profiles was performed using miRNAs common among all PCCs/PGLs (FDR $<0.05$) and nAMs and then to each genetic group, filtering those applied to all PCCs/PGLs regardless of genetic background experimental group. First, this integration analysis was carried out in triplicate.

### PC12 cells data set

A miRNA microarray data set for PC12 cells was obtained from a publicly available database, Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/gds), from the study of Hamada et al. (2012), which included expression of 350 miRNAs after NGF stimulation at four time points (0, 12, 24, and 48 h).

### Cell culture

PC12 cells (provided by Marcos Malumbres, CNIO, Madrid, Spain) were grown in a humidified 5% CO$_2$ atmosphere at 37°C in a complete medium, DMEM supplemented with 5% horse serum (Sigma) and 10% bovine calf serum (Sigma). To assess differentiation, cells were grown in a differentiating medium, DMEM supplemented with 0.5% horse serum and 1% bovine calf serum.

For stimulation, PC12 cells were plated on poly-L-lysine-coated 96-well plates ($0.5 \times 10^4$ cells/well) in the complete medium for 24 h prior cotransfection and, after 12 h, treated with the differentiating medium in the presence of low-dose NGF (10 ng/ml; Sigma). For negative (undifferentiated) and positive (differentiating) controls, we used PC12 cells transfected with miR-cel-67 in the absence and presence of NGF respectively.

### miRNA mimics and transfections

Twenty-four hours after plating, cells were cotransfected with miRNA mimics and pcGFP-1 using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions (Life Technologies). The pcGFP-1 green fluorescent protein reporter was used to indicate positively transfected cells. Caenorhabditis elegans cel-miR-67 mimic does not target any gene in human and rat and was used for control transfections. The final concentration of miRNA mimic and pcGFP-1 used for cotransfections was $30 \text{ nM}$ and $0.2 \text{ ng/\mu l}$ respectively. Three independent experiments were performed and experimental conditions carried out in triplicate.

### Quantitative analysis of cellular morphology and differentiation

PC12 cells ($0.5 \times 10^4$ cells/well) were fixed with 4% formalin solution (Merck KLaA) for 10 min. Cells were washed with PBS and incubated with 0.2 $\mu$g/ml DAPI (Life Technologies) and CellMask:PBS (1:3; Life Technologies) for 40 min at room temperature and then washed with PBS. Images were captured in a laser scanning confocal TCS-SP2 (Leica Microsystems, Wetzlar, Germany) using LCS acquisition software (v2.61, Leica Microsystems) and magnification was $63 \times$ oil immersion objective with 1.4 NA.

Using the CellMask signal as the cell image, the DAPI signal as the nuclear image, and GFP signal as the neuronal cell image to discriminate transfected cells, cellular morphology was assessed by the Definiens Developer XD Software (v2.0; Munich, Germany). Only GFP-positive PC12 cells were considered for subsequent analysis. Cellular morphology was quantified using three parameters: length ($\mu$m), border length ($\mu$m), and roundness (unitless values $0 \rightarrow \infty$; more round (0) $\rightarrow$ less round ($\infty$)).

NGF-negative and -positive controls were used to define neuron-like differentiation using the above Definiens parameters. The NGF-negative group represented cells with a less differentiated phenotype, more round...
with few projections, and with lower values for cellular length, border length, and roundness. On the other hand, cells in the NGF-positive group displayed a neuronal-like differentiated phenotype, with neurite elongation and higher values for these parameters. This way we were able to assess the level of differentiation of cells in the miR-183- and/or miR-96-transfected groups.

Proteomic analysis

Four PCC/PGL tumors (SDHB_3, SDHB_9, MAX_1, and MAX_3) were subjected to label-free proteome analysis. Samples were extracted and proteins were digested using a standard FASP protocol (Wisniewski et al. 2009). The resulting peptides were separated by online nano-LC and analyzed by electrospray MS/MS using a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA, USA). The same protein amount was injected in triplicates. Raw files were searched against UniProtKB/Swiss-Prot human database (release date: April 18, 2012; 73 579 sequences) using MaxQuant Software (v1.3.0.3; Martinsried, Germany; Cox & Mann 2008). For protein assessment, at least two unique peptides with a FDR = 0.01 were required. Label-free analysis was performed using the label-free quantitation (LFQ) values (Luber et al. 2010) determined by MaxQuant. Further analysis was done with Perseus Software (v1.3.0.3; Martinsried, Germany). A more detailed description of methodology is provided in the Supplementary Materials and methods, see section on supplementary data given at the end of this article.

Results

Unsupervised analysis of miRNA expression profiles

A full listing of the microarray results has been deposited in the National Center for Biotechnology Information GEO database under accession no. GSE29742. A total of 230 miRNAs were identified as significantly differently expressed among the PCC/PGL classes and nAM (ANOVA, FDR < 0.05). Unsupervised hierarchical cluster analysis revealed a great homogeneity among cases with an alteration in the same gene (Fig. 1). Two main clusters were identified, mainly defined by SDHx/VHL/nAM and RET/NF1/TMEM127/MAX specimen profiles respectively. Of 69 samples, only two from the RET/NF1/TMEM127/MAX-related cluster were allocated to the VHL/SDHx/nAM branch. Both adrenal and extra-adrenal tumors were represented in both clusters, whereas the head and neck tumors were grouped within the VHL/SDHx/nAM branch.

With the exception of two tumors (SP_1 and SP_10: abdominal and carotid PGLs respectively), the remaining sporadic PCCs/PGLs (two abdominal and ten adrenal) clustered within the RET/NF1/TMEM127/MAX branch. The nAMs also included in the unsupervised hierarchical cluster analysis clustered together and formed a separate subcluster, indicating that these samples have very similar miRNA signatures and that this signature was different from those of tumor samples. It is known that obtaining nAM without cortical contamination is extremely difficult. However, we used these nAMs available solely as a calibrator because they resemble normal adrenal tissue more than the commercial RNA reference, and any effect related to possible cortical contamination would cancel out during comparisons between experimental groups.

miRNAs can be used as genetic group markers

Owing to the limited number of malignant tumors in our series, we could not identify any differentially expressed miRNAs during the comparison between malignant and benign tumors (data not shown). Supervised analysis results of miRNA expression data comparing individual tumor classes with nAM are listed in Supplementary Table 1, see section on supplementary data given at the end of this article. We found that 51 miRNAs were significantly differently expressed in VHL-related tumors, 54 in SDHB, 32 in SDHD, 50 in RET, 35 in NF1, 60 in TMEM127, 64 in MAX, and 49 in sporadic tumors compared with nAM. Five miRNAs (miR-193b, miR-424, miR-365, miR-493*, and miR-99a) were identified as commonly deregulated among all PCCs/PGLs (Fig. 2, Supplementary Table 1). Besides identifying miRNAs commonly deregulated, we determined group-specific miRNA markers: 12 for VHL-related tumors, 5 for SDHB, 6 for SDHD, 6 for RET, 1 for NF1, 10 for TMEM127, and 31 for MAX (Fig. 2).

miRNA validation

Three miRNAs (miR-137, miR-382, and miR-210) common among some PCC/PGL experimental groups and five group-specific miRNAs (miR-133b, miR-183, miR-488, miR-885-5p, and miR-96) were selected for validation. Upregulation of miR-137 was a common feature among experimental groups with the exception of MAX-related tumors and was selected as a general PGL/PCC tumor marker. While significantly downregulated in MAX-related PCCs/PGLs, miR-382 was upregulated in nearly all tumors, but especially in VHL-, SDHB-, SDHD-, and RET-related tumors and
Hierarchical clustering of the nine experimental groups based on their microRNA (miRNA) expression profiles. Those 93 miRNAs with significant differences in expression (ANOVA, \( P < 0.002 \)) were subjected to unsupervised hierarchical cluster analysis. Both genes and samples were clustered by average linkage clustering method. Overexpression is shown in red, whereas under-expression is indicated in green. Blue dots indicate mis-clustered samples (max2 and ret1).

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0183
Published by Bioscientifica Ltd.
therefore selected for validation. Upregulation of miRNA-210 was unique to both SDHB- and VHL-related tumors and selected for validation to discriminate SDHB and VHL tumors from other experimental groups. Upregulation of miR-133b was specific to the VHL-mutant group, while upregulation of miR-488 and miR-885-5p was specific to RET-related tumors. miR-183 and miR-96 were selected for validation, as their robust upregulation was associated exclusively with SDHB-related tumors. All the above miRNAs were confirmed by RT-qPCR in an independent series (ANOVA, \( P < 0.05 \); Supplementary Figure 1, see section on supplementary data given at the end of this article), which allowed for further interpretation and integration of miRNA profiling data.

**Integrative analysis of miRNA/mRNA expression**

The negative and positive correlations of miRNA–mRNA interactions predicted by MAGIA are provided in Supplementary Table 2. The actual number of unique target genes was lower than the predicted miRNA–mRNA interactions as several genes can be targeted by multiple miRNAs (Table 2). Predicted miRNA–mRNA interactions for individual miRNAs were further explored to infer their biological function.

Because of the poor prognosis associated with SDHB-related tumors, we were interested in examining those genes predicted as miR-183 and miR-96 targets. Among the 101 potential miR-183 and miR-96 targets, there were genes involved with neuronal or neuroendocrine-like differentiation, such as EZR; Rho GTPase-activating protein 18 (ARHGAP18); CTD small phosphatase 1 (CTDSP1); FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (FARP1); and leucine-rich, glioma inactivated 1 (LGI1).

Predicted miRNA target gene interactions with reported functions in neuronal or neuroendocrine-like differentiation was not only unique to SDHB-related PCCs/PGLs, but in fact observed in all experimental groups, including those belonging to cluster 2, such as MAX-mutant tumors. Some examples for the latter included brain-derived neurotrophic factor (BDNF) targeted by miRs-370/381/382/495, CREB5 by miRs-539/543/495, PIK3R1 by miRs-376a/376b/495, and sortilin-related VPS10 domain containing receptor 1 (SORCS1) by miR-382/495.

After obtaining the miRNA–mRNA interactions predicted by MAGIA, we applied IPA enrichment analysis to identify pathways potentially regulated by the five commonly under-expressed miRNAs (Supplementary Table 3, see section on supplementary data given at the end of this article). ‘CREB signaling in neurons’, ‘breast cancer regulation by Stathmin 1 (STMN1)’, and ‘ERK5 signaling’ were included among these pathways.
Endocrine-Related Cancer was enriched in RET pathways, while the protein kinase A signaling pathway, lipase C signaling, and LPS-stimulated MAPK signaling alterations in mTOR signaling, PTEN signaling, phospho-RET/PCCs/PGLs.

For instance, in the same cluster. Experimental groups belonging to cluster 2 or similar among experimental groups belonging to the purigenic receptor signaling pathways. In neurons, ‘neurotrophin/TRK signaling’, and ‘P2Y tumors showed significant enrichment in ‘CREB signaling’, ‘synaptic long term potentiation’, and ‘dopamine-DARPP32 feedback in cAMP signaling’, while existing differentiation as a common feature. For instance, those SDHB-related pathways were involved in neuronal and neuroendocrine-like differentiation as a common feature. For instance, those SDHB-related pathways included ‘axonal guidance signaling’, ‘synaptic long term potentiation’, and ‘dopamine-DARPP32 feedback in cAMP signaling’, while MAX-related tumors showed significant enrichment in ‘CREB signaling in neurons’, ‘neurotrophin/TRK signaling’, and ‘P2Y purigenic receptor signaling’ pathways.

Predicted miRNA-regulated pathways were the same or similar among experimental groups belonging to the same cluster. Experimental groups belonging to cluster 2 showed enrichment in pathways associated with PI3K/AKT, RAS, and mTOR signaling. For instance, in RET-, NF1-, and TMEM127-related PCCs/PGLs, we detected alterations in mTOR signaling, PTEN signaling, phospholipase C signaling, and LPS-stimulated MAPK signaling pathways, while the protein kinase A signaling pathway was enriched in RET-, TMEM127-, and MAX-related PCCs/PGLs. RET- and TMEM127-related tumors were enriched in the NGF signaling pathway, and the SAPK/JNK signaling pathway was enriched in RET- and NF1-related PCCs/PGLs. Interestingly, the ERK/MAPK and p70S6K signaling pathways were significantly enriched in RET-, NF1-, and TMEM127-related tumors, as well as in VHL-related tumors, while the PI3K/AKT signaling pathway was uniquely enriched in TMEM127-related tumors.

**Table 2** Number of potential targets for the miRNAs with significant differences in expression for each experimental group of PCCs/PGLs relative to nAM. Percent of genes potentially regulated by the microRNAs was calculated using the total number of unique genes present on the Agilent Human 4×4k array (n = 19,620).

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Total interactions</th>
<th>Unique genes</th>
<th>Total genes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL</td>
<td>2583</td>
<td>1710</td>
<td>8.72</td>
</tr>
<tr>
<td>SDHB</td>
<td>2193</td>
<td>1410</td>
<td>7.19</td>
</tr>
<tr>
<td>SDHD</td>
<td>749</td>
<td>638</td>
<td>3.25</td>
</tr>
<tr>
<td>RET</td>
<td>2942</td>
<td>1981</td>
<td>10.10</td>
</tr>
<tr>
<td>NF1</td>
<td>1749</td>
<td>1244</td>
<td>6.34</td>
</tr>
<tr>
<td>TMEM127</td>
<td>3412</td>
<td>2084</td>
<td>10.62</td>
</tr>
<tr>
<td>MAX</td>
<td>1160</td>
<td>682</td>
<td>3.48</td>
</tr>
<tr>
<td>14q32.2 miRNAs in MAX</td>
<td>800</td>
<td>500</td>
<td>2.55</td>
</tr>
</tbody>
</table>

IPA enrichment analyses of the differentially expressed miRNAs associated with each experimental group, as well as for the downregulation of large portion of miRNAs clustered at chromosome 14q32.2 (DLK/MEG3 cluster) in MAX-related PCCs/PGLs, are summarized in Supplementary Tables 4 and 5 respectively. Interestingly, we observed that potential miRNA-regulated pathways were involved in neuronal and neuroendocrine-like differentiation as a common feature. For instance, those SDHB-related pathways included ‘axonal guidance signaling’, ‘synaptic long term potentiation’, and ‘dopamine-DARPP32 feedback in cAMP signaling’, while MAX-related tumors showed significant enrichment in ‘CREB signaling in neurons’, ‘neurotrophin/TRK signaling’, and ‘P2Y purigenic receptor signaling’ pathways.

Predicted miRNA-regulated pathways were the same or similar among experimental groups belonging to the same cluster. Experimental groups belonging to cluster 2 showed enrichment in pathways associated with PI3K/AKT, RAS, and mTOR signaling. For instance, in RET-, NF1-, and TMEM127-related PCCs/PGLs, we detected alterations in mTOR signaling, PTEN signaling, phospholipase C signaling, and LPS-stimulated MAPK signaling pathways, while the protein kinase A signaling pathway was enriched in RET-, TMEM127-, and MAX-related PCCs/PGLs. RET- and TMEM127-related tumors were enriched in the NGF signaling pathway, and the SAPK/JNK signaling pathway was enriched in RET- and NF1-related PCCs/PGLs. Interestingly, the ERK/MAPK and p70S6K signaling pathways were significantly enriched in RET-, NF1-, and TMEM127-related tumors, as well as in VHL-related tumors, while the PI3K/AKT signaling pathway was uniquely enriched in TMEM127-related tumors.

**miR-183 and/or miR-96 impede differentiation in the presence of low-dose NGF in PC12 cells**

As our integration analysis suggested that miR-183 and miR-96 could be implicated in neuronal differentiation, we further examined the role of these miRNAs in PCC/PGL differentiation. Using PC12 miRNA expression data from the Hamada et al. (2012) study, we determined that miR-183 and miR-96 were not overexpressed and that expression levels remained constant throughout NGF-induced differentiation (data not shown). After determining that the PC12 cell line was suitable, we measured the effect of miR-183 and/or miR-96 on neuronal differentiation in PC12 cells in the presence of low-dose NGF. Using the cellular length, border length, and roundness of PC12 cells as parameters to quantify neuronal differentiation, we determined that miR-183- and/or miR-96-transfected cells were significantly different from NGF-positive control PC12 cells (Fig. 3). In fact, miR-183- and/or miR-96-transfected cells in the presence of low-dose NGF closely resembled NGF-negative control PC12 cells. These results indicate that miR-183 and/or miR-96 hinder neuronal differentiation of PC12 in the presence of low-dose NGF.

**Proteomic profiles**

To validate our miRNA–mRNA integration results and assess possible posttranscriptional miRNA regulation, we utilized mass spectrometry to analyze protein levels in two SDHB- and two MAX-related PCCs/PGLs. MAX-related PCCs/PGLs were selected for proteomic analysis because the PC12 cell line has been firmly established as a model system MAX-mutant tumors. We identified a total of 1808 proteins in one or both PCC/PGL experimental groups (data not shown).

Having identified miRNA, mRNA, and protein signatures for these four tumors, we ascertained the relationship between the transcript expression and protein abundance. For the 1637 genes for which both transcript and protein levels were measured between SDHB and MAX, the correlation was moderate but significant (Fig. 4A). Thus, to evaluate the global structure of the data, the expression of the genes measured at both transcript and protein levels were subjected to unsupervised hierarchical clustering (Fig. 4B). This analysis...
showed a clear division of the profiles into branches according to genetic background as mRNA and protein samples from the same tumor were consistently clustered together. This demonstrated that mRNA and proteomic profiles were compatible.

As miRNAs have been reported to regulate gene expression by inducing target mRNA degradation, translational truncation, or both, we separately compared positively and negatively correlated miRNA–mRNA interactions with the corresponding proteomic data. In total, protein data were available for 36 negatively and 25 positively correlated miRNA–mRNA interactions between SDHB- and MAX-related tumors. None of the 25 positively correlated interactions were confirmed at the protein level. Interestingly, among the 36 negatively correlated interactions, four were confirmed at the protein level. These included the following miRNA–mRNA pairs: miR183/96-EZR, miR183-PPP2R5C, miR410-HTRA2, and miR433-OXCT1.

Discussion

miRNA signature in PCCs/PGLs reflects genetic background

As described for mRNA profiling (Eisenhofer et al. 2004, Dahia 2006, Favier et al. 2009, Lopez-Jimenez et al. 2010), our findings demonstrate that miRNA expression profiles are capable of classifying PCC/PGL specimens into different tumor subgroups according to genetic background. Of our reduced list of candidates, eight miRNAs specific to or common among genetic group(s) were identified and validated in this study. Among them, upregulation of miR-885-5p and miR-488 was unique to RET-related PCCs/PGLs; the former miRNA was also described by Tombol et al. (2010) as a RET-specific miRNA and reported to suppress cell migration through modulation of focal adhesion activity (Song et al. 2011). In neuroblastoma, miR-885-5p has been reported as a tumor suppressor gene, which targets cyclin-dependent kinase 2 (CDK2) and mini-chromosome maintenance protein 5 (MCM5) (Afanasyeva et al. 2011). Although further studies are necessary to determine the precise roles played by these miRNAs in these tumors, it is clear that upregulation of miR-488 and miR-885-5p is RET specific and could in part explain their relatively benign nature.

Upregulation of miR-133b, whose function appears to be cell-type specific, was confirmed as unique to VHL-related PCCs/PGLs. It has been reported that this miRNA targets Ptx3 to regulate the maturation and function in midbrain dopaminergic neurons (Kim et al. 2007), while it suppresses BMP2-induced osteogenesis by targeting runt-related transcription factor 2 (RUNX2; Li et al. 2008).
Moreover, its downregulation promoted tumorigenesis in esophageal squamous cell carcinoma and colorectal cancer by targeting fascin homolog 1 (FSCN1; Kano et al. 2010) and hepatocyte growth factor receptor (MET; Hu et al. 2010). Thus, it is difficult to speculate as to whether miR-133b functions as an oncogene or a tumor suppressor gene in VHL-related PCCs/PGLs, and further studies are warranted to determine its role in the context of these tumor cells.

miR-210 was found robustly and moderately upregulated in VHL- and SDHB-related PCCs/PGLs respectively. Overexpression of miR-210 has been described in many hypoxic tumors (Miko et al. 2009, Chan & Loscalzo 2010, Puisségur et al. 2010), such that its upregulation in VHL- and SDHB-related PCCs/PGLs was not surprising given their pseudohypoxic gene signature (Baysal et al. 2000, Maher & Eng 2002, Dahia 2006, Favier et al. 2009, Lopez-Jimenez et al. 2010, Raimundo et al. 2011). miR-210 modulates the cellular hypoxic response through a wide range of actions. Its promoter contains a functioning hypoxia response element, recognized by HIF1α, which induces its transcription upon exposure to hypoxia (Chan & Loscalzo 2010). The higher expression of miR-210 in VHL- vs SDHB-related PCCs/PGLs reported here is also consistent with our previous mRNA expression profiling results (Lopez-Jimenez et al. 2010), which indicated a predominant activation of HIF1α in VHL- compared with SDHB-related PCCs/PGLs, as reflected by the more robust induction of HIF1α target genes, including miR-210, in the former.

In addition to miR-183, which was recently reported in PCCs/PGLs (Patterson et al. 2012), we also identified robust overexpression of miR-96 uniquely in SDHB-related tumors. Previously, Vohwinkel et al. (2011) reported that high CO2 levels induced miR-183 and observed a decrease in IDH2 mRNA and protein, but the miR-183–IDH2 interaction was not conclusively confirmed. Although the exact role of IDH1/2 in tumorigenesis is unclear, these enzymes convert isocitrate to α-ketoglutarate, which is a citric acid cycle metabolite and a cofactor for more than 60 enzymes, including EGL-Nine homologs (Raimundo et al. 2011, Borodovsky et al. 2012). IDH1/2 mutations decrease availability of α-ketoglutarate resulting in both altered cellular metabolism and inhibition of enzymes that use it as a cofactor (Raimundo et al. 2011, Borodovsky et al. 2012). Taking into account the fundamental role of EGLN3 in mediating neuronal apoptosis during normal development (Lee et al. 2005, Schlissel et al. 2008) and that succinate accumulation due to an SDHB mutation competitively inhibits EGLN3 activity (Schlissel et al. 2008, Raimundo et al. 2011), we suggest that miR-183 overexpression in SDHB-related PCCs/PGLs could further contribute to EGLN3 inhibition, by downregulating IDH2 levels and thus decreasing α-ketoglutarate availability, in agreement with the recent findings reported by Tanaka et al. (2013) in glioma cells.

With the exception of MAX-related tumors, we observed a general upregulation of miR-137. It has been shown that miR-137 modulates differentiation, maturation, and proliferation of neurons by targeting several genes, such as RUNX2 and histone H3 Lys4 demethylase (KDM5B; Tarantino et al. 2010). Our integration results predict that IDH1 is potentially downregulated by miR-137, which may contribute to PCC/PGL tumorigenesis by...
Further interfering with the activity of EGLN enzymes, as described earlier.

The 14q32.2 miRNA cluster member, miR-382, was generally upregulated in nearly all tumors (especially in VHL-, SDHB-, SDHD-, and RET-related samples) but not in MAX-related PCCs/PGLs. In osteosarcoma cells, miR-382 has been shown to induce both differentiation followed by apoptosis (Thayanithy et al. 2012) and loss of epithelial characteristics in renal cells (Kriegel et al. 2010). Superoxide dismutase 2 (SOD2; Kriegel et al. 2010) and c-MYC (MYC; Thayanithy et al. 2012) are among the validated miR-382 targets.

Integration reveals potential miRNA-regulated pathways involved in neuronal differentiation

Clustering of miRNA genes is common within the genome, with 38% of known miRNA genes residing in clusters (Altuvia et al. 2005). Our profiling data showed deregulation of several miRNA clusters, miR-193b/365 (chromosome 16p13.12), miR-183/96 (on chromosome 7q32.2), and DLK–MEG3 miRNA cluster (chromosome 14q32.2) in all PCCs/PGLs and SDHB- and MAX-related tumors respectively. Although there is no a clear explanation in regard to the first two clusters, the down-regulation of DLK/MEG3 cluster miRNAs in MAX-related PCCs/PGLs reflects conventional and copy-neutral LOH events at chromosome 14 frequently observed in these tumors (Comino-Mendez et al. 2011, Burnichon et al. 2012b). Evolutionary conservation of clustered miRNA genes suggests an important common biological function, co-regulating identical targets or components in the same pathway (Yuan et al. 2009). In fact, several miRNAs mapping to 14q32.2 were predicted to target the same target genes. Loss of expression of this miRNA cluster or other genes in close proximity has been previously reported in PCC (Astuti et al. 2005) as well as other cancers (Thayanithy et al. 2012). In osteosarcoma, downregulation of 14q32.2 miRNAs stabilizes c-MYC, facilitates apoptotic escape, and sustains tumorigenesis (Thayanithy et al. 2012). Altogether, this suggests that loss of expression of miRNAs clustered at 14q32.2 further deregulates the MYC network and likely contributes to MAX-related PCC/PGL development.

Commonly deregulated miRNAs in PCCs/PGLs showed enrichment in pathways implicated in neuronal and neuroendocrine-like differentiation. As shown in Supplementary Table 3, the ‘breast cancer regulation by STMNI’ pathway was among those potentially regulated by common differentially expressed miRNAs in PCCs/PGLs. Our results predicted that STMNI is a potential miR-193b target, and in fact, this regulatory interaction was recently confirmed (Ikeda et al. 2012). STMNI is highly expressed in the developing nervous system and plays a role in axonal elongation and neuronal regeneration (Grenningloh et al. 2004), and its overexpression was reported in malignant PCCs/PGLs (Bjorklund et al. 2010). Although we observed an inverse correlation between miR-193b and STMNI expression, it was not possible to assess a potential relationship between miR-193b, STMNI, and malignancy because our series lacked sufficient numbers of malignant tumors. IPA analysis of the commonly downregulated miRNAs also showed enrichment in ‘CREB signaling in neurons’ and ‘ERK5 signaling’ pathways. In response to factors that elevate intracellular cAMP or Ca²⁺ levels, CREB signaling has been shown to mediate survival, proliferation, and glucose metabolism (Siu & Jin 2007). In PC12 cells, EGF and NGF have been shown to activate Erk5 that in turn stabilizes tyrosine hydroxylase and promotes survival (Obara et al. 2009).

Although the miRNA signature associated with each of PCC/PGL genetic group differed, there was some overlap between significantly enriched pathways. In this regards, in agreement with current knowledge about gene signature associated with cluster 2 tumors, containing RET-, NF1-, TMEM127-, and MAX-related PCCs/PGLs (Jiang & Dahia 2011), we observed common enrichment of predicted miRNA-regulated pathways related to PI3K/AKT, RAS, and mTOR signaling.

miR183/96 contribute to PCC/PGL tumorigenesis by interfering with differentiation

Here, we provide evidence that SDHB-specific miRNAs, miR183 and/or miR96, contribute to tumorigenesis in PCCs/PGLs by interfering with neuronal differentiation upon stimulation with NGF. In cells capable of neuronal differentiation, such as PC12 cell, stimulation with NGF initiates a signal cascade that culminates in the transcriptional activation/repression of targets, as well as post-translational modification of the activity for already present proteins (Watanabe et al. 2012). According to our results, the overexpression of miR-183 and/or miR-96 in PC12 cells possibly downregulates genes induced by NGF stimulation necessary for neuronal differentiation. In this regard, Weeraratne et al. (2012) showed reduced viability and migration in medulloblastoma cells after miR-183 and miR-96 knockdown. These cells acquired a more flattened appearance with projections indicative of neurite
outgrowth, as well as presented an increased preneuronal gene expression signature (Weeraratne et al. 2012). Furthermore, our results also suggested that miR-183 and miR-96 may target the same or similar genes, as cotransfection of both miRNAs did not produce an additive or synergistic effect in cellular length, border length, and roundness as individual miR-183 or miR-96 transfections. These results were in agreement with our integration analysis, through which we identified numerous potential miR-183/96 targets with reported involvement in neuronal differentiation. For instance, CTDSP1, an inversely correlated potential miR-183 target, has been shown to be involved in silencing neuronal genes through interaction with REST/NRSE, and CTDSP1 inactivation was found to promote neuronal differentiation of P19 stem cells (Yeo et al. 2005). On the other hand, knockdown of ARHGAP18, a potential miR-183 target, enhanced stress fiber formation and induced rounding of cells (Maeda et al. 2011). Likewise, potential miR-96 target genes included FARPI that has been shown to promote dendritic growth of spinal motor neurons subtypes (Zhuang et al. 2009) and LGI1 that has been implicated in the formation, differentiation, maintenance, and plasticity of neuronal synapses (Ko & Kim 2007). Altogether, these experimental evidences are consistent with our results showing that upregulation of miR-183 and miR-96 would have a negative effect on neuronal differentiation.

**Proteomic analysis**

The global proteomic profile for two SDHB- and two MAX-related PCCs/PGLs allowed us to evaluate the robustness of our miRNA–mRNA integration and assess possible post-transcriptional regulation by miRNAs. The correlation found between both strategies suggests that miRNAs regulate gene expression by triggering mRNA degradation rather than translational truncation.

Approximately 11% of the MAGIA-predicted miRNA–mRNA interactions were confirmed at the protein level. This low proportion could be due to an inherent bias of mass spectrometry detection toward most abundant proteins. As we focused on miRNAs that were generally upregulated in one experimental group relative to the others, their corresponding targets would have lower levels of expression at both mRNA and protein levels.

Nevertheless, we confirmed the predicted miR-183/96 interaction with EZR mRNA at the protein level. This is highly relevant given that EZR together with radixin and/or moesin form ERM complexes that connect actin to other membrane proteins (Sperka et al. 2011). Sperka et al. (2011) showed that activation of RAS requires the essential participation of ERM complexes and actin and that disrupting either the interaction of the ERM proteins with co-receptors or by downregulation of ERM proteins abolished growth factor-induced RAS activation. The NGF-induced neuronal differentiation initiates a signal cascade requiring activation of RAS signaling, and interfering interference with the activity or function of RAS has been shown to impede or abolish NGF-induced differentiation (Klesse et al. 1999, Sperka et al. 2011). Taken altogether, disruption of RAS signaling by downregulation of EZR by miR-183 and/or miR-96 could in part explain our results showing that these miRNAs impede NGF-induced differentiation in PC12 cells. Thus, aberrant upregulation of miR-183 and miR-96 in SDHB-related PCCs/PGLs could contribute to their resistance to the process of differentiation and apoptosis that occurs naturally as mature sympathoadrenal precursors and acquire a chromaffin or sympathetic neuron phenotype.

In summary, we have demonstrated that PCCs/PGLs express different miRNA signatures depending on the genetic background. These signatures grouped into two clusters: one was enriched in SDHx/VHL-related tumors, while the other contained RET/NF1/TMEM127/MAX-related tumors, in accordance with the results obtained for previous mRNA transcriptional profiling studies (Eisenhofer et al. 2004, Dahia 2006, Favier et al. 2009, Lopez-Jimenez et al. 2010). In fact, it was possible to identify several miRNAs associated with the primary mutation, as well as miRNAs common among PCCs/PGLs, which could be used to guide genetic study. Through integration with matched mRNA profiles, we found a number of potentially miRNA-regulated pathways involved in neuronal differentiation that may contribute to the development of these tumors. Functional assays showed that miR-183 and/or miR-96 overexpression impeded neuronal differentiation of PC12 cells in the presence of NGF, and global proteomic analysis suggested that this could be due in part by disruption of growth factor-induced RAS activation.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0183.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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