miR-192, miR-194, miR-215, miR-200c and miR-141 are downregulated and their common target ACVR2B is strongly expressed in renal childhood neoplasms.

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Micro RNAs (miRNAs) play an important role during renal development and show a tissue-specific enrichment in the kidney. Nephroblastomas, embryonal renal neoplasms of childhood, are considered to develop from nephrogenic rests (NRs) and resemble morphologically and genetically developing kidney. We therefore investigated the role of kidney-enriched miRNAs in the pathogenesis of nephroblastomas. miR-192, miR-215 and miR-194 had a significantly lower expression in nephroblastomas regardless of the subtype compared with mature kidney measured by quantitative real-time–PCR. miR-141 and miR-200c showed a significantly lower expression in blastema-type and mixed-type tumors. In comparison with NRs, a significantly lower expression of miR-192, miR-194 and miR-215 was identified in blastema-type, mixed-type and stroma-type nephroblastomas and of miR-141 and miR-200c in blastema-type tumors. Kidney parenchyma had a significantly higher expression of miR-192, miR-194, miR-215 and miR-200c compared with NRs. In this study, the activin receptor type II B (ACVR2B), a member of the transforming growth factor (TGF)-β pathway, was identified as single common target gene for miR-192, miR-215, miR-194 and miR-200c in silico for the first time. The interaction between all five miRNAs and ACVR2B was also verified by an in vitro assay. Additionally, a distinct protein expression of ACVR2B was detected in 53 of 55 nephroblastomas paralleled by an upregulation of ACVR2B messenger RNA demonstrated in 25 nephroblastomas of all subtypes. A differential regulation of ACVR2B by miRNAs in NRs and nephroblastomas appears to be an important step in the pathogenesis of nephroblastomas implicating for the first time the TGF-β pathway in this process.

Introduction

Micro RNAs (miRNAs) are small non-coding fragments of RNA influencing the expression levels of messenger RNA (mRNA) by posttranscriptional mechanisms (1). They bind to the 3' untranslated region (UTR) of the respective target thereby leading to degradation of the RNA or suppression of protein translation (2,3). During kidney development, expression of miRNAs seems to play a role predominantly in later stages. In terminally differentiated epithelia, several miRNAs can be detected. The miR-200 family has been described to be involved in the process of mesenchymal–epithelial transition during renal development (4). In an animal model, the enzyme responsible for maturation of miRNAs, Dicer, was specifically suppressed in podocytes. At 3 weeks of age, the transgenic animals showed proteinuria and end-stage kidney disease (5). In an additional animal model with deletion of Dicer in podocytes, mmu-miR23b, mmu-miR24 and mmu-miR26b were identified to be responsible for glomerular filtration.

Abbreviations: miRNA, micro RNA; mRNA, messenger RNA; NR, nephrogenic rest; TGF, transforming growth factor; UTR, untranslated region.
miRNAs and their target ACVR2B in nephroblastomas

<table>
<thead>
<tr>
<th>Predicted target regions</th>
<th>Seed match</th>
<th>3'-UTR position</th>
<th>Predicted consequential pairing of target region (top) and miRNA (bottom)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-192 7mer</td>
<td>2582–2610</td>
<td></td>
<td>JUR: y A GUAAGU U 3</td>
</tr>
<tr>
<td>hsa-miR-192 7mer</td>
<td>8021–8049</td>
<td></td>
<td>miRNA: C C A AGU 5</td>
</tr>
<tr>
<td>hsa-miR-192 7mer</td>
<td>9188–9216</td>
<td></td>
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</tr>
<tr>
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<td>2582–2610</td>
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<td>miRNA: C C A AGU 5</td>
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<td></td>
<td>JUR: y A GUAACG U 3</td>
</tr>
<tr>
<td>hsa-miR-194 8mer</td>
<td>9328–9356</td>
<td></td>
<td>miRNA: G A AAGC U 3</td>
</tr>
<tr>
<td>hsa-miR-141 7mer</td>
<td>1872–1900</td>
<td></td>
<td>JUR: y GUAU A 3</td>
</tr>
<tr>
<td>hsa-miR-200c 7mer</td>
<td>5632–5660</td>
<td></td>
<td>miRNA: C UAU A 3</td>
</tr>
<tr>
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</table>

Materials and methods

Tissue samples

All tumors were obtained from the Kiel Paediatric Tumor Registry at the Department of Paediatric Pathology, University of Kiel, Germany. Tissue samples had been fixed in buffered formaldehyde and embedded in paraffin wax. Three micrometer sections of the samples were obtained for further analysis.

miRNA target prediction

Different web servers of DIANA lab were used to predict the target genes and their target regions. A common target gene for the miRNAs analyzed in the TGF β pathway was identified by the DIANA-mirPath programme, which is a tool to identify molecular pathways targeted by single or multiple miRNAs (25). DIANA-mirPath programme based on DIANA-micro-T-4.0 (beta version) was used to identify biological pathways targeted by miR-192, miR-194, miR-215, miR-141 and miR-200c (http://diana.cslab.ece.ntua.gr/pathways/). Computer mouse over option was used to demarcate a common target gene for all five miRNAs in possible targeted biological pathways predicted by the programme according to −In P values. The identification of target regions of miR-192, miR-194, miR-215, miR141 and miR200c in the 3'-UTR region of the common target gene was performed by the DIANA-micro-T-3.0 (http://diana.cslab.ece.ntua.gr/microT/) program. All targeting regions of the miRNAs analyzed in the 3'-UTR of the ACVR2B gene (see Table I) were identified by DIANA-microT 3.0 (26).

Cell lines

WT-CLS1 cells (Cell lines services, Heidelberg, Germany), an epithelial nephroblastoma cell line, were cultured in Isocoves medium (Sigma Aldrich, St Louis, MO) supplemented with 15% fetal bovine serum (PAA, Pasching, Austria), 1% penicillin and streptomycin (PAA) and 2 mM L-Glutamine (PAA). HEK293 cells (American type culture collection, Manassas, VA), an embryonic kidney cell line, were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (PAA), 1% penicillin and streptomycin (PAA) and 2mM L-Glutamine (PAA). Both cell lines were cultured at 37°C in a humidified atmosphere with 5% CO2.

miRNA and miRNA isolation

Total cellular RNA from formalin-fixed paraffin-embedded samples was isolated using the RNeasy FFPE kit (Qiagen, Hilden, Germany) and from cultured cells by TRIZOL (Invitrogen) according to the manufacturer’s instructions.

miRNA quantitative real-time–PCR

Expressions of mature miR-192, miR-194, miR-215, miR-141 and miR-200c were analyzed using the TaqMan miRNA Assay (Applied Biosystems, Carlsbad, CA). Expression of RNU6B (Applied Biosystems) was used as endogenous control. All measurements were performed in triplicate and relative expressions were calculated as ΔΔCT.

mRNA quantitative real-time–PCR

Up to 500 ng of total RNA were reverse transcribed into complementary DNA using the High Capacity complementary DNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative real-time–PCR was performed in triplicates with primers specific for ACVR2B and 18s RNA (Supplementary material, available at Carcinogenesis Online). Quantitative real-time–PCR was carried out on a 7900T Fast real-time PCR system (Applied Biosystems) using SYBR green as the detection fluorophore. Quantitative real-time–PCR was performed in triplicates with primers specific for 18s RNA (Supplementary material, available at Carcinogenesis Online). Expression of RNU6B (Applied Biosystems) was used as endogenous control. All measurements were performed in triplicate and relative expressions were calculated as ΔΔCT.

Immunohistochemistry

ACVR2B (Abnova, Taipei City, Taiwan) antibodies were diluted 1:25 in antibody diluent (Dako, Glostrup, Denmark). The immunohistochemical staining procedure was performed using the automated VENTANA staining system (Ventana, Tucson, AZ).

Transfections of miRNA precursor molecules with the target regions and luciferase reporter assay

WT-CLS1 cells were grown in six-well plates up to 50% confluence. Luciferase plasmid constructs were created by ligating oligonucleotides containing the target sites of the ACVR2B 3'-UTR downstream of the luciferase gene in the pMIR-REPORT™ Reporter Luciferase vector (Ambion, Carlsbad, CA) (Supplementary material, available at Carcinogenesis Online). Transfection was performed with a solution consisting of 2 μg firefly luciferase reporter vector containing the target sequence, 500 ng of pMIR-REPORT™ beta-gal control vector (Ambion) and pre-miRNA precursor molecules of miR-192, miR-194, miR-215, miR-141, miR-200c (Ambion) or pre-miR scrambled miRNA (Ambion). Pre-miRNA molecules were used at a final concentration of 100 nM and mixed with 12 μl lipofectamine 2000 (Invitrogen). A final volume of 100 μl was achieved by adding Opti-MEM® 1 reduced-medium (Invitrogen) per well. The transfection solution was incubated at 37°C for 30 min. Then, the mixture was added to 900 μl of serum-free medium covering the cells and 4 h after transfection, another 1 ml of medium containing serum was added. The medium was changed to standard growth medium after washing the cells with phosphate-buffered saline 24 h after the transfection.

Luciferase activity was measured 50 h after transfection (Bright Glo Luciferase Assay System; Promega, Madison, WI) using β-galactosidase for normalization (β-galactosidase Enzyme Assay System; Promega). Changes in the luciferase activity were calculated by dividing the luciferase activity of the test miRNA by the luciferase activity of the scrambled miRNA.

Western blot analysis

HEK293 cells were grown in six-well plates up to 70% confluence and transfected with 100 nM pre-miRNA precursor molecules of miR-192, miR-194, miR-215, miR-141, miR-200c (Ambion) or pre-miR scrambled miRNA (Ambion). Transfections were performed as described above.

vector containing the target sequence, 500 ng of pMIR-REPORT™ beta-gal control vector (Ambion) and pre-miRNA precursor molecules of miR-192, miR-194, miR-215, miR-141, miR-200c (Ambion) or pre-miR scrambled miRNA (Ambion). Pre-miRNA molecules were used at a final concentration of 100 nM and mixed with 12 μl lipofectamine 2000 (Invitrogen). A final volume of 100 μl was achieved by adding Opti-MEM® 1 reduced-medium (Invitrogen) per well. The transfection solution was incubated at 37°C for 30 min. Then, the mixture was added to 900 μl of serum-free medium covering the cells and 4 h after transfection, another 1 ml of medium containing serum was added. The medium was changed to standard growth medium after washing the cells with phosphate-buffered saline 24 h after the transfection.

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Total cellular proteins were isolated 60 h after transfection by resuspending the cell pellets in RIPA buffer (Thermo Scientific, Rockford, IL) containing 1× protease inhibitors (Halt protease and phosphatase inhibitor cocktail; Thermo Scientific). After keeping the samples on ice for 15 min, cells were sonicated and extracts were cleared by centrifugation for 10 min at 13 000 r.p.m. Supernatants were transferred into fresh tubes and protein concentrations were measured by the Lowry protein assay.

Thirty micrograms of proteins were loaded onto a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis 1.5 mm gels for electrophoresis. Then, the proteins were transferred electrophoretically to a nitrocellulose membrane at 240 mA for 120 min. The membrane was blocked with 5% (vol/vol) skimmed milk powder (Bio-Rad, Hercules, CA) in TBST [0.2mM Tris, 15 mM NaCl, pH 7.4, 0.2% (vol/vol) Tween 20] for 1 h at room temperature. For immunodetection, the membrane was incubated with antibodies against ACVR2B (Abnova) diluted 1:100 in 1% (vol/vol) skimmed milk powder with TBST for 12 h at room temperature. Afterward, the membrane was washed in TBST three times for 5 min each at room temperature before treating the membrane with horseradish peroxidase conjugated anti-rabbit secondary antibody (Dako). The membrane was developed by exposure to a photosensitive film using Pierce ECL western blotting substrate (Thermo scientific). For normalization of the protein content, the membranes were stripped using stripping buffer (Bio-Rad) and washed in TBST three times for 5 min each at room temperature. The membranes were blocked as described above and incubated with antibodies against glyceraldehyde 3-phosphate dehydrogenase diluted 1:500 in 1% (vol/vol) skimmed milk powder with TBST and incubated overnight at 4°C. Detection was carried out as described above. Densitometric analysis was performed to quantify protein expression using ImageJ application.

Statistical analysis
All data were analyzed using Graphpad Prism software V5 (Graph pad, San Diego, CA). Statistical analysis for the comparison of the expression of miRNA and mRNA was calculated by the Mann–Whitney test (non-parametric analysis) and the difference in the change of luciferase activity and protein content analyzed using western blot by the Student’s t-test (parametric analysis), whereby P < 0.05 was considered as significant (27).

Results
miR-192, miR-194, miR-215, miR-141 and miR-200c are downregulated in most nephroblastosomas regardless of the subtype
Quantitative real-time–PCR was carried out to determine the expression levels of miR-192, miR-194, miR-215, miR-141 and miR-200c in 6 blastema-type, 4 epithelial-type, 11 mixed-type and 3 stroma-type nephroblastomas in relation to 7 samples of mature kidney.

miR-192, miR-194 and miR-215 had a significantly lower expression in all nephroblastomas regardless of subtype and morphology. All blastema-type and mixed-type nephroblastomas showed additionally a significantly lower expression of miR-141 in contrast to stroma-type nephroblastomas. Three epithelial-type tumors showed a similar level of expression like blastema-type and mixed nephroblastomas; however, one epithelial-type nephroblastoma had a very high expression of miR-141. Therefore, the difference between epithelial- and stromal-type tumors and mature kidney did not reach a level of significance. The same pattern of expression was observed for miR-200c (Figure 1).

NRs have a higher expression of miR-192, miR-194, miR-215, miR-141 and miR-200c compared with most nephroblastomas
MiR-192, miR-194, miR-215, miR-141 and miR-200c expressions were evaluated in nine incidentally detected NRs in comparison with nephroblastomas and mature kidney. Expression of miR-192, miR-194 and miR-215 was significantly lower in all NRs compared with mature renal parenchyma and significantly higher compared with blastema-type, mixed-type and stroma-type nephroblastomas, but not in comparison with epithelial-type nephroblastomas (Figure 1).

Expression of miR-141 was significantly higher in NRs compared with blastema-type nephroblastomas but did not differ significantly compared with epithelial-type, mixed-type, stroma-type nephroblastomas or mature kidney. Only one NR had a very high expression of miR-141 and miR-200c in comparison with all other NRs (Figure 1).

Interestingly, miR-200c had a significantly lower expression in NRs compared with mature kidney and a significantly higher expression in relation to blastema-type nephroblastomas. In contrast, the expression of miR200c did not differ significantly between NRs and the other subtypes of nephroblastomas (see Figure 1).

All five miRNAs are targeting the ACVR2B gene, which is part of the TGF-β pathway according to an in silico analysis
A web-based computational tool, Diana web path software, was used to predict target genes in the TGF-β pathway (25, 26). Names of the five miRNAs were included as input in the DIANA-miRPath analysis based on DIANA-micro-T-4.0 (β-version). This web-based computational programme performs enrichment analysis of data sets with all available pathways provided by the Kyoto Encyclopedia of Genes (28). Interactions of genes in biological pathways are diagrammatically represented in the KEGG database. The enrichment analysis of the input miRNAs regarding pathways is performed by a Pearson’s chi-square test (25). The combinatorial effect of the five miRNAs (Union data set) on the TGF-β pathway was 9.17 (−ln P value). Computer mouse over option displayed that miR-192, miR-194, miR-215, miR-141 and miR-200c are targeting a common gene ACVR2B. The analysis of other pathways, which had −ln P value of >9.17, did not show a common target gene for all five miRNAs (see Table I).

mRNA and protein expression of ACVR2B are upregulated in most nephroblastomas compared with normal kidney
Samples of 25 nephroblastomas including 4 blastema-type, 4 epithelial-type, 3 stroma-type, 13 mixed-type and 1 regressive nephroblastoma as well as 6 samples of mature kidney were analyzed for mRNA expression of ACVR2B. Compared with mature renal parenchyma, nephroblastomas had a significantly higher (P < 0.0013) mRNA expression (Figure 2).

Membrane and cytoplasmic protein expression for ACVR2B was assessed immunohistochemically in 55 tumors, comprising 20 mixed-type, 4 epithelial-type, 4 blastema-type, 6 stroma-type and 21 regressive nephroblastomas. Additionally, protein expression of six incidentally detected NRs was analyzed. Corresponding nephroblastomas of the NRs were two regressive, three mixed-type and one stroma-type nephroblastoma.

The staining pattern of ACVR2B was assessed in all the different morphological components of nephroblastomas. Importantly, ACVR2B protein expression was detected in the main morphological components of nephroblastomas, in all blastemal areas, regardless of the subtype (Figure 3A), and in all tubular structures (Figure 3B) with the exception of one mixed nephroblastoma, which showed negative tubular structures, but positivity in the blastema. Undifferentiated mesenchyme showed negativity in 19 and positivity in 9 cases. A minority of nephroblastomas also contained some more specialized structures. These included immature glomeruloid bodies with expression of ACVR2B in three of five cases and skeletal muscle with expression of ACVR2B in four of six cases. Two mixed nephroblastomas were entirely negative in all tumor components.

A direct comparison between staining intensity of the tumor and the adjacent parenchyma was possible in 25 cases. Twenty-one tumors had a stronger protein expression of ACVR2B compared with adjacent normal kidney, two had a lower and one regressive and one mixed nephroblastoma the same intensity. In all other samples, a direct comparison with non-tumoral renal parenchyma was not possible, as sections contained exclusively areas of tumor. Correlation of mRNA and protein expression levels showed that the tumors with high mRNA expression of ACVR2B also had a strong protein expression.

All six NRs were positive for ACVR2B (Figure 3). A direct comparison on the same section with corresponding nephroblastomas was possible in two cases, with corresponding nephroblastomas and normal kidney parenchyma in one case and with normal parenchyma in three cases. In all cases, NRs showed a higher expression than the corresponding normal parenchyma and a lower expression in comparison with the corresponding nephroblastomas.

U.Senanayake et al.
ACVR2B is a direct target of miR-192, miR-215, miR-194, miR-141 and miR-200c

Luciferase reporter assays were performed to determine whether the 3′ UTR regions of ACVR2B were indeed functional target regions of miR-192, miR-215, miR-194, miR-141 and miR-200c. Luciferase reporter plasmids harboring the target regions were transiently transfected using a WT-CLS1 cell line, showing a low expression of ACVR2B.

The miR-192 precursor co-transfected with target regions 2582–2610, 8021–8049 and 9188–9216, respectively, led to a significant decrease in luciferase activity compared with scrambled control (Figure 4 A–C). Similarly, the 2582–2610 and 9188–9216 target regions of miR-215 showed a significant downregulation of the luciferase activity following the transfection of the miR-215 precursor (Figure 4D and E).
renal parenchyma.

Fig. 2. Relative ACVR2B mRNA expression was analyzed in nephroblastomas \((n = 25)\) and mature renal parenchyma \((n = 6)\) by quantitative real-time–PCR. Tumors showed a statistically significantly higher ACVR2B expression than renal parenchyma (vertical bars represent ±SEM, \(\star P < 0.05, \star\star P < 0.01, \star\star\star P < 0.001\)).

Fig. 3. Protein expression of ACVR2B was assessed by immunohistochemistry in various nephroblastoma subtypes \((n = 55)\). Blasstal regions, epithelial regions, mesenchyme (mixed type tumor-A, mixed type- B, \(\times 400\)) and NRs \((C, \times 400)\) showed an intense membranous and cytoplasmic staining for ACVR2B compared with corresponding mature renal parenchyma.

Out of the two target regions of miR-194, only 9328–9356 showed a downregulation of luciferase activity compared with scrambled control (Figure 4F and G).

The target regions of miR-200c, 5632–5660 and 7567–7593, showed a significant downregulation following transfection with miR-200c precursors (Figure 4H and I).

miR-141 had a single target region 1872–1900, which showed a significant downregulation of luciferase activity compared with control (Figure 4J). Additionally, the regulation of protein expression by all miRNAs was investigated in HEK293 cells showing a high level of ACVR2B expression. Western blot analysis was performed in triplicates after transfecting pre-miRNAs and pre-scrambled control miRNA. A statistically significant downregulation of the protein expression of ACVR2B by miR-141 \((P = 0.0039)\), miR-200c \((P = 0.0028)\), miR-215 \((P = 0.0038)\), miR-194 \((P = 0.0054)\) and miR-192 \((P = 0.04)\) compared with scrambled control was identified by densitometric analysis (Figure 5).

Discussion

miRNAs are involved in a wide variety of biological cellular functions including differentiation, proliferation and apoptosis (29,30). In addition, aberrant expression of miRNAs plays a role in the pathogenesis, progression and metastasis of malignant tumors. miRNAs can function as oncogenes or tumor suppressors and might even represent targets for therapeutic approaches (30–36).

In our study, we demonstrate a downregulation of miR-192, miR-194 and miR-215 in all nephroblastomas regardless of the subtype. A controversial role of these miRNAs has already been discussed in other malignant tumor entities. Expression levels of miR-192 were found to be significantly reduced in adenocarcinomas in a rat model (37). miR-192, miR-194 and miR-215 were also shown to be decreased in colon cancer samples in comparison with normal colon (38). In contrast, high levels of miR-194 were detected in Barrett’s epithelium and adenocarcinoma in comparison with normal gastric and oesophageal mucosa (39).

We also show that miR-200c and miR-141 are downregulated in the majority of nephroblastomas with the exception of stroma-type nephroblastomas and one epithelial-type tumor consisting of mature tubules, which showed expression comparable with mature kidney parenchyma. Large international studies have revealed that stroma-type and highly differentiated epithelial-type nephroblastomas characteristically have a very good outcome (40), which might also be reflected by a distinct gene expression pattern since epithelial-type nephroblastomas with differentiated tubular growth pattern harbored a gene expression pattern analogous to an arrest following mesenchymal to epithelial transition (41). The role of miR-141 and miR-200c has also been investigated in several other tumor entities. Comparison of gastric cancer cell lines, gastric cancer samples and adjacent non-neoplastic tissue revealed a significantly reduced expression of miR-141 in most of the neoplastic samples (42). A comparison of clear cell renal carcinomas with corresponding non-malignant samples showed a decreased expression of miR-200c and miR-141. A comparison of the miRNA expression profiles also enabled a clear distinction between malignant and normal tissue according to the miRNA profile (34,43–46). Differences in expression levels of several miRNAs including miR-200c were also able to discriminate between oncocytoma and chromophobe carcinoma in up to 93% (47). In contrast, in a different study, high levels of miR-141 and miR-200c were found in renal clear cell carcinomas compared with normal renal tissue (48). A comparison of ovarian cancer and normal tissue revealed overexpression of numerous miRNAs including miR-200c and miR-141 (49).

The divergent results of expression of these miRNAs in different studies and tumor entities might also be related to the wide range of different functions and targets of miRNAs. Our in silico analysis demonstrated that all five miRNAs with low expression in the large majority of the nephroblastomas investigated target the same gene, ACVR2B, which has not been identified as a target gene of these
miRNAs so far. In vitro interaction assays verified ACVR2B as a target gene of all five miRNAs for the first time.

Analysis of the expression of ACVR2B on mRNA level demonstrated a significantly increased expression in all nephroblastomas compared with mature kidney and on protein level a distinct expression in 53 of 55 nephroblastomas. So far, an expression in 10% of cells in five cases of primary nephroblastomas and in the low passage culture of these tumors has been noted (50).

**Fig. 4.** Direct targeting of ACVR2B mRNA 3'-UTR by miR-192, miR-215, miR-194, miR-141 and miR-200c was analyzed by luciferase reporter assays. Assays were performed by transfecting WT-CLS1 cells with firefly luciferase vector containing different target regions for miRNAs and precursor miRNAs for different target regions or scrambled miRNA. β-galactosidase activity was used for normalization and results were depicted as fold change in luciferase activity compared with scrambled control. miR-192 (A, B, C), miR-215 (D and E), miR-200c (H and I) and miR-141 (J) were downregulating luciferase activity significantly by binding with all their target regions. miR-194 decreased the luciferase activity by binding one target region out of two target regions (F and G) (vertical bars represent +SEM, n = 3, *P < 0.05, **P < 0.01).
Activins are part of the TGF-β pathway and act on cell growth and differentiation in different biological functions. The activity of activin is regulated by dimerization of two different receptors, activin receptor type I and II (21). Activation of the TGF-β pathway plays a pivotal role in human tumorigenesis, acting as tumor suppressor or oncogene (22,51–53). The role of ACVR2B in different malignant neoplasms is controversial and might influence the role of the TGF-β pathway during tumorigenesis. ACVR2B showed a lower expression in colorectal carcinomas compared with normal epithelia. In addition, antibodies against ACVR2B were more abundant in normal sera compared with patients suffering from colorectal carcinoma (54). ACVR2B was also higher expressed in a well differentiated endometrial adenocarcinoma cell line than in a cell line derived from a poorly differentiated endometrial adenocarcinoma (55). In a breast carcinomacell-line, estradiol induced mitosis at the same time decreasing the expression of ACVR2B (56). In contrast, the ovarian cancer cell line OVCAR-3 demonstrated a higher expression of ACVR2B compared with normal ovarian surface epithelium and showed proliferative induction by activin (57). A distinct expression of ACVR2B was identified in 4 of 10 clear cell adenocarcinomas of the ovary (58). Although the sample size in this study is rather low, it is interesting to note that the expression of ACVR2B is not only found in ovarian cancer cell lines but also in tumor samples. According to our analysis, nephroblastomas are by far the most homogenous group of neoplasms (59). We show that miRNAs and their target gene ACVR2B exhibit an important pathogenetic role in the tumorigenesis of nephroblastomas.

miRNAs emphasizing an important role of microRNAs and the TGF-β pathway in the pathogenesis of nephroblastomas at the same time. However, genetic evidence for this assumption has so far been sparse (59). We show that miRNAs and their target gene ACVR2B exhibit an expression level in between nephroblastomas and mature renal parenchyma. Typically, precursor lesions of malignant tumors harbor some but not all genetic lesions identified in the tumors, such as typhical adenomatous hyperplasia of the prostate and prostate adenocarcinoma (60) or the adenoma to carcinoma sequence of the colon (61).

In our study, we demonstrate an important role of ACVR2B expression and the TGF-β pathway in the tumorigenesis of nephroblastomas. Additionally, we verify the regulation of ACVR2B by five different miRNAs emphasizing an important role of microRNAs and the TGF-β pathway in the pathogenesis of nephroblastomas at the same time.

Supplementary material
Supplementary Material can be found at http://carcin.oxfordjournals.org/.

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References
miRNAs and their target ACVR2B in nephroblastomas


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