

***BMPRII* is a direct target of miR-21**

Wenming Qin¹, Botao Zhao¹, Yi Shi¹, Chengguo Yao¹, Li Jin², and Youxin Jin^{1*}

¹State Key Laboratory of Molecular Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

²St. Luke's Hospital, 786 Yuyuan Road, Shanghai 200050, China

*Correspondence address. Tel: +86-21-54921222; Fax: +86-21-54921011; E-mail: yxjin@sibs.ac.cn

MicroRNAs (miRNAs) are a type of small non-coding RNAs that regulate cognate mRNA expressions at the post-transcriptional stage. Although several miRNAs are known to be involved in various biological processes, including developmental timing, patterning, embryogenesis, differentiation and organogenesis, growth control, and apoptosis, many target genes and the functions of most miRNAs are still unclear. Since there is only a partial complementarity between miRNAs and their targets in animal cells, it is difficult to identify the specific target genes for a given miRNA and elucidate its function. In this study, we confirmed that bone morphogenetic protein receptor II (*BMPRII*) is a direct target of miR-21, and also showed that the protein level of *BMPRII* correlates inversely with the amount of miR-21 in PC3 and Lncap cells. These findings suggest that miR-21 may have a potential role in regulating the malignancy and metastatic abilities of prostate cancer cells and in self-renewal of stem cells by regulating the expression of *BMPRII*.

Keywords microRNA; miR-21; *BMPRII*; target; prostate cancer

Received: March 20, 2009 Accepted: April 7, 2009

Introduction

MicroRNAs (miRNAs) are a recently discovered class of approximately 21 nucleotide long RNA molecules that negatively regulate the expression of target mRNAs at the post-transcriptional level [1–3]. Their importance in a variety of biological processes has become apparent during the last decade [4–7]. Recent advances in miRNA research have provided evidence of aberrant gene expressions in different kinds of diseases such as

cancer, cardiovascular diseases, psychological disorders, and others [8–11].

MiR-21 has been identified as the most expressed miRNA in a number of profiling experiments designed for the detection of miRNAs dysregulated in cancer. MiR-21 is abundantly expressed in most cancer cell lines [12]. It has been found that miR-21 plays a role in various fields such as development, oncology, stem cell biology, and aging. It is one of the most studied miRNAs [13]. Since there is only a partial complementarity between miRNAs and their targets in animal cells, it is difficult to identify the specific target genes for a given miRNA and elucidate its function. Although miR-21 expression is generally associated with cancer, it has several other direct targets such as programmed cell death 4, tropomyosin 1, and phosphatase and tensin homolog deleted on chromosome 10 [14–16].

Bone morphogenetic proteins (BMPs) are the members of the tumor growth factor (TGF)- β super family. BMP-Smad signaling regulates stem cell renewal, cell proliferation, differentiation, migration, and apoptosis, and controls embryo development and post-natal tissue homeostasis [17–19]. BMPs exert their effects via a heteromeric receptor complex, which consists of two types of serine-threonine kinase transmembrane receptors. *BMPRII* is the type 2 receptor of BMPs; mutations in *BMPRII* lead to the development of hereditary pulmonary hypertension, and its knockout results in early embryonic lethality [20,21]. *BMPRII* initiates intracellular signaling in response to the following specific ligands: BMP-2, BMP-4, BMP-6, BMP-7, growth and differentiation factor (GDF)-5, and GDF-6 [22]. *BMPRII* plays a critical role in the maintenance of normal pulmonary vascular physiology, cell proliferation, cell death, and stem cell differentiation and self-renewal [23,24]. Loss of *BMPRII* expression in both prostate

cancer tissues and cell lines has been shown to be associated with the progression of prostate cancer [25].

In this study, we investigated whether *BMPRII* is the direct target of miR-21 and found that the protein level of BMPRII correlated inversely with miR-21 levels in PC3 and Lncap cells, two different kinds of prostate cancer cells with different malignancy and metastasis status. These findings suggest that miR-21 might be involved in the progression of prostate cancer and self-renewal of stem cells.

Materials and Methods

Cell culture

293T, PC3, and Lncap cells were obtained from the Cell Bank, China Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, and RPMI 1640 medium (Invitrogen, Carlsbad, USA), respectively. All the media were supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, USA) and maintained at 37°C and in 5% CO₂.

Construction of 3'-untranslated region-luciferase plasmid

The two upstream and the two downstream predicted binding sites on the 3'-untranslated region (UTR) of *BMPRII* along with ~1 kb of the contiguous sequences were cloned into the *EcoRI*-*NdeI* and *NdeI*-*PstI* sites of pGL3 (Promega, Madison, USA) and designated as Luc-BS12-*BMPRII* and Luc-BS34-*BMPRII*, respectively. In addition, the two downstream predicted binding sites, which also included 1 kb of the contiguous sequences, were cloned into the *NdeI* and *PstI* sites of Luc-BS12-*BMPRII*; this plasmid was designated as Luc-B1234-*BMPRII*. The following primers were used for plasmid construction. BS12-sense primer: 5'-TCAG AATCAATCAGTTTGGACCAGTTTC-3'; BS12-antisense primer: 5'-TCACATATGGTTTGATAGCAGC CCTTC-3'; BS34-sense primer: 5'-TCA CATATGAATCC TCGTAAGTATGTAAAGGA-3'; BS34-antisense primer: 5'-TCACTGCAGAAACGGAATAACGCACCA-3'.

Luciferase assay

For reporter assays, the cells were transiently cotransfected into 12-well plates with the luciferase vector Luc-BS12-*BMPRII*, Luc-BS34-*BMPRII*, Luc-B1234-*BMPRII*, or pGL3 reporter plasmid and either miR-21-mimics (Ambion, Austin, USA) or miR-21-antisense oligonucleotide (ASO, Takara, Dalian, China)

using lipofectamineTM 2000 (Invitrogen, Carlsbad, USA). Seeding region-mutated miR-21 mimics and miR-21-ASO were used as negative controls (NC-mimics and NC-ASO). To determine the transfection efficiency, pRL-SV40 plasmid was cotransfected as a control. Reporter assays were performed at 24 h post-transfection using the Dual-luciferase assay system (Promega, Madison, USA).

RNA isolation

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Contaminating genomic DNA was removed from the isolated RNA by treatment with amplification-grade DNase I (Invitrogen, Carlsbad, USA) for 2 h at 37°C. RNA was precipitated with 3 M NaAc (pH 5.5) and 2.5 volumes of ethanol and quantified spectrophotometrically.

NCodeTM quantitative polymerase chain reaction

Quantitative polymerase chain reaction (PCR) was performed on unamplified total RNA using the NCodeTM quantitative PCR kit (Invitrogen) according to the manufacturer's instructions. The differences in the miRNA expression between samples were determined using the relative quantification method. The PCR program is: 94°C for 5 min; 94°C for 15 s, 57°C for 20 s, 72°C for 10 s, 45 cycles; 72°C for 2 min. And we did the melting curve procedure at the first time. The cycle threshold (Ct) values of the samples were normalized to the Ct values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene. The fold difference in the gene expressions of the samples was calculated using the equation $2^{-\Delta\Delta Ct}$.

Protein extraction and western blot

Protein was extracted with protein extraction reagent (Sigma, St. Louis, USA) according to the manufacturer's instructions. Then proteins were suspended in 1× SDS loading buffer (Sigma, St. Louis, USA). And 20 μg proteins were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. Then, the membrane was blocked with Poly(butylene succinate-co-terephthalate) containing 5% milk powder for 1 h at room temperature, followed by incubation overnight at 4°C in TBS containing 0.1% Tween20, 1% milk powder and primary antibodies. Primary antibodies were detected using a peroxidase-coupled secondary antibody (1:2000, Sigma, St. Louis, USA) and chemiluminescence (Pierce, Rockford, USA). The following

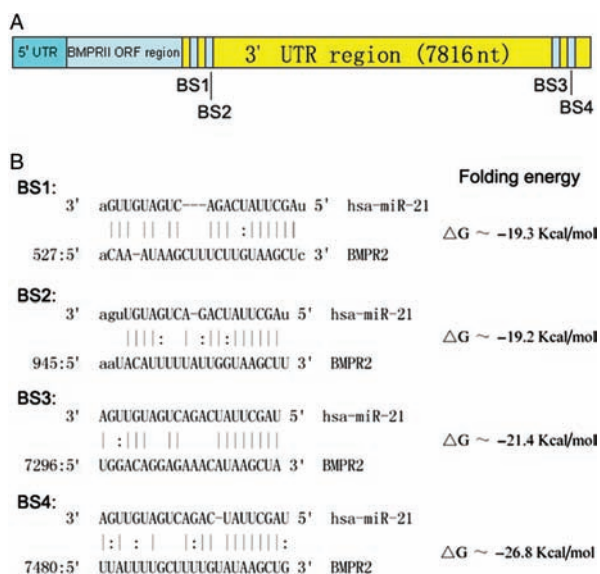


Figure 1 Four binding sites were predicted on the 3' UTR region of *BMPRII* mRNA (A) Schematic representation of the four putative binding sites in the *BMPRII* 3' UTR region that are regulated by miR-21. (B) The miRNA-target pairing sequences and calculated free energies.

primary antibodies were used: rabbit anti-BMPRII (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA) and GAPDH (1:2000, Abcam, Cambridge, UK).

Results

BMPRII is a predicted target of miR-21

Using the TargetScan software (<http://www.targetscan.org/>), it was predicted that there are 4 binding sites in the 3' UTR region of the *BMPRII* gene [Fig. 1(A)]. These four predicted binding sites were located at the 527, 945, 7296, and 7480 nt in the 3' UTR region of the *BMPRII* gene, respectively. The free energy values for the 4 BSs were approximately -19.3 , -19.2 , -21.4 , and -26.8 Kcal/mol [Fig. 1(B)]. Generally, all the miR-21 seed region (2–8 nt position) and corresponding pairing sites make near perfect complementarity, which is a common accepted principle in miRNA target prediction.

Two predicted binding sites on *BMPRII* are broadly conserved among vertebrates

By comparing the human sequence of the BSs for interspecies homology, we found that two of the predicted miR-21 binding sequences, BS3 and BS4, are highly conserved among several vertebrate species, indicating this kind of regulation via miR-21 is a conserved

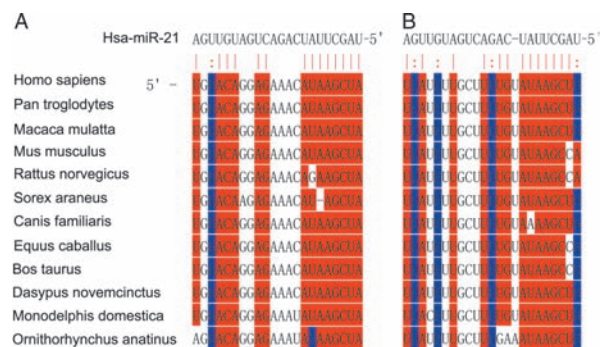


Figure 2 Sequence alignment of the conserved binding sites among the different species (A) The binding site BS3 is conserved among different vertebrate species. (B) The binding site BS4 is conserved among different vertebrate species. Red: G:C pair or A:U pair; Blue: G:U pair.

mechanism for fine tuning its expression level in evolution (Fig. 2).

Luciferase assay confirmed that *BMPRII* could be regulated by miR-21

Since the distance between the two upstream and the two downstream predicted binding sites was very long, we first cloned these two upstream and downstream sites separately along with ~ 1 kb of the contiguous sequences and designated them B12 and B34, respectively. To determine the predicted binding sites that could be regulated by miR-21, the fragment containing the sequences for BS12 and BS34 was cloned separately into the 3' UTR region of the luciferase gene in a reporter vector pGL3. Subsequently, the two luciferase reporter vectors with the miR-21 response elements were transfected into 293T cells. We used the seeding region-mutated miR-21-mimics as the negative control (NC-mimics). NC-mimics control or miR-21-mimics were also cotransfected into these cells. Then, the activity of luciferase and Renilla in these cells was measured. It was found that miR-21 decreased the luciferase activity of the reporter vector containing either the miR-21 response elements B12 or B34 [Fig. 3(A,B)]. These data suggest that miR-21 could regulate the expression of *BMPRII* by binding to not only the two downstream conserved binding sites but also the two upstream predicted binding sites. To further investigate whether all the four predicted binding sites are required for the regulation of miR-21, all the four binding sites regions were cloned into the 3' UTR of the luciferase gene in the pGL3 plasmid. The results of the luciferase assay revealed that the expression of luciferase gene containing the predicted binding sites was downregulated by $\sim 50\%$ in the

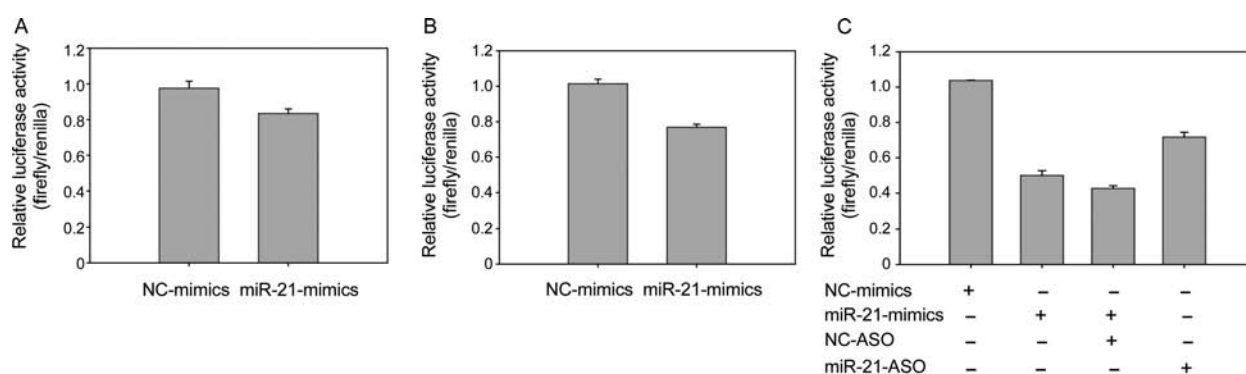


Figure 3 Luciferase reporter assay for the examination of miR-21:BMPRII interaction proved that BMPRII could be regulated by miR-21. 293T cells were transfected with a reporter vector containing the indicated binding sites sequences. CMV-Renilla luciferase vector was also cotransfected as the internal standard in each case. miR-21 mimics or ASO was also cotransfected to analyze its effect on the putative binding sites compared with NC-mimics or NC-ASO, respectively. Relative luciferase activity were calculated after normalization of the internal signal ($n = 3$). (A) Luciferase, containing the two upstream binding sites BS1 and BS2 on its 3' UTR region, could be down regulated about 20% by miR-21-mimics. (B) Luciferase, containing the two downstream binding sites BS3 and BS4 on its 3' UTR region, could be down regulated about 30% by miR-21-mimics. (C) Luciferase, containing all the four binding sites on its 3' UTR region, could be down regulated about 40% by miR-21-mimics. And the down-regulation by miR-21-mimics could be up-regulated by miR-21-ASO to about 70%.

presence of the miR-21 mimics. The downregulation of the luciferase gene was $\sim 30\%$ in the presence of ASO-miR-21 [Fig. 3(C)]. Taken together, these results confirmed that *BMPRII* is regulated by miR-21.

The protein level of *BMPRII* correlates inversely with the amount of miR-21 in PC3 and Lncap cells

Using NCodeTM quantitative PCR, it was found that the expression of miR-21 is approximately eight times higher in PC3 cells than in Lncap cells [Fig. 4(A)]. On the other hand, the results of the western blot showed that the expression of BMPRII was lower in PC3 cells than in Lncap cells [Fig. 4(B,C)]. These data showed that the protein level of BMPRII correlates inversely with the miR-21 levels in PC3 and Lncap cells.

BMPRII has several predicted conserved site on its 3' UTR region for miRNA families broadly conserved among vertebrates

Since *BMPRII* has a 7816-nt long 3' UTR region, we hypothesized that it might be regulated by several miRNAs at the post transcription level. We used TargetScan software to predict miRNAs which could bind on the 3' UTR region of *BMPRII*. As predicted, a lot of miRNAs were predicted to have binding sites on the 3' UTR region of *BMPRII*. Among them, several conserved sites for miRNA families that are broadly

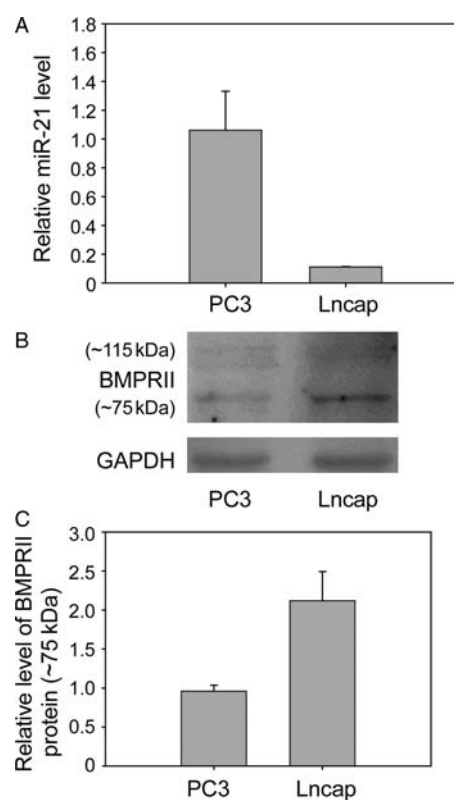


Figure 4 The inverse expression level of miR-21 and BMPRII between PC3 and Lncap cells (A) MiR-21 level was examined with NCodeTM quantitative PCR. (B) The BMPRII expression level was detected with western blot. (C) Relative protein level of BMPRII was normalized to that of GAPDH ($n = 3$).

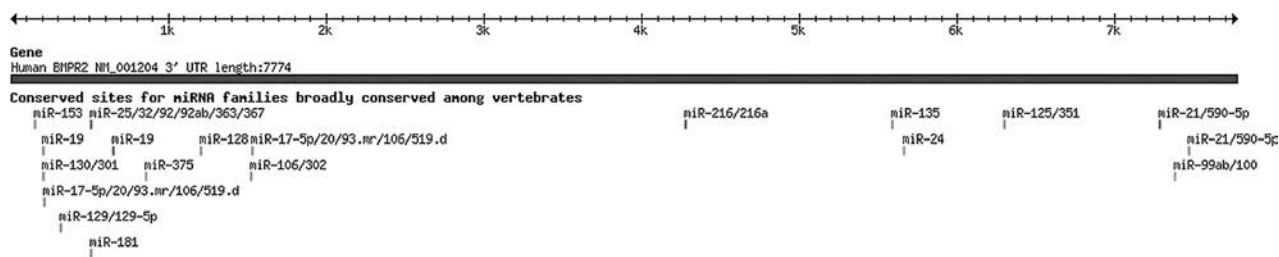


Figure 5 Schematic display predicted conserved site on its 3' UTR region for miRNA families broadly conserved among vertebrates Using TargetScan software, miRNAs which could bind on the 3' UTR region of *BMPRII* and are broadly conserved among vertebrates were predicted.

conserved among vertebrates have been predicted (Fig. 5). These data provided a clue that *BMPRII* might be regulated by different miRNAs under different condition.

Discussion

BMPs are one of the largest groups of cytokines within the TGF- β super family. BMPs were originally identified as molecules that regulate the growth and differentiation of bones and cartilage [26,27]. *BMPRII* is a type II serine/threonine kinase receptor of BMPs that is required to initiate the intracellular signaling pathway. Mutations in *BMPRII* lead to hereditary pulmonary hypertension, and knock out of *BMPRII* results in early embryonic lethality [28,]. miR-21 is well-known as an oncogene in a variety of tumor types. A previous study has determined several targets of miR-21, most of which are associated with cell growth [29]. In this study, we provided evidence that *BMPRII* is a direct target of miR-21 and has four predicted target sites in its 3' UTR region. Among the four predicted binding sites, two downstream sites are conserved among several vertebrate species. Our data showed that miR-21 could regulate the expression of *BMPRII* by binding to not only the two downstream conserved binding sites but also the two upstream predicted binding sites. We speculate that these four binding sites could function together to enhance the regulation ability of miR-21. We confirmed that *BMPRII*, which is associated with self-renewal and differentiation of stem cells, is a target of miR-21. These data suggest that miR-21 not only regulates cell growth and stem cell differentiation but is also involved in many other biological processes.

In this study, it was found that the protein level of *BMPRII* correlated inversely with miR-21 levels in PC3 and Lncap cells. Lncap cells are androgen-sensitive human prostate adenocarcinoma cells derived from the

left supraclavicular lymph node metastasis. PC3 cell lines were originally derived from the advanced androgen-independent bone metastasis. The metastatic and malignancy potential of PC3 cells is higher than that of Lncap cells, which have moderate metastatic potential [30]. Our data showed that the expression level of miR-21 was higher in PC3 than in Lncap cells, suggesting a possibility that miR-21 could have potential therapeutic applications in cancer. *BMPRII* has been reported to be expressed at low levels in prostate cancers, and loss of *BMPRII* in both prostate cancer tissues and cell lines have been shown to have an association with the progression of prostate cancers [31]. Our study suggested that miR-21 might be associated with the malignance and metastatic abilities of cancer cells by regulating *BMPRII*.

In conclusion, we have confirmed that *BMPRII* is a direct target of miR-21. In addition, we found that the protein level of *BMPRII* correlates inversely with miR-21 levels in PC3 and Lncap cells. The results of this study provided further evidence of the extensive role of miR-21 in the regulation of prostate cancer progression.

Funding

This work was supported by grants from the National Key Basic Research and Development Program (2005CB724602) and the Programs from Chinese Academy of Sciences (KSCX-2-SW-228 and KSCX1-YW-R-64).

References

- 1 Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004, 116: 281–297.
- 2 Eulalio A, Huntzinger E and Izaurralde E. Getting to the root of miRNA-mediated gene silencing. *Cell* 2008, 132: 9–14.

- 3 Yao C, Zhao B, Li W, Li Y, Qin W, Huang B and Jin Y. Cloning of novel repeat-associated small RNAs derived from hairpin precursors in *Oryza sativa*. *Acta Biochim Biophys Sin* 2007, 39: 829–834.
- 4 Wu L and Belasco JG. Let me count the ways: mechanisms of gene regulation by miRNAs and siRNAs. *Mol Cell* 2008, 29: 1–7.
- 5 Yao C, Ge L, Li Y, Zhao B, Li C, Ruan K and Lin H, *et al.* Expression and characterization of rice putative PAUSED gene. *Acta Biochim Biophys Sin* 2008, 40: 893–900.
- 6 Filipowicz W, Bhattacharyya SN and Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008, 9: 102–114.
- 7 Asli NS, Pitulescu ME and Kessel M. MicroRNAs in organogenesis and disease. *Curr Mol Med* 2008, 8: 698–710.
- 8 Osaki M, Takeshita F and Ochiya T. MicroRNAs as biomarkers and therapeutic drugs in human cancer. *Biomarkers* 2008, 13: 658–670.
- 9 Yang B, Lu Y and Wang Z. Control of cardiac excitability by microRNAs. *Cardiovasc Res* 2008, 79: 571–580.
- 10 Huang B, Li W, Zhao B, Xia C, Liang R, Ruan K and Jing N, *et al.* MicroRNA expression profiling during neural differentiation of mouse embryonic carcinoma P19 cells. *Acta Biochim Biophys Sin* 2009, 41: 231–236.
- 11 Rogaev EI, Borinskaia SA, Islamgulov DV and Grigorenko AP. Human microRNA in norm and pathology. *Mol Biol (Mosk)* 2008, 42: 751–764.
- 12 Tong AW and Nemunaitis J. Modulation of miRNA activity in human cancer: a new paradigm for cancer gene therapy? *Cancer Gene Ther* 2008, 15: 341–355.
- 13 Urbich C, Kuehbachner A and Dimmeler S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc Res* 2008, 79: 581–588.
- 14 Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A and Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J Biol Chem* 2008, 283: 1026–1033.
- 15 Zhu S, Si ML, Wu H and Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem* 2007, 282: 14328–14336.
- 16 Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST and Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 2007, 133: 647–658.
- 17 Li B. Bone morphogenetic protein-Smad pathway as drug targets for osteoporosis and cancer therapy. *Endocr Metab Immune Disord Drug Targets* 2008, 8: 208–219.
- 18 Nie X, Luukko K and Kettunen P. BMP signalling in craniofacial development. *Int J Dev Biol* 2006, 50: 511–521.
- 19 Granjeiro JM, Oliveira RC, Bustos-Valenzuela JC, Sogayar MC and Taga R. Bone morphogenetic proteins: from structure to clinical use. *Braz J Med Biol Res* 2005, 38: 1463–1473.
- 20 Rigelsky CM, Jennings C, Lehtonen R, Minai OA, Eng C and Aldred MA. BMPR2 mutation in a patient with pulmonary arterial hypertension and suspected hereditary hemorrhagic telangiectasia. *Am J Med Genet A* 2008, 146: 2551–2556.
- 21 Hassel S, Eichner A, Yakymovych M, Hellman U, Knaus P and Souchelnytskyi S. Proteins associated with type II bone morphogenetic protein receptor (BMPR-II) and identified by two-dimensional gel electrophoresis and mass spectrometry. *Proteomics* 2004, 4: 1346–1358.
- 22 Lavery K, Swain P, Falb D and Alaoui-Ismaili MH. BMP-2/4 and BMP-6/7 differentially utilize cell surface receptors to induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. *J Biol Chem* 2008, 283: 20948–20958.
- 23 Southwood M, Jeffery TK, Yang X, Upton PD, Hall SM, Atkinson C and Haworth SG, *et al.* Regulation of bone morphogenetic protein signalling in human pulmonary vascular development. *J Pathol* 2008, 214: 85–95.
- 24 McKay RD. Stem cell biology and neurodegenerative disease. *Philos Trans R Soc Lond B Biol Sci* 2004, 359: 851–856.
- 25 Ye L, Lewis-Russell JM, Kyanaston HG and Jiang WG. Bone morphogenetic proteins and their receptor signaling in prostate cancer. *Histol Histopathol* 2007, 22: 1129–1147.
- 26 Weston AD, Rosen V, Chandraratna RA and Underhill TM. Regulation of skeletal progenitor differentiation by the BMP and retinoid signaling pathways. *J Cell Biol* 2000, 148: 679–690.
- 27 Chen D, Zhao M and Mundy GR. Bone morphogenetic proteins. *Growth Factors* 2004, 22: 233–241.
- 28 Abramowicz MJ, Van Haecke P, Demedts M and Delcroix M. Primary pulmonary hypertension after amfepramone (diethylpropion) with BMPR2 mutation. *Eur Respir J* 2003, 22: 560–562.
- 29 Krichevsky AM and Gabriely G. miR-21: a small multi-faceted RNA. *J Cell Mol Med* 2009, 13: 39–53.
- 30 Hoosein NM. Neuroendocrine and immune mediators in prostate cancer progression. *Front Biosci* 1998, 3: 1274–1279.
- 31 Kim IY, Lee DH, Lee DK, Ahn HJ, Kim MM, Kim SJ and Morton RA. Loss of expression of bone morphogenetic protein receptor type II in human prostate cancer cells. *Oncogene* 2004, 23: 7651–7659.