RESEARCH ARTICLE

Roles of microRNA-206 in Osteosarcoma Pathogenesis and Progression

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Abstract

Backgroud and Aims: MicroRNA-206 has proven to be down-regulated in many human malignancies in correlation with tumour progression. Our study aimed to characterize miR-206 contributions to initiation and malignant progression of human osteosarcoma. Methods: MiR-206 expression was detected in human osteosarcoma cell 1ine MG63, human normal osteoblastic cell line hFOB 1.19, and paired osteosarcoma and normal adjacent tissues from 65 patients using quantitative RT-PCR. Relationships of miR-206 levels to clinicopathological characteristics were also investigated. Moreover, miR-206 mimics and negative control siRNA were transfected into MG63 cells to observe effects on cell viability, apoptosis, invasion and migration. Results: We found that miR-206 was down-regulated in the osteosarcoma cell line MG63 and primary tumor samples, and decreased miR-206 expression was significantly associated with advanced clinical stage, T classification, metastasis and poor histological differentiation. Additionally, transfection of miR-206 mimics could reduce MG-63 cell viability, promote cell apoptosis, and inhibit cell invasion and migration. Conclusions: These findings indicate that miR-206 may have a key role in osteosarcoma pathogenesis and development. It could serve as a useful biomarker for prediction of osteosarcoma progression, and provide a potential target for gene therapy.

Keywords: MicroRNA-206 - osteosarcoma - tumor stage - metastasis - invasion - migration

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Introduction

Osteosarcoma (OS) is the most common human primary malignant bone tumor in children and young adults, which accounts for approximately 60% of malignant bone tumors in the first 2 decades of life (Geller and Gorlick, 2010). With combined treatment (neoadjuvant chemotherapy, surgery, and adjuvant chemotherapy), the 5-year survival of patients with OS has significantly improved in the past thirty years (Cho et al., 2011; Lewis, 2009). However, about 80% of patients would eventually develop metastatic disease following surgical treatment (Marina et al., 2004), and outcome remains poor for these patients. The fundamental molecular mechanisms underlying the initiation, drug resistance, and development of metastasis in OS remain obscure. Therefore, the identification of the effector molecules and/or signal pathways responsible for OS pathogenesis and progression is needed to further optimize treatment strategies.

MicroRNAs (miRNAs) are small (20–24 nucleotides) noncoding RNA gene products that post-transcriptionally modulate gene expression by negatively regulating the stability or translational efficiency of their target mRNAs (Ambros, 2003). The importance of miRNA in cancer was first recognized when miRNA genes were found to be specifically deleted in leukemia (Calin et al., 2002). Subsequent reports have shown that miRNAs are differentially expressed in many cancers (Calin et al., 2004; Song et al., 2010). miRNAs are now widely believed to play an essential role in many malignancies, acting as either tumor suppressors or oncogenes (Fabian and Sonenberg, 2012; Liu et al., 2011). Recent studies have also analyzed various miRNAs that might contributing to invasion and metastasis in OS (Kobayashi et al., 2012).

One of the tumor suppressive miRNAs is miR-206. Using high-throughput technology, such as miRNA oligonucleotide arrays and quantitative RT-PCR for validation, previous studies have corroborated aberrant miR-206 expression in human malignancies such as lung cancer (Wang et al., 2011), rhabdomyosarcoma (Missiaglia et al., 2010), breast cancer (Iorio et al., 2005), and endometrioid adenocarcinoma (Chen et al., 2012). However, what function miR-206 exerts in OS is largely unknown. Therefore, in this study, the expression of miR-206 in OS tissue and cell line MG63 was examined by real-time PCR. In addition, the effects of miR-206 on MG63 cell proliferation, apoptosis, invasion and migration were also investigated.

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Materials and Methods

Human tissues and cell lines

A total of 65 histopathologically diagnosed OS patients who received neoadjuvant chemotherapy and wide resection at The First People's Hospital of Jingzhou, China between January 2009 and June 2011 were enrolled in this study. Patients were excluded if they had a previous or secondary malignancy, and/or had previously undergone radiation therapy, chemotherapy, or immunotherapy. This study was approved by the Medical Ethics Committee of our institution, and signed informed consent was obtained from all patients. Paired OS tumor tissues and normal adjacent tissues (NATs) were biopsy obtained from each patient prior to any treatment. The fresh specimens were stored at 4°C for 24 h in RNA Later (Ambion Inc.), then at −80°C liquid nitrogen until further use. Human OS cell 1ine MG63 and human normal osteoblastic cell line hFOB 1.19 were obtained from China Center for Type Culture Collection (Wuhan, China). Cells were cultured in DMEM with 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO_2.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using miRNeasy kit (Qiagen) according to the manufacturer's instructions. To detect miRNA expression, real-time quantitative RT-PCR (qRT-PCR) analysis was performed using LightCycler (Roche) and SYBR RT-PCR kit (Takara). cDNA was synthesized from total RNA using miR-206RT primer. The miR-206 RT primer was 5'-GCGCGTGAGCAGGCTG GAGAAA TTAACCACGCGC-5'. The miR-206 PCR primers were: sense: 5'-GGAATGTAAGGAAGTGTG-3'; antisense: 5'-GAGCAGGCTGGAGAA-3'. The reaction was incubated at 94 °C for 4 minutes followed by 35 cycles of 20 seconds at 94 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C. U6 small nuclear RNA was used as an internal control. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. All reactions were run in triplicate. The relative expression level of miR-206 was normalized to that of internal control U6 by using $2^{-\Delta\Delta Ct}$ cycle threshold method (Schmittgen and Livak, 2008).

miR-206 transfection

Double-stranded miR-206 mimics were purchased from GenePharma Co. Ltd, Shanghai, China. Primers for miR-206 were: sense: 5'-UGGAAUGUAAGGAAGUGUGUGG-3'; antisense: 5'-ACACACUUCCUUACAUUCCAUU-3'; negative control (NC) siRNA: sense: 5'-UUCUCCGAACGUGU CACGUTT-3'; antisense: 5'-GCGUGACACGUUCGGA GAATT-3'. LipofectamineTM 2000 (Invitrogen) was used to transfect miR-206 mimics into cells. Complete medium without antibiotics was used to culture cells and serum-free medium was used to dilute LipofectamineTM 2000 and miR-206 mimics.

Analysis of cell viability in vitro

The in vitro cell viability of MG63 transfected with NC or miR-206 mimics was measured using the MTT method (Alley et al., 1988). In brief, cells were seeded into 96-well plates and transfected. In the indicated time periods, 0.1 ml of spent medium was replaced with an equal volume of fresh medium containing MTT 0.5 mg/ml. Plates were incubated at 37˚C for 4 h, then the medium was replaced by 0.1 ml of DMSO (Sigma) and plates shaken at room temperature for 10 min. The absorbance was measured at 490 nm.

Detection of apoptosis

Osteosarcoma MG63 cells were transfected with NC or miR-206 mimics respectively. At 48 h post-transfection, spent cell culture medium was replaced with serum-free DMEM. In the indicated time periods post serum deprivation, cells were harvested, washed, resuspended in the staining buffer, and examined with Vybrant Apoptosis Assay kit (Invitrogen). Stained cells were detected by FACSCalibur and data were analyzed with CellQuest software (both from Becton-Dickinson). The Annexin V-positive and propidium iodide (PI)-negative cells were regarded as apoptotic cells.

Invasion assay

Invasion assays were performed in triplicate using Transwell invasion chambers coated with Matrigel (50 ul per filter) (BD, USA) as described in the manufacturer's protocol. MG-63 cells were transfected with either NC or miR-206 mimics, cultured for 48 h, and transferred on the top of Matrigel-coated invasion chambers in a serum-free DMEM (1×10^5 cells per Transwell). DMEM containing 10% fetal calf serum was added to the lower chambers. After incubation for 24 h, cells that remained on the top of the filter were scrubbed off and cells that migrated to the lower surface were fixed in 90% alcohol and followed by crystal violet stain. The number of migrated cells on the lower surface of the membrane was counted under a microscope in 10 fields with magnification of × 400.

Scratch migration assay

When MG-63 cells transfected with NC or miR-206 mimics were seeded and grown to confluence, a scratch was set with a pipette tip running though the dish and cultured under standard conditions for 24 h. Plates were washed twice with fresh medium to remove nonadherent cells and then photographed. The cell migration was evaluated by counting cells that migrated from the wound edge.

Statistics

Data are expressed as the mean ± standard error. The differences between groups were analyzed using the Student's t-test or one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Results

miR-206 is down-regulated in OS cell line MG-63 and primary tumor samples

In order to gain insight into the biological roles of miR-206 in human OS pathogenesis and progression, we compared miR-206 expression in human normal

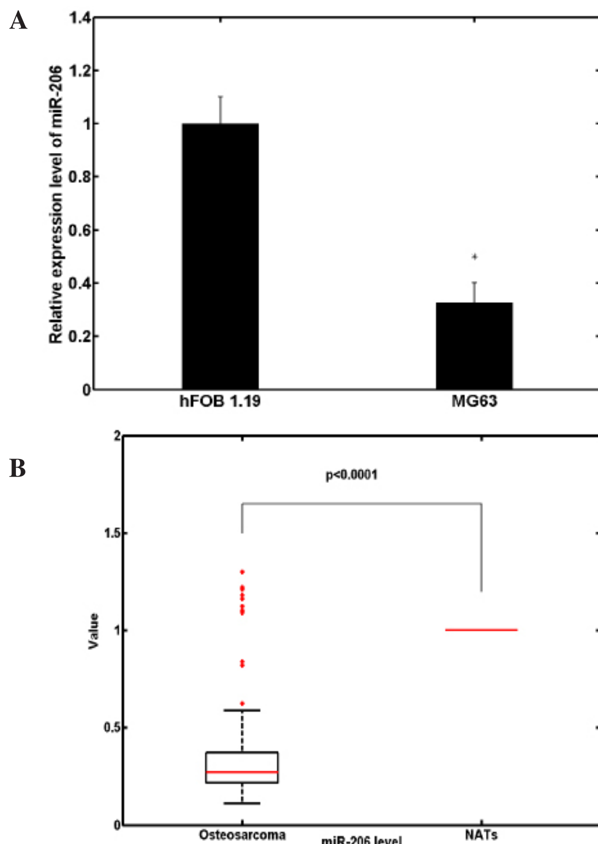

Figure 1. MiR-206 was Down-regulated in Osteosarcoma Cell Line MG63 (A) and Primary Tumor Samples (B)

Table 1. The Relationship of miR-206 Expression Level and Clinicopathological Features

Factors	No. of patients	MiR-206 (Average fold-change±SD)	*p*-value
Age (year)			
≤24	46	0.33±0.14	0.388
>25	19	0.26±0.19	
Gender			
Male	38	0.38±0.35	0.215
Female	27	0.24±0.16	
Clinical stage			
I	26	0.87±0.71	<0.001[a]
II	32	0.40±0.25	0.002[b]
III	7	0.13±0.11	
T classification			
T_1	25	0.49±0.44	0.003
T_2	40	0.18±0.15	
M classification			
M_0	56	0.45±0.30	0.011
M_1	9	0.20±0.17	
Histological differentiation			
G_1	30	0.59±0.46	<0.001
G_2	35	0.16±0.10	

[a]Comparing with stage II and III; [b]Comparing with stage III

osteoblastic cell line hFOB 1.19 and human OS cell line MG63 by real-time qRT-PCR. Compared to normal hFOB 1.19 cells, miR-206 expression was significantly decreased in OS MG63 cells (Figure 1A). Furthermore, we collected 65 pairs of primary OS tumor samples and NATs. Expression of miR-206 was significantly suppressed in tumor samples compared to NATs (Figure 1B).

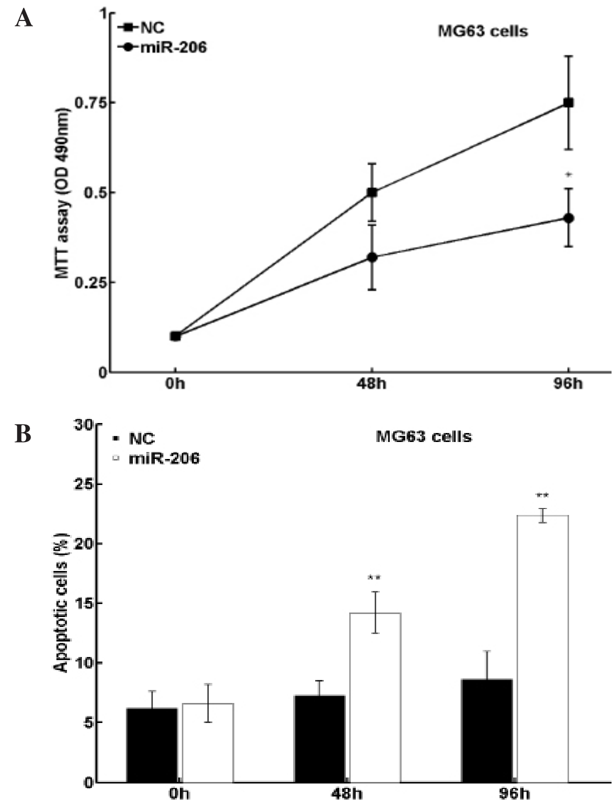

Figure 2. MiR-206 Could Reduce Osteosarcoma Cell Line MG63 Viability (A) and Promote Cell Apoptosis (B)

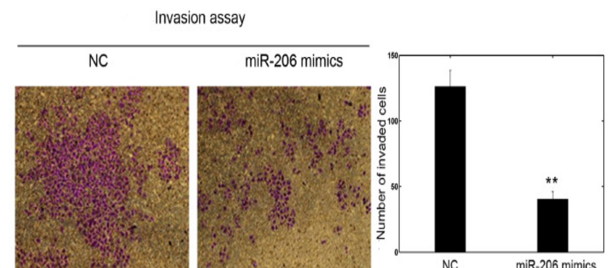

Figure 3. MiR-206 Regulates Cell Invasion in MG-63 Cells

Decreased miR-206 expression is associated with clinical features and poor survival of OS

Correlation between miR-206 expression level and clinicopathologic characteristics of OS is summarized in Table 1. We found that miR-206 expression levels were significantly associated with clinical stage, T classification, metastasis and histological differentiation, suggesting that lower miR-206 expression is correlated with OS development.

miR-206 reduces cell viability and promotes cell apoptosis

The suppression of miR-206 prompted us to investigate whether miR-206 functions as a tumor suppressor in OS. We found that transfection of miR-206 mimics reduced cell viability in MG63 cells (Figure 2A). Furthermore, restoration of miR-206 expression promoted cell apoptosis in MG63 cells (Figure 2B). These results demonstrate that miR-206 inhibits OS growth in vitro.

miR-206 affects cell invasion and migration in vitro

To corroborate the effect of miR-206 on cell invasion

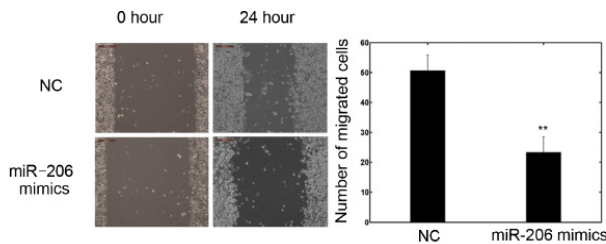

Figure 4. MiR-206 Affects the Ability of MG-63 Cell Migration

and migration, we employed the cell invasion assay and scratch migration assay. MG-63 was transfected with miR-206 mimics or NC and we observed a significant downregulation of invasion into Matrigel in miR-206-transfected MG-63 cells (Figure 3). Meanwhile we noticed that the number of the migrated cells transfected with miR-206 mimics were significantly fewer than NC (Figure 4). These two observations emphasize the function of miR-206 in the invasion and metastasis of MG-63 cells.

Discussion

Osteosarcoma is the most common bone tumor seen in the pediatric and adolescent age group. Although progress has been made on OS diagnosis and treatment, there are still many unexplored areas, and patients with metastasis or recurrent diseases still have a poor prognosis. The miRNAs are involved in a majority of biological processes, including cell cycle regulation, cell growth, apoptosis, cell differentiation and stress response (Jovanovic and Hengartner, 2006). More specifically, miRNAs can either modulate oncogenic or tumor suppressor pathways (Casalini and Iorio, 2009). Deeper comprehension of miRNA activity in the human body can lead to promising new therapies for the management of human malignancies, including OS.

In the present study, we reported for the first time that miR-206 was down-regulated in OS cell line MG-63 and primary tumor samples. We also showed that miR-206 could reduce MG-63 cell viability, promote cell apoptosis, and affect cell invasion and migration. Moreover, the expression levels of miR-206 was strongly associated with clinical and pathological features, such as differentiation, clinical stage, T classification, and metastasis. These raise the possibility that miR-206 may be a target for therapeutic intervention and a biomarker for the prediction of OS progression and prognosis.

Located on chromosome 6p12.2, miR-206 has been proven to play a tumor suppressive function in many previous studies. MiR-206 inhibits cell growth and induces apoptosis in rhabdomyosarcoma (Missiaglia et al., 2010), breast cancer (Leivonen et al., 2009), endometrial endometorioid carcinoma (Chen et al., 2012) and lung cancer cells (Wang et al., 2011). Cell migration and invasion activities are also inhibited by miR-206 in rhabdomyosarcoma, endometrial endometorioid carcinoma and lung cancer cells. In vivo, a tumor suppressive function for miR-206 has been shown in rhabdomyosarcoma in xenotransplanted mice (Taulli et al., 2009). Kondo et al confirmed that miR-206 expression was markedly decreased in estrogen receptor alpha-positive human breast cancer tissues, and transfection of miR-206 into MCF-7 breast cancer cells would inhibit cell growth in a dose-dependent manner (Kondo et al., 2008). Zhang and collegues revealed that miR-206 was significantly down regulated in the laryngeal suamous cell carcinoma tissues (Zhang et al., 2011). Inverse correlation of miR-206 expression was found with the T grade, nodal metastasis and clinical stage of laryngeal suamous cell carcinoma. Vickers et al reported that miR-206 was one of five miRNAs that exhibited stage-dependent differential expression in human colorectal cancers (Vickers et al., 2012). Furthermore, miR-206 expression levels in serum might be used to distinguish rhabdomyosarcoma from non- rhabdomyosarcoma tumors (Miyachi et al., 2010). Taken together, these findings suggest that miR-206 might play an important role not only in tumour initiation but also in the development and progression of malignancy.

Conducting qPCR, western blotting, and reporter assays and using bioinformatic prediction programs, recent research has identified several targets of miR-206, such as MET (Taulli et al., 2009), notch 3 (NOTCH3) (Song et al., 2009), estrogen receptor alpha (Kondo et al., 2008), and VEGF (Zhang et al., 2011). However, the molecular genetic basis of carcinogenesis and cancer progression is complex, and it is not a ‘‘one to one’’ connection between miRNAs and target mRNAs. An average miRNA can have more than 100 targets (Bartel and Chen, 2004), and one mRNA can be regulated by a variety of miRNAs (Krek et al., 2005). So the potential regulatory circuitry afforded by miR-206 may be enormous. It is probable that we are still far from unveiling the last target of miR-206, and some of these potential targets may be still unknown in osteosarcoma carcinogenesis and progression. According to this presumption, interesting future work is required to identify the targetome and entire roles of miR-206 in osteosarcoma.

In summary, our study was the first to show that miR-206 was frequently down-regulated in osteosarcoma and decreased miR-206 expression was associated with advanced clinical stage and poor histological differentiation. Moreover, miR-206 plays a crucial role in osteosarcoma cell MG-63 viability, apoptosis, invasion and migration. Therefore, miR-206 could be a useful biomarker for the prediction of osteosarcoma progression, and a potential target for gene therapy.

Acknowledgements

The author(s) declare that they have no competing interests.

References

Alley MC, Scudiero DA, Monks A, et al (1988). Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res*, **48**, 589-601.

Ambros V (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell*, **113**, 673-6.

Bartel DP, and Chen CZ (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat Rev Genet*, **5**, 396-400.

Calin GA, Dumitru CD, Shimizu M, et al (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*, **99**, 15524-9.

Calin GA, Sevignani C, Dumitru CD, et al (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A*, **101**, 2999-3004.

Casalini P, and Iorio MV (2009). MicroRNAs and future therapeutic applications in cancer. *J Buon*, **14**, S17-22.

Chen X, Yan Q, Li S, et al (2012). Expression of the tumor suppressor miR-206 is associated with cellular proliferative inhibition and impairs invasion in ERalpha-positive endometrioid adenocarcinoma. *Cancer Lett*, **314**, 41-53.

Cho Y, Jung GH, Chung SH, et al (2011). Long-term survivals of stage IIb osteosarcoma: a 20-year experience in a single institution. *Clin Orthop Surg*, **3**, 48-54.

Fabian MR, and Sonenberg N (2012). The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. *Nat Struct Mol Biol*, **19**, 586-93.

Geller DS, and Gorlick R (2010). Osteosarcoma: a review of diagnosis, management, and treatment strategies. *Clin Adv Hematol Oncol*, **8**, 705-18.

Iorio MV, Ferracin M, Liu CG, et al (2005). MicroRNA gene expression deregulation in human breast cancer. *Cancer Res*, **65**, 7065-70.

Jovanovic M, and Hengartner MO (2006). miRNAs and apoptosis: RNAs to die for. *Oncogene*, **25**, 6176-87.

Kobayashi E, Hornicek FJ, and Duan Z (2012). MicroRNA Involvement in Osteosarcoma. *Sarcoma*, **2012**, 359739.

Kondo N, Toyama T, Sugiura H, et al (2008). miR-206 Expression is down-regulated in estrogen receptor alpha-positive human breast cancer. *Cancer Res*, **68**, 5004-8.

Krek A, Grun D, Poy MN, et al (2005). Combinatorial microRNA target predictions. *Nat Genet*, 37, 495-500.

Leivonen SK, Makela R, Ostling P, et al (2009). Protein lysate microarray analysis to identify microRNAs regulating estrogen receptor signaling in breast cancer cell lines. *Oncogene*, **28**, 3926-36.

Lewis VO (2009). What's new in musculoskeletal oncology. *J Bone Joint Surg Am*, **91**, 1546-56.

Liu ZY, Zhang GL, Wang MM, et al (2011). MicroRNA-663 targets TGFB1 and regulates lung cancer proliferation. *Asian Pac J Cancer Prev*, **12**, 2819-23.

Marina N, Gebhardt M, Teot L, and Gorlick R (2004). Biology and therapeutic advances for pediatric osteosarcoma. *Oncologist*, **9**, 422-41.

Missiaglia E, Shepherd CJ, Patel S, et al (2010). MicroRNA-206 expression levels correlate with clinical behaviour of rhabdomyosarcomas. *Br J Cancer*, **102**, 1769-77.

Miyachi M, Tsuchiya K, Yoshida H, et al (2010). Circulating muscle-specific microRNA, miR-206, as a potential diagnostic marker for rhabdomyosarcoma. *Biochem Biophys Res Commun*, **400**, 89-93.

Schmittgen TD, and Livak KJ (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*, **3**, 1101-8.

Song G, Zhang Y, and Wang L (2009). MicroRNA-206 targets notch3, activates apoptosis, and inhibits tumor cell migration and focus formation. *J Biol Chem*, **284**, 31921-7.

Song T, Xia W, Shao N, et al (2010). Differential miRNA expression profiles in bladder urothelial carcinomas. *Asian Pac J Cancer Prev*, **11**, 905-11.

Taulli R, Bersani F, Foglizzo V, et al (2009). The muscle-specific microRNA miR-206 blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation. *J Clin Invest*, **119**, 2366-78.

Vickers MM, Bar J, Gorn-Hondermann I, et al (2012). Stage-dependent differential expression of microRNAs in colorectal cancer: potential role as markers of metastatic disease. *Clin Exp Metastasis*, **29**, 123-32.

Wang X, Ling C, Bai Y, and Zhao J (2011). MicroRNA-206 is associated with invasion and metastasis of lung cancer. *Anat Rec*, **294**, 88-92.

Zhang T, Liu M, Wang C, et al (2011). Down-regulation of MiR-206 promotes proliferation and invasion of laryngeal cancer by regulating VEGF expression. *Anticancer Res*, **31**, 3859-63.