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# Direct targeting of SUZ12/ROCK2 by miR-200b/c inhibits cholangiocarcinoma tumourigenesis and metastasis

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**Background:** The multidrug resistance and distant metastasis of cholangiocarcinoma result in high postoperative recurrence and low long-term survival rates. It has been demonstrated that the ectopic expression of miR-200 suppresses the multidrug resistance and metastasis of cancer. However, the expression and function of miR-200 in cholangiocarcinoma has not yet been described.

**Methods:** In this study, we identified dysregulated microRNAs (miRNAs, miR) in cholangiocarcinoma tissue by microarray analysis, and subsequent real-time PCR and northern blot analyses validated the expression of candidate miR. We performed functional analyses and investigated the relationship between miR-200b/c expression and the properties of cholangiocarcinoma cells. A dual luciferase assay was applied to examine the effect of miRNAs on the 3'-UTR of target genes, and we demonstrated the function of the target gene by siRNA transfection identifying the downstream pathway via western blotting.

**Results:** We found significantly downregulated expression of four miR-200 family members (miR-200a/b/c/429) and then confirmed that ectopic miR-200b/200c inhibits the migration and invasion of cholangiocarcinoma cells both *in vitro* and *in vivo*. We found that miR-200b/c influenced the tumourigenesis of cholangiocarcinoma cells including their tumour-initiating capacity, sphere formation, and drug resistance. We further found that miR-200b/c regulated migration and invasion capacities by directly targeting rho-kinase 2 and regulated tumorigenic properties by directly targeting SUZ12 (a subunit of a polycomb repressor complex).

**Conclusion:** Our study shows that miR-200b/c has a critical role in the regulation of the tumorigenic and metastatic capacity of cholangiocarcinoma and reveals the probable underlying mechanisms.

Cholangiocarcinoma is generally acknowledged as being difficult to diagnose and treat. Despite improvements in treatment, the survival of patients remains poor (Khan *et al*, 2008), which is attributed to early extensive local tumour invasion and distant metastasis, as well as multichemoresistance (Khan *et al*, 2005; Blechacz and Gores, 2008). The development of cholangiocarcinoma is a multistep process,

with an accumulation of genetic and epigenetic alterations (Evert *et al*, 2013; Timmer *et al*, 2013). Therefore, a better understanding of the molecular mechanisms of the multidrug resistance, distant metastasis, and tumorigenic properties of cholangiocarcinoma is likely to lead to novel therapeutic strategies for cholangiocarcinoma (Visvader and Lindeman, 2008).

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MicroRNAs (miRNAs) are a well-known class of small RNAs that inhibit gene expression at the post-transcriptional level by directly targeting regions of sequence complementarity in the 3'-untranslated regions (3'-UTRs) of mRNAs (He and Hannon, 2004). The miR-200 family, including the miR-200a/141 and miR-200b/c/429 clusters, is involved in neurogenesis (Karres *et al*, 2007), the regulation of embryonic and adult stem cells, and cancer stem cells (CSCs) (Shimono *et al*, 2009; Wellner *et al*, 2009; Samavarchi-Tehrani *et al*, 2010), chemosensitivity, and apoptosis (Cochrane *et al*, 2010; Schickel *et al*, 2010). The miR-200 family also influences the epithelial-to-mesenchymal transition (EMT) through the *Zeb-E-cadherin* axis (Burk *et al*, 2008; Korpál *et al*, 2008; Park *et al*, 2008). However, recent studies have yielded conflicting results in terms of the tumour suppressor function of this family (Dykxhoorn *et al*, 2009), it remains unclear whether the metastasis suppressor functions of the miR-200 family are mediated entirely or only partially through the *Zeb-E-cadherin* axis (Korpál *et al*, 2011). Indeed, although the potential of other cancer-related functions of the miR-200 family warrants further research, few studies on the relationship between the expression levels of miRNAs and cholangiocarcinoma cells have been published. Therefore, we conducted miRNA profiling in cholangiocarcinoma tissues and found a differential expression of miRNAs, including four of the five miR-200 family members, which have been shown to have an important role in regulating the properties of metastasis and invasion. Nonetheless, the function of miR-200 in cholangiocarcinoma tumorigenesis has not been clearly elucidated.

Here, we demonstrate that miR-200b/c regulates the metastatic capacity and tumorigenic properties of cholangiocarcinoma, including tumour-initiating capacity, sphere formation, and drug resistance. We also show that miR-200b/c inhibits invasion and migration by directly targeting other genes such as rho-kinase2 (*ROCK2*). Moreover, miR-200b/c influences cholangiocarcinoma tumorigenesis by silencing *SUZ12* (a subunit of a polycomb repressor complex). These results provide insight into the molecular mechanisms of cholangiocarcinoma regulation and may suggest a novel therapeutic strategy for cholangiocarcinoma treatment.

## MATERIALS AND METHODS

**Tumours.** Human cholangiocarcinoma tissues were obtained from 14 patients with postoperative pathological diagnosed perihilar or distal biliary cholangiocarcinoma from September 2010 to May 2011 at the Department of Biliary-Pancreatic Surgery, Affiliated Tongji Hospital (Hubei, China) (Supplementary Table 1). Human sample collection procedures were approved by the China Ethical Review Committee.

**MiRNA microarrays.** Total RNA was extracted from three cholangiocarcinoma tissues and their corresponding normal bile ducts using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. An Agilent Human miRNA Microarray Kit (V2) (Agilent Inc, Santa Clara, CA, USA) (Sanger database v.10.1) was used for analysis. RNA labelling and hybridisation on Agilent miRNA microarray chips were performed with an miRNA Labelling Reagent and Hybridization Kit (Agilent Technologies). Microarray data analyses including GO analysis, Pathway Analysis, Path-net, Signal-net, and MicroRNA-gene network were performed (Genminix Company, Shanghai, China). The microarray data were deposited in NCBI's Gene Expression Omnibus (GEO) public database (<http://www.ncbi.nlm.nih.gov/geo/>; Gene Expression Omnibus accession number, GSE47764).

**Cell culture and transfections.** The TFK-1 cell line was purchased from DSMZ by Professor Shengquan Zou of the Affiliated Tongji Hospital. The QBC939 cell line was provided by Professor Shu-guang Wang of The Third Military Medical University, Chongqing, China. All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, and 1% penicillin/streptomycin at 37°C in a 5% CO<sub>2</sub> incubator. Cells were seeded in 6-well plates and transfected with miR-200b/c mimics/antagomir or negative control (NC) (Ambion) at a final concentration of 50 nM using siPORT NeoFX Transfection Agent (Ambion). Small interfering RNA and the NC were purchased from RiboBio (Guangzhou, China). The sequences of siRNA could be found in Supplementary Table 4. MiRNA-200b/c overexpression and knockdown lentiviruses, sh-SUZ12 and sh-ROCK2, were constructed by and purchased from Genechem (Shanghai, China). All transfections were carried out according to the manufacturer's instructions.

**RNA extraction and RT-qPCR.** Total RNA was isolated as described above. Real-time polymerase chain reaction was carried out using 50 ng or 500 ng of total RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) or PrimeScript Reverse Transcription Kit (TAKARA, Dalian, China) for miRNA and mRNA analyses, respectively. Real-time fluorescence quantitative polymerase chain reaction was performed using TaqMan MicroRNA Assays (Applied Biosystems) or SYBR Premix Ex Taq (TAKARA) for miRNA and mRNA analyses, respectively. All values were normalised to human RNU6B or  $\beta$ -actin endogenous controls, respectively, and run in triplicate. The sequences of primers could be found in Supplementary Table 4. Amplification and detection were performed using a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA).

**Northern blot.** MiRNA northern blot assays was performed using the MiRNA Northern Blot Assay Kit (Signosis, Sunnyvale, CA, USA) according to the manufacturer's protocol and as described in Yu *et al* (2007). Total RNAs were extracted as described above. High-sensitive miRNA probe used was follows: miR-200b: 5'-TCA TCATTACCAGGCAGTATTA-3'; miR-200c: 5'-TCCATCATTAC CCGGCAGTATTA-3'; RNU6B: 5'-AGTATATGTGCTGCCGAA GCGAGCAC-3'. The image was acquired using a FluorChem FC2 imager (Alpha Innotech Corp, San Leandro, CA, USA). Expression of *SUZ12/ROCK2* mRNA was assessed using DIG luminescent detection kit (Roche, Shanghai, China) as described (Almontashiri *et al*, 2013).

**Wound healing assay.** Cells ( $1 \times 10^6$  per well) were cultured in 6-well plates overnight, and then transfected with miR-200b/c mimics or antagomir and the respective controls. After 24 h of transfection, the cell layer was scratched with a sterile plastic tip, washed with PBS two times, and then cultured for 24 h with medium containing 1% FBS. Images of the plates were taken under a microscope.

**Invasion assay.** Transwell inserts (24-well insert, 8  $\mu$ m pore size; Corning Inc., Corning, NY, USA) were used to determine the invasion capacity *in vitro*. Inserts were precoated with extracellular matrix gel (BD Biosciences, Bedford, MA, USA). After transfection, cells were serum starved in serum-free medium overnight, and then resuspended in medium containing 0.1% bovine serum albumin and placed in the upper chambers in triplicate. Lower chambers were filled with 10% FBS as the attractant, and the cells were incubated for 48 h. Then, the cells were removed from the upper surface of the membrane, and cells on the lower surface were fixed and stained with 0.4% paraformaldehyde and 0.1% crystal violet, respectively. Then, the stained cells were counted under a light microscope.

**Liver metastasis model of cholangiocarcinoma cells in BALB/c nude mice.** Female BALB/c nude mice, 4–6 weeks old, were purchased from the Animal Laboratory Unit of Peking Union Medical College (Beijing, China). The mice were maintained under standard conditions and reared according to the institutional guidelines for animal care. Lentivirus-transfected cells ( $1 \times 10^5$ ) were suspended in serum-free medium and then injected into the involucre of the spleen. The mice underwent a lienectomy at 20 min after injection. The weight of the mice was measured once a week.

**Xenograft experiments.** A total of  $1 \times 10^5$  transfected cells were subcutaneously injected into the right armpit of BALB/c nude mice. The weight of the mice and the diameter of tumours were measured every week.

**Drug resistance experiments.** A total of  $2 \times 10^5$  transfected cells were first seeded into one well of a 24-well plate in the normal medium and cultured overnight. Then, the cells were treated with 5-fluorouracil at the indicated concentrations. At 24, 48, and 72 h, the survival rate was detected using a WST Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), and IC<sub>50</sub> values were calculated following the manufacturer's instructions.

**Sphere culture and differentiation.** Cells (1000 cells per ml) were cultured in suspension in serum-free DMEM-F12 (Hyclone, Logan, UT, USA), supplemented with B27 (1:50; Invitrogen, Carlsbad, CA, USA), 20 ng ml<sup>-1</sup> epidermal growth factor (PeproTech EC, London, UK), 100 ng ml<sup>-1</sup> basic fibroblast growth factor (PeproTech EC), and 100 ng ml<sup>-1</sup> leukaemia inhibitory factor (Chemicon, Billerica, MA, USA) as described elsewhere (Hermann *et al.*, 2007). Differentiation experiments were carried out as described (Yu *et al.*, 2007).

**Flow cytometry.** Transfected cells were dissociated, washed two times with BD PharMingen Stain Buffer (BD Bioscience), and resuspended. Antibodies were added to the cells followed by incubation for 30 min at 4 °C. The antibodies used were CD24-FITC, CD44-APC, and CD133-PE (BD Bioscience) each at 10 µl per  $1 \times 10^6$  cells. Flow cytometry was performed using a FACSaria (BD Immunocytometry Systems, Franklin Lakes, NJ, USA).

**Luciferase reporter assay.** The 3'-UTRs of eight putative *SUZ12/ROCK2* genes were selected according to the genomic fragment of TargetScan containing the predicted miR-200b/c/429 binding sites. The mutant *SUZ12/ROCK2* 3'-UTR construct was designed to mutate three intermittent nucleotides complementary to the miR-200b/c/429 seed region. The TFK-1 cells were cultured in 24-well plates and transfected with 400 ng of either wild-type and one mutant pMIR/*SUZ12* or three mutant pMIR/*ROCK2* plasmids carrying firefly luciferase, together with 100 ng pRL-TK vector (Promega, Madison, WI, USA) carrying renilla luciferase. Transfection was performed using Lipofectamine 2000 (Invitrogen). Relative luciferase activity was calculated at 48 h post-transfection by a Dual Luciferase Reporter Assay (Promega).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard error. Statistically significant differences were determined by the Student's *t*-test. Statistical analyses were performed by SPSS Software (version 19.0) (SPSS Inc, Chicago, IL, USA). A two-tailed value of  $P < 0.05$  was considered to indicate a statistically significant result.

## RESULTS

**Differential expression of miRNAs in cholangiocarcinoma patients, and validation of microarray results by RT-qPCR.** To identify miRNAs that are differentially expressed in cholangiocarcinoma, we compared the expression of miRNAs in three

cholangiocarcinoma samples and the corresponding normal bile duct tissues using an Agilent miRNA Microarray. We found that 15 miRNAs were overexpressed and six miRNAs were underexpressed in the cholangiocarcinoma tumour group compared with that in the controls (Figure 1A). The 15 overexpressed miRNAs in the cholangiocarcinoma group were 2.25- to 42 222-fold overexpressed compared with the control group, and the six underexpressed miRNAs were 6.39- to 4032-fold underexpressed compared with the controls (Supplementary Table 2). Next, we predicted the possible targets of this differential expression and identified 2844 target genes. These target genes were then analysed for biological process and molecular function using information available in the Gene Ontology (GO) database. As shown in Supplementary Figure 1B and Supplementary Table 5, there were 78 upregulated GO terms and 82 downregulated terms. To determine whether the abnormal expression of these genes was due to dysregulated signalling pathways, we performed pathway analyses and found 20 upregulated pathways and 30 downregulated pathways in relation to the dysregulated genes (Supplementary Figure 1C and Supplementary Table 6). Using the significant pathway terms, a network of pathways and genes was generated using the KEGG database (Supplementary Figure 2A and B). Lastly, we identified the crucial target genes regulated by the differentially expressed miRNAs, including miR-200b and miR-200c (Supplementary Figure 1A). Three miRNAs, miR-200b, miR-200c, and miR-429, were selected for validation of the microarray results by RT-qPCR and northern blotting. The data analysis showed that miR-200b, miR-200c, and miR-429 were underexpressed in the cholangiocarcinoma group compared with the controls (Figure 1B and Supplementary Figure 7A). The data showed that the reduced expression of miR-200b and miR-200c was significant (Figure 1C,  $P = 0.038$  and  $P = 0.019$ , respectively), whereas that of miR-429 was not (Figure 1C,  $P = 0.057$ ). The RT-PCR and northern results were consistent with those of the microarray analysis. Therefore, miR-200b and miR-200c were selected for further study.

**Ectopic miR-200b/c inhibits the migration and invasion of cholangiocarcinoma cells.** We determined whether miR-200b/c can alter the migration and invasion capacities of cholangiocarcinoma cells. As expected, the transfection of miR-200b/c mimics and antagomir into TFK-1 cells resulted in a substantial increase (by mimics) and decrease (by antagomir) of miR-200b/c expression, respectively, compared with that in the NC-transfected cells (Figure 2A,  $P < 0.001$ ). An invasion assay showed that the overexpression of miR-200b/c resulted in a reduction in the invasion rate of TFK-1 cells compared with that of the control (Figures 2C and B,  $5.04 \pm 0.11$ - and  $3.12 \pm 0.03$ -fold, respectively,  $P < 0.001$ ). The TFK-1 cells transfected with the miR-200b/c mimics also slowly closed wound gaps compared with the control (Figure 2D and Supplementary Figure 4,  $2.13 \pm 0.24$ - and  $1.92 \pm 0.03$ -fold, respectively,  $P < 0.001$ ). Similar results were observed using QBC939 cells (Figure 2E and Supplementary Figure 3A and B). In contrast, the knockdown of miR-200b/c promoted the invasion of the cholangiocarcinoma cells (Figure 2C): miR-200b/c knockdown in TFK-1 cells resulted in significant gap closure in the wound healing assay (Figure 2D,  $0.69 \pm 0.01$ - and  $0.65 \pm 0.03$ -fold, respectively,  $P < 0.001$ ) and an increase in cell invasion compared with that of the antagomir-NC group (Figure 2B,  $1.96 \pm 0.10$ - and  $3.07 \pm 0.19$ -fold, respectively,  $P < 0.001$ ). Moreover, we confirmed an effect of miR-200b/c on cholangiocarcinoma's metastatic capacity *in vivo*. We transfected TFK-1 cells with a lentivirus-based expression vector for either the overexpression or downregulation miR-200b/c, and then established a liver metastasis model of cholangiocarcinoma using these

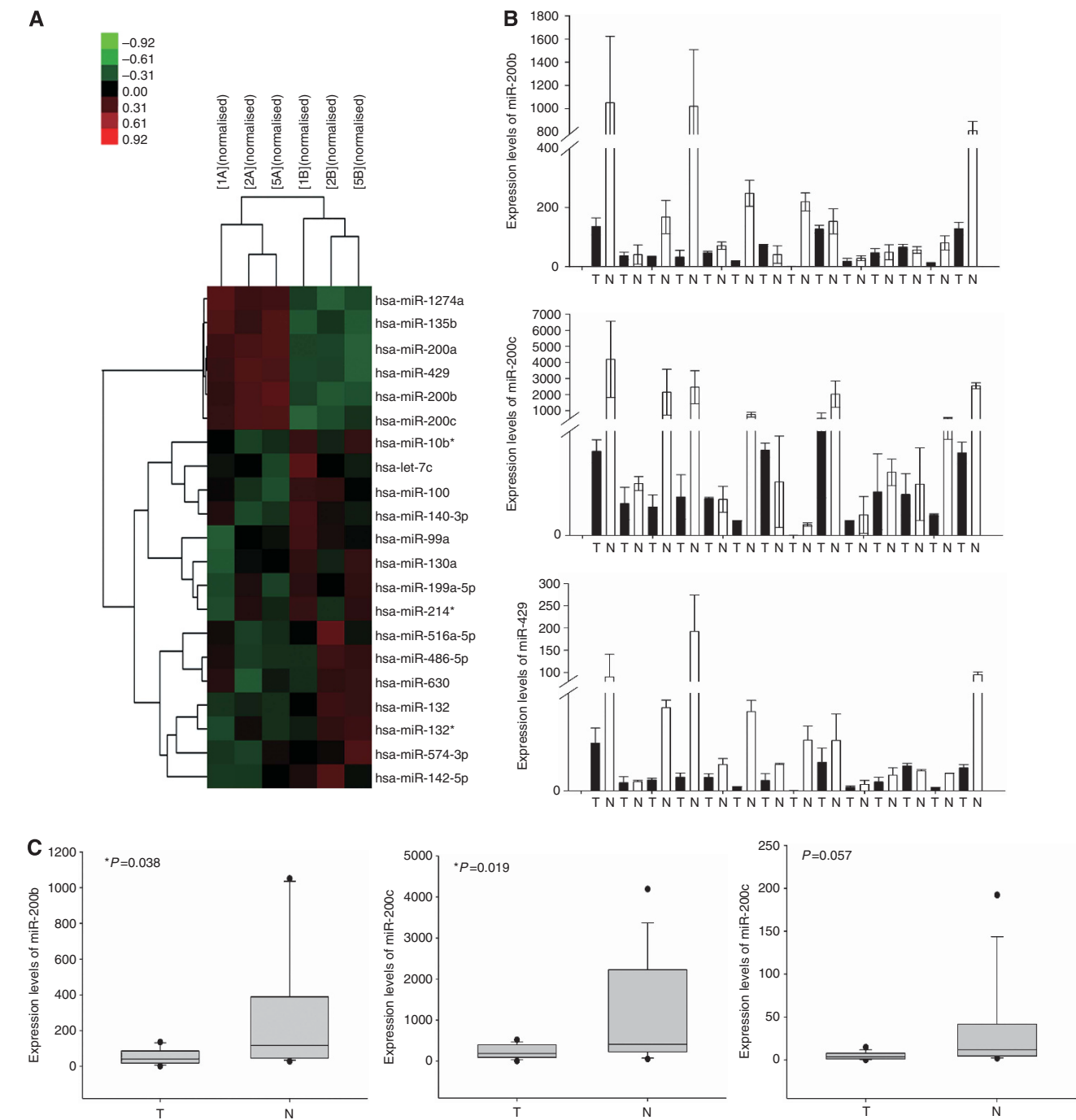


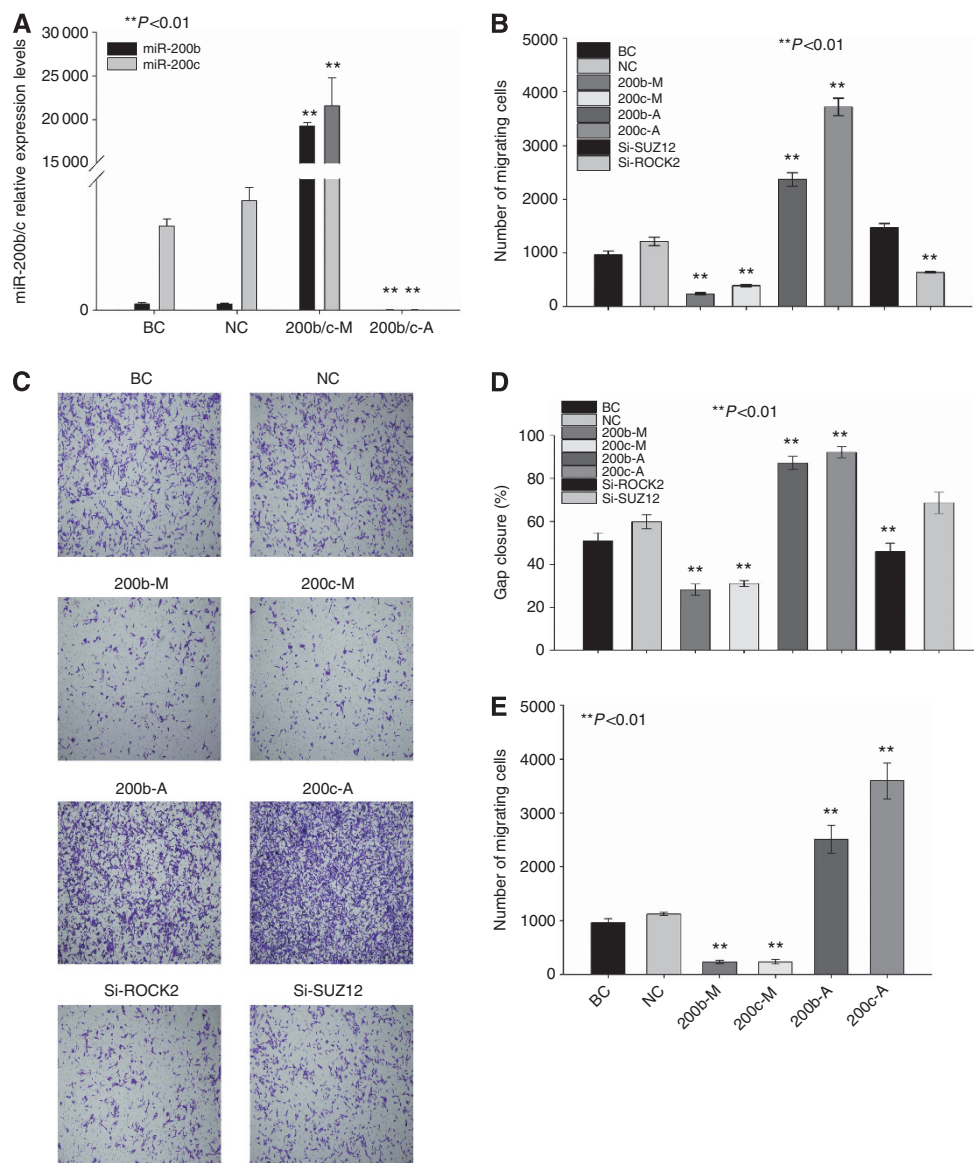
Figure 1. Differential expression of miRNAs in three cholangiocarcinoma samples and matching normal bile duct tissues as determined by an Agilent miRNA microarray. (A) Heatmap of miRNA profiling. A line indicates a microRNA gene, and a column indicates a sample. Red reflects a high expression value, whereas green reflects a low expression value. (B) Validation of differential miR-200b/c/429 expression in 14 cholangiocarcinoma and matching normal bile duct tissues by RT-qPCR. The expression levels of miR-200b, miR-200c, and miR-429 are shown. (C) Collected data from 14 pairs of cholangiocarcinoma patient tissues show the expression of miR-200b/c/429 compared with that in their normal bile duct tissue  $*P<0.05$ . Abbreviations: N = Normal bile duct tissue; T = cholangiocarcinoma (tumour group).

transfected cells. The results showed that the upregulation of miR-200b/c inhibited the distant metastasis of TFK-1 cells, whereas the knockdown of miR-200b/c promoted this metastatic process (Figure 3A). Because of the low resolution of MRI, we could not scan all the liver tumour metastases. Thus, we prepared serial sections of whole liver for haematoxylin and eosin (HE) staining, and observed the sections under a light microscope. According to the results, more micrometastases were detected in the miR-200b/c-knockdown group, and miR-200b/c upregulation reduced the formation of micrometastases compared with the NC groups

(Figure 3A). Interestingly, miR-200c might have more of an influence on distant metastasis than miR-200b. In conclusion, these data show that miR-200b/c is an important participant in the regulation of the migration and invasion potentials of cholangiocarcinoma cells.

**MiR-200b/c expression is associated with 5-fluorouracil chemoresistance in cholangiocarcinoma cells.** To determine whether miR-200b/c expression is also associated with chemoresistance in cholangiocarcinoma, we measured the



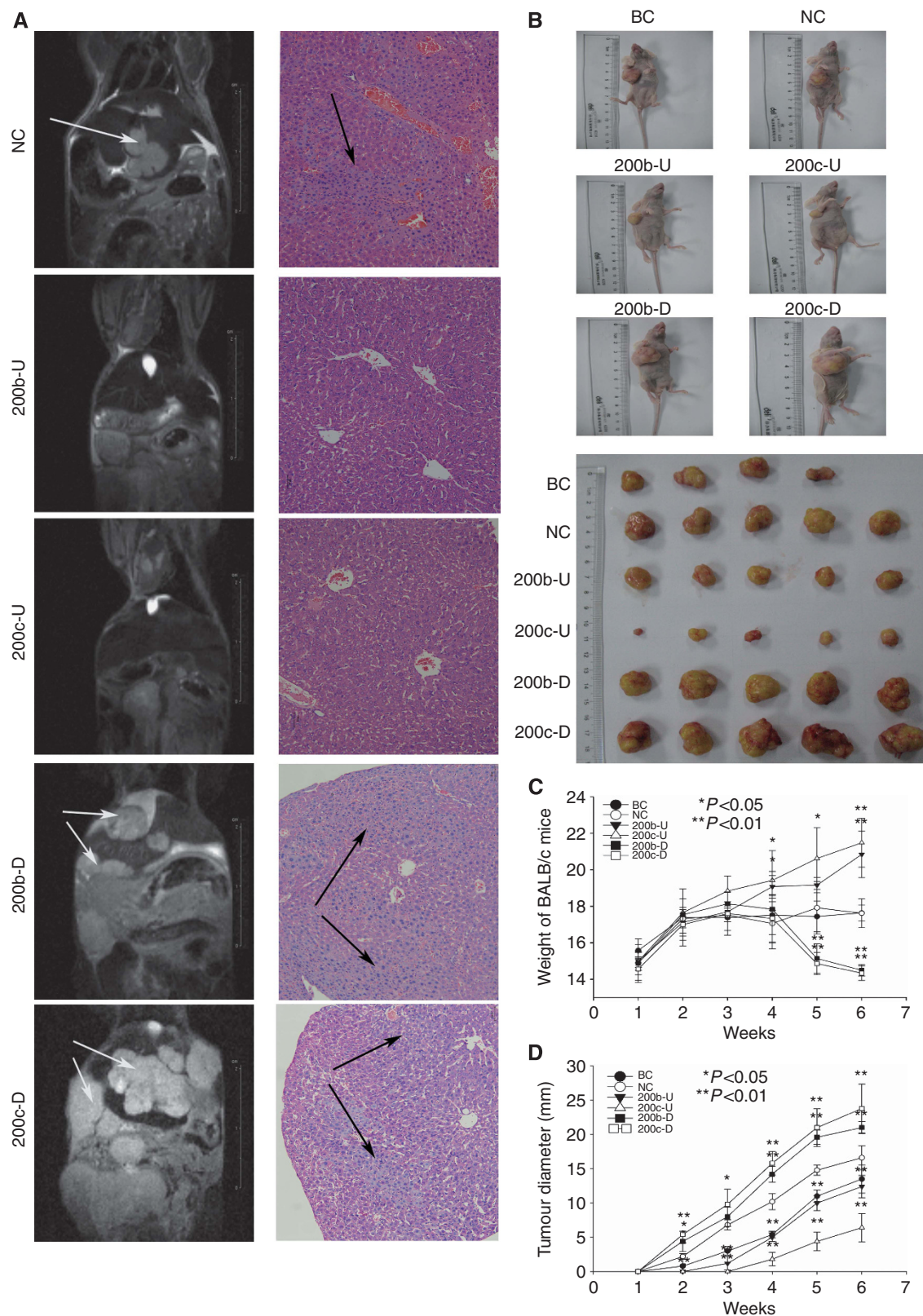


**Figure 2.** Ectopic miR-200b/c expression inhibits the invasion and migration of cholangiocarcinoma cells. The effects of miR-200b/c mimics and an antagomir are shown in (A). (C) Upregulated miR-200b/c expression inhibited the invasion capacity of TFK-1 cells, whereas downregulated miR-200b/c expression promoted the capacity. The number of invasive cells in the wound healing assay is shown in (D). All the data were compared with those from the NC group. MiR-200b/c upregulation inhibited the invasion capacity of QBC939 cells, whereas downregulated miR-200b/c promoted this capacity. The number of invasive cells is shown in (E). Abbreviations: BC = blank control group; NC = negative control group; 200b/c-M = 200b/c-mimics group; 200b/c-A = 200b/c-antagomir group.

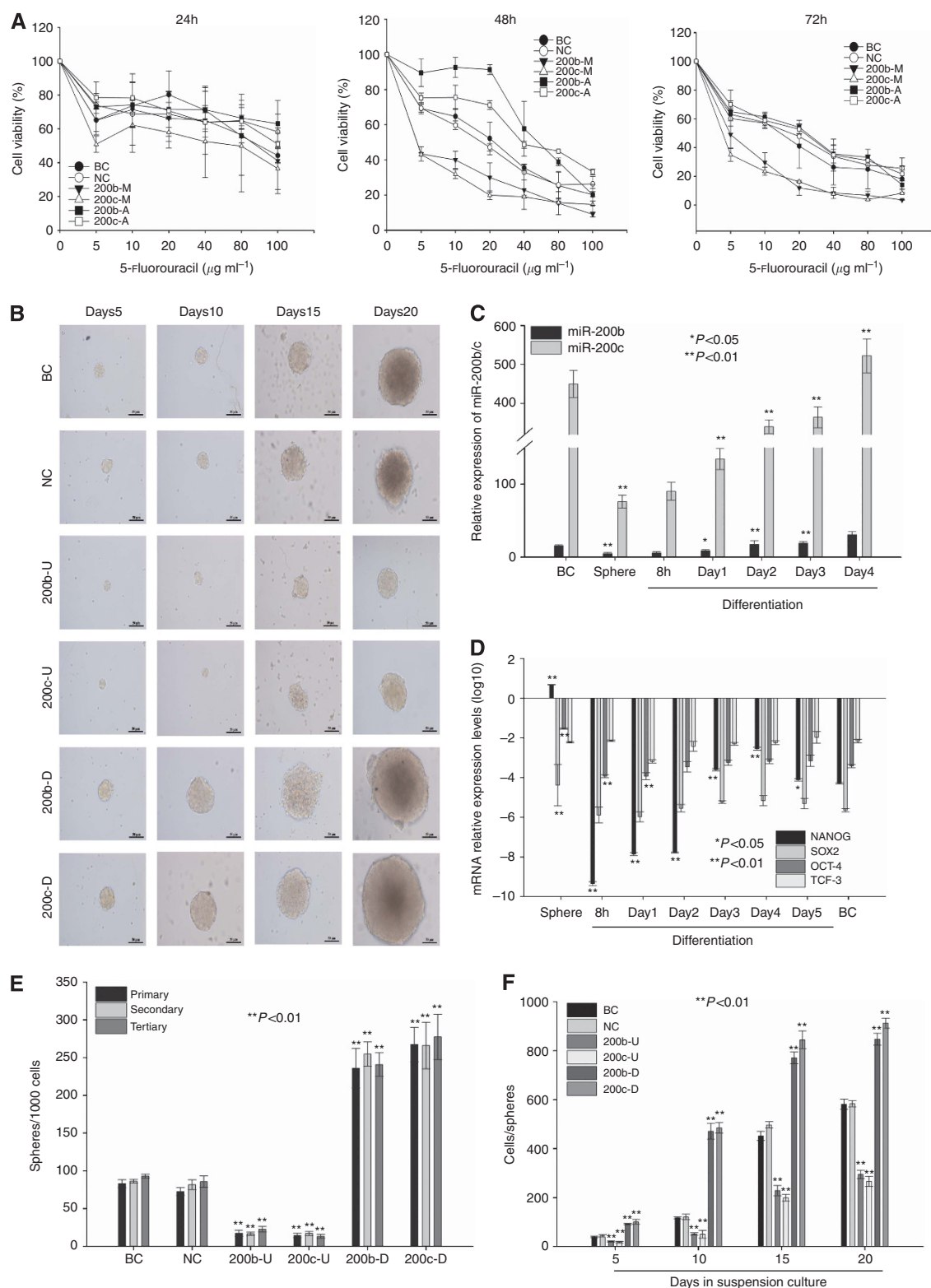
expression of miR-200b/c in TFK-1 cells pretreated with 5-fluorouracil, which is widely used for cholangiocarcinoma treatment. The expression level of miR-200b/c in the 5-fluorouracil-pretreated TFK-1 cells was lower than that in the untreated controls (Supplementary Figure 3C). We then transfected TFK-1 cells as described above and found that pretreatment with miR-200b/c mimics promoted the cytotoxic effects of 5-fluorouracil on TFK-1 cells compared with the 5-fluorouracil + NC (Figure 4A). The IC<sub>50</sub> values for the pre-miR-200b mimics + 5-fluorouracil and pre-miR-200c mimics + 5-fluorouracil groups at various time points are shown in Supplementary Table 3, with the IC<sub>50</sub> value for the miR-200b/c mimics + 5-fluorouracil group being significantly lower than that of the NC group. Conversely, the knockdown of miR-200b/c by an antagomir aggravated the chemoresistance against 5-fluorouracil. As shown in Supplementary Table 3, the IC<sub>50</sub> values for the pre-miR-200b antagomir + 5-fluorouracil and

pre-miR-200c antagomir + 5-fluorouracil groups at various time points were significantly higher than those of the NC + 5-fluorouracil. These data illustrate that miR-200b/c significantly enhances the cytotoxic effect of 5-fluorouracil on cholangiocarcinoma cells.

**Ectopic miR-200b/c expression in cholangiocarcinoma cells influences their self-renewal *in vitro* and tumorigenic potential *in vivo*, and CD133 expression can be influenced by exogenous miR-200b/c expression.** The relationship between miR-200b/c and metastasis/chemoresistance had been demonstrated, and it is well-known that there are multiple steps in tumourigenesis. To demonstrate whether miR-200b/c expression affects other aspects of cholangiocarcinoma tumorigenicity *in vivo* and *in vitro*, we transfected TFK-1 cells with the lentivirus-based expression vector, as described above. To investigate the effects of miR-200b/c on tumour formation, we next subcutaneously injected TFK-1 cells



**Figure 3.** Ectopic miR-200b/c expression inhibits the migration and distant metastasis capacities of TFK-1 cells. **(A)** Lentivirus-transfected TFK-1 cells were used to construct a liver metastasis model of cholangiocarcinoma cancer cells. Images of the MRI scans are shown on the left line. Whole-liver serial sectioning for HE staining of the liver metastasis BALB/c-nude mice model. The micrometastases are shown on the right line. **(B)** Different tumour-initiating capacity of transfected TFK-1 cells. All the BALB/c-nude mice were killed at 6 weeks after injection. The weight data are shown in **(C)**. The tumour diameters are shown in **(D)**. All of the data were compared with those from the NC group. Abbreviations: BC = blank control group; NC = negative control group; 200b/c-U = 200b/c-upregulated lentivirus group; 200b/c-D = 200b/c downregulated lentivirus group.



**Figure 4.** (A) 5-Fluorouracil chemoresistance. The proportion of cell viability is shown at 24, 48, and 72 h. (B) Different transfected TFK-1 cells were used for sphere formation and are shown at various time points (5, 10, 15, and 20 days). The expression of miR-200b and miR-200c (C) was detected during sphere formation and differentiation culture. The expression of NANOG, SOX2, TCF3, and OCT-4 mRNA during sphere formation and differentiation culture is shown in (D). The cell per sphere values (F) indicate the dimensions of the spheres. Sphere per 1000 cells (E) shows the capacity for sphere formation. All the data were compared with the data from the NC group.

transfected with the control, miR-200b/c overexpression, and miR-200b/c knockdown vectors into the right armpit of BALB/c nude mice. Strikingly, even after 3 weeks, injection of  $1 \times 10^5$  miR-200b/c-knockdown cells resulted in large tumours, whereas the miR-

200b/c-overexpressing cells formed smaller tumours in five out of five mice compared with the control groups (Figure 3B,  $P < 0.001$ ). The tumour size was also dramatically altered, as shown in Figure 3D, the miR-200b/c-overexpressing cells formed tumours at



a much slower rate than the NC group. The miR-200b/c-knockdown cells showed the opposite trend ( $P < 0.001$ ). The weight of the mice was also statistically significance from week 4 (Figure 3C,  $P < 0.01$ ). Figure 3B shows the different tumour-initiating capacities of the transfected cholangiocarcinoma cells. All these results confirm that the tumour-initiating capacity of TFK-1 cells is associated with low miR-200b/c expression, in support of our future research objectives.

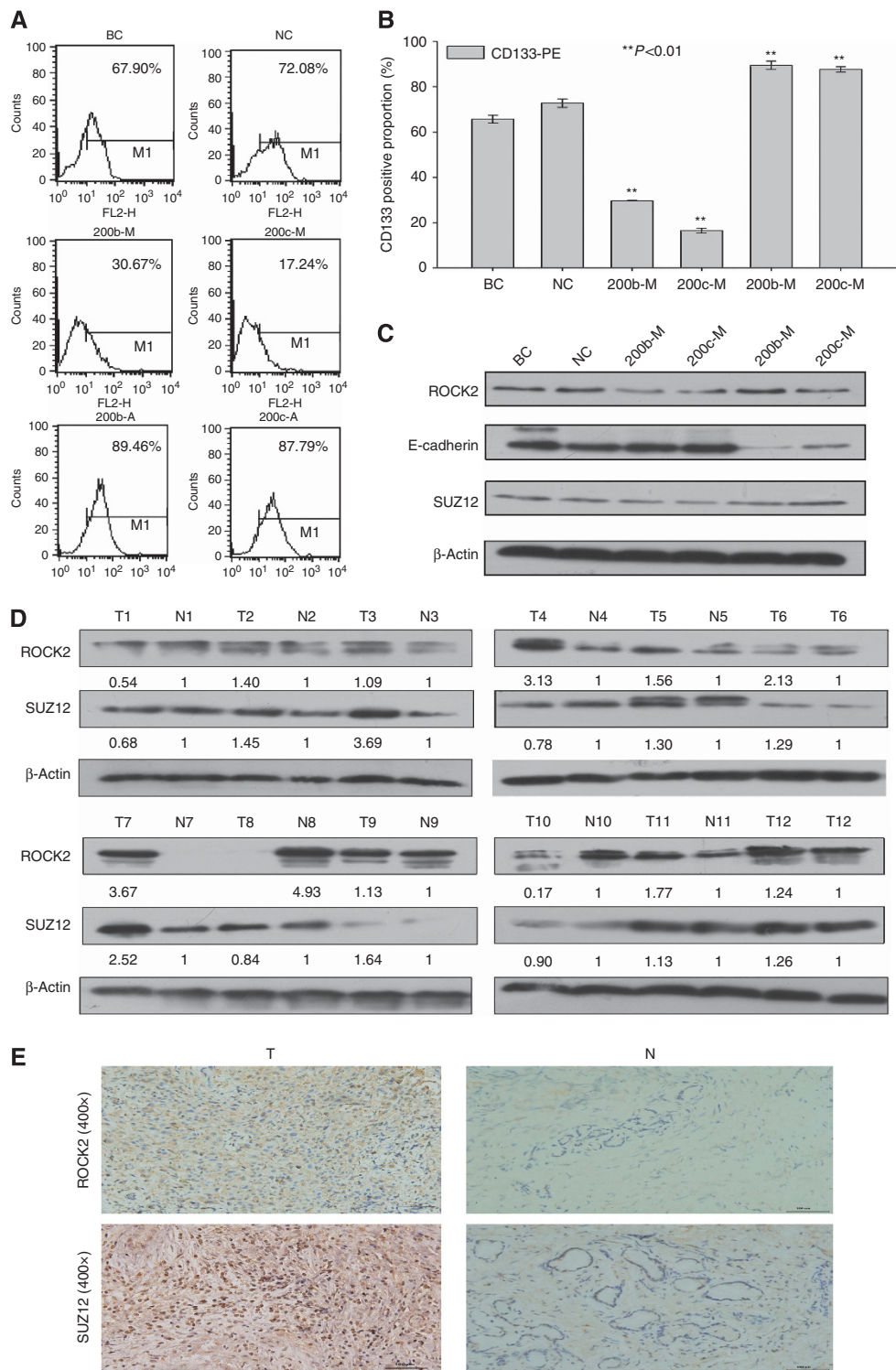
To investigate whether miR-200b/c is important for influencing the tumorigenic properties of cholangiocarcinoma cells, we transfected TFK-1 cells with the lentiviral vectors described above and cultured the transfected cells in suspension under serum-free conditions to generate spheres. The number of spheres reflects the self-renewal capacity of the cells *in vitro*, whereas the number of cells per spheres indicates the self-renewal capacity of each sphere-generating cell (Yu *et al*, 2007). Over three passages, the miR-200b/c-overexpressing cells formed fewer spheres than the cells in the NC group (Figure 4E,  $17.33 \pm 4.04$ ,  $16.67 \pm 2.08$ , and  $22.67 \pm 3.51$  for each passage of miR-200b-overexpressing cells,  $14.33 \pm 3.05$ ,  $17.0 \pm 2.65$ , and  $13.33 \pm 2.52$  for each passage of miR-200c-overexpressing cells, and  $72.67 \pm 5.13$ ,  $81.67 \pm 6.5$ , and  $85.67 \pm 7.5$  for each passage of NC cells,  $P < 0.001$ ). In contrast, the miR-200b/c-knockdown cells formed more spheres than the NC group over three passages (Figure 4E,  $236 \pm 26$ ,  $254.7 \pm 16$ , and  $240 \pm 15.6$  for each passage of miR-200b-knockdown cells, and  $267 \pm 22.7$ ,  $266 \pm 30$ , and  $277.3 \pm 29.96$  for each passage of miR-200c-knockdown cells,  $P < 0.001$ ). As shown in Figures 4B and F, after 20 days of culture, the miR-200b/c-knockdown cells formed larger spheres than the controls cells ( $847 \pm 24.6$  and  $912.67 \pm 20.31$  vs  $583.67 \pm 12.4$ , respectively,  $P < 0.001$ ), whereas the miR-200b/c-overexpressing cells formed smaller spheres than the controls cells ( $294.67 \pm 17.04$  and  $266.33 \pm 20.6$ , respectively,  $P < 0.001$ ). To imitate the differentiation of cancer cells, the dissociated spheres were plated on collagen IV in serum under differentiating conditions. After 6 days under differentiating conditions, the expression levels of differentiation markers *OCT-4*, *NANOG*, and *SOX2* were decreased (Figure 4D), whereas the levels of miR-200b/c decreased after sphere formation and increased during differentiation (Figure 4C). Taken together with the slower growth rate and size of the miR-200b/c-overexpressing spheres and the inability to passage the cells as spheres, these data suggest that miR-200b/c-overexpressing spheres may have undergone the first step toward losing their self-renewal capacity. Conversely, the miR-200b/c-knockdown spheres appeared to have a greater capacity for sphere formation, indicating a higher self-renewal capacity.

Self-renewal capacity is an important characteristic of tumorigenesis and is also a potential stem-like property of cancer cells. Our previous studies showed that CD24, CD44, and CD133 are probable stem cell surface markers of cholangiocarcinoma (Wang *et al*, 2011a, b). As our above results confirmed that miR-200b/c expression might be associated with the self-renewal capacity of cholangiocarcinoma, miR-200b/c may have a potential effect on stem-like cholangiocarcinoma cells. Therefore, we validated these results using flow cytometry by incubating the transfected TFK-1 cells, as described above, with antibodies against CD24, CD44, and CD133. As shown in Figures 5A and B, the proportion of CD133+ cells among the miR-200b/c-overexpressing cells was lower than that in the NC ( $29.72 \pm 0.15\%$ ,  $16.55 \pm 0.11\%$  vs  $72.77 \pm 1.89\%$ ). However, the proportion of CD133+ cells among the miR-200b/c-knockdown cells was high ( $89.47 \pm 1.81$  and  $87.66 \pm 1.19\%$ ). Furthermore, the proportion of CD24+ and CD44+ cells was not related to miR-200b/c expression (Supplementary Figure 6B). Collectively, these data show that CD133 is a potential CSC marker of cholangiocarcinoma (Wang *et al*, 2011a), which can be inhibited by miR-200b/c.

**SUZ12 and ROCK2 are direct targets of miR-200b/c.** According to our results, miR-200b/c affects migration, invasion, chemoresistance, tumour growth, and self-renewal capacities. Previous studies have shown that *SUZ12* is a crucial CSC gene and a direct target of miR-200b in breast cancer cells. *ROCK2* is an important downstream gene of the *Rho/ROCK2* pathway, participating in the regulation of cytoskeletal signalling events and is crucial for cell motility (Supplementary Figures 2A and B). A TargetScan analysis (TargetScan Human 6.0) revealed that *SUZ12* contains a classical and evolutionarily conserved miR-200b/c binding site in its 3'-UTR; *ROCK2* contains one conserved and two poorly conserved miR-200b/c binding sites (Supplementary Figure 5). The ectopic expression of miR-200b/c in TFK-1 cells resulted in a reduction in *SUZ12* and *ROCK2* expression, whereas miR-200b/c inhibition led to increased expression (Figure 5C). However, their mRNA expression levels were unaffected by transfection (Supplementary Figure 7D). To further confirm whether *SUZ12* and *ROCK2* are direct targets of miR-200b/c, we detected the expression of the *SUZ12* and *ROCK2* proteins in 12 cholangiocarcinoma samples and the corresponding normal bile duct tissues using western blotting and immunohistochemistry. The results showed that both *SUZ12* and *ROCK2* were overexpressed in the cholangiocarcinoma tissues: 8 of 12 and 9 of 12 tumour tissues showed increased *SUZ12* and *ROCK2* levels, respectively, compared with the matched normal bile duct tissues (Figure 5D). Immunohistochemistry also showed that the protein expression levels of *SUZ12* and *ROCK2* were higher in the tumour tissues than in the normal bile duct tissues (Figure 5E, Supplementary Figures 3D and E,  $P = 0.018$  and  $< 0.001$ , respectively). Regardless, there was little significant difference in the mRNA expression levels of *SUZ12/ROCK2* between the cholangiocarcinoma and normal bile duct groups (Supplementary Figure 7B), suggesting that miR-200b/c might silence these two target only in a post-transcriptional manner. To further confirm our results, the miR-200b/c binding sites of *SUZ12* and *ROCK2* were each cloned into a luciferase reporter. When the *SUZ12* wild-type sequence, but not a mutant sequence, was transfected into TFK-1 cells, we found that the ectopic expression of miR-200c resulted in a decrease in luciferase activity (*SUZ12* has been reported to be direct target of miR-200b; Iliopoulos *et al*, 2010). Similar data were observed for *ROCK2*, showing that the forced expression of miR-200b/c in TFK-1 cells results in a decrease in luciferase activity using conserved wild-type binding sites compared with a mutant sequence (Figures 6A and B) and also indicating that the conserved predicted binding sites of miR-200b/c in the 3'-UTR of *ROCK2* were functional. In conclusion, the above data demonstrate that *SUZ12* and *ROCK2* are direct target genes of miR-200b/c.

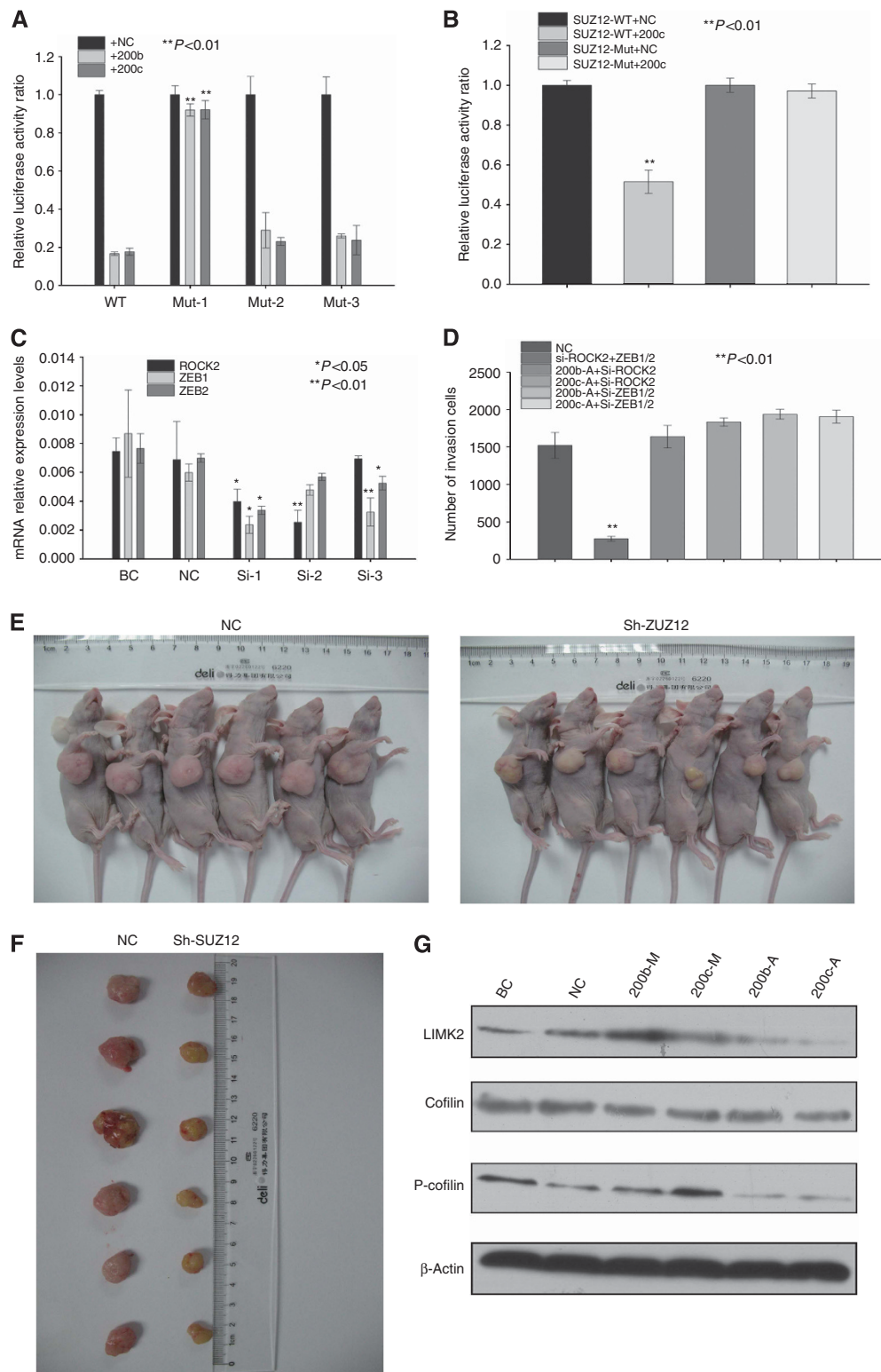
**SUZ12 and ROCK2 are potentially involved in the miR-200b/c-regulated properties of cholangiocarcinoma.** Previous studies have concluded that miR-200-*Zeb1/2-E-cadherin* is correlated with the migration and invasion capacities of cancer cells. To determine whether *SUZ12* and *ROCK2* are related to these capacities, we transfected TFK-1 cells with *SUZ12* and *ROCK2* siRNAs to knockdown their expression and then detected the invasion capacity using a Transwell assay. The knockdown of *SUZ12* did not affect the invasion capacity of TFK-1 cells: the number of invasive cells had no statistical significance compared to that of the control. In contrast, *ROCK2* knockdown reduced the number of invasive cells compared with the controls (Figures 2B and C). Furthermore, we transfected TFK-1 cells with *ROCK2* and *ZEB1/2* siRNAs (Figure 6C), to confirm their involvement in the migration and invasion capacities of cancer cells. However, the silencing of neither *ROCK2* nor *ZEB1/2* in the miR-200b/c-knockdown cells could recover the invasive capacity compared with that of the controls. In addition, the silencing of both





**Figure 5. (A)** Proportion of CD133 + cells among transfected TFK-1 cells. At 48 h after transfection with miR-200b/c mimics/antagomir, the TFK-1 cells were resuspended, and the expression of CD133 was detected on the cell membrane. All the proportions are relative to the blank control and are shown in **(B)**. **(C)** Protein expression of SUZ12, ROCK2, and E-cadherin. β-Actin was used as the control. All the data were compared with the data from the NC group. **(D)** Protein expression of SUZ12 and ROCK2 in 12 cholangiocarcinoma and matching normal bile duct tissues. **(E)** Immunohistochemistry of normal bile duct and cholangiocarcinoma tissues. The left and right images are the cholangiocarcinoma and normal bile duct tissues, respectively.

ROCK2 and ZEB1/2 resulted in the lowest number of invasive cells (Supplementary Figures 6A and D). Collectively, the results showed that the effect of miR-200b/c on the invasion capacities of cholangiocarcinoma cells is mediated by both the Zeb1/2-E-cadherin axis and also the ROCK2 pathway, whereas SUZ12 is not associated with the migration and invasion capacities of cholangiocarcinoma cells. Furthermore, we transfected TFK-1 cells with miR-200b/c mimics/antagomir and detected selected downstream genes of the ROCK2 pathway. As shown in Figures 5C and 6G, the overexpression of miR-200b/c decreased the protein expression level of ROCK2 and then increased the expression level of LIMK2. Increased LIMK2 could phosphorylate



**Figure 6.** Luciferase reporter assays of *SUZ12* and *ROCK2* expression are shown in (A) and (B). The mRNA expression of *ROCK2* and *ZEB1/2* after transfection of siRNA is shown in (C). Effect on the invasion capacity of TFK-1 cells after transfection with si-*ROCK2* + miR-200b/c antagonist, si-*ZEB1/2* + miR-200b/c antagonist and si-*ROCK2* + si-*ZEB1/2*. The number of invasive cells is shown in (D). (E) and (F) Lentivirus-based sh-*SUZ12*-transfected TFK-1 cells were used for tumour initiation. The BALB/c-nude mice were killed at 6 weeks after injection. All the data were compared with the NC group. (G) Downstream gene expression of *ROCK2* after transfection with miR-200b/c mimics and antagonist.

*Cofilin* and thus might reduce the invasion capacity of cholangiocarcinoma cells.

To confirm the mechanism regulating the effect of *SUZ12* on the tumorigenesis properties of cholangiocarcinoma, we transfected TFK-1 cells with a lentivirus-based shRNA vector to silence the *SUZ12* and *ROCK2* genes (Supplementary Figure 6C) and we repeated the sphere formation and tumour-initiating assays. The *SUZ12* shRNA group formed smaller spheres than the NC

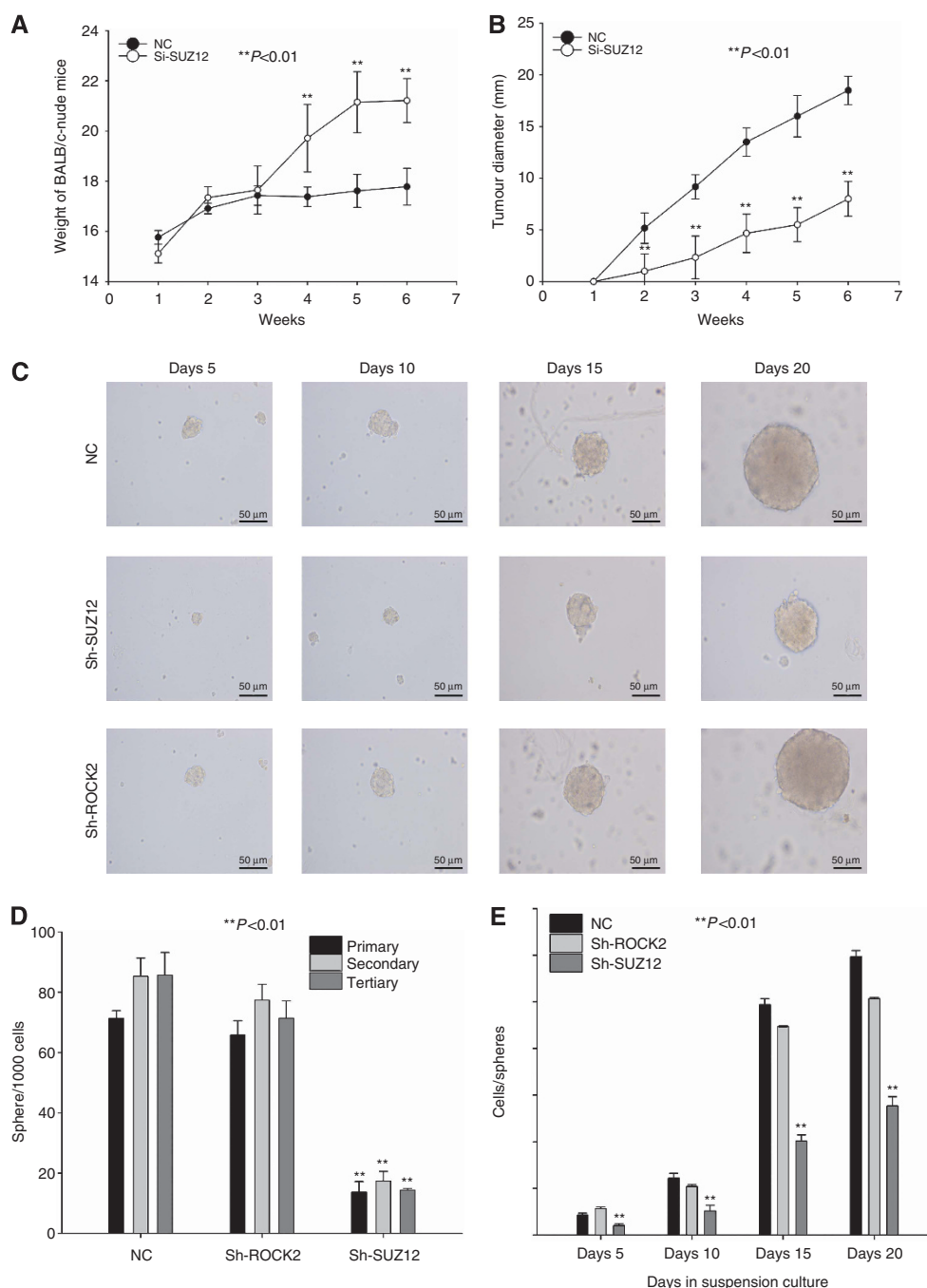


Figure 7. The weight data for the BALB/c-nude mice from the sh-SUZ12 tumour-initiating assay are shown in (A). The data for the tumour diameters are shown in (B). Lentivirus-based si-SUZ12-transfected TFK-1 cells were used for the sphere culture and differentiation assay (C). The cell per sphere values (D) and sphere per 1000 cell (E) values were determined.

(Figure 7C), and the spheres of the control group increased in size and cell number more quickly than those of the *SUZ12* shRNA group (Figure 7D and E). However, the sphere formation capacity of the sh-ROCK2 shRNA group showed no statistical significance compared with the NC group (Figure 7C–E). These changes were also associated with the self-renewal capacity, and the blank vector group formed tumours much more rapidly than the *SUZ12*-shRNA group (Figures 6E, F and 7A, B). However, the chemoresistance capacities of the *SUZ12* shRNA group were not significant in comparison to the NC group. Collectively, the results indicate that *SUZ12* is an important gene that is associated with some cholangiocarcinoma properties, such as sphere formation and tumour initiation, and is regulated by miR-200b/c.

## DISCUSSION

The initial studies on cholangiocarcinoma focused on its multi-chemoresistance and distant metastasis. To investigate the possible mechanism of the malignant biological behaviour of cholangiocarcinoma and to identify crucial regulators, we established that there was a dysregulated miRNA profile in cholangiocarcinoma tissues and found four miR-200 family members among the top six down-regulated miRNAs. Although the miR-200 family members have been demonstrated to be cancer suppressors in many forms of cancer, the function of miR-200 in cholangiocarcinoma cells has not been elucidated to date. Here, we investigated the dysregulation



of miR-200b/c as a possible cause of the invasion and metastasis capacities and tumorigenic properties of cholangiocarcinoma, and we clarify a novel downstream mechanism. Overall, our results suggest a novel therapeutic strategy for cholangiocarcinoma treatment.

In comparison to normal controls, miR-200 family members are downregulated in many human cancers, including breast cancer (Teng *et al*, 2013), pancreatic tumours (Li *et al*, 2010), and hepatic cancer (Kim *et al*, 2011; Zhang *et al*, 2013). Most researchers have demonstrated that the expression of miR-200 is negatively correlated with the invasion and metastasis capacities of cancer (Hur *et al*, 2012; Soubani *et al*, 2012; Guo *et al*, 2013). Although the existence of the miR-200-*ZEB1/2-E-cadherin* axis during the EMT process was confirmed in initial studies (Teng *et al*, 2013), several recent studies have reported conflicting roles of miR-200 in metastatic progression. These contradictory findings may in part be due to the different source organisation and models (Korpál *et al*, 2011); indeed, there are different expression levels and mechanisms in different tissues. Thus, in our study, we demonstrated that miR-200b/c is downregulated in cholangiocarcinoma and that the knockdown of miR-200b/c promotes the invasion and metastasis capacities of cholangiocarcinoma cells. Similarly, we demonstrated that inhibiting the expression of *ROCK2* reduces the invasion of cholangiocarcinoma cells (although the inhibition of *SUZ12* had no effect). Both the overexpression of miR-200b/c and inhibition of *ROCK2* can reduce the invasion of cholangiocarcinoma cells, yet *ROCK2*-knockdown cholangiocarcinoma cells may retain a stronger invasion capacity. The *ROCK2*-knockdown cholangiocarcinoma cells exhibited enhanced invasion and metastasis capacities in comparison to the miR-200b/c-overexpression cells, indicating that the influence of miR-200 on cholangiocarcinoma invasion extends beyond the regulation of *ROCK2* and most likely involves other targets. We found that the combined suppression of *ZEB1/2* and *ROCK2* resulted in a similar inhibition effect as that of miR-200b/c overexpression. Therefore, both *ZEB1/2* and *ROCK2* are crucial regulatory proteins of the invasion capacity of cholangiocarcinoma cells and are silenced by miR-200b/c.

In addition to the invasion and metastasis capacities, many researchers have demonstrated that the expression of miR-200 is associated with the stemness of cancer cells. Indeed, our experiments examining sphere formation, drug resistance, and tumour-initiating capacity addressed CSC-like properties, suggesting a possible link between miR-200b/c and cholangiocarcinoma stem-like cells. Previous studies have shown that stemness-related genes such as those of the *ZEB* family, which have been demonstrated to be direct targets of miR-200, consist of transcriptional repressors, including *ZEB1* and *ZEB2*. The functions of this protein family are extensive and include development, the activation of the transforming growth factor (TGF)- $\beta$  signalling pathway in EMT, and stem cell pathways (Vandewalle *et al*, 2009; Sanchez-Tillo *et al*, 2011; Gheldof *et al*, 2012). According to the miR-200-*ZEB* dual loop theory, not only does the miR-200 family directly target *ZEB1/2* but the overexpression of *ZEB1/2* also reduces miR-200 levels. Based on the above findings, there may be two pathways by which the miR-200 family influences the stemness of cancer: (1) the suppression of *ZEB1/2*, thereby influencing the stemness of CSCs via downstream proteins, or (2) the direct targeting of certain stemness-related proteins, thereby directly influencing CSC properties. There are many studies supporting the hypotheses of pathway 1, but less experimental evidence supports pathway 2. Remarkably, our results show that the protein expression level of *ZEB1* in TFK-1 cells is deficient, suggesting that the proportion of *ZEB* family related CSC-like cells in the TFK-1 cell line may be very low. Our integrated analysis of miR-200 and stemness shows that miR-200 regulates a very large number of target mRNA sequences. Thus, it appears likely that the

effect of miR-200 on cholangiocarcinoma tumorigenic or stem cell-like properties is not mediated solely through the miR-200-*ZEB* axis and that there are some target mRNAs directly regulated by miR-200. The miR-200 family members may influence the stemness of cancer via direct suppression. Our results establish that miR-200b/c influences the drug resistance, tumour-initiating capacity, sphere formation, and CD133 expression level, a potential CSC marker, of cholangiocarcinoma cells. *SUZ12* has been reported to be a direct target of miR-200b in breast CSCs, and the direct targeting of *SUZ12* by miR-200c was demonstrated in our study. The direct knockdown of *SUZ12* by shRNA achieved a similar suppressive effect on the sphere formation and tumour-initiating capacities of cholangiocarcinoma as that of miR-200b/c. This result confirms that *SUZ12* is important in tumourigenesis and that related genes may be directly regulated by miR-200b/c. Based on the enrichment of low-differentiation cholangiocarcinoma cells by sphere formation and drug resistance, we considered that the proportion of cholangiocarcinoma stem-like cells had been increased, and we next validated the miR-200b/c expression levels of the parental and differentiated spheres and drug-resistant cells. Interestingly, the expression of miR-200b/c was decreased during low-differentiation cell enrichment and increased upon differentiation, indicating that there may be negative feedback between miR-200b/c and cholangiocarcinoma stem-like cells.

In conclusion, our findings demonstrate that miR-200b/c inhibits the invasion and metastasis capacities, and tumorigenic capacity (or the so-called CSC-like properties), including drug resistance, tumour-initiating capacity, sphere formation, and CD133 expression level, of cholangiocarcinoma cells. Such a suppression of these properties can be imitated by directly repressing *ROCK2* and *SUZ12*, two direct targets of miR-200b/c. Thus, our observations provide very strong experimental results regarding the involvement of miR-200 in the invasion and metastasis capacities of cholangiocarcinoma, suggesting a novel therapeutic strategy for cholangiocarcinoma treatment and proposing a potential surface marker for cholangiocarcinoma stem-like cells.

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