MiR-19b/20a/92a regulates the self-renewal and proliferation of gastric cancer stem cells

Running title: miR-19b/20a/92a regulate self-renewal

Qiong Wu¹,³, Zhiping Yang¹,³, Fang Wang², Sijun Hu¹, Li Yang², Yongquan Shi¹*, Daiming Fan¹*

¹ State Key Laboratory of Cancer Biology and Xijing Hospital of Digestive Diseases, Fourth Military Medical University, Xi’an, Shaanxi, 710032, China
² Ningxia Medical University Affiliated Hospital, Yinchuan, Ningxia, 750004, China.
³ These authors contributed equally to this work.

*Correspondence

State Key Laboratory of Cancer Biology and Xijing Hospital of Digestive Diseases, Fourth Military Medical University, Xi’an 710032, China
Tel: +86 29 84771501
Fax: +86 29 82539041
E-mail: daimingfan@fmmu.edu.cn (Daiming Fan), shiyquan@fmmu.edu.cn (Yongquan Shi)

Key words: miR-17-92, Wnt/β-catenin, gastric cancer, cancer stem cell, self-renewal
**Abbreviations:**
miRNA: microRNA  
CSC: cancer stem cell  
GCSC: gastric cancer stem cell  
TIC: tumor initiating cell  
SP: side population  
Luc: luciferase labeled  
NOD-SCID mice: severe combined immunodeficient mice  
H&E: hematoxylin and eosin  
Pre-miR: precursor microRNAs

**Summary**

Human gastric cancers contain a population of gastric cancer stem cells (GCSCs) that can undergo self-renewal and multipotent differentiation. GCSCs can be enriched by EpCAM+/CD44+ gastric cancer cells. However, the mechanisms underlying how GCSCs balance self-renewal and differentiation remain to be explored. Because miRNAs can regulate cancer cell fates, we compared tumorspheric cancer cells enriched for GCSCs with more differentiated cells in terms of miRNA expression. We found that the miR-17-92 cluster members miR-19b, miR-20a and miR-92a were gradually reduced during the differentiation of GCSCs. Herein, we speculated that miR-17-92 members might function as regulators to sustain the self-renewal ability of GCSCs. By down-regulating miR-19b, miR-20a and miR-92a in EpCAM+/CD44+ GCSCs, or over-expressing them in EpCAM-/CD44- non-GCSC populations, we found that miR-19b, miR-20a and miR-92a could sustain the self-renewal function of GCSCs. Furthermore, we found that miR-19b, miR-20a and miR-92a could also promote the proliferation of gastric cancer cells. Moreover, miR-17-92 targeted the E2F1 and HIPK1 proteins, which suppressed Wnt-β-catenin signaling. A real-time PCR analysis of miR-19b, miR-20a and miR-92a expression in 97 gastric cancer specimens suggested that miR-92a could be used as an independent prognostic factor.
in gastric cancer. This study indicated that several members of the miR-17-92 cluster, miR-19b, miR-20a and miR-92a, might play significant roles in the development of gastric cancer stem cells and that miR-92a has the potential to be used as a predictive prognostic marker in gastric cancer.

Introduction

In the past several years, a growing body of evidence has shown that cancers are organized in a population of heterogeneous cells with different biological properties that help sustain tumor formation and development. A small proportion of cells that are self-renewing and have the ability to differentiate into multiple lineages are termed cancer stem cells (CSCs) or tumor-initiating cells (TICs) (Dalerba et al., 2007). CSCs have been identified in many cancers (Al-Hajj et al., 2003; Singh et al., 2004). They are not only the source of tumors, but have also been associated with tumor aggressiveness and metastasis (Wicha, 2006). Furthermore, CSCs mediate chemoresistance and subsequent tumor recurrence (Al-Hajj, 2007). CSCs can be enriched by sorting for stemness markers (Han et al., 2011) or by selecting for side-population (SP) cells exhibiting Hoechst dyes efflux (Fukuda et al., 2009). A third method for CSC accumulation is isolating spherical populations of tumorspheres from suspension cultures. For example, sphere-forming cells can be cultured from human glioblastomas using conditions that enrich for neural stem cells and display increasing tumorigenicity and resistance to radiation treatment (Bao et al., 2006). Breast CSCs can also be isolated via spherical culture based on stem cell properties (Ponti et al., 2005).

miRNAs are small, non-coding RNAs that can regulate target genes post-transcriptionally through complementary binding to their target mRNAs (Bartel, 2009). In cancers, miRNAs can function either as oncogenes or tumor-suppressors to regulate carcinogenesis and cancer development (Croce, 2009), while in stem cells,
miRNAs can either promote self-renewal or promote differentiation to determine stem cell fates (Shimono et al., 2009). In cancer stem cells, miRNAs can exert the functions they present in both cancers and stem cells. One of the best-studied miRNAs, let-7, can act as a tumor-suppressor in breast cancer and can impede the self-renewal of breast tumor-initiating cells (Yu et al., 2007). miR-200c not only inhibits the clonal expansion of breast cancer cells, but also strongly suppresses tumor formation driven by human breast cancer stem cells (Shimono et al., 2009).

The existence of CSCs in most organ systems has been demonstrated, and some molecular links between CSCs and cancers have been putatively established. However, it is still not clearly known how CSC activities such as self-renewal are controlled by different genetic molecular factors. In the present study, we used gastric cancer as a research model to explore the roles that miRNAs play in the self-renewal of CSCs and the potential associated mechanisms.

Results

Gastric cancer cells separated by EpCAM+/CD44+ are enriched for GCSCs

Gastric cancer stem cells (GCSCs) were identified using the cell surface markers, EpCAM+ and CD44+, from primary tissues (Han et al., 2011). To examine whether EpCAM+/CD44+ cells from gastric cancer cell lines might also enrich for GCSCs, we compared the tumorspheric ability of two gastric cancer cell lines that exhibit great differences in the levels of EpCAM and CD44 expression. In the gastric cancer cell line, SGC7901, 80% of the cells expressed EpCAM and CD44. Additionally, the SGC7901 cells formed tumorspheres more often than MKN28 cells, which contained only 0.2% EpCAM+/CD44+ cells (Figure 1A and B). Because tumorspheric generation represents an in vitro assay for determining self-renewal potential and a method for culturing CSCs, we isolated EpCAM+/CD44+ cells and non-EpCAM+/CD44+ cells via FACS and cultured them in suspension to generate tumorspheres. After 15 days in culture, 85-95% of the EpCAM+/CD44+ cells formed tumorspheres, compared with 15-20% tumorsphere formation by
non-EpCAM+/CD44+ cells (data not shown). Furthermore, the spheres formed by the EpCAM+/CD44+ cells contained more cells than the spheres formed by the non-EpCAM+/CD44+ cells (Supplementary Figure 1 A and B). In BALB/C nude mice, $10^4$ EpCAM+/CD44+ cells generated tumors that showed a more than two-fold stronger luciferase signal compared with tumors generated by $10^5$ non-EpCAM+/CD44+ cells, as detected on days 15, 21 and 28 using the IVIS 100 Imaging System (Supplementary Figure 1 C and D). These data suggested that EpCAM and CD44 are also GCSC markers in cultured gastric cancer cell lines and can be used as an in vitro model for the exploration of GCSCs.

An important property of CSCs is multipotency. We took advantage of the above findings to determine whether GCSCs would differentiate when cultured in serum-containing medium. As expected, the cells proliferated more rapidly when attached to flasks than tumorspheric cells via the label of CFSE after 8 days’ culture (Figure 1C). Furthermore, the tumorspheric cells expressed lower levels of the myoepithelial marker, CK14, and the luminal epithelial marker, CK18. However, after further attachment to the flasks and differentiation in serum-containing medium, these cells developed into elongated cells with a higher percentage expressing either CK14 or CK18 based on real-time PCR analysis (Figure 1D and E) and immunofluorescence (Supplementary Figure 1E). The potent differentiation capability of tumorspheric EpCAM+/CD44+ cells after being cultured and adhered in serum-containing medium indicated the multipotency of the GCSCs and suggested that there must be specific mechanisms that regulate this process.

**miR-19b/20a/92a are reduced during GCSC differentiation**

Because miRNAs can regulate the self-renewal and differentiation of stem cells, we exploited our ability to obtain a large number of self-renewing tumorspheric cells to compare miRNA expression in these cells with cells that were further differentiated. Among the cell lines showing increased EpCAM+/CD44+ expression, tumorspheric cells that were freshly dissociated (Figure 2A Lane1) or had only briefly attached...
(Figure 2A, 8h, Lane2) expressed higher levels of some miRNAs compared with cells that were differentiated under serum-containing conditions (Figure 2A, 12h, Lane3; 1 day, Lane4; 8 days, Lane5). We considered the miRNAs that gradually decreased during the attachment of the tumorspheric cells to be miRNAs that might control the self-renewal of GCSCs. Based on ANOVA analysis on normalized chip data, we identified a number of miRNAs whose expression was significantly different in tumorspheric cells compared with differentiated cells. Among these miRNAs, miR-19b, miR-20a and miR-92a emerged as the most significant miRNAs that were gradually reduced during the attachment of the tumorspheric GCSCs (Figure 2B). A number of other miRNAs, such as miR-106a and miR-30d, showed similar expression patterns as miR-19b, miR-20a and miR-92a (Figure 2A).

To confirm the decreased expression of miR-17-92 miRNAs during the attachment of tumorspheric cells, we performed real-time PCR using miRNA-specific primers. miR-19b, miR-20a and miR-92a were detected at higher levels in tumorspheric cells; their expression levels were briefly reduced after 8 h of attachment and began to decrease within 1 day of attachment, then decreased further over 8 days of differentiation (Figure 2C). The over-expression of two other miRNAs related to tumors, miR-106a and miR-30d, was confirmed using miRNA-specific primers (Supplementary Figure 2).

To test whether GCSCs from human gastric cancer tissues also have a higher expression of miR-19b/20a/92a, cells were isolated from human gastric cancer tissues and cultured in non-serum conditions in low attached 96-well plates. After three weeks of culture, GCSCs formed tumorspheres as shown in supplementary figure 6A. The total RNA of GCSCs and non-GCSCs was harvested and miR-19b/20a/92a expression was detected. The expression of miR-19b/20a/92a was also higher in tissue GCSCs than non-GCSCs as indicated in supplementary figure 6B.

**Over-expression of miR-19b/20a/92a is required to maintain tumorspheres in**
To investigate whether miR-19b/20a/92a could sustain the self-renewal of GCSCs, we stably infected EpCAM-/CD44- (separated from SGC7901 cells) and MKN28 cells with lenti-viruses containing pre-miR-19b, pre-miR-20a and pre-miR-92a. The successful over-expression of mature miRNAs was confirmed via real-time PCR (Supplementary Figure 3A and B). The over-expression of the miRNAs lenti-miR-19b, lenti-miR-20a and lenti-miR-92a resulted in markedly greater self-renewal ability in gastric cancer cells, as indicated by the high intensity of tumorspheric cells compared with lenti-NC-infected cells (Figure 3A, Supplementary Figure 3C). Furthermore, the cell numbers within each sphere of lenti-miR-19b, lenti-miR-20a and lenti-miR-92a-infected cells were much higher than observed for the NC-infected cells (Figure 3B). To avoid experimental artifacts related to cellular changes, miRNA precursors were transiently transfected into EpCAM-/CD44- and MKN28 cells; transcription was confirmed using specific miR-17-92 primers by real-time PCR (Supplementary Figure 3D, E, F and G). Consistently to stable cell lines, the transient over-expression of the miR-19b, miR-20a and miR-92a precursors significantly increased the tumorsphere numbers of EpCAM-/CD44- and MKN28 gastric cancer cells (Supplementary Figure 3H and I). To detect the effects from knocking down miR-19b/20a/92a, we transient transfected miRNA inhibitors to EpCAM+/CD44+ cells or SGC7901 cells. After testing the tumorspheric abilities of these cells, we found miR-19b/20a/92a inhibitors markedly impeded and delayed the formation of tumorspheres in EpCAM+/CD44+ and SGC7901 cells (Figure 3 C and D). Our results implied that the miRNAs of the miR-17-92 cluster regulated the self-renewal ability of GCSCs under non-adherent conditions by promoting the number of tumorspheres among gastric cancer cells and the number of cells in each sphere.

To test whether these miRNAs can work synergistic or can counteract with each other, we did co-transfection of pre-miR-92a with pre-miR-20a and pre-miR-19b separately in MKN28 cells. Compared with pre-miR-92a transfection alone, the co-transfection of miR-92a with miR-19b had a higher tumorsphere percentage.
(Supplementary Figure 7) which indicated that miR-92a and miR-19b might work synergistic in this cluster.

It has been reported that CSCs are resistant to chemotherapy, which leads to multidrug resistance and the subsequent recurrence of cancer. In the present study, using MTT assay, we found that miR-17-92-infected tumorspheric cells were more resistant to the chemotherapeutic drug 5-FU than negative control-infected cells (Figure 3E). Our results indicated that miR-19b/20a/92a not only sustains the self-renewal of GCSCs by increasing the number of tumorspheric cells under non-adherent conditions but also maintains the stemness of GCSCs by inducing resistance to chemotherapeutic drugs.

Because GCSCs are cells presenting the cell surface markers EpCAM+/CD44+, we performed FACS to determine whether pre-miR-19b/20a/92a-transfected cells exhibit more EpCAM+/CD44+ GCSCs than the pre-NC cells. We found that the transient transfection of pre-miR-19b, pre-miR-20a and pre-miR-92a into SGC7901 and MKN28 gastric cancer cells could markedly increased EpCAM+/CD44+ cell percentage (Supplementary Figure 4A, while the inhibitors of miR-19b, miR-20a and miR-92a decreased the percentage of EpCAM+/CD44+ cells in SGC7901 cells (Supplementary Figure 4B).

**Over-expression of miR-19b/20a/92a up-regulates the self-renewal of GCSCs in NOD-SCID mice**

We next assessed the effects of induced miR-17-92 expression on self-renewal in vivo. To facilitate detection, the luciferase-labeled SGC7901-Luc cell line established in our laboratory (SGC7901-luc) was used to create stable transfectants expressing miR-17-92 miRNAs and negative controls. The positive expression of mature miR-17-92 was confirmed using real-time PCR (Supplementary Figure 4C). Each mouse was inoculated with 2 X 10^3 tumorspheric miR-19b/20a/92a-infected cells or negative control (NC)-infected cells. All of the mice injected with
lenti-miR-19b/20a/92a-infected cells formed tumors, while only one mouse (p<0.001) formed tumors from lenti-NC-infected cells (Table 1). The miR-19b/20a/92a-infected cells formed tumors that exhibited stronger luciferase activity under the IVIS system on the 28th day after injection compared with the NC group cells (Figure 4A and B). Moreover, the tumors that expressed lenti-miR-19b/20a/92a grew to at least 3 cm in diameter only 28 days after becoming palpable, while the lenti-NC-expressing cells did not generate tumors more than 2 cm in diameter after 49 days, which was the end of the experimental period (Supplementary Figure 4D). Furthermore, mice injected subcutaneously with miR-19b/20a/92a-infected cells presented liver and lung metastasis (Table 1), as detected by H&E stain (Figure 4C), which is consistent with the hypothesis that CSCs are prone to metastasis. The histological analyses of subcutaneous tumors were confirmed using H&E (Figure 4D) to verify that the miR-17-92 miRNAs sustained the self-renewal ability of GCSCs in vivo. Because the lenti-virus contains an EGFP expressing vector, the lenti-miR stable cell lines should express EGFP. We thus did EGFP staining using immune-histochemistry on livers, lungs and tumors of NOD-SCID mice as indicated in figures 4C and D.

**miR-19b/20a/92a miRNAs promote tumor growth in vitro and in vivo**

SC-regulatory genes generally also have effects on the proliferation of cells. Therefore, we next defined the roles of miR-19b/20a/92a miRNAs in the regulation of gastric cancer cell growth and progression. Using MTT assay and colony formation assay, we found lenti-miR-19b/20a/92a stable expressing gastric cancer cell lines proliferated faster compared with lenti-NC expressing cells (Figure 5A left, B and C). To test the knock down function of these miRNAs, we transiently transfected miR-19b/20a/92a inhibitors into gastric cancer cell lines SGC7901 and MKN28 and tested them using proliferation assays. Conversely, we found miR-19b/20a/92a inhibitors made cancer cells proliferated slower than negative control transfection using MTT assay (Figure 5A right and Supplementary figure 5B) and colony formation assay (Supplementary figure 5C lower and D right). To avoid the cellular changes by experimental artifacts, we also did sense transient transfection using
miRNA precursors and tested the cell proliferative functions. Consistently to stable expressing cells, the transient transfection of miR-19b/20a/92a promoted proliferation in both MTT assay and colony formation assay as indicated in Supplementary figure 5A, upper C and left D.

To validate the results of the cell proliferation assays in vitro, we performed in vivo assays to evaluate the tumorigenetic effects of the miR-17-92 miRNAs in BALB/C nude mice using a luciferase-labeled lenti-miR-17-92-infected SGC7901-Luc cell line. The lenti-miR-19b/20a/92a-infected cells showed a proliferative tendency in nude mice, and all of them significantly promoted tumor growth in nude mice compared with the lenti-NC group (Figure 5D and E). The histologic analyses of the tumors were confirmed using H&E and EGFP staining to verify that the miR-17-92 miRNAs promoted tumor growth in vivo (Figure 5F).

**The miR-17-92 cluster members targets E2F1 and HIPK1 directly at the post-transcriptional level and activates the β-catenin signaling pathway**

In silico analysis using MiRanda software (http://www.microrna.org/microrna/home.do) showed that the 3’-UTR of human E2F1 contains two conserved putative target sites for miR-20a, and the 3’UTR of human HIPK1 contains one conserved site for each of miR-19b and miR-92a (Figure 6A). E2F1 was previously reported to be a target of miR-20a and can inversely induce the expression of the miR-17-92 cluster. To further validate these target sites, the 3’-UTRs of human E2F1 and HIPK1 were inserted in both orientations downstream of the luciferase gene in the pGL3-Control vector, providing sense (Luc-1S, 2S and S) and antisense (Luc-1AnS, 2AnS and AS) constructs, collectively referred to as Luc-1, Luc-2, Luc-3 and Luc-4 in Figure 6. Transfecting HEK-293 cells with miR-20a significantly decreased the expression of Luc-1S, while displaying no effect on Luc-1AnS expression (Figure 6B). In contrast, miR-150 did not exhibit any effect on the expression of Luc-1S (Figure 6B), in accordance with the fact that the E2F1 3’-UTR contains no miR-150 target sites. The expression of Luc-2S was reduced by
either miR-19b or miR-92a, but not by miR-150, as a result of the lack of common target sites (Figure 6C). Moreover, miR-19b could target Luc-3S, and miR-92a could target Luc-4S (Figure 6D).

To evaluate the down-regulating effect of the miR-17-92 cluster on E2F1 and HIPK1, we performed western blot analysis using anti-E2F1 and anti-HIPK1 antibodies. As would be expected, transfecting SGC7901 cells with pre-miR-20a decreased E2F1 levels (Figure 6E), whereas transfecting cells with miR-20a inhibitory RNAs had the opposite effect. The cells infected with pre-miR-19b and pre-miR-92a showed reduced HIPK1 levels compared with NC-infected cells, which suggests that the miR-17-92 miRNAs regulate E2F1 and HIPK1 expression in vivo at the post-transcriptional level.

It was previously reported that E2F1 can trans-activate CTNNBIP1 (β-catenin-interacting protein 1), also known as ICAT (inhibitor of β-catenin and TCF4), which is required to inhibit β-catenin activity. E2F1 can also induce axin2, which could cause β-catenin degradation. Furthermore, HIPK1, whose expression is frequently altered in cancers, represses Wnt/β-catenin target gene activation. To determine whether the β-catenin pathway was activated by enforced miR-17-92 expression, we performed a western-blotting assay using anti-β-catenin antibodies in miR-19b/20a/92a-transfected cells and GCSCs from human GC tissues. The nucleoprotein of miR-19b/20a/92a-transfected cells and GCSCs was used for the detection. We observed an up-regulation of β-catenin proteins in miR-19b/20a/92a-transfected cells (Figure 6F) and GCSCs from human GC tissues (Supplementary Figure 6C).
underwent gastrectomy and were followed up at Xijing Hospital during the years 2006 to 2008. Furthermore, we ranked the patients on the basis of the relative expression levels obtained via of real-time PCR and divided them into a high-expression group and low-expression group using the 50th percentile (median) as the cut-off point. HR from the univariate Cox regression analysis showed that the tumor stage and the expression of miR-20a and miR-92a were correlated with death from any cause (Figure 7, Supplementary Table). A multivariate Cox regression analysis identified the level of miR-92a as an independent factor that was associated with overall survival (Supplementary Table).

**Discussion**

GCSCs were recently identified using the cell surface markers CD44 and EpCAM by Han et al. and Takaishi et al. (Takaishi et al., 2009; Han et al., 2011). However, the small number of CSCs increases the difficulty of studying how they sustain self-renewal and initiate cancers without differentiating. Through the application of miRNA arrays, we compared tumorspherical GCSCs with more differentiated gastric cancer cells based on CSC properties. We found that the expression of the miR-17-92 cluster, especially of miR-19b/20a/92a, was reduced gradually during GCSC differentiation. These results indicated that miR-19b/20a/92a might have the ability to sustain the self-renewal of GCSCs.

The miRNA miR-17-92 cluster was first identified in the year 2005 as a potential human oncogene by He L et al., and it was demonstrated that tumors derived from hematopoietic stem cells expressed a subset of the mir-17-92 cluster (He et al., 2005). miR-17-92 members have been identified as being over-expressed in numerous types of stem cells, such as hematopoietic stem cells (Jin et al., 2008), embryonal carcinoma SCs (Gallagher et al., 2009), mouse embryonic SCs (Gunaratne, 2009) and neuronal SCs (Chen et al., 2010), and are down-regulated during neuronal lineage differentiation in unrestricted somatic stem cells (Iwaniuk et al., 2011) and in unrestricted somatic stem cells differentiated into neuronal lineages (Trompeter et al., 2011).
2011). Additionally, miR-17-92 was shown to be highly induced during early reprogramming stages in induced pluripotent stem cells (iPSCs) (Li et al., 2011). However, the exact roles of the miR-17-92 cluster in stem cells and CSCs have not been fully elucidated. In the present study, we demonstrated the ability of the miR-17-92 cluster to regulate self-renewal of GCSCs using in vitro and in vivo assays.

The earliest studies addressing miR-17-92 and stemness maintenance in stem cells were associated with the whole gene function of the miR-17-92 cluster, prior to the year 2009. There were two studies that reported the function of single miRNAs in this cluster. Mu et al. (Mu et al., 2009) used the whole cluster as a positive control and deleted every member of this cluster and found that miR-19 acted as the most important miRNA in the cluster in B cell lymphoma. At the same time, through the over-expression of single miRNAs of the miR-17-92 cluster, Olive et al. (Olive et al., 2009) also demonstrated that miR-19 is the most functional member of the whole cluster. In the present study, we used gastric cancer as a research model and discovered that in addition to miR-19b miR-20a and miR-92a also performed similar significant function in regulating the self-renewal of GCSCs. The difference between previous studies and this study indicated that members of the miR-17-92 cluster might perform different roles in different cancers.

Recently, E2F1 was found to suppress Wnt/β-catenin signaling via trans-activating the β-catenin-interacting protein ICAT (Wu et al., 2011) or by up-regulating axin2 (Hughes and Brady, 2006), which promotes β-catenin degradation (Fancy et al., 2011). In the present study, exogenous miR-17-92 significantly knocked down E2F1 expression. The Wnt/β-catenin signaling pathway has been reported to regulate the self-renewal and multipotency of stem cells. Mice that lack β-catenin in their hematopoietic cells form HSCs, but are deficient in long-term growth and maintenance (Zhao et al., 2007), indicating an essential role of Wnt/β-catenin in the self-renewal of normal and neoplastic stem cells in the hematopoietic system.
Activation of Wnt signaling using a GSK-3-specific inhibitor can maintain the pluripotency of human and mouse embryonic stem cells (Sato et al., 2004). More importantly, activation of Wnt/β-catenin signaling enriches the EpCAM(+) cell population, whereas RNA interference-based blockage of EpCAM, a Wnt/β-catenin signaling target, attenuates the activities of these cells (Yamashita et al., 2009; Terris et al., 2010).

Through a bioinformatics analysis, we first identified another suppressor of Wnt/β-catenin signaling HIPK1 that can be targeted by the miR-17-92 cluster. A previous report indicated that HIPK1 can repress Wnt/β-catenin target gene activation (Louie et al., 2009), as demonstrated through β-catenin reporter assays in human embryonic kidney cells and by indicators of dorsal specification in X. laevis embryos at the late blastula stage. In addition, a subset of Wnt-responsive genes subsequently requires HIPK1 for activation in the involuting mesoderm during gastrulation. The present study not only demonstrated that miR-19b/20a/92a can target HIPK1 but also showed that transfection of miR-19b/20a/92a decreased the expression of β-catenin protein. Based on our findings, it can be speculated that the miR-17-92 cluster might activate the Wnt/β-catenin signaling pathway through down-regulating its direct suppressors E2F1 and HIPK1, which subsequently activates Wnt/β-catenin signaling and increases the abundance of EpCAM+ GCSCs(Yamashita et al., 2007).

Wnt/β-catenin signaling (wnt5a) has previously been reported to be associated with a poor prognosis in gastric cancer (Kurayoshi et al., 2006). miR-17-92 has been reported to contribute to tumorigenesis and a poor prognosis in multiple myeloma (Chen et al., 2011). Herein, we performed real-time PCR in tissue samples from patients to examine the prognostic predictive abilities of the cluster and each individual miRNA. Our results showed that miR-92a could act as an independent factor associated with overall survival.

In conclusion, the results of the present study suggest that members of the
miR-17-92 cluster, miR-19b, miR-20a and miR-92a, act as important miRNAs that regulated the self-renewal of GCSCs by targeting E2F1 and HIPK1 and subsequently activating Wnt/β-catenin signaling, increasing the expression of the GCSC marker EpCAM. This study also showed that miR-19b, miR-20a and miR-92a are over-expressed in human GCSCs and that the expression of miR-20a and miR-92a is negatively associated with overall survival in gastric cancer patients. Furthermore, miR-92a acts as an independent factor that might be used as a prognostic marker in gastric cancer. Thus, this study shed light on the potential clinical application of miR-19b/20a/92a. Targeting miR-19b/20a/92a might effectively restrict the growth of gastric cancer.

Materials and Methods

Ethics Statement

For the analyzed tissue specimens, all patients gave informed consent to use excess pathological specimens for research purposes. The protocols employed in this study were approved by the hospital’s Protection of Human Subjects Committee. The use of human tissues was approved by the institutional review board of the Fourth Military Medical University and conformed to the Helsinki Declaration, and to local legislation. Patients offering samples for the study signed informed consent forms. In the animal experiments, all procedures for animal experimentation were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the Experimental Animal center of the Fourth Military Medical University. The approval ID for using the animals was No.12039 from Experimental Animal Center of the Fourth Military Medical University.

Cell culture and reagents

All of the cells were grown in RPMI1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C under 5% CO2 in a humidified incubator (Forma Scientific, Marietta, OH). The miRNA precursors,
inhibitors and negative controls were obtained from Ambion (Applied Biosystems, USA).

**Lentiviral miR-17-92 infection and stable cells**

The immunodeficiency virus lenti-viral system with EGFP-expressing miR-19b/20a/92a or lenti-NC lentiviruses was purchased from Genechem (Shanghai Genechem CO.LTD). CD44+/EpCAM+ and CD44-/EpCAM- cells were separated from SGC7901 and SGC7901-Luc cells, and used as hosts for lenti-virus infection. Gastric cancer cells were infected with a lenti-viral system expressing miR-19b/20a/92a or lenti-NC according to the manufacturer’s instructions, and stable cells were obtained by sorting GFP-positive cells.

**Microarray**

Microarray analysis was performed using a service provider (LC Sciences), as previously reported (Kort et al., 2008), in 2 to 5 µg of total RNA from tumorsphere non-adherent GCSCs that were attached to the bottoms of flasks for 8 h, 12 h, 1 day or 8 days. Total RNA was isolated using TRIzol (Invitrogen). Hybridization was performed overnight on a micro-fluidic chip. The data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (locally weighted regression). ANOVA was conducted among the 5 samples by LC Science. miRNAs showing p values < 0.01 were considered as differentially expressed miRNAs.

**Tumorsphere culture**

Tumorsphere culturing of GCSCs was performed as described previously (Takaishi et al., 2009). Cells were suspended and inoculated in serum-free culture medium, RPMI-1640 medium (Hyclone), containing human recombinant epidermal growth factor (EGF) (Invitrogen) at a concentration of 20ng/ml and human recombinant basic fibroblast growth factor (bFGF) (Invitrogen) at a concentration of 10ng/ml. After 3 weeks, each well was examined under a light microscope, and the
total cell numbers in spheroid colonies and cell numbers within one tumorsphere colony were counted.

**In vivo tumorigenicity in BALB/C nude mice and severe combined immunodeficient mice**

BALB/C nude mice and severe combined immunodeficient (NOD-SCID) mice at 4-6 weeks of age were handled using best humane practices and cared for in accordance with NIH Animal Care Institutional Guidelines in the Experimental Animal Center of the Fourth Military Medical University (Xi’an, Shanxi Province, P. R. China). Nude mice were used to test the proliferation ability using $5 \times 10^6$ cells while NOD-SCID mice were used to test the self-renewal ability of gastric cancer stem cells using $5 \times 10^3$ cells. CD44+/EpCAM+ and CD44+/EpCAM+ cells separated from SGC7901-Luc cells were used to inoculate mice. For the primary experiments which aimed to test the self-renewal in nude mice, $10^4$ CD44+/EpCAM+ cells and $10^5$ non-CD44+/EpCAM+ cells were injected subcutaneously into the right or left upper back at a single site. Five mice were injected for each group. To investigate self-renewal in NOD-SCID mice, $5 \times 10^3$ cells infected with either lenti-NC or lenti-miR-19b/20a/92a were injected subcutaneously into the back at a single site. Each group contained 8 mice. To detect tumorigenicity in nude mice, $5 \times 10^6$ cells containing lenti-miRs were injected subcutaneously into the back of each mouse; each group contained six mice. The mice were injected with 100mg/kg D-luciferin intraperitoneally five minutes before imaging. Bioluminescent signals were detected twice a week using the IVIS 100 Imaging System (Xenogen, Hopkinton, MA). The nude mice were killed after 28 days and NOD-SCID mice were killed after 28 days (miR groups) or 49 days (NC group). Their subcutaneous tumors were harvested and fixed in 10% formalin before paraffin embedding, then sectioned and stained in H&E.

**Reporter gene assay**
A reporter gene assay was performed as described previously (Tie et al., 2010). Cells were cotransfected with pre-miR-19b/20a/92a (150 nM, Ambion) and 20ng of the Renilla luciferase control vector (pRL-TK, Promega). Firefly luciferase activities were normalized to Renilla luciferase activities. Pre-NC from Ambion was used as one of the negative control miRNAs.

**In vitro drug sensitivity assay**

To conduct drug sensitivity assays, the water-soluble tetrazolium salt WST-8 was employed. At 24 hours after transfection with pre-miR-19b/20a/92a or pre-NC, cells were treated with the serially diluted chemotherapeutic drug 5-fluorouracil (5-FU) at a concentration of 0.25ug/ml. Optical density was measured at 450nm and 690nm using a micro-plate reader.

**Cell proliferation assay with CFSE labeling**

Cells were adjusted to a density of 10^6 cells/ml and treated with CFSE at a final concentration of 10µM. After incubation at 37°C for 10min, labeling was blocked by the addition of RPMI medium with 10% FCS. The fluorescence intensity was determined via flow cytometry analysis.

**In vitro cell proliferation assay**

The in vitro cell proliferation assay was performed as described previously (Wu et al., 2010). A total of 10^3 miR-17-92-infected cells or cells transiently transfected with a 150nM concentration of miR-17-92 precursors or inhibitors were used for the assays in 200 µl of complete medium. The cultures were assayed each day and read at a 490 nm absorbance (A490) on a micro-plate reader (168-1000 Model 680, Bio-Rad, Hercules, USA). Each experiment was performed in triplicate and repeated 3 times.

**Plate colony formation assay**

The plate colony formation assay was performed as described previously (Wu et al., 2010), and transfection was performed as described above. Cells were plated at a
density of 300 cells/well in 12-well plates and cultured in RPMI 1640 medium with 10% fetal calf serum for 3 weeks. The colonies were stained with crystal violet solution, and the number of colonies was counted. Each assay was performed three times.

**Immunofluorescence**

The attached cells were washed using PBS and then fixed by 4% paraformaldehyde for 20 minutes at room temperature. Cells were then rinsed three times using PBS for 5 minutes and permeabilized with PBS-T solution for 10 minutes. After 5 minutes’ wash with PBS, cells were blocked using 1% BSA for 30 minutes. Primary antibodies anti-CK14 and anti-CK18 were added to cells and incubated at 4°C overnight. After three times wash, cells were incubated with secondary antibodies.

**Western blot analysis**

Western blotting was performed as described previously (Wu et al., 2010). Log-phase cells were harvested and resolved on SDS-PAGE gels, followed by blotting on nitrocellulose membranes (Amersham, Pittsburgh, PA, USA). The membranes were blocked with 10% non-fat milk and incubated overnight with primary antibodies: anti-E2F1, anti-β-catenin and anti-HIPK1 (1:1,000; ABcam, USA) or an anti-beta-actin antibody (1:2,000; Sigma–Aldrich, USA).

**RNA extraction and real-time PCR**

Total RNA was extracted from FFPE-impeded tissue sections according to the manufacturer’s instructions (AM1975, Ambion, USA). Total RNA was extracted from cells as described previously (Wu et al., 2010). Reverse transcription was performed according to the manufacturer’s instructions (D350A, TaKaRa Biotechnology, Co.,Ltd). Quantitative real-time PCR was performed to determine the expression levels of each miRNA using the exact sequences (U to T) of these miRNAs as the forward primer and the unique q-PCR primer from the cDNA Synthesis Kit. U6 was used as an internal control, and each plate contained one cDNA sample for each
primer as a calibration sample.

**Statistical Analysis**

Continuous variables were compared with ANOVA tests. If the test of homogeneity of variances between the groups was significant, the Mann-Whitney U test and Kruskal-Wallis H test were adopted as appropriate. A Chi-square or Fisher’s test was used for categorical variables. The independent predictors of survival were calculated using the Cox regression model. The covariates incorporated into the multivariate analysis were the variables reaching $p<0.05$ in a univariate analysis. Cumulative survival was assessed with Kaplan-Meier curves and compared using the log-rank test. Two-tailed $p$-values $<0.05$ were considered statistically significant. All statistical analyses were conducted using SPSS software, 14.0 (Chicago, Illinois, USA).

**Acknowledgements**

We thank Zhen Chen and Taidong Qiao for the common supply of the lab issues. This work was supported by National Basic Research Program of China (No. 2010CB732400, No.2010CB529300) and National Natural Science Foundation of China (No. 81172062, No. 81030044, No. 81000988).

**Conflict of interest.**

The authors declare no conflict of interest.

**Reference**


Figure legend

Figure 1 Gastric cancer cells separated based on EpCAM+/CD44+ are enriched for GCSCs.
(A) The gastric cancer cell lines SGC7901 and MKN28 show distinct abilities to generate tumorspheres in non-adherent conditions. Representative images show tumorspheres of SGC7901 and MKN28 cells in low adherence flasks with non-serum medium. After 15 days of culture, a higher percentage of SGC7901 cells formed tumorspheres compared with MKN28 cells.
(B) FACS analysis of EpCAM/CD44 expression in SGC7901 (80%) and MKN28 (0.2%) cells.
(C) CFSE analysis of the proliferative ability of tumorspherical GCSCs and adherent differentiated cells using the SGC7901 cell line. Representative images show the results of FACS analysis of the proliferative ability of tumorspherical GCSCs after 8 days and the tumorspherical cells that attached to the flasks after 8h, 12h, 1 day and 8 days using CFSE labeling.
(D and E) Real-time PCR analysis of CK14 (D) and CK18 (E) expression in tumorspherical GCSCs from the SGC7901 cell line and the more differentiated cells attached to the bottom of the flasks for 8h, 12h, 1 day and 8 days. The presented values correspond to the mean±SD (n=4). *p<0.05, **p<0.01 compared with tumorspheres. Error bars correspond to the mean±SD. Each experiment contained three replicates and three independent experiments were performed.

Figure 2 miRNA array analyses of GCSCs and differentiated GCSCs. An miRNA array analysis was performed in tumorspherical gastric cancer cells, and differentiated spherical-tumor cells adhered for 8 h, 12 h, 1 day and 8 days.
(A) The miRNA array showed reductions of miRNA levels, including miR-19b, miR-20a and miR-92a, during GCSC differentiation. Lane1: freshly dissociated tumorspheric cells; lane 2: 8h after tumorspheric cells were re-suspended in normal cell culture conditions; lane 3: 12h after re-suspension; lane 4: 1 day after re-suspension; lane 5: 8 days after re-suspension.
(B) The relative expression of each member of the miR-17-92 cluster (miR-19b, miR-20a and miR-92a) in the miRNA array.
(C) Real-time PCR confirmation of the expression of miR-19b, miR-20a and miR-92a in cells under the same treatment applied in the miRNA array analysis **p<0.01 compared with tumorsphere cells. Each experiment contained three replicates and three independent experiments were performed.

Figure 3 Over-expression of miR-19b/20a/92a is required to maintain tumorspheres in
gastric cancer cells
(A) There is a greater percentage of CD44-/EpCAM- cells, in a tumorsphere infected with miR-19b, miR-20a and miR-92a have more tumorspherical cells compared with lenti-NC-infected cells out of a total of 1,000 cells. **p<0.01 compared with the lenti-NC group.
(B) Tumorspheres derived from miR-19b, miR-20a and miR-92a-infected cells, show an increase in the number of cells. **p<0.01 compared with the NC group.
(C and D) Conversely, transient transfection of miR-19b/20a/92a-inhibitors in CD44+/EpCAM+ cells and SGC7901 cells generated much fewer tumorspheres out of a total of 1,000 cells. **p<0.01 compared with the NC group.
(E) Growth curves of lenti-miR-17-92-infected cells treated with 0.25ug/ml 5-FU on the first day of proliferation. **p<0.01 compared with NC group.

Figure 4 Over-expression of miR-19b/20a/92a up-regulated the self-renewal of GCSCs in NOD-SCID mice
(A and B) Luciferase signals are stronger in tumors from miR-19b, miR-20a and miR-92a -infected CD44-/EpCAM- cells compared with tumors from lenti-NC-infected CD44-/EpCAM- cells in NOD-SCID mice. Representative images show the luciferase intensity in mice on the 28th day (A) and the detected luciferase signals (B).
(C) Representative images show H&E staining and EGFP staining of lung (×100) and liver (×400) tissues from mice with metastases. Arrows indicate the focal metastasis.
(D) Representative images show H&E staining of the tumors (×400, D) generated in nude mice from lenti-miR or lenti-NC-infected cells and EGFP staining of these tumors (×400, E).

Figure 5 miR-19b/20a/92a miRNAs promoted tumor growth in vitro and in vivo
(A) Cell proliferation was measured using lenti-miR- and lenti-NC-infected SGC7901 and MKN28 gastric cancer cells. Lenti-miR-19b/20a/92a-infected cells grew faster compared with lenti-NC-infected cells, **p<0.01.
(B and C) A colony formation assay was performed to evaluate the proliferative functions of lenti-miR-infected cells. Lenti-miR-19b/20a/92a-infected cells formed more colonies compared with lenti-NC-infected cells, **p<0.01. Representative images show the colony intensities of lenti-miR and lenti-NC-infected cells using 150cells/well in 24-well plates (B) and the obtained colony numbers (C). Error bars correspond to the mean±SD.
(D and E) In vivo tumorigenecity was evaluated in BALB/C nude mice. Luciferase signals were detected using the IVIS system. Tumors generated from lenti-miR-infected cells exhibited a stronger signal compared with the lenti-NC group of mice on the 28th day. Representative images show the luciferase intensities in tumors from mice (D) and the luciferase signals detected (E).
(F) H&E staining and EGPF staining of tumors (×400) from mice injected with 5X106 cells showed a similar histology.

Figure 6 E2F1 and Hipk1 are direct targets of miR-17-92 miRNAs
(A) Schematic representation of the E2F1 and HIPK1 3’-UTR constructs in pGL3-Control (Luc-1,-2,-3 and -4). Conserved putative target sites for the miR-19b, miR-20a and miR-92a miRNAs are indicated.

(B-D) Luciferase assays were performed with Luc-1 through Luc-4, as indicated, in both sense (S) and antisense (AS) orientations. Bars indicate the ratio of firefly luciferase (normalized to Renilla luciferase) activity measured following transfection with miR-19b/20a/92a pre-miRNA compared with the activity measured following transfection with the pre-miR-control (pre-NC) for the same construct. **p<0.01 compared with the antisense results.

(E) Western blot analysis of E2F1 protein levels in pre-NC or pre-miR-20a-expressing cells (NC, 20a) and cells transiently transfected with miR-20a inhibitors (NC, 20ai) as well as Hipk1 protein levels in pre-NC-and pre-19b/92a-expressing cells (NC, 19b, 92a). β-actin was used as an internal control. Three independent experiments were performed.

(F) Western blot analysis of beta-catenin protein levels in pre-NC or pre-miR-19b/20a/92a expressing cells (NC, 19b, 20a, 92a) Three independent experiments were performed.

Figure 7 Kaplan-Meier survival curves of miR-20a and miR-92a in gastric cancer patients.

(A-B) Gastric cancer patients were ranked on the basis of the relative expression values obtained via real-time PCR and were divided into a high-expression group and low-expression group using the 50th percentile (median) as the cut-off point.
Table 1 Number of tumors formed and metastasized of lenti-NC or lenti-miRs infected cells in NOD-SCID mice

<table>
<thead>
<tr>
<th></th>
<th>Number of mice injected cells</th>
<th>Number of mice generated tumors</th>
<th>Number of mice had liver metastasis</th>
<th>Number of mice had lung metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lenti-NC</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lenti-miR-19b</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Lenti-miR-20a</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Lenti-miR-92a</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>