Large-scale Identification of Functional Genes Regulating Cancer Cell Migration and Metastasis Using the Self-assembled Cell Microarray

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Large-scale Identification of Functional Genes Regulating Cancer Cell Migration and Metastasis Using the Self-assembled Cell Microarray

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

SAMcell	self-assembled cell microarray
miRNA	microRNA
ECM	extracellular matrix
RNAi	RNA interference
mRNA	messenger RNA
siRNA	small interfering RNA
pri-miRNA	primary microRNA
pre-miRNA	precursor microRNA
RISC	RNA induced silencing complex
UTR	untranslated regions
HTS	high-throughput screening
SNP	single nucleotide polymorphism
EMT	epithelial–mesenchymal transition
RKIs	Rho-kinase inhibitors
GFP	green fluorescent protein
PNI	Poly (N-isopropylacrylamide)
PR	positive representative
NR	negative representative
FC	fold change
NC	negative control
No.	number
DEGs	differentially expressed genes
FDR	false discovery rate
GO	gene ontology
PPIs	protein-protein interactions
LR	likelihood ratio

SUMMARY

Metastasis is one of the critical hallmarks of malignancy tumor and the principal cause of death in patients with cancer. Cell migration is the basic and essential step in cancer metastasis process. To systematically investigate functional genes regulating cell migration and cancer metastasis on large scale, we developed a novel on-chip method, SAMcell (self-assembled cell microarray). This method was demonstrated to be particularly suitable for loss-of-function high-throughput screening because of its unique advantages. The first application of SAMcell was to screen human genome miRNAs, considering that more and more miRNAs had been proved to govern cancer metastasis. We found that over 20 % of miRNAs have migratory regulation activity in diverse cell types, indicating a general involvement of miRNAs in migratory regulation. Through triple-round screenings, we discovered miR-23b, which is down-regulated in human colon cancer samples, potently mediates the multiple steps of metastasis, including cell motility, cell growth and cell survival. In parallel, the second application of SAMcell was to screen human genome kinase genes, considering that more and more kinase genes had become successful diagnostic marker or drug targets. We found over 11% migratory kinase genes, suggesting the important role of kinase group in metastasis regulation. Through both functional screening and bioinformatics analysis, we discovered and validated 6 prospective metastasis-related kinase genes, which can be new potential targets in cancer therapy. These findings allow the understanding of regulation mechanism in human cancer progression, especially metastasis and provide the new insight into the biological and therapeutical importance of miRNAs or kinases in cancer.

Key Words: Cancer metastasis; High-throughput screening; SAMcell; Functional genes.

CHAPTER 1

INTRODUCTION

1.1 Cancer metastasis

Metastasis is one of the critical hallmarks of malignancy tumor and the principal cause of death in patients with cancer[1]. By now, statistical data indicate that metastases account for 90% of human cancer deaths[2]. However, our understanding of the molecular circuitry that regulates metastatic dissemination remains obscure. The knowledge of detailed metastasis mechanisms may lead to promising opportunities for drug discovery and clinical therapy in cancer treatment[3].

1.1.1 The metastasis cascade

Metastasis, or metastatic disease, is the spread of a cancer from one organ or part to another non-adjacent organ or part[4]. It was previously thought that only malignant tumor cells and infections have the capacity of metastasis; however, this has been reconsidered due to the discovery that normal mammary epithelial cells may establish residence in the lung once they have entered the bloodstream circulation and may assume malignant growth once oncogene activation[5]. Cancer metastasis is a complex and multi-step process. First of all, cells at the tumor edge break away from the primary tumor and locally migrate into neighboring ECMs (extracellular matrix). Secondly, cancer cells invade into the adjacent tissues. Thirdly, some cancer cells acquire the ability to penetrate the walls of lymphatic or blood vessels, after which they are able to circulate and survive during bloodstreams. Next, cancer cells are arrested at distant site and extravasate to reach foreign tissue parenchyma. Finally,

they proliferate from microscopic growth into macroscopic secondary tumor.[6]

1.1.2 Cell migration involving in metastasis

Cell migration refers to the translation of cells from one location to another. Cells often migrate in response to specific external signals, including chemical signals and mechanical signals.[7] Migration process begins with polarization to form protrusion at the front and constriction at the rear, then the tail detaches and contracts to generate a movement[8].

Cell migration plays a central role in the development and maintenance of biological processes, especially metastasis. Human cells exhibit three kinds of motion, such as collective motility, mesenchymal-type movement, and amoeboid movement. Cancer cells can opportunistically switch between different kinds of motion.[9] Metastasis process requires a strong adaptability of cells to the different microenvironments, such as within primary tumors, in the ECM, in blood or lymphatic streams and finally in the metastatic niche. Cells in the core of the tumor mainly move using a mesenchymal-type movement, while cells at the tumor edge escape the tumor limit and enter the ECM using an amoeboid motility.[10]

1.2 RNA interference technology

The central tool we used is RNA interference (RNAi) technology to operate gene's expression and study gene's function. RNAi is a biological process in which RNA molecules inhibit gene expression, typically by resulting in the degradation of messenger RNA (mRNA) molecules with specific sequences. In 2006, Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine for their work "*Discovery of RNA interference - gene silencing by double-stranded RNA"* on RNA

interference in the nematode worm C. elegans[11]. There are two types of small ribonucleic acid (RNA) molecules which play central roles in RNAi process, including microRNA (miRNA) and small interfering RNA (siRNA). According to the central dogma, RNA transcripts are the direct products of genes, and mRNAs guide the protein translation[12]. These small RNAs can bind to specific mRNA molecules and usually decrease their activity, either by causing cleavage of mRNAs, or by preventing mRNAs from producing proteins[13].

1.2.1 Biogenesis and mechanism of miRNA

Majority of primary microRNA(pri-miRNA) transcripts are typically produced by RNA Polymerase Ⅱ[14, 15]. The length of these pri-miRNAs is from hundreds to thousands of nucleotides with one or more double-stranded regions. Some pri-miRNAs have independent transcriptional unite with mono-(e.g. miR-21[16]) or polycistronic miRNA-precursor structure (e.g.the miR-17-92-1 cluster[17]), whereas others locate in the intron (e.g. miR-10b[18]) or exon (e.g. miR-198[16]) of messenger RNAs (mRNAs). These polymerase Ⅱ-directed pri-miRNAs are post-transcriptionally polyadenylated and capped at 5'end and 3'end respectively. However, recent data indicate that a subset of miRNAs may be transcribed by RNA Polymerase Ⅲ, instead of RNA Polymerase Ⅱ, such as the largest human miRNA cluster, C19MC[19]. Expression of individual miRNA is controlled by transcription factors, for example c-MYC or P53[20, 21] or lies on the methylation of promoter region. However, the regulatory mechanism of miRNAs at the transcriptional level is various.

Next, the pri-miRNA is cleaved by a complex of RNase Ⅲ endonuclease Drosha and the DGCR8 protein (also known as Pasha)[22] which contain two double-stranded RNA-binding domains[23-26]. First, the DGCR8 directly interacts with the pri-miRNA and determines the accurate cleavage site, and then the Drosha finish the

cleavage processing. Consequently, an imperfect stem-loop structure of ~50-70 nt in length is released, with a hairpin stem, a terminal loop and two single-stranded flanking regions, which is named precursor microRNA (pre-miRNA). The double-stranded stem and the flanking regions are indispensable for DGCR8 binding and Drosha cleavage, in contrast, the loop region is less critical for this step[27-29].

After nuclear processing，The pre-miRNAs are transported to the cytoplasm by the nucleocytoplasmic shuttle Exportin-5, which recognizes a two-nucleotide overhang left by the RNase III enzyme Drosha at the 3' end of the pre-miRNA hairpin, rather than its sequence or the loop structure. Exportin-5-mediated transport to the cytoplasm is energy-dependent, using GTP bound to the Ran protein[30]. In the nuclear, with the help of high concentration of RanGTP, the Exportin-5 induces the Drosha complex to release pre-miRNA, and transport them off nuclear. Oppositely, in the cytoplasm, the low concentration of RanGTP results in the pre-miRNA being separated from Exportin-5. During the subsequent processing, the RNase Ⅲ enzyme-Dicer is in charge of cutting off the loop portion of the hairpin structure and yielding ~22nt small RNA duplexes consisting of the mature miRNA and a partially complementary strand. Knockout Dicer leads to a decreased or abrogates amount of mature miRNAs, which demonstrate this cleavage is essential for miRNA biogenesis processing[31-34]. Dicer cleavage activity is regulated by binding its amino-terminal DExD/H-box helicase domain to TRBP which activates Dicer through a conformational rearrangement[35].

Besides of canonical miRNA biogenesis pathway, several alternative pathway has been discovered. For example, many Drosha/DGCR8- independent pathways can generate pre-miRNA-like hairpin structure, which serve as Dicer substrate without cleaving by Drosha/DGCR8 complex (e.g. miR-62, mirR-1071) [36-39]. Additionally, pri-miR-451 is processing by Drosha/DGCR8 to format a short pre-miRNA with only~18nt of duplex stem, which is too short to recognize by Dicer. Instead, pre-mir-451 is directly cleaved by Ago protein and some unidentified proteins[40-42].

After the finial miRNA duplex was generated, one strand was loaded into a protein complex called RNA induced silencing complex (RISC)[43]. This strand is the mature miRNA (named guide strand), while the other strand (named passenger strand) gets degraded by cleavage or a bypass mechanism. An essential core protein in RISC is the argonaute protein (Ago), which has four related members in human. Typically, the mature RISCs bind to 3'UTR (untranslated regions) of mRNAs containing a partially complementary sequence with the seed region of miRNAs (the 2^{nd} to 8^{th} nt of the mature miRNA). If the seed region of miRNA can anneal with the mRNA, RISC cleave the mRNA or suppresses the translation without affecting the mRNA transcriptional level.

1.2.2 RNAi high-throughput screening

According to the specific sequence of mRNA from a gene, we can selectively design and synthesize exogenous siRNA duplex with specificity. By introducing siRNA duplex into either cultured cells or living organisms, the gene expression can be suppressed robustly. RNAi has now become a valuable research tool, especially used for large-scale screening to identify the components necessary for a particular cellular process or an event such as cell migration and cell proliferation[44, 45], by systematically knocking down each gene in the cell. This method is also used as a practical tool in biotechnology and medicine.

The RNAi high-throughput screening (HTS) technology allows genome-wide loss-of-function screening and is broadly used to identify genes associated with specific biological phenotypes[46]. This technology has been hailed as the second genomics wave, following the first genomics wave of gene expression microarray and single nucleotide polymorphism (SNP) platforms[46]. One of the major advantages of the RNAi HTS technology is the ability to simultaneously test thousands of genes,

which lead to an explosion in the rate of generating data. Consequently, one of the most fundamental challenges in the RNAi HTS studies is to glean biological significance from huge scale of data, which requires the adoption of appropriate statistical and bioinformatics methods[47]. We can divide the analytic process of a cell-based RNAi screening into several steps[47]. First of all, we should choose the suitable RNAi library, including the vector and the gene range. Secondly, we should select the robust and stable type of cells. Thirdly, we should use the perfect transfection methods or reagents to introduce the RNAi library into cells. Fourthly, we should implement the necessary experimental treatment and signal detection. Finally, we should figure out the determination of important genes or therapeutical targets by statistical and bioinformatics analysis.

1.3 miRNAs governing cancer metastasis

miRNAs are post-transcriptional regulators that bind to complementary sequences on target mRNA transcripts, usually resulting in translational repression or target degradation and gene silencing[48, 49]. The human genome may encode over 1000 miRNAs[50], which may target about 60% of mammalian genes[51, 52] and are abundant in many human cell types[53]. As a new class of signalling modulators, miRNAs have attracted great attention for certain unique features, including multi-target regulation, tissue specificity, and evolutionary conservation[54-56]. These small endogenous RNAs are able to interact with many physiologically essential genes, such as c-MYC, P53 and RAS, and play critical roles in a wide range of biological processes, including cell development, cell proliferation and differentiation, as well as cell migration and cancer metastasis[20, 21, 57, 58]. Strikingly, these important modulators are globally dysregulated in human cancer samples[59]. A small number of miRNAs have been demonstrated to actively participate in the regulation of tumor development[60-64]. With the latest deciphering of roles for miRNAs in the metastatic progress, there are new hopes that we can interpret the molecular pathways

underlying each step through miRNAs. Since miRNAs were involved in cancer pathogenesis[65], accumulating data have pointed to a central regulatory role for miRNAs in the initiation and progression of most cancers analyzed thus far[64].

1.3.1 miRNA as metastasis activator

In Weinberg's group, using a combination of mouse and human cells, they showed that miR-10b is highly expressed in metastatic breast cancer cells and positively regulates cell migration and invasion[62]. Importantly, over-expression of this miR-10b in otherwise non-metastatic breast cancer cells enables them to acquire invasive and metastatic capability. Expression of miR-10b is induced by the transcription factor TWIST, which binds directly to the putative promoter of mir-10b, and is essential for TWIST induced epithelial–mesenchymal transition (EMT) that promotes cell motility[66]. The miR-10b induced by TWIST binds directly to mRNA of HOXD10 and represses translation of HOXD10, resulting in up-regulated expression of a famous pro-metastatic gene, RHOC. Significantly, the level of miR-10b expression in primary breast carcinomas correlates with clinical progression6. These findings suggest an undescribed signalling pathway, in which a pleiotropic transcription factor (TWIST) induces expression of a specific miRNA (miR-10b), which suppresses its direct target (HOXD10) and activates another pro-metastatic gene (RHOC), leading to cell invasion and tumor metastasis.

In a collaborative effort between Huang and Agami's groups, to identify potential metastasis promoting miRNAs, through a genetic screen using a nonmetastatic, human breast tumor cell line that was transduced with a miRNA-expression library and subjected to a trans-well migration assay, miR-373 and miR-520c were also identified as metastasis promoting genes[61]. In MCF7 breast cancer cells, over-expression of miR-373 or miR-520c promoted an in vitro migration and invasion. Furthermore, nude mice transplanted with these cells induced metastatic nodules,

whereas control cells did not. miR-373 and miR-520c were found to share a similar 'seed' sequence and to have a common direct target CD44, which encodes a cell surface receptor for hyaluronan. CD44 is lost in breast cancer with high metastatic potential and acts as a metastatic suppressor in also prostate and colon cancer. It was found that significant up-regulation of miR-373 in clinical breast cancer metastasis samples that correlated inversely with CD44 expression[61].

According to the study of Ma and colleagues, the research reveal that miR-9 is up-regulated in breast cancer cells, directly targets CDH1, the E-cadherin-encoding messenger RNA, leading to increased cell motility and invasiveness[67]. miR-9-regulated E-cadherin down expression induces the activation of β-catenin signaling pathway, which results in up expression of the gene encoding VEGF, which leads to tumor angiogenesis. miR-9 is positively associated with malignancy of human cancers. miR-9 expression is significantly up-regulated in both clinical breast cancers[68] and in c-myc-induced mouse mammary tumors[69]. Conversely, inhibiting miR-9 expression in highly malignant cells inhibits metastasic capability. MYC and MYCN oncoproteins, acting on the *mir-9-3* locus, activate miR-9 expression in tumor cells. Significantly, in human cancers, miR-9 levels correlate with *MYCN* amplification, tumor grade and metastatic status[67].

1.3.2 miRNA as metastasis suppressor

HMGA2, a member of the high mobility group AT-hook family of nonhistone chromatin proteins, is an architectural transcription factor[70], which plays a critical role in growth during embryonic development[71]. Knockdown of Dicer in HeLa cells revealed that the HMGA2 gene is transcriptionally active, but its mRNA is destabilized in the cytoplasm through the miRNA pathway[72]. HMGA2 was repressed by let-7 in cells. Ectopic expression of miRNA let-7 down regulates HMGA2 and cell proliferation in lung cancer cells through directly binds to the target

sites in the 3’ untranslated region (UTR). At the same time, The 3’ UTR of the human RAS gene, which is also a famous oncogene, contains multiple complementary sites, allowing let-7 to regulate RAS expression[58]. let-7 expression is lower in lung tumors than in normal lung tissue, while RAS protein is significantly higher in lung tumors. These findings suggest that let-7 can serve as a tumor suppressor gene through inhibiting the expression of oncogenes HMGA2 and RAS.

Weinberg’s group identified an antimetastatic human miRNA, miR-31, that acts at multiple steps of the invasion-metastasis cascade via repression of a cohort of prometastatic targets[73]. Genome-wide studies have described miR-31 down-regulation or deletion of the miR-31 genomic locus in human breast cancers[74, 75]. miR-31 represses metastasis without contributing to influence on primary tumor development. As such, mir-31 might aptly be categorized as a ‘metastasis suppressor gene’[76]. Of significance, down regulation of miR-31 enhances migration, invasion, and anoikis resistance in human breast epithelial cancer cells. Conversely, increasing expression of miR-31 reduces migratory, invasive and metastasic capability both *in vitro* and *in vivo* and represses EMT markedly. The direct targets of miR-31 are Fzd3, ITGA5, MMP16, RDX, and RhoA, which are famous pro-metastasic proteins described in human tumors[77-79]. miR-31 expression was diminished in primary tumors that subsequently metastasized, when compared to normal breast tissue and primary tumors that did not recur; moreover, low miR-31 levels correlated strongly with reduced distant disease-free survival relative to tumors with high miR-31[78]. This suggests one putative mechanism by which the invasion-metastasis cascade could be initiated very early during the course of tumor progression, a phenomenon that has recently been observed in clinical breast tumors[80].

1.3.3 miRNA involved in EMT

Epithelial-mesenchymal transition (EMT) describes a reversible series of events

during which an epithelial cell loses cell-cell contacts and acquires mesenchymal characteristics. These events involve molecular reprogramming of the cell, including loss or redistribution of epithelial-specific junctional proteins such as E-cadherin and turning on of mesenchymal markers including vimentin and N-cadherin[81]. The embryonic programme 'epithelial–mesenchymal transition' (EMT) is thought to promote malignant tumor progression[82]. EMT induces detachment of cells from each other and increasing cell mobility, both of which are necessary for tumor cell dissemination. Metastases often contain the differentiated phenotype of the primary tumor; therefore, EMT seems to be activated by the invasive tumor environment, but is reversed in growing metastases[83]. Activators of EMT, such as TGFβ, TNFα and HGF, are produced by penetrating cells or the tumor cells themselves, and enhance expression of EMT transcriptional repressors[81], including the Snail family, the basic helix–loop–helix family, Goosecoid and members of the ZFH family, ZEB1 and ZEB2[84, 85]. ZEB is a crucial inducer of EMT in various human tumors, and was recently shown to promote invasion and metastasis of tumor cells.

Goodall's group and others shown that the miR-200 family is down regulated in metaplastic and basal breast cancers respectively, relative to ductal breast tumors[86, 87]. These differences most likely reflect the more poorly differentiated grade of these tumors compared to ductal tumors, and in the case of metaplastic tumors are indicative of EMT-like changes having taken place as observed in their characteristic sarcomatoid (mesenchymal) morphology and expression profile[77, 87]. These findings indicate miR-200 family participates in a signalling network with the E-cadherin transcriptional repressors ZEB, and TGFβ2. In fact, miR-200 family directly binds to mRNAs of ZEB1 and ZEB2 and represses the translation process.

Interestingly, Thomas Brabletz's group reported that ZEB1 directly suppresses transcription of microRNA-200 family members miR-141 and miR-200c, by binding to at least two highly conserved sites in their putative promoter, which strongly activate epithelial differentiation in pancreatic, colorectal and breast cancer cells[86].

Notably, as above, the EMT activators TGFβ2 and ZEB1 are the predominant targets down-regulated by these microRNAs. These results indicate that ZEB1 induces a miRNA-mediated feed forward loop that stabilizes EMT and promotes invasion and metastasis of cancer cells.

1.4 Protein kinase modulating cancer metastasis

Protein kinase is involved extensively in transmitting signals and controlling complex processes in cells. More than five hundred different kinases have been identified in humans and they constitute about 2% of all human genes.[88] Recent data have pointed to a central regulatory role of kinase genes in cell migration and cancer mestastasis. For example, PI-3-kinase and MAPK regulate cell proliferation and migration in response to PDGF[89]. FAK functions as a key regulator of fibronectin receptor stimulated cell migration events through the recruitment of both SH2 and SH3 domain containing signaling proteins to sites of integrin receptor clustering[90]. However, comprehensive understanding of kinases' function involved in cancer metastasis is essential for drug discovery and clinical medicine.

1.4.1 Protein kinase as biomarker for diagnosis

The ERBB2 (another name is HER2) oncogene is a member of the erbb-like oncogene family, and is related to, but distinct from, the epidermal growth factor receptor[91]. This gene was found to be amplified from 2- to more than 20-fold in 30% of the tumors[92]. Correlations between gene amplification and several disease parameters were evaluated, hence expression level of ERBB2 was a significant predictor of both survival and relapse in patients with breast cancer[93]. Recent studies highlighted the distinguishing status between primary tumor and metastatic sites, with a discordance rate of 10–17.5%[94]. This gene plays an important role in both biology behavior and pathogenesis of human breast cancer.

1.4.2 Protein kinase as drug target for therapy

ROCK1 and ROCK2 support the metastatic growth of a spectrum of human cancer types, making them potential targets for the development of antitumor drugs[95]. ROCK is activated in cancer when bound to the GTP-bound form of Rho GTPase. Recently, Sebti's group reported on the design and synthesis of novel Rho-kinase inhibitors (RKIs), using a fragment-based approach and X-ray crystallography[96]. They selected several RKIs to test in cancer and found that inhibition of ROCK1 with RKI-18 results in preventing migration, invasion and anchorage-independent growth. Virtually, protein kinase has now become the second most important group of drug targets[97].

1.5 Research approaches for migratory genes

Usually, research of cancer metastasis begins with investigation of cell migratory behavior. However, the traditional methods for cell migration studies, such as wound healing assay or Transwell assay, cannot be scaled up for high-throughput screening easily. So the motivation of next generation HTS is to create an on-chip approach.

1.5.1 Conventional migration assays

The wound healing assay is an easy, low-cost and well-developed method to measure cell migration in vitro[98]. The basic step is to create a ''scratch'' in a cell monolayer, capturing the images at different times during cell migration to close the scratch, and comparing the images to calculate the migration rate of the cells[99]. The Transwell assay, originally introduced by Boyden for the analysis of leukocyte chemotaxis, is based on a chamber of two medium-filled compartments separated by a

microporous membrane[100]. In general, cells are placed in the upper chamber and are allowed to migrate through the pores of the membrane into the lower chamber, in which chemotactic agents are present. After an appropriate incubation time, the number of cells that have migrated to the lower side of the membrane is determined.

1.5.2 HTS assays for identification of migratory genes

Recently, several groups have focused on developing a new approach. Simpson created a robotic-driven pin to deliver a precise scratch in confluent cell monolayers to perform a wound healing screen with MCF-10A breast epithelial cells[44]. This method demands specific equipments, yet the boundaries of the edges generated by the bins are not clear enough. This results in poor veracity and big error.

Miyake's idea of printing both the labeled fibronectin, for identification of the starting region of a cell, and the green fluorescent protein (GFP) expression vector, for identification of cells that had been transfected with miRNA and of the end point of migration, brings a rapid and efficient high-throughput screening procedure[3]. This approach requires peculiar cell line, and diffusion of cells will bring out remarkable cross-contamination. Furthermore, the signals are not propitious to statistically analysis.

Agami set up a genetic screen using a nonmetastatic, human breast tumor cell line that was transduced with a miRNA-expression library and subjected to a transwell migration assay[61]. This is an excellent idea. However, a blemish in an otherwise perfect thing lies that the results just contain migration-promoting miRNAs but without migration-suppressing miRNAs. Although these methods have improved experimental throughput to a certain degree, they are still either technically demanding or only applicable to specific cell lines, thus hampering their application to large-scale screening.

1.6 Problem statement and content of this thesis

By far, we have two central problems: how to systematically investigate functional genes regulating cancer metastasis and how to identify genes which play central roles and can be potential targets of cancer therapy. To address these questions, we should develop a new HTS assay suitable to study cancer cell behaviors, especially migration and make sure the determination of important genes and therapeutical targets.

At first, we developed a novel method, SAMcell (self-assembled cell microarray), for investigation of cell migration behaviors on large scale. The current assays are not suitable for HTS. And there is no previous report that HTS was performed through an on-chip approach to investigate functional genes regulating cancer. Based on SAMcell, we also set up a pipelining of HTS platform integrated with high-throughput devices.

The first application of SAMcell based HTS system was to screen human genome miRNAs regulating cancer metastasis, considering that more and more miRNAs had been proved to govern cancer metastasis. According to what we are informed, there was no previous report that so many miRNAs were investigated through such a systematic screen. We found 20% migratory miRNAs out of more than 900 human miRNAs. Before our research, no evidence can help us make an overall judgment of migratory miRNA distribution and no research can identify so many functional genes at one time. In the traditional screening strategy, researchers picked up the most significant gene after one-round screening. But in our study, we performed triple-round screenings for three phenotypes and picked up the powerful gene miR-23b based on its performances in three rounds of screenings.

In parallel, the second application of SAMcell based HTS system was to screen human genome kinase genes regulating cancer metastasis, considering that more and

more kinase genes had become successful diagnostic marker or drug targets. There was no previous research that focused on the group of kinase and performed both functional screening and bioinformatics analysis for functional genes regulating cancer metastasis. We found 11% migratory genes out of more than 700 human kinase genes and 16 genes as potential clinical targets.

CHAPTER 2

DEVELOPMENT OF SELF-ASSEMBLED CELL MICROARRAY

2.1 Fabrication of SAMcell

We developed a self-assembled cell microarray (SAMcell) to examine the capacity of miRNA or siRNA to mediate cell migration. The fabrication of a self-assembled cell microarray is schematically shown in Figure 2.1a. Glass slides (2.5 cm × 2.5 cm) were washed with detergent and miliQ water. After dryness, the slides were covered with Poly (N-isopropylacrylamide) (For short PNI, Aldrich) dissolved in ethanol (6 % w/v). The slides were etched via a shadow mask by oxygen plasma for 5 min at 200 w Power. The protocol refers to previous description[101]. In brief, 3 μl of OptiMEM (Invitrogen), containing sucrose and 2.5 μl LipofectamineTM 2000 (Invitrogen) were transferred to each tube and mixed thoroughly. Then, 2 μg siRNA/ 1 μg plasmid was added to each tube and the mixture was incubated for 20 min at room temperature. Finally, 7.25 μl of a 0.2 % (w/v) gelatin (Sigma, Type B) solution was added to each tube and mixed thoroughly. After UV sterilization, the reverse transfection reagent was printed on the chip via nanodispenser (Phoenix, Art Robbins Instruments, US). Next, the slides were fixed in a 6-well plate by melted wax. About 3 ml 37 °C medium containing 5×10^5 cells were transferred in each well. Cells self-assembled within each circle, since they grew very well on the glass slide, but rather poorly on PNI. PNI has been previously demonstrated to have a sharply lower critical solution temperature, ~32 °C [102]. That is, PNI undergoes a solid–liquid phase transition as it is cooled below 32 °C, and dissolves in the surrounding aqueous medium. About 24-48 h later, the dishes were moved at room temperature for 5 min and washed with PBS for three times to ensure the total removal of the polymer. Therefore, the subsequent removal of the dissolved polymer results in the formation of individual

cell islands, enabling them to acquire the freedom to move. Cell chips were recorded by a microscope system (Image Xpress, Molecular Devices, US). Areas of cell islands were measured by the software of Image J. Polygon selections were used to recognize the boundary of cells, and both Analyze and Measure were used to calculate the area of the islands. Four repeats were performed in each group.

By now, we set up a SAMcell based pipelining for RNAi high-throughput screening (Figure 2.1b). To detect the transfection efficiency and cross-contamination of this method, we alternatively printed GFP (Green Fluorescent Protein) expressing plasmid and NC (negative control) plasmid in an array of 10 by 10 on one chip. We addressed the issue of cross-contamination by adjusting the size and number of siRNA spots prior to large-scale screening. When a circle had a diameter of approximately 800 μm and its center-center distance was no less than 2.25 mm, no detectable cross-contamination was observed on our chip (Figure 2.1c).

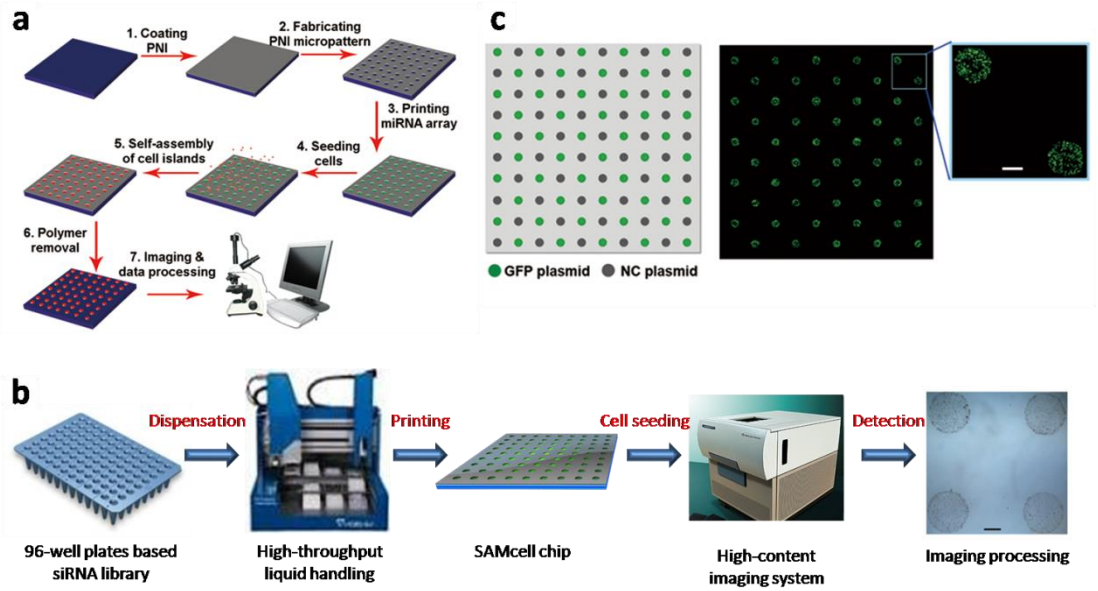


Figure 2.1 Self-assembled cell microarray

a. Fabrication of the self-assembled cell microarray (SAMcell).

b. Pipelining of SAMcell based high-throughput screening. Scale bar: 500 μm.

c. No detectable cross-contamination in neighbouring diagonal positions. GFP expressing plasmid and NC plasmid were alternatively spotted on 10 X 10 arrays in

the microarray. Schematic diagram and fluorescent microscopic images are shown in the left and right panels, respectively. Scale bar: 500 μm.

2.2 SAMcell studying cell migration

We first investigated the capacity of this microarray to effectively detect cell migration. To minimize the effect of cell proliferation on migration, we did not remove PNI until the cells within a given island had become confluent. The data showed that within a certain period, such as 9 hours, the island area increased as a linear function of time, so that the slopes could represent the migratory activities, at least for the three cell lines Hela, HepG2, U2OS (Figure 2.2a). Given the facts that PNI patterns were micro-fabricated on the basis of semiconductor technology and that the self-assembled cell islands were incubated under the same culture conditions, this assay demonstrates clear advantages in reliability and accuracy over the traditional scratch wound healing assays. Five parallel experiments resulted in no more than a 5 % standard deviation for each cell line (Figure 2.2b).

Next, we synthesized a number of siRNAs, each targeting PTEN, RHOG or SGEF, as well as a scrambled siRNA as negative control, and then printed them on a glass slide. The quantitative real-time PCR results validated the silencing efficiency of these siRNAs (Figure 2.2c). As expected, the assay showed that both Hela and U2OS cells moved faster when PTEN was silenced, whereas both cell types migrated more slowly when either RHOG or SGEF was inhibited (Figure 2.2d)[103, 104]. Therefore, through the incorporation of RNAi technology, this microarrary is applicable to large-scale screening of the functional genes regulating cell migration.

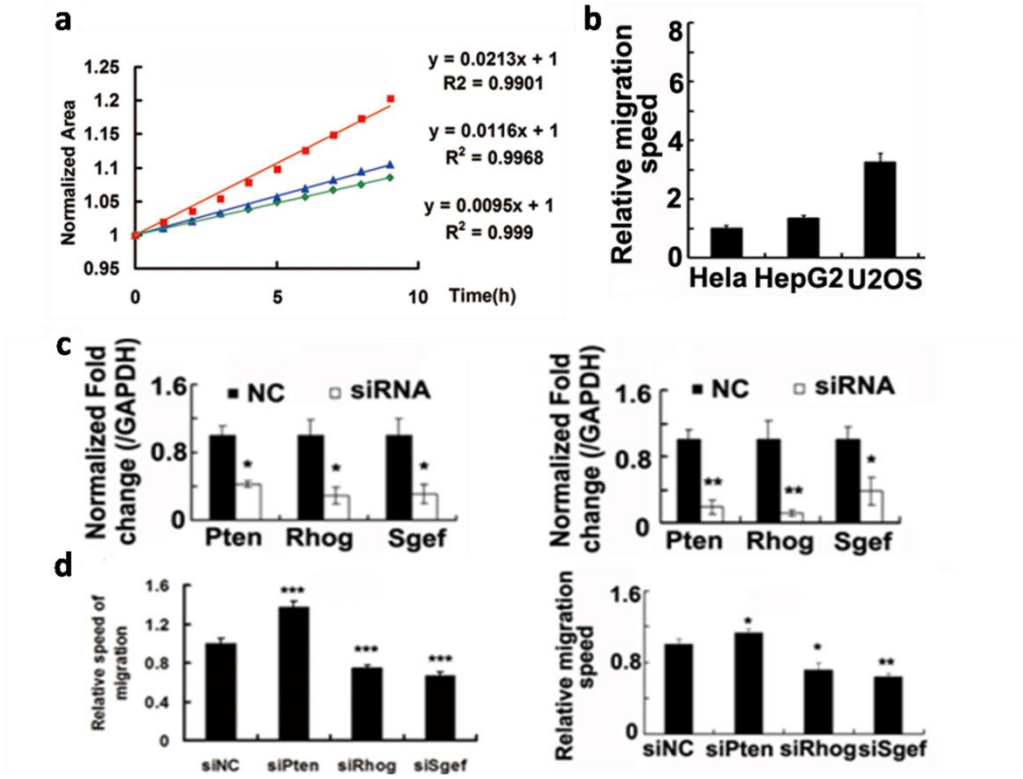


Figure 2.2 The study of cell migration by use of SAMcell

a. The self-assembled cell microarray was applied to study the migration of three types of cells, Hela (green), HepG2 (blue) and U2OS (red).

b. Reliability of the microarray. The migration speed referred to the ratio of island area to time. All speeds were normalized to Hela. n = 5.

c. Real-time PCR results showed that PTEN, RHOG and SGEF could be efficiently silenced by the corresponding siRNAs in Hela (left) or U2OS cells (right), respectively. GAPDH was used as an internal quantification control, and scrambled siRNA as NC. n=3.

d. Cell migration was examined when various genes were silenced in Hela (left) or U2OS cells (right), respectively. n =5.

2.3 Statistical preparation for HTS

We first concluded a formula to calculate the cell island's migration speed, MS= $(A_f-A_i)/T$, where A_f is the final area, A_i is the initial area, and T is the time of the

process. The migration speed treated with one specific siRNA/miRNA was normalized to that treated with scrambled siRNA which played as the negative control. Consequently, we could use the fold change, which is equal to the normalized migration speed, to represent the migratory activity influenced by siRNAs/miRNAs.

Then we printed 40 repeats of scrambled siRNAs in parallel on one chip to calculate the fold changes of cell migration and drew the frequency histogram (Figure 2.3a). Range of fold change is from 0.9 to 1.1, so cut-off for SAMcell migration assay is <0.9 or >1.1. To determine whether data sets come from normal distribution, we used Shapiro-Wilk W-test and Kolmogorov–Smirnov Z-test (JMP9.0 Software), with the null hypothesis that the sample came from a normally distributed population. P(W)=0.854, P(Z)=0.978. The possibilities were so big that we could accept the null hypothesis. That is, the distribution of population was closed to normal distribution.

Similarly, we examined the distributions of the datasets from Transwell migration assay and Transwell invasion assay. We also performed 40 repeats of scrambled siRNAs in parallel and drew the frequency histograms (Figure 2.3b and c). After W-test and Z-test, they were closed to normal distribution. However, the ranges of fold change from both Transwell assays are from 0.8 to 1.2, so cut-off for Transwell migration/invasion assay is <0.8 or >1.2.

When comparing two groups, we took use of unpaired Student's t-tests (two-tailed) to calculate the significance. For all tests, a P value <0.05 was considered significant. Benjamini and Hochberg false discovery rate was used as a correction for multiple testing.

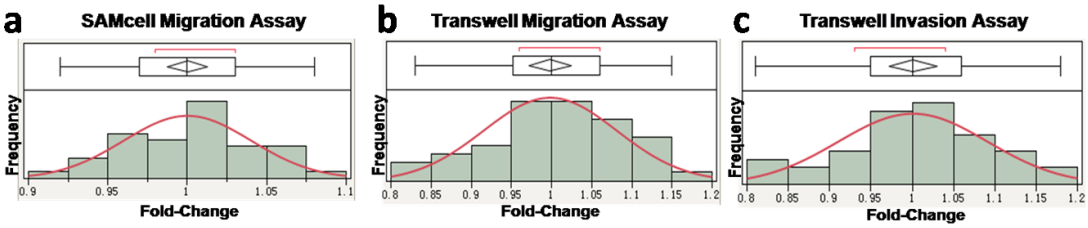


Figure 2.3 Frequency histograms of three assays.

a. SAMcell migration assay. P(W)=0.854, P(Z)=0.978. Range of fold change is from 0.9 to 1.1.

b. Transwell migration assay. P(W)=0.596, P(Z)=0.984. Range of fold change is from 0.8 to 1.2.

c. Transwell invasion assay. P(W)=0.596, P(Z)=0.984. Range of fold change is from 0.8 to 1.2.

2.4 Summary of SAMcell method

In order to perform the migration screening on a large-scale level, we developed a self-assembled cell microarray. Compared to the traditional Transwell assay and wound healing assay, this method demonstrates certain important advantages, including lower cost of transfection reagents, RNAs, antibodies or dyes, higher throughput and more reliable accuracy. Due to a thermally responsive polymer, this microarray has additional important features, such as tailored cell patterns, as well as a clear cell boundary and hence no interference from neighbouring cells. This cell chip is an open system and very easy to use, so all of the procedures are performed only once, but not well by well if using multi-well plates. Cells on one chip are cultured and treated with the same condition, which results in low variation.

We demonstrated this method for investigation of cell migration study. Furthermore, it will be readily adapted to perform large scale screens of functional genes regulating

other phenotypes, such as cell apoptosis, cell autophagy, sugar metabolism, and mitochondria morphology (Figure 2.4).

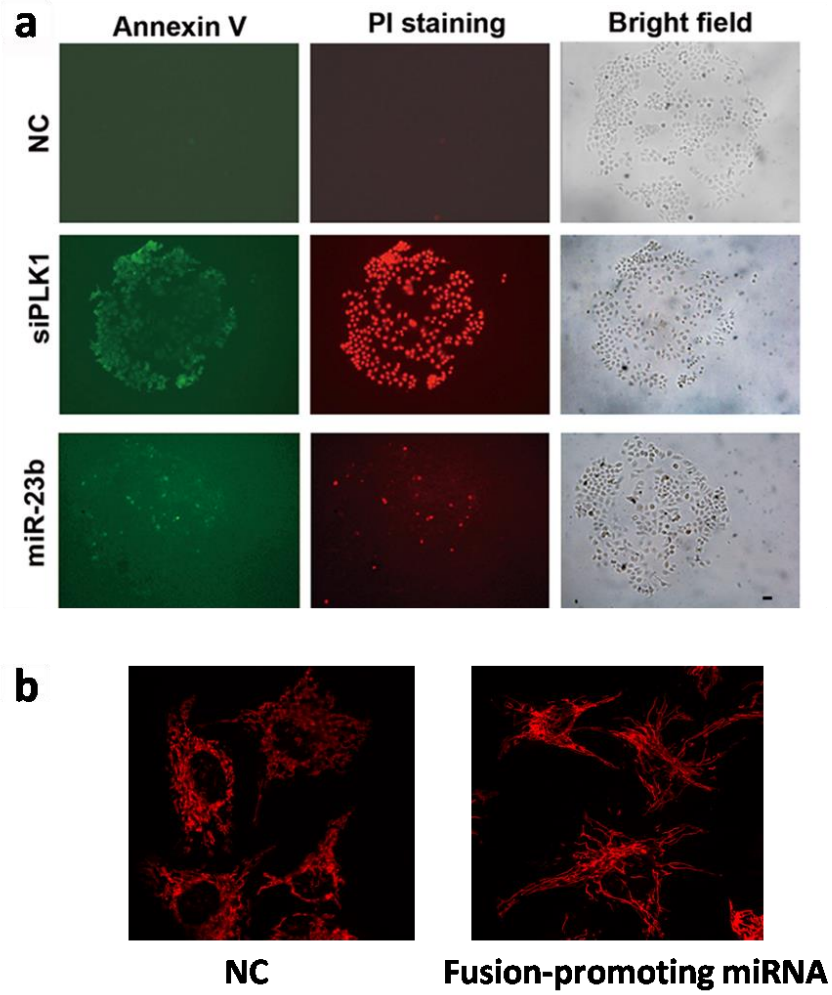


Figure 2.4 Applications of SAMcell

a. Annexin V/PI co-staining assay to detect cell apoptosis. Annexin V (green) indicated cells with apoptosis. PI (red) indicated cells with necrosis.

b. MitoFluorRed589 staining to detect mitochondria morphology.

CHAPTER 3

SCREENING OF HUMAN GENOME MIRNAS REGULATING CANCER METASTASIS

3.1 Screening of miRNAs regulating cell migration

Thanks to the development of SAMcell chip, we set up a HTS system to investigate miRNAs involved in cancer metastasis. We took use of Hela cells (human cervix cancer cells) to perform the screens, because Hela is one of the most popular and stable cell lines. As shown in Figure 3.1a, the functional screen started with migration assays including SAMcell assay for primary screen and Transwell migration assay for validation, followed by gel-coated Transwell invasion assay and apoptosis assay. In total, over 900 miRNAs were investigated (Table 3.1A). The primary screen indicated that 201 miRNA genes are able to regulate cell migration. To assess the quality of our screening method, we validated the positive representative miRNAs (PR-miRNAs) through conventional Transwell migration assay. In total, 182 miRNA genes (20%) demonstrated consistent behaviour in both assays (Figure 3.1b and Table 3.1B). Analysis of the 19 miRNAs exhibiting non-concordant behaviour found that 18 of them exerted a potent effect on cell proliferation, that is, less than 2 % of the false positive results being due to the influence of proliferation in this assay (Table 3.1C). Additionally, we also randomly selected 10 miRNAs incapable of regulating cells migration (negative representative miRNAs, or NR-miRNAs) and individually examined the migratory regulation capability of each miRNA by means of the Transwell migration assay. Unsurprisingly, none of them exhibited any significant change in cell mobility (Figure 3.1c).

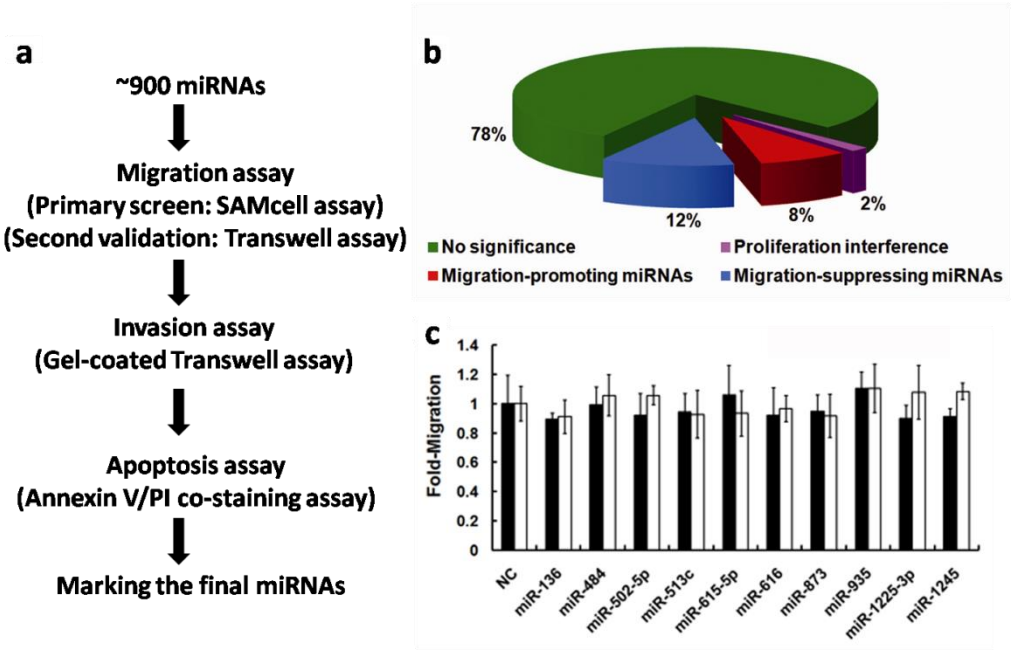


Figure 3.1 Screening of miRNAs regulating cancer cell migration

a. Screening strategy used to perform the functional screen.

b. Pie diagram showed the distribution of the migratory miRNAs. The 2% false positives are mainly due to proliferation.

c. Ten NR miRNAs were confirmed to have no effect on the migration of Hela cells (black bar) by use of the Transwell migration assay. It was also observed that these miRNAs demonstrated the same incapacity to regulate the mobility of MCF-7 cells (white bar).

Table 3.1A List of human miRNAs examined in this work

miR-1	miR-570	miR-1259	miR-106b	miR-28-5p	miR-514b-3p
miR-7	miR-571	miR-1260	miR-106b*	miR-296-3p	miR-514b-5p
miR-9	miR-572	miR-1261	miR-10a	miR-296-5p	miR-515-3p
miR-16	miR-573	miR-1262	miR-10b	miR-299-3p	miR-515-5p
miR-17	miR-575	miR-1263	miR-10b*	miR-299-5p	miR-516a-3p
miR-21	miR-577	miR-1264	miR-1207-3p	miR-29a	miR-516a-5p
miR-22	miR-578	miR-1265	miR-1207-5p	miR-29a*	miR-516b
miR-24	miR-579	miR-1266	miR-122*	miR-29b	miR-516b*
miR-25	miR-580	miR-1267	miR-1224-3p	miR-29c	miR-517*
miR-31	miR-581	miR-1268	miR-1224-5p	miR-301a	miR-517a
miR-32	miR-583	miR-1269	miR-1225-3p	miR-301b	miR-517b
miR-93	miR-584	miR-1270	miR-1225-5p	miR-302a	miR-517c
miR-95	miR-585	miR-1271	miR-1226*	miR-302a*	miR-518a-3p

Table 3.1A Continued					
miR-96	miR-586	miR-1272	miR-1228*	miR-302b	miR-518a-5p
miR-98	miR-587	miR-1273	miR-124*	miR-302b*	miR-518b
miR-100	miR-588	miR-1275	miR-1255a	miR-302c	miR-518c
miR-101	miR-589	miR-1276	miR-1255b	miR-302c*	miR-518c*
miR-103	miR-591	miR-1277	miR-125a-3p	miR-302d	miR-518d-3p
miR-105	miR-592	miR-1278	miR-125a-5p	miR-302d*	miR-518d-5p
miR-107	miR-593	miR-1279	miR-125b	miR-302e	miR-518e
miR-122	miR-595	miR-1280	miR-126*	miR-302f	miR-518e*
miR-124	miR-596	miR-1281	miR-127-3p	miR-3065-3p	miR-518f
miR-126	miR-597	miR-1282	miR-1274a	miR-3065-5p	miR-518f*
miR-128	miR-598	miR-1283	miR-1274b	miR-30a	miR-519a
miR-132	miR-599	miR-1284	miR-127-5p	miR-30b	miR-519a*
miR-134	miR-600	miR-1285	miR-129*	miR-30b*	miR-519b-3p
miR-136	miR-601	miR-1286	miR-129-3p	miR-30c	miR-519b-5p
miR-137	miR-602	miR-1287	miR-129-5p	miR-30c-1*	miR-519c-3p
miR-138	miR-603	miR-1288	miR-130a	miR-30c-2*	miR-519c-5p
miR-141	miR-604	miR-1289	miR-130a*	miR-30d	miR-519d
miR-143	miR-605	miR-1290	miR-130b	miR-30d*	miR-519e
miR-144	miR-606	miR-1291	miR-130b*	miR-30e	miR-519e*
miR-145	miR-607	miR-1292	miR-132*	miR-30e*	miR-520a-3p
miR-147	miR-608	miR-1293	miR-133a	miR-31*	miR-520a-5p
miR-149	miR-609	miR-1294	miR-133b	miR-3126-3p	miR-520b
miR-150	miR-610	miR-1295	miR-135a	miR-3130-3p	miR-520c-3p
miR-152	miR-611	miR-1296	miR-135a*	miR-3130-5p	miR-520c-5p
miR-153	miR-612	miR-1297	miR-135b	miR-3144-3p	miR-520d-3p
miR-154	miR-613	miR-1298	miR-135b*	miR-3144-5p	miR-520d-5p
miR-155	miR-614	miR-1299	miR-136*	miR-3186-5p	miR-520e
miR-182	miR-616	miR-1301	miR-138-1*	miR-3190-5p	miR-520f
miR-184	miR-617	miR-1302	miR-138-2*	miR-320a	miR-520g
miR-185	miR-618	miR-1303	miR-139-3p	miR-320b	miR-520h
miR-186	miR-619	miR-1304	miR-139-5p	miR-320c	miR-522*
miR-187	miR-620	miR-1305	miR-140-3p	miR-323-3p	miR-523*
miR-190	miR-621	miR-1306	miR-140-5p	miR-323-5p	miR-524-3p
miR-191	miR-622	miR-1307	miR-141*	miR-323b-5p	miR-524-5p
miR-192	miR-623	miR-1308	miR-142-3p	miR-324-3p	miR-525-3p
miR-194	miR-624	miR-1321	miR-142-5p	miR-324-5p	miR-526a
miR-195	miR-625	miR-1322	miR-143*	miR-330-3p	miR-526b
miR-197	miR-626	miR-1323	miR-144*	miR-330-5p	miR-526b*
miR-198	miR-627	miR-1324	miR-145*	miR-331-3p	miR-532-3p
miR-202	miR-629	miR-1468	miR-146a	miR-331-5p	miR-532-5p
miR-203	miR-632	miR-1469	miR-146a*	miR-335*	miR-541*
miR-204	miR-634	miR-1470	miR-146b-3p	miR-337-3p	miR-542-3p

Table 3.1A Continued					
miR-206	miR-635	miR-1471	miR-146b-5p	miR-337-5p	miR-542-5p
miR-210	miR-637	miR-1537	miR-148a	miR-338-3p	miR-544b
miR-211	miR-639	miR-1538	miR-148a*	miR-338-5p	miR-545*
miR-212	miR-640	miR-1539	miR-148b	miR-339-3p	miR-548a-3p
miR-214	miR-643	miR-1825	miR-148b*	miR-339-5p	miR-548a-5p
miR-215	miR-644	miR-1826	miR-149*	miR-33a	miR-548b-3p
miR-217	miR-646	miR-1827	miR-150*	miR-33a*	miR-548b-5p
miR-218	miR-647	miR-1908	miR-151-3p	miR-33b	miR-548c-3p
miR-221	miR-649	miR-1909	miR-151-5p	miR-340*	miR-548c-5p
miR-222	miR-650	miR-1910	miR-154*	miR-342-3p	miR-548d-3p
miR-223	miR-655	miR-1911	miR-155*	miR-342-5p	miR-548d-5p
miR-224	miR-656	miR-1912	miR-15a	miR-34a	miR-548e
miR-297	miR-657	miR-1913	miR-15a*	miR-34a*	miR-548f
miR-300	miR-658	miR-1915	miR-15b	miR-34b	miR-548g
miR-325	miR-659	miR-1972	miR-15b*	miR-34b*	miR-548h
miR-328	miR-661	miR-1973	miR-16-1*	miR-34c-3p	miR-548i
miR-329	miR-663	miR-1975	miR-16-2*	miR-34c-5p	miR-548j
miR-335	miR-664	miR-1976	miR-17*	miR-361-3p	miR-548k
miR-340	miR-665	miR-1979	miR-181a	miR-361-5p	miR-548l
miR-345	miR-668	miR-2052	miR-181a*	miR-362-3p	miR-548m
miR-346	miR-670	miR-2053	miR-181a-2*	miR-362-5p	miR-548n
miR-363	miR-675	miR-2054	miR-181b	miR-363*	miR-548o
miR-365	miR-711	miR-2110	miR-181c	miR-365*	miR-548p
miR-367	miR-718	miR-2113	miR-181d	miR-367*	miR-548q
miR-370	miR-720	miR-2114	miR-182*	miR-369-3p	miR-548s
miR-372	miR-758	miR-2115	miR-183*	miR-369-5p	miR-548t
miR-373	miR-759	miR-2116	miR-186*	miR-371-3p	miR-548w
miR-375	miR-761	miR-2117	miR-187*	miR-371-5p	miR-548x
miR-377	miR-762	miR-2276	miR-188-3p	miR-373*	miR-550*
miR-378	miR-764	miR-2277	miR-18a	miR-374a	miR-551a
miR-379	miR-765	miR-2278	miR-18a*	miR-374a*	miR-551b
miR-380	miR-766	miR-2909	miR-18b	miR-374b	miR-551b*
miR-381	miR-802	miR-3119	miR-1909*	miR-374b*	miR-556-3p
miR-382	miR-873	miR-3123	miR-190b	miR-376a	miR-556-5p
miR-383	miR-874	miR-3124	miR-191*	miR-376a*	miR-574-3p
miR-384	miR-887	miR-3127	miR-1915*	miR-376b	miR-574-5p
miR-410	miR-888	miR-3128	miR-193a-3p	miR-376c	miR-576-3p
miR-411	miR-889	miR-3131	miR-193a-5p	miR-377*	miR-576-5p
miR-412	miR-890	miR-3132	miR-193b	miR-378*	miR-582-3p
miR-421	miR-920	miR-3133	miR-195*	miR-378b	miR-582-5p
miR-424	miR-921	miR-3134	miR-196a	miR-379*	miR-589*
miR-425	miR-922	miR-3135	miR-196a*	miR-380*	miR-590-3p

Table 3.1A Continued					
miR-429	miR-924	miR-3136	miR-196b	miR-409-3p	miR-590-5p
miR-431	miR-933	miR-3137	miR-196b*	miR-409-5p	miR-593*
miR-432	miR-934	miR-3138	miR-199a-3p	miR-422a	miR-615-3p
miR-433	miR-935	miR-3139	miR-199a-5p	miR-423-3p	miR-615-5p
miR-448	miR-936	miR-3140	miR-199b-3p	miR-423-5p	miR-616*
miR-451	miR-937	miR-3141	miR-199b-5p	miR-424*	miR-624*
miR-452	miR-938	miR-3142	miR-19a	miR-425*	miR-625*
miR-454	miR-939	miR-3143	miR-19a*	miR-431*	miR-628-3p
miR-466	miR-940	miR-3145	miR-19b	miR-432*	miR-654-3p
miR-484	miR-941	miR-3147	miR-200a	miR-449a	miR-654-5p
miR-488	miR-942	miR-3149	miR-200b	miR-449b	miR-663b
miR-489	miR-943	miR-3150	miR-200b*	miR-449b*	miR-664*
miR-492	miR-944	miR-3151	miR-200c	miR-449c	miR-671-3p
miR-493	miR-1178	miR-3152	miR-202*	miR-449c*	miR-671-5p
miR-494	miR-1179	miR-3153	miR-205*	miR-450a	miR-675*
miR-495	miR-1180	miR-3155	miR-208a	miR-450b-3p	miR-7-2*
miR-496	miR-1181	miR-3156	miR-208b	miR-450b-5p	miR-767-3p
miR-497	miR-1182	miR-3157	miR-20a	miR-452*	miR-767-5p
miR-498	miR-1183	miR-3158	miR-20a*	miR-454*	miR-769-3p
miR-500	miR-1184	miR-3159	miR-20b	miR-455-3p	miR-769-5p
miR-503	miR-1185	miR-3160	miR-20b*	miR-455-5p	miR-770-5p
miR-504	miR-1197	miR-3162	miR-2115*	miR-483-3p	miR-875-3p
miR-505	miR-1200	miR-3163	miR-2116*	miR-483-5p	miR-875-5p
miR-506	miR-1201	miR-3164	miR-216a	miR-485-3p	miR-876-3p
miR-507	miR-1202	miR-3165	miR-216b	miR-485-5p	miR-876-5p
miR-510	miR-1203	miR-3166	miR-218-1*	miR-486-3p	miR-877*
miR-511	miR-1204	miR-3167	miR-218-2*	miR-486-5p	miR-885-3p
miR-514	miR-1205	miR-3168	miR-219-1-3p	miR-487a	miR-885-5p
miR-521	miR-1206	miR-3170	miR-219-2-3p	miR-487b	miR-886-3p
miR-522	miR-1208	miR-3171	miR-219-5p	miR-488*	miR-886-5p
miR-523	miR-1226	miR-3172	miR-22*	miR-490-3p	miR-891a
miR-527	miR-1227	miR-3174	miR-220a	miR-490-5p	miR-891b
miR-539	miR-1228	miR-3175	miR-220b	miR-491-3p	miR-892b
miR-541	miR-1229	miR-3176	miR-220c	miR-491-5p	miR-9*
miR-543	miR-1231	miR-3177	miR-221*	miR-493*	miR-92a
miR-544	miR-1233	miR-3178	miR-222*	miR-497*	miR-92a-1*
miR-545	miR-1234	miR-3181	miR-223*	miR-499-3p	miR-92a-2*
miR-549	miR-1236	miR-3182	miR-224*	miR-499-5p	miR-92b
miR-550	miR-1237	miR-3183	miR-23a	miR-500*	miR-93*
miR-552	miR-1238	miR-3184	miR-23a*	miR-501-3p	miR-96*
miR-553	miR-1243	miR-3187	miR-23b	miR-501-5p	miR-99a
miR-554	miR-1244	miR-3188	miR-23b*	miR-502-3p	miR-99a*

Table 3.1A Continued					
miR-555	miR-1245	miR-3189	miR-24-1*	miR-502-5p	miR-99b
miR-557	miR-1246	miR-3197	miR-24-2*	miR-505*	miR-99b*
miR-558	miR-1247	miR-3198	miR-25*	miR-508-3p	let-7a
miR-559	miR-1248	miR-3199	miR-26a	miR-508-5p	let-7b
miR-561	miR-1249	miR-3200	miR-26a-1*	miR-509-3p	let-7c
miR-562	miR-1250	miR-100*	miR-26a-2*	miR-509-5p	let-7d
miR-563	miR-1251	miR-101*	miR-26b	miR-512-3p	let-7e
miR-564	miR-1252	miR-103-2*	miR-27a	miR-512-5p	let-7f
miR-566	miR-1253	miR-103-as	miR-27a*	miR-513a-3p	let-7g
miR-567	miR-1254	miR-105*	miR-27b	miR-513a-5p	let-7i
miR-568	miR-1256	miR-106a	miR-27b*	miR-513b	
miR-569	miR-1257	miR-106a*	miR-28-3p	miR-513c	

Table 3.1B List of miRNAs capable of regulating cell migration

No.	miRNA	SAMcell chip	Transwell
1	miR-1287	Inhibit ***	Inhibit **
2	miR-1306	Inhibit ***	Inhibit **
3	miR-658	Inhibit ***	Inhibit **
4	miR-1295	Inhibit ***	Inhibit **
5	miR-185	Inhibit ***	Inhibit ***
6	miR-1255a	Inhibit ***	Inhibit **
7	miR-421	Inhibit **	Inhibit **
8	miR-32	Inhibit **	Inhibit **
9	miR-132	Inhibit **	Inhibit **
10	miR-let-7f	Inhibit **	Inhibit *
11	miR-95	Inhibit **	Inhibit **
12	miR-206	Inhibit **	Inhibit ***
13	miR-593*	Inhibit **	Inhibit ***
14	miR-498	Inhibit **	Inhibit **
15	miR-596	Inhibit **	Inhibit **
16	miR-485-5p	Inhibit **	Inhibit *
17	miR-222	Inhibit **	Inhibit *
18	miR-188-3p	Inhibit **	Inhibit **
19	miR-302b	Inhibit **	Inhibit **
20	miR-124	Inhibit **	Inhibit **
21	miR-143	Inhibit **	Inhibit ***
22	miR-145	Inhibit **	Inhibit ***
23	miR-497	Inhibit **	Inhibit **
24	miR-450b-5p	Inhibit **	Inhibit **
25	miR-1469	Inhibit **	Inhibit **
26	miR-let-7e	Inhibit **	Inhibit *

Table 3.1B Continued			
27	miR-1915*	Inhibit **	Inhibit *
28	miR-128	Inhibit *	Inhibit *
29	miR-192	Inhibit *	Inhibit **
30	miR-215	Inhibit *	Inhibit *
31	miR-126*	Inhibit *	Inhibit *
32	miR-33b	Inhibit *	Inhibit *
33	miR-17	Inhibit *	Inhibit *
34	miR-25	Inhibit *	Inhibit **
35	miR-451	Inhibit *	Inhibit ***
36	miR-663	Inhibit *	Inhibit *
37	miR-133a	Inhibit *	Inhibit *
38	miR-23b	Inhibit *	Inhibit **
39	miR-29a*	Inhibit *	Inhibit *
40	miR-320a	Inhibit *	Inhibit *
41	miR-34b	Inhibit *	Inhibit *
42	miR-767-3p	Inhibit *	Inhibit *
43	miR-98	Inhibit *	Inhibit *
44	miR-555	Inhibit *	Inhibit *
45	miR-606	Inhibit *	Inhibit *
46	miR-611	Inhibit *	Inhibit *
47	miR-25*	Inhibit *	Inhibit *
48	miR-204	Inhibit *	Inhibit **
49	miR-221	Inhibit *	Inhibit *
50	miR-1183	Inhibit *	Inhibit *
51	miR-1266	Inhibit *	Inhibit *
52	miR-1324	Inhibit *	Inhibit **
53	miR-512-3p	Inhibit *	Inhibit *
54	miR-34c-5p	Inhibit *	Inhibit *
55	miR-9	Inhibit *	Inhibit *
56	miR-101	Inhibit *	Inhibit **
57	miR-559	Inhibit *	Inhibit *
58	miR-1975	Inhibit *	Inhibit *
59	miR-1207-5p	Inhibit *	Inhibit *
60	miR-200c	Inhibit *	Inhibit **
61	miR-29b	Inhibit *	Inhibit *
62	miR-34a	Inhibit *	Inhibit **
63	miR-671-5p	Inhibit *	Inhibit *
64	miR-let-7b	Inhibit *	Inhibit *
65	miR-548m	Inhibit *	Inhibit **
66	miR-190	Inhibit *	Inhibit **
67	miR-31	Inhibit *	Inhibit **
68	miR-661	Inhibit *	Inhibit *

Table 3.1B Continued			
69	miR-125a-5p	Inhibit *	Inhibit *
70	miR-200b	Inhibit *	Inhibit *
71	miR-490-5p	Inhibit *	Inhibit *
72	miR-517a	Inhibit *	Inhibit *
73	miR-548d-3p	Inhibit *	Inhibit *
74	miR-let-7a	Inhibit *	Inhibit **
75	miR-133b	Inhibit *	Inhibit **
76	miR-200a	Inhibit *	Inhibit **
77	miR-22	Inhibit *	Inhibit **
78	miR-1201	Inhibit *	Inhibit *
79	miR-30c-1*	Inhibit *	Inhibit *
80	miR-320c	Inhibit *	Inhibit *
81	miR-548e	Inhibit *	Inhibit *
82	miR-126	Inhibit *	Inhibit **
83	miR-138	Inhibit *	Inhibit **
84	miR-586	Inhibit *	Inhibit *
85	miR-1307	Inhibit *	Inhibit *
86	miR-29a	Inhibit *	Inhibit **
87	miR-29c	Inhibit *	Inhibit **
88	miR-373*	Inhibit *	Inhibit **
89	miR-548h	Inhibit *	Inhibit *
90	miR-1181	Inhibit *	Inhibit *
91	miR-320b	Inhibit *	Inhibit *
92	miR-1	Inhibit *	Inhibit *
93	miR-7	Inhibit *	Inhibit *
94	miR-297	Inhibit *	Inhibit **
95	miR-433	Inhibit *	Inhibit *
96	miR-492	Inhibit *	Inhibit *
97	miR-592	Inhibit *	Inhibit *
98	miR-18b	Inhibit *	Inhibit **
99	miR-208b	Inhibit *	Inhibit *
100	miR-22*	Inhibit *	Inhibit **
101	miR-26a	Inhibit *	Inhibit **
102	miR-552	Inhibit *	Inhibit *
103	miR-554	Inhibit *	Inhibit **
104	miR-1262	Inhibit *	Inhibit *
105	miR-1979	Inhibit *	Inhibit *
106	miR-199b-5p	Inhibit *	Inhibit *
107	miR-let-7g	Inhibit *	Inhibit *
108	miR-937	Inhibit *	Inhibit *
109	miR-523*	Inhibit *	Inhibit *
110	miR-20a*	Inhibit *	Inhibit *

Table 3.1B Continued			
111	miR-19b	Promote *	Promote **
112	miR-20a	Promote *	Promote *
113	miR-518d-5p	Promote *	Promote *
114	miR-516b*	Promote *	Promote **
115	miR-548g	Promote *	Promote *
116	miR-367	Promote *	Promote **
117	miR-575	Promote *	Promote **
118	miR-612	Promote *	Promote *
119	miR-1283	Promote *	Promote *
120	miR-202*	Promote *	Promote **
121	miR-513a-3p	Promote *	Promote ***
122	miR-548f	Promote *	Promote *
123	miR-103	Promote *	Promote *
124	miR-106b	Promote *	Promote *
125	miR-135b	Promote *	Promote *
126	miR-30d	Promote *	Promote *
127	miR-518f*	Promote *	Promote *
128	miR-548k	Promote *	Promote **
129	miR-210	Promote *	Promote *
130	miR-218	Promote *	Promote **
131	miR-219-5p	Promote *	Promote *
132	miR-28-5p	Promote *	Promote *
133	miR-296-3p	Promote *	Promote *
134	miR-302d	Promote *	Promote *
135	miR-574-5p	Promote *	Promote *
136	miR-590-5p	Promote *	Promote *
137	miR-107	Promote *	Promote **
138	miR-539	Promote *	Promote **
139	miR-550	Promote *	Promote **
140	miR-1250	Promote *	Promote **
141	miR-193a-3p	Promote *	Promote **
142	miR-335*	Promote *	Promote ***
143	miR-483-5p	Promote *	Promote **
144	miR-1323	Promote *	Promote *
145	miR-181b	Promote *	Promote ***
146	miR-511	Promote *	Promote *
147	miR-1227	Promote *	Promote *
148	miR-151-5p	Promote *	Promote *
149	miR-520c-5p	Promote *	Promote **
150	miR-99a	Promote *	Promote *
151	miR-373	Promote *	Promote **
152	miR-501-3p	Promote *	Promote **

Table 3.1B Continued			
153	miR-153	Promote **	Promote *
154	miR-340	Promote **	Promote *
155	miR-665	Promote **	Promote *
156	miR-93	Promote **	Promote **
157	miR-155	Promote **	Promote **
158	miR-429	Promote **	Promote *
159	miR-130b	Promote **	Promote *
160	miR-196a	Promote **	Promote **
161	miR-27b	Promote **	Promote **
162	miR-302b*	Promote **	Promote *
163	miR-30b	Promote **	Promote *
164	miR-211	Promote **	Promote *
165	miR-494	Promote **	Promote **
166	miR-20b	Promote **	Promote *
167	miR-369-5p	Promote **	Promote **
168	miR-522*	Promote **	Promote ***
169	miR-520c-3p	Promote **	Promote **
170	miR-99b	Promote **	Promote **
171	miR-154	Promote **	Promote **
172	miR-1913	Promote **	Promote *
173	miR-593	Promote **	Promote *
174	miR-367*	Promote **	Promote **
175	miR-339-5p	Promote **	Promote *
176	miR-632	Promote **	Promote ***
177	miR-214	Promote ***	Promote ***
178	miR-510	Promote ***	Promote **
179	miR-639	Promote ***	Promote ***
180	miR-10a	Promote ***	Promote **
181	miR-21	Promote ***	Promote ***
182	miR-10b	Promote ***	Promote ***

Note: SAMcell chip, * 10%-20%; ** 20%-30%; *** >30%. Transwell, * 15%-30%; ** 30%-60%; *** >60%.

Table 3.1C List of miRNAs demonstrating non-concordant behaviors

No.	miRNA	SAMcell chip	Transwell	Proliferation
1	miR-345	Inhibit *	-	-
2	miR-222*	Inhibit *	-	Inhibit *
3	miR-217	Inhibit *	-	Inhibit *
4	miR-579	Inhibit *	-	Inhibit *

Table 3.1C Continued				
5	miR-335	Inhibit *	-	Inhibit *
6	miR-583	Inhibit *	Promote *	Inhibit *
7	miR-518a-5p	Inhibit *	-	Inhibit *
8	miR-302c*	Inhibit *	-	Inhibit *
9	miR-524-5p	Inhibit *	-	Inhibit *
10	miR-let-7c	Inhibit *	-	Inhibit *
11	miR-376c	Inhibit *	Promote *	Inhibit *
12	miR-28-3p	Promote *	Inhibit *	Promote *
13	miR-520e	Promote *	-	Promote *
14	miR-20b*	Promote *	Inhibit *	Promote *
15	miR-26b	Promote *	Inhibit *	Promote *
16	miR-194	Promote *	-	Promote **
17	miR-134	Promote *	-	Promote *
18	miR-220a	Promote **	Inhibit *	Promote **
19	miR-591	Promote **	Inhibit *	Promote **

Note: SAMcell chip, * 10%-20%; ** 20%-30%; *** >30%. Transwell, * 15%-30%; ** 30%-60%; *** >60%. Proliferation, *10%-20%; ** >20%.

3.2 General regulation of miRNA on cell migration

To investigate whether the screening results, including both the negative and positive ones, are applicable to other cells in addition to Hela cells, we first delivered the above-mentioned NR-miRNAs into MCF-7 cells, which are human breast cancer cells. Unsurprisingly, these miRNAs consistently demonstrated an inability to regulate the mobility of MCF-7 (Figure 3.1c). We then randomly selected 20 miRNAs from the screening list as positive representative miRNAs (PR-miRNAs), and examined their migratory regulation capacity in three other epithelial cancer cell lines, MCF-7 (human breast cancer cell), Panc-1 (human pancreas cancer cell) and HCT 116 (human colon cancer cell). It was observed that almost all of them exhibited the same regulatory capacity in these three cell lines as in Hela cell line, with the exception of a few miRNAs which did not have any evident impact (Figure 3.2a). Taken together, these results imply that the screening results based on Hela cells may be applicable to other epithelial cancer cell lines, and thus are of potentially general use. These

migratory miRNAs exhibit versatility in diverse cell types, including four types of epithelial cells as well as HUVEC cells (Figure 3.2b), which are human umbilical vein endothelial cells, thus being of general functionality. Given the fact that cell migration is a basic biological process, we hypothesize that a general regulation of migratory behaviour seems to be another distinctive feature of miRNAs.

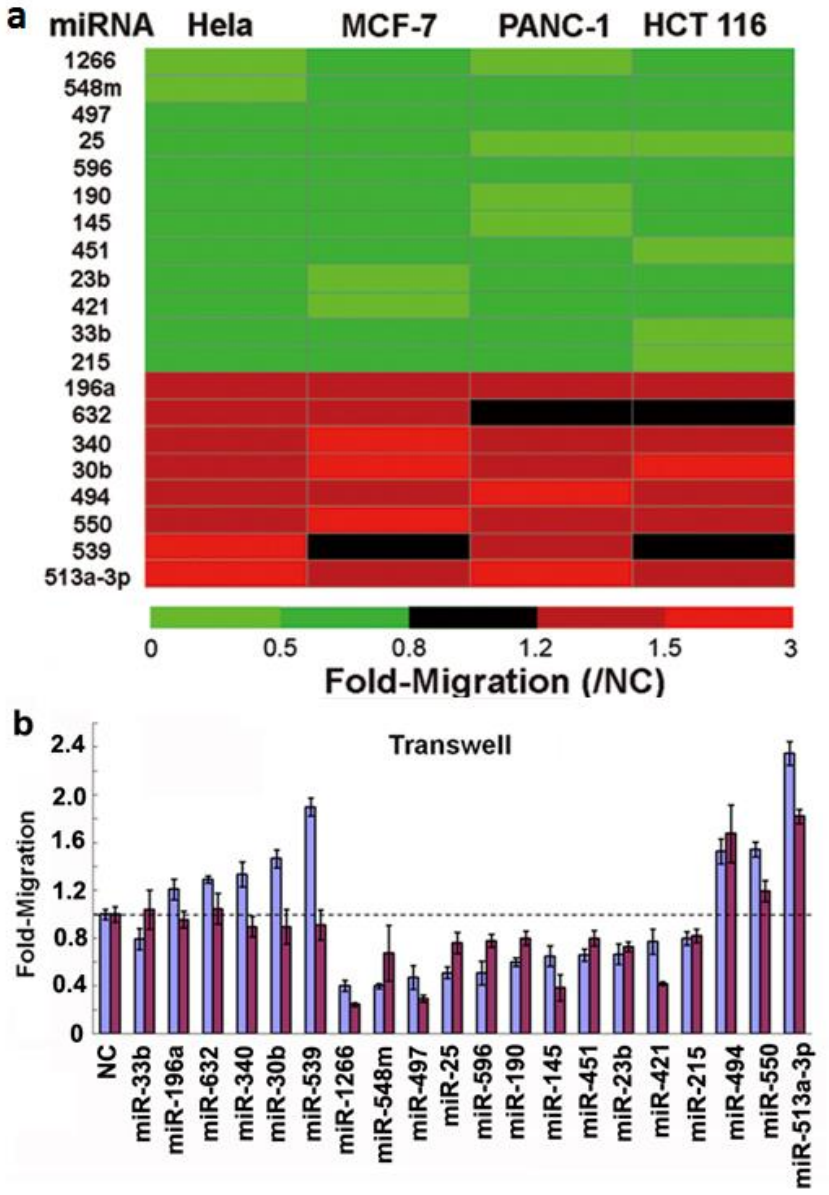


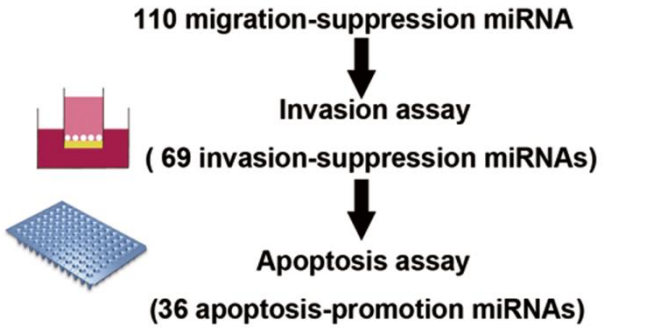
Figure 3.2 General regulation of miRNA on cell migration

a. General regulation of miRNA on the migratory behaviours. Noticeably, no miRNAs exhibited a contrasting migratory regulation capacity. Transwell cell migration assay was used here. Red, green or black means up-regulated activity, down-regulated activity, or no significant change, respectively.

b. Migration assays of Hela (blue bar) and HUVEC cells (red bar) transfected with indicated miRNAs. Noticeably, no miRNAs exhibited a contrasting migratory regulation capacity.

3.3 Results of the miRNA functional screens

Next, we concentrated on the migration-suppressing miRNAs and investigated their capacity to regulate cell invasion or apoptosis, since most miRNAs are pathologically down-expressed in cancer samples. The results showed that 69 of these miRNAs significantly suppressed invasion *in vitro* (Table 3.3A), among which 36 miRNAs were demonstrated to be capable of inducing apoptosis (Figure 3.3 and Table 3.3B). Importantly, dozens of well characterized miRNAs, including miR-101, the miR-200 family and let-7a/b are included in our screening results. Each of the miRNAs was ranked by the capacity to regulate migration, invasion and apoptosis activity. Our data show that miR-23b is capable of not only inhibiting both migration and invasion, but also inducing apoptosis effectively. According to the formula defined in Table 3.3B, miR-23b presents the highest score among all the miRNAs identified.



miR-22	miR-101	miR-128	miR-138	miR-143	miR-145
miR-185	miR-190	miR-206	miR-421	miR-433	miR-451
miR-497	miR-559	miR-1181	miR-1201	miR-1266	miR-1469
miR-1979	miR-1255a	miR-200a	miR-200b	miR-200c	miR-22*
miR-23b	miR-26a	miR-29a	miR-29a*	miR-29c	miR-33b
miR-34a	miR-34b	miR-34c-5p	miR-593*	let-7a	let-7b

Figure 3.3 Results of the miRNA functional screens.

miRNAs previously reported to regulate cell migration, invasion or apoptosis are highlighted in blue. miR-23b in red is the final gene with the highest scores.

Table 3.3A List of miRNAs capable of inhibiting cell invasion

No.	miRNA	Migration	Invasion
1	miR-1	Inhibit *	-
2	miR-7	Inhibit *	Inhibit *
3	miR-9	Inhibit *	-
4	miR-17	Inhibit *	Inhibit **
5	miR-22	Inhibit **	Inhibit *
6	miR-25	Inhibit **	Inhibit **
7	miR-31	Inhibit **	Inhibit **
8	miR-32	Inhibit **	Inhibit ***
9	miR-95	Inhibit **	-
10	miR-98	Inhibit *	Inhibit *
11	miR-101	Inhibit **	Inhibit *
12	miR-124	Inhibit **	Inhibit **
13	miR-126	Inhibit **	Inhibit *
14	miR-128	Inhibit *	Inhibit ***
15	miR-132	Inhibit **	-
16	miR-138	Inhibit **	Inhibit ***
17	miR-143	Inhibit ***	Inhibit **
18	miR-145	Inhibit ***	Inhibit **
19	miR-185	Inhibit ***	Inhibit ***
20	miR-190	Inhibit **	Inhibit **
21	miR-192	Inhibit **	Inhibit *
22	miR-204	Inhibit **	-
23	miR-206	Inhibit ***	Inhibit **
24	miR-215	Inhibit *	Inhibit **
25	miR-221	Inhibit *	-
26	miR-222	Inhibit *	-
27	miR-297	Inhibit **	-
28	miR-421	Inhibit **	Inhibit **
29	miR-433	Inhibit *	Inhibit *
30	miR-451	Inhibit ***	Inhibit ***
31	miR-492	Inhibit *	Inhibit *
32	miR-497	Inhibit **	Inhibit **
33	miR-498	Inhibit **	Inhibit **
34	miR-552	Inhibit *	Inhibit ***
35	miR-554	Inhibit **	-

Table 3.3A Continued			
36	miR-555	Inhibit *	-
37	miR-559	Inhibit *	Inhibit *
38	miR-586	Inhibit *	-
39	miR-592	Inhibit *	-
40	miR-596	Inhibit **	Inhibit **
41	miR-606	Inhibit *	-
42	miR-611	Inhibit *	Inhibit **
43	miR-658	Inhibit **	-
44	miR-661	Inhibit *	-
45	miR-663	Inhibit *	-
46	miR-937	Inhibit *	Inhibit **
47	miR-1181	Inhibit *	Inhibit ***
48	miR-1183	Inhibit *	-
49	miR-1201	Inhibit *	Inhibit **
50	miR-1262	Inhibit *	-
51	miR-1266	Inhibit *	Inhibit ***
52	miR-1287	Inhibit **	-
53	miR-1295	Inhibit **	-
54	miR-1306	Inhibit **	Inhibit ***
55	miR-1307	Inhibit *	Inhibit ***
56	miR-1324	Inhibit **	-
57	miR-1469	Inhibit **	Inhibit ***
58	miR-1975	Inhibit *	Inhibit ***
59	miR-1979	Inhibit *	Inhibit ***
60	miR-1207-5p	Inhibit *	Inhibit ***
61	miR-1255a	Inhibit **	Inhibit ***
62	miR-125a-5p	Inhibit *	Inhibit **
63	miR-126*	Inhibit *	-
64	miR-133a	Inhibit *	-
65	miR-133b	Inhibit **	-
66	miR-188-3p	Inhibit **	Inhibit **
67	miR-18b	Inhibit **	-
68	miR-1915*	Inhibit *	-
69	miR-199b-5p	Inhibit *	-
70	miR-200a	Inhibit **	Inhibit **
71	miR-200b	Inhibit *	Inhibit **
72	miR-200c	Inhibit **	Inhibit **
73	miR-208b	Inhibit *	Inhibit **
74	miR-20a*	Inhibit *	-
75	miR-22*	Inhibit **	Inhibit **
76	miR-23b	Inhibit **	Inhibit ***
77	miR-25*	Inhibit *	Inhibit **

Table 3.3A Continued			
78	miR-26a	Inhibit **	Inhibit *
79	miR-29a	Inhibit **	Inhibit *
80	miR-29a*	Inhibit *	Inhibit *
81	miR-29b	Inhibit *	-
82	miR-29c	Inhibit **	Inhibit **
83	miR-302b	Inhibit **	-
84	miR-30c-1*	Inhibit *	-
85	miR-320a	Inhibit *	-
86	miR-320b	Inhibit *	-
87	miR-320c	Inhibit *	-
88	miR-33b	Inhibit *	Inhibit **
89	miR-34a	Inhibit **	Inhibit *
90	miR-34b	Inhibit *	Inhibit *
91	miR-34c-5p	Inhibit *	Inhibit *
92	miR-373*	Inhibit **	Inhibit *
93	miR-450b-5p	Inhibit **	-
94	miR-485-5p	Inhibit *	Inhibit **
95	miR-490-5p	Inhibit *	Inhibit **
96	miR-512-3p	Inhibit *	Inhibit *
97	miR-517a	Inhibit *	-
98	miR-523*	Inhibit *	-
99	miR-548d-3p	Inhibit *	Inhibit *
100	miR-548e	Inhibit *	Inhibit *
101	miR-548h	Inhibit *	Inhibit *
102	miR-548m	Inhibit **	Inhibit ***
103	miR-593*	Inhibit ***	Inhibit ***
104	miR-671-5p	Inhibit *	-
105	miR-767-3p	Inhibit *	-
106	miR-let-7a	Inhibit **	Inhibit **
107	miR-let-7b	Inhibit *	Inhibit **
108	miR-let-7e	Inhibit *	Inhibit **
109	miR-let-7f	Inhibit *	-
110	miR-let-7g	Inhibit *	-

Note: Migration & Invasion, - no significance; * 15%-30%; ** 30%-60%; *** >60%.

Table 3.3B List of miRNAs capable of inhibiting cell migration, invasion and promoting apoptosis

No.	miRNA	Migration	Invasion	Apoptosis	Total scores

Table 3.3B Continued					
1	miR-22	Inhibit **	Inhibit *	Promote **	5*
2	miR-101	Inhibit **	Inhibit *	Promote **	5*
3	miR-128	Inhibit *	Inhibit ***	Promote **	6*
4	miR-138	Inhibit **	Inhibit ***	Promote *	6*
5	miR-143	Inhibit ***	Inhibit **	Promote **	7*
6	miR-145	Inhibit ***	Inhibit **	Promote **	7*
7	miR-185	Inhibit ***	Inhibit ***	Promote *	7*
8	miR-190	Inhibit **	Inhibit **	Promote *	5*
9	miR-206	Inhibit ***	Inhibit **	Promote *	6*
10	miR-421	Inhibit **	Inhibit **	Promote *	5*
11	miR-433	Inhibit *	Inhibit *	Promote *	3*
12	miR-451	Inhibit ***	Inhibit ***	Promote *	7*
13	miR-497	Inhibit **	Inhibit **	Promote *	5*
14	miR-559	Inhibit *	Inhibit *	Promote *	3*
15	miR-1181	Inhibit *	Inhibit ***	Promote *	5*
16	miR-1201	Inhibit *	Inhibit **	Promote *	4*
17	miR-1266	Inhibit *	Inhibit ***	Promote *	5*
18	miR-1469	Inhibit **	Inhibit ***	Promote *	6*
19	miR-1979	Inhibit *	Inhibit ***	Promote *	5*
20	miR-1255a	Inhibit **	Inhibit ***	Promote *	6*
21	miR-200a	Inhibit **	Inhibit **	Promote *	5*
22	miR-200b	Inhibit *	Inhibit **	Promote *	4*
23	miR-200c	Inhibit **	Inhibit **	Promote *	5*
24	miR-22*	Inhibit **	Inhibit **	Promote *	5*
25	**miR-23b**	**Inhibit ** **	**Inhibit *** **	**Promote *** **	**8***
26	miR-26a	Inhibit **	Inhibit *	Promote ***	6*
27	miR-29a	Inhibit **	Inhibit *	Promote **	5*
28	miR-29a*	Inhibit *	Inhibit *	Promote *	3*
29	miR-29c	Inhibit **	Inhibit **	Promote **	6*
30	miR-33b	Inhibit *	Inhibit **	Promote *	4*
31	miR-34a	Inhibit **	Inhibit *	Promote **	5*
32	miR-34b	Inhibit *	Inhibit *	Promote *	3*
33	miR-34c-5p	Inhibit *	Inhibit *	Promote **	4*
34	miR-593*	Inhibit ***	Inhibit ***	Promote *	7*
35	miR-let-7a	Inhibit **	Inhibit **	Promote **	6*
36	miR-let-7b	Inhibit *	Inhibit **	Promote **	5*

Note: Migration & Invasion, - no significance; * 15%-30%; ** 30%-60%; *** >60%. Apoptosis, * 25%-50%; ** 50%-100%; *** >100%. All data presented here stands for the decrement or increment compared to NC experimental data (scrambled siRNA): first normalized to NC data, and subtracted to 100 %. Total

score= Star No. of migration + Star No. of invasion + Star of apoptosis. miR-23b highlighted in red is the molecule with the highest score.

3.4 miR-23b inhibiting metastasis-related traits

Since miR-23b was the most powerful molecule to inhibiting cell motility and apoptosis in our screening results, we then deeply investigated its impact on many metastasis-related traits. An antagomir is a small synthetic RNA that is perfectly complementary to the specific miRNA target with either mispairing at the cleavage site of Ago2 or some sort of base modification to inhibit Ago2 cleavage[105]. We took use of miR-23b's antagomir to silence endogenous miRNA. Ectopic expression of miR-23b produced a potent inhibition of the cell migration and invasion, but its antagomir increased the migratory and invasive activities by more than 40 % (Figure 3.4a). Next, we also investigated the capability of cell proliferation regulated by miR-23b and its antagomir. We found that miR-23b resulted in a lower cell number over time but its antagomir distinctly increased the proliferation activity after 48 hours (Figure 3.4b and c). Furthermore, we took use of Annexin V/PI co-staining assay to detect whether miR-23b regulated cell apoptosis. Obviously, over-expression of miR-23b induced increasing ratio of cells with apoptosis and necrosis (Figure 3.4d). Anoikis is a form of programmed cell death which is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix[106]. Our results showed that miR-23b resulted in higher ratio of anoikis-mediated cell death by more than 40% (Figure 3.4e).

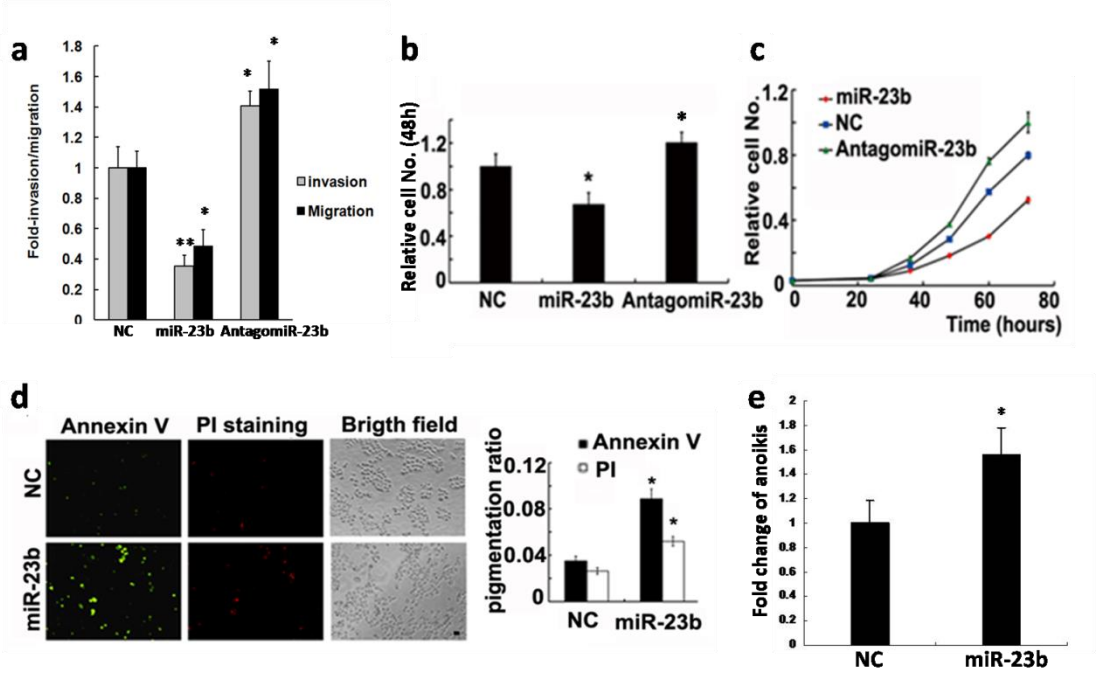


Figure 3.4 miR-23b inhibiting metastasis-related traits

a. Migration (black bar) or invasion (grey bar) assay after transfection with the indicated RNAs in HCT 116 cells. n = 3.

b. Proliferation assay after transfection with the indicated RNAs in HCT 116 cells at 48 hours. n=3.

c. Proliferation assay after transfection with the indicated RNAs in HCT 116 cells at different time points. n=3.

d. Apoptosis assay after transfection with the indicated RNAs in HCT 116 cells after 48 hours (quantified left). Annexin V (green) indicated cells with apoptosis. PI (red) indicated cells with necrosis. n=3. Scale bar: 50 μm.

e. Anoikis assay after transfection with the indicated RNAs in HCT 116 cells after 48 hours. n=3.

3.5 miR-23b repressing EMT

Since epithelial to mesenchymal transition (EMT) is viewed as an essential early step in cancer metastasis, we examined whether miR-23b might regulate EMT through immunofluorescene assay. Immunofluorescene visualisation showed that miR-23b expression up-regulated E-cadherin (marker of epithelial stage) and down-regulated Vimentin (marker of mesenchymal stage), suggesting that the miR-23b may repress EMT process in cells (Figure 3.5a). We also took use of western blot assays to confirm this discovery. Obviously, over-expression of miR-23b resulted in higher E-cadherin level by 100% and lower Vimentin level by 60% (Figure 3.5b). These findings suggested that miR-23b might work at the early stage of cancer metastasis.

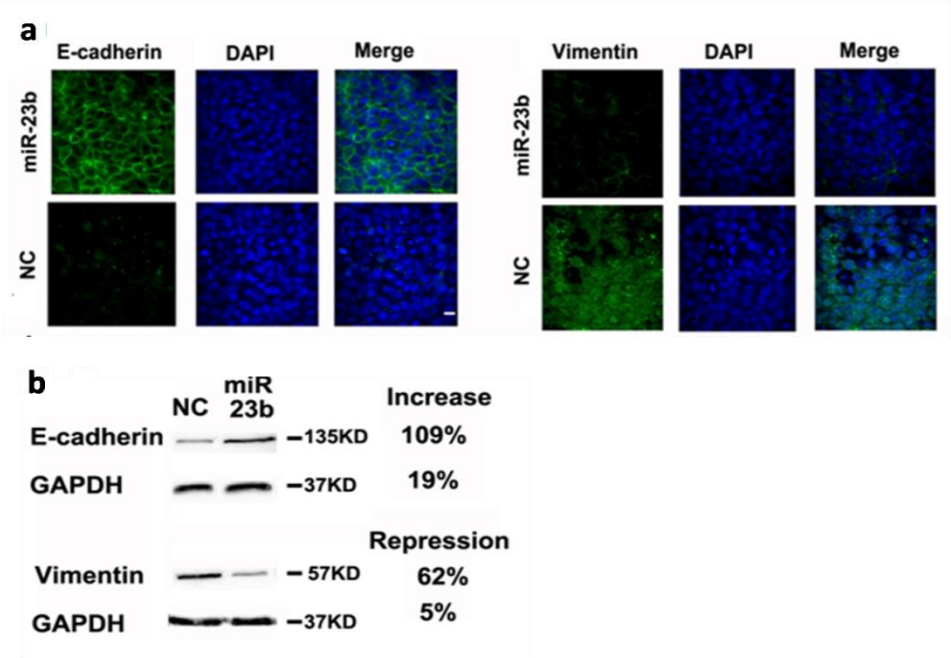


Figure 3.5 miR-23b repressing EMT

a. Manipulation of the miR-23b levels repressed the EMT. E-cadherin and Vimentin were detected by antibodies (green). Nuclei were detected by DAPI (blue). Scale bar: 50 μm.

b. Western blot assays showed the expression levels of E-cadherin and Vimentin in response to miR-23b. GAPDH was used as loading control.

3.6 Expression patterns of miR-23b

miR-23b is expressed in diverse colon cell lines. To determine whether the expression levels of miR-23b affect metastasis-relevant activities in human colon cancer, we selected seven human colon cancer cell lines, including HT29, LOVO, SW480, HCT116, HCT8, LS174T and SW620. HCT 116 has a lower expression level of miR-23b than HT29, LOVO or SW480, but a higher expression level than HCT29, LS174T or SW620 (Figure 3.6a). These cell lines demonstrated the reverse capability of migration and invasion to miR-23b levels (Figure 3.6b).

To statistically investigate the expression profile of miR-23b, we collected more than 100 of human colorectal tumor and normal adjunct tissue samples. Through a real-time detection method[107], expression analysis of miR-23b in these samples showed a higher mean expression in the normal samples ($P < 3.0 \text{ X } 10^{-4}$) (Figure 3.6c). Furthermore, we divided the tumor samples into two groups according to whether they are obtained from metastasis-free or metastasis-positive patients, where metastasis positive means metastasic tumor has been identified, metastasis free means none metastasic tumor has been identified in surrounding ECM. We found that the mean expression level of miR-23b was 2-fold lower in the metastatic-free samples than the normal samples ($P_{13} < 2.0 \text{ X } 10^{-6}$) (Figure 3.6d). Unexpectedly, the mean expression of miR-23b was higher in the metastasis-positive samples compared to the metastasis-free samples ($P_{23} < 0.01$), although it was still lower compared to the levels in the normal samples ($P_{12} < 0.05$). Mann-Whitney U-test was used for calculating P-values, when two groups were compared. The P-value of the metastasis-free samples relative to the normal samples was much lower than the metastatic samples relative to either the normal or metastasis-free ones (that is, $P_{13} << P_{12}$ or P_{23}) (Figure 3.6d), thus indicating a significant association between the expression level of miR-23b and the early stage of colon cell progression towards tumor states.

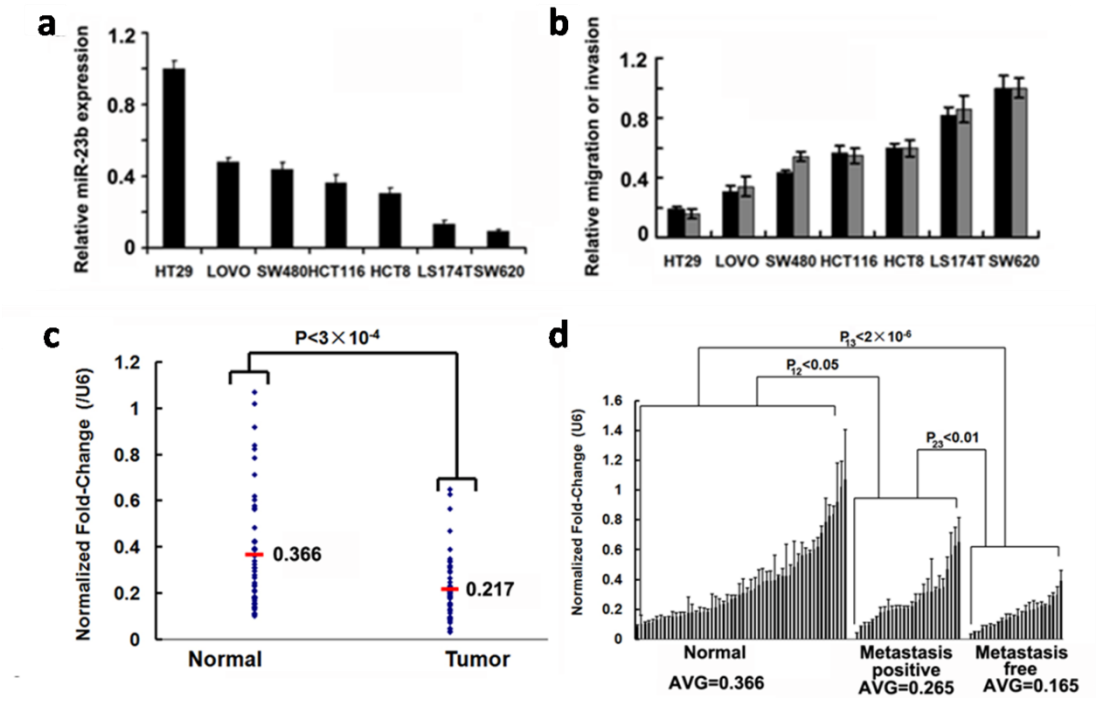


Figure 3.6 miR-23b levels in human colon cancer cells and samples

a. miR-23b levels in human colon cancer cell lines. U6 was used as an internal quantification control. n=3.

b. The migration (black bar) or invasion (grey bar) activities of human colon cancer cell lines. n=5.

c. Real-time PCR results for miR-23b in 104 human colon tumors or normal adjunct samples. There was a significant difference in miR-23b expression between these two groups (P < 3.0 X 10-4) (Mann-Whitney U-test). n=3.

d. The same samples as in panel c were divided into three groups: normal adjunct samples, metastasis-positive cancer sample, and metastasis-free samples. There was a significant difference in miR-23b expression between these three groups ($P_{13} < 2X10^{-6}$, $P_{23} < 0.05$, $P_{12} < 0.01$) (Mann-Whitney U-test). n=3.

3.7 miR-23b regulating a cohort of prometastatic genes

To unveil the molecular mechanism of miR-23b, we employed three algorithms that predict the targets of miRNA, such as PicTar[108], TargetScan [109] and miRDB

[110]. More than 100 genes may be regulated by miR-23b on the basis of an agreement between at least two algorithms. We cloned the 3' UTR of 28 putative miR-23b targets into a luciferase construct and performed quantitative examination of the luciferase activities. Reporter assays with miR-23b expressing HCT 116 cells revealed that a dozen putative targets were silenced by 35-75 % (Figure 3.7a). Mutation of the putative miR-23b site(s) in the 3' UTR of FZD7, MAP3K1 (MEKK1), PAK2, TGFβR2, RRAS2 or uPA resulted in an abrogated responsiveness to miR-23b (Figure 3.7b). The mutation of the miR-23b site(s) leads to almost identical activity to the wild-type reporters, suggesting the putative site(s) in the UTR of those genes is the main site targeted by miR-23b. Furthermore, the protein levels of these six genes were assayed in HCT 116 cells. Over-expression of miR-23b reduced the levels of these proteins by 35-70 % (Figure 3.7c).

By now, we concluded the signaling pathways mediated by miR-23b in cancer cells (Figure 3.7d). miR-23b directly targets MAP3K1, RRAS2, PAK2, TGFBR2 and FZD7, resulting in negative regulation of NFKB, JNK, ERK, PI3K and ROCK1 pathways. Consequently, cancer cell migration, invasion, and proliferation are promoted, whereas apoptosis is repressed.

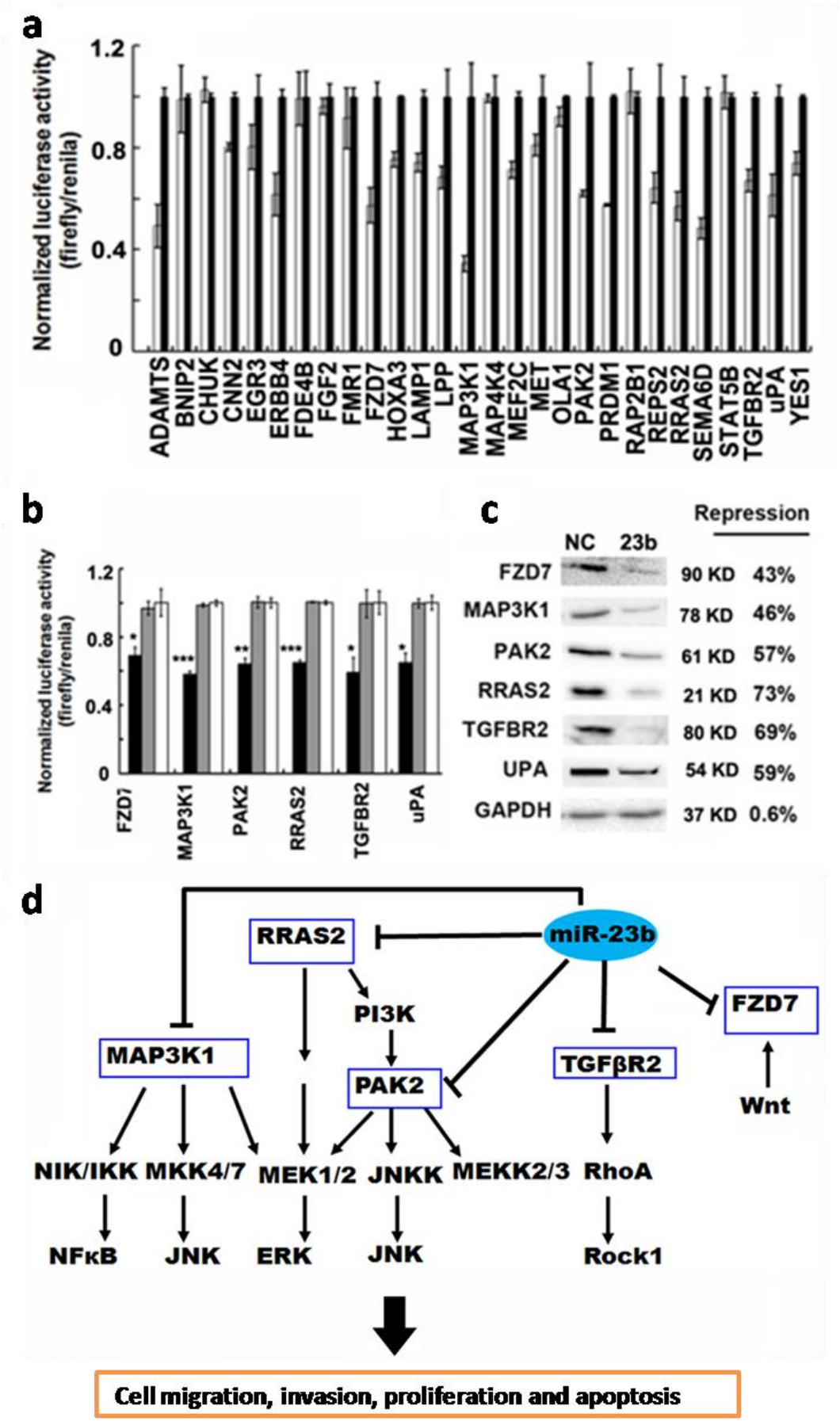

a
Normalized luciferase activity (firefly/renila)
1.2
0.8
0.4
0
ADAMTS
BNIP2
CHUK
CNN2
EGR3
ERBB4
FDE4B
FGF2
FMR1
FZD7
HOXA3
LAMP1
LPP
MAP3K1
MAP4K4
MEF2C
MET
OLA1
PAK2
PRDM1
RAP2B1
REPS2
RRAS2
SEMA6D
STAT5B
TGFBR2
uPA
YES1
b
Normalized luciferase activity (firefly/renila)
1.2
0.8
0.4
0
FZD7
MAP3K1
PAK2
RRAS2
TGFBR2
uPA
c
Repression
NC
23b
FZD7
90 KD
43%
MAP3K1
78 KD
46%
PAK2
61 KD
57%
RRAS2
21 KD
73%
TGFBR2
80 KD
69%
UPA
54 KD
59%
GAPDH
37 KD
0.6%
d
RRAS2
miR-23b
PI3K
FZD7
MAP3K1
TGFβR2
PAK2
Wnt
NIK/IKK
MKK4/7
MEK1/2
JNKK
MEKK2/3
RhoA
NFκB
JNK
ERK
JNK
Rock1
Cell migration, invasion, proliferation and apoptosis

Figure 3.7 miR-23b regulating a cohort of prometastatic genes

a. Luciferase activity in HCT 116 cells infected with miR-23b (white bar) or control vector (black bar) after transfection with the indicated 3' UTR-driven reporter constructs. n = 3.

b. Luciferase activity in HCT 116 cells infected with miR-23b or control vector after transfection with the indicated 3' UTR-driven reporter constructs. Black bar: 3'UTR + miR-23b; grey bar: mutation 3'UTR + miR-23b; white bar: 3'UTR + NC. n = 3.

c. Immunoblots results for FZD7, MAP3K1, PAK2, RRAS2, TGFBR2 and UPA in the indicated HCT 116 cells. GAPDH was use as the loading control. Repression: protein levels in miR-23b-expressing cells relative to controls were repressed.

d. Model of miR-23b mediated pathways in tumor growth and cancer metastasis. The key proteins directly targeted by miR-23b were highlighted in the pathways of NFKB, JNK, ERK, PI3K, ROCK1 and Wnt.

3.8 Summary of miRNA screening

More and more evidence has accumulated showing that miRNAs participate in cancerogenesis. It has thus become an essential task to identify physiologically relevant and therapeutically promising miRNAs[73, 111]. In this study, we systematically investigated the capacity of individual miRNAs in cancer cell migration, invasion and apoptosis. Through quantitatively examining the impact of all known human miRNAs on cell migration, we found that over 20 % miRNAs demonstrated a capacity to regulate cell mobility. What is also noted, these migratory miRNAs exhibited versatility in diverse cell types, including four types of epithelial cells as well as HUVECs, thus being of general functionality. Given the fact that cell migration is a basic biological process, we hypothesize that a general regulation of migratory behaviour seems to be another distinctive feature of miRNAs.

According to the best of our knowledge, approximately twenty miRNAs have been described as functionally regulating cell migration or metastasis in previous studies [64, 73]; almost all of which are included in our screening list, and these miRNAs exhibited exactly the same prometastic or antimetastatic activities in our screening assay as reported previously. For example, miR-10b or miR-21 promotes cell migration, while miR-31 or miR-101 has a suppressive impact[62, 73, 112, 113]. Further implementation of invasion or apoptosis assays resulted in the identification of 36 miRNAs capable of suppressing cancer cell migration, invasion and growth, among which more than half of them have not been characterized yet. These results suggest that more miRNAs might be identified which have potential as therapeutic reagents against cancer metastasis and development.

miR-23b is highly conserved in all vertebrates. The data reported here demonstrated that miR-23b, which is down-regulated in human colon cancer samples, can potently repress cancer cell migration, invasion, growth and survival. It directly regulates a cohort of prometastatic genes or oncogenes, including FZD7, MAP3K1, PAK2, TGFβR2, RRAS2 and UPA. These six genes participate in certain critical signalling pathways, including the ERK, JNK, NFKB, PI3K, TGFB and Wnt pathways. To the best of our best knowledge, this is the first demonstration that so many critical pathways are interconnected in the regulation of metastasis under the control of a single miRNA-miR-23b.

Collectively, the findings of the present study have important implications for the understanding of the universal dysregulation of miRNAs in human cancer progression, especially metastasis. We believe that these findings provide new insight into the physiological and therapeutical importance of miRNAs in cancer.

CHAPTER 4

SCREENING OF HUMAN GENOME KINASE GENES REGULATING CANCER METASTASIS

4.1 Strategy of systematic investigation

The strategy of investigation was schematically shown in Figure 4.1. At first, we used a RNAi-based on-chip assay, SAMcell[114], to systematically investigate 710 human kinase genes involved in cell migration. Then we validated the primary results by Transwell migration assay. Next, we further screened the invasion activity using the gel-coated Transwell invasion assay. Hela epithelial carcinoma cells were used in such functional screenings.

Bio-informatics analysis started with differential expression strategy based on microarray dataset GSE6791[115] from cervix samples. Then gene ontology analysis was implemented by AmiGO[116] and KEGG[117]. Next, prePPI database[118] was used to predict protein-protein interactions. We identified sixteen important genes, including ten well-characterized genes and six prospective metastasis-related genes. Finally, all six prospective genes were examined by proliferation assay, apoptosis assay and anoikis assay.

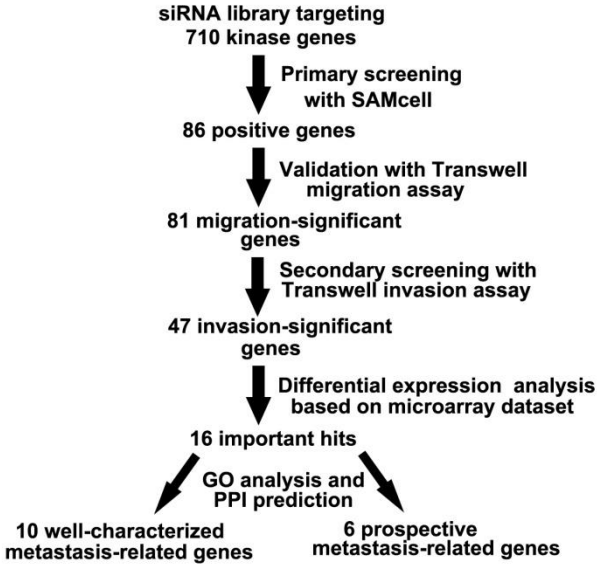


Figure 4.1 Strategy of functional screening and bio-informatics analysis.

4.2 Loss-of-function study of kinase genes regulating cell migration

We firstly used the SAMcell assay for high-throughput screening based on the cell migration performance. The assay conditions were established using siRNAs targeting *AKT1*, *ROCK1* and *ERBB2*, which are famous metastasis-related kinase genes. The quantitative real-time RT–PCR results validated the silencing efficiency of these siRNAs (Figure 4.2a). As expected, the assay showed that cells moved faster when *AKT1* was silenced, whereas cells migrated more slowly when either *ROCK1* or *ERBB2* was inhibited[95, 119, 120] (Figure 4.2b). By far, we set up a high-throughput system based on SAMcell assay and RNAi technology applying to loss-of-function study for cell migration (Figure 4.2c).

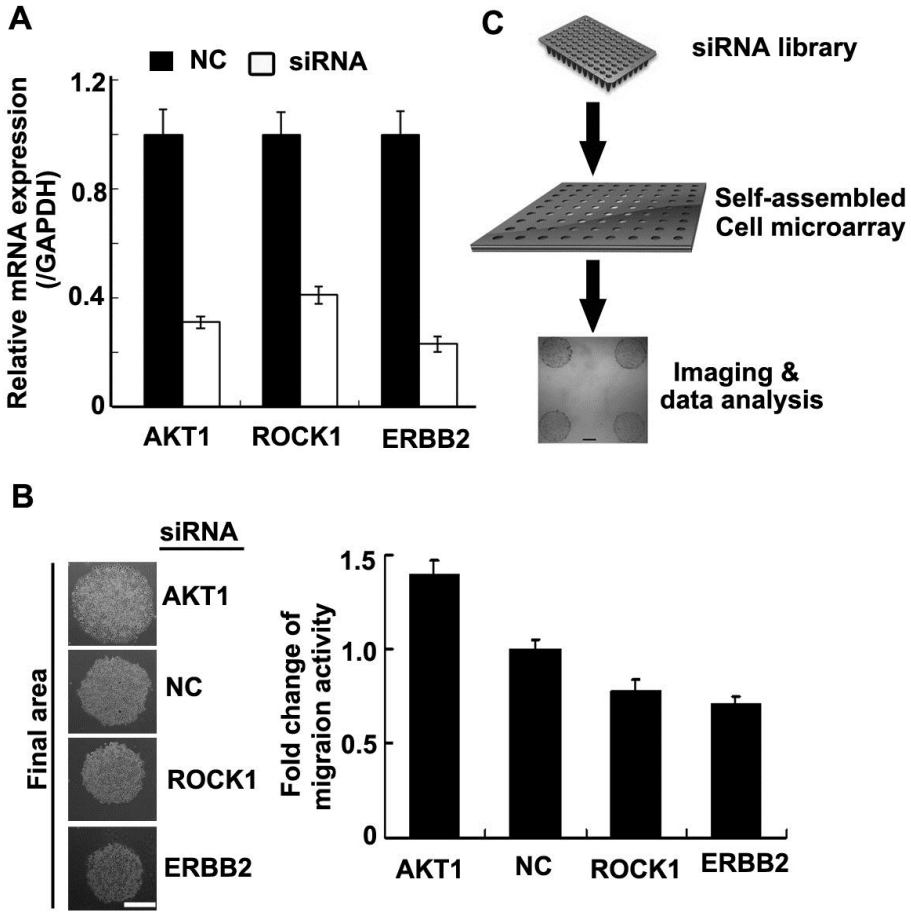


Figure 4.2 Development of high-throughput system for screening cell migration.

a. real-time PCR results indicated these genes can be efficiently silenced by the corresponding siRNAs in Hela. GAPDH was used as an internal quantification control. n=3.

b. Representative Hela cell island images showed migration phenotypes. Scale bar: 500um. Statistical results are shown in histogram on the right. n=4.

c. Flow chart showed the pipelining of loss-of-function screening system.

4.3 Functional screening for cell migration and invasion

We performed the primary screening with SAMcell assay for cell migratory activities. In total, 710 kinase or kinase-like genes were investigated (Table 4.3A). The empirical fold-change cut-off was selected based on the fold-change distribution. That is, the significant genes should have the fold-change of migration less than 0.9 or

more than 1.1. With adjusted p-value threshold of 0.05, 86 genes were found to be PR genes (positive-representative genes) (Figure 4.3a).

Next, we took use of Transwell assay, the most commonly used method for cell migration study, to validate the primary screening results. Among the 86 PR genes, 81 demonstrated consistent behavior in both assays (Table 4.3B). Analysis of the other 5 showing non-concordant behavior found that all of them exerted a potent effect on cell proliferation or apoptosis (Table 4.3C). Hence, 11% of kinase genes are able to regulate cancer cell migration (Figure 4.3b). Additionally, we randomly selected 15 NR genes (negative representative genes) and examined their migration activities by Transwell assay. Unsurprisingly, none of them exhibited any significant change (Figure 4.3c). In total, there were 101 genes detected by both assays. By comparing the fold changes and sample deviations between the two assays, we observed that Transwell assay had wider fold-change range but higher variance, while SAMcell assay had smaller fold-change range but higher repeatability (Figure 4.3e). These findings suggest that SAMcell assay has higher stability than Transwell assay in cell migration study.

The second round of functional screening was to detect the invasion activities. We screened the 81 migration-significant genes by gel-coated Transwell invasion assay. The significant genes had the fold-change of migration less than 0.8 or more than 1.2. With adjusted p-value threshold of 0.05, 47 genes were found to be significant genes regulating cell invasion (Figure 4.3d & Table 4.3D). Considering that migration and invasion are initial steps in the invasion-metastasis cascade, we hypothesized that these genes could play basic roles in regulation of cancer metastasis.

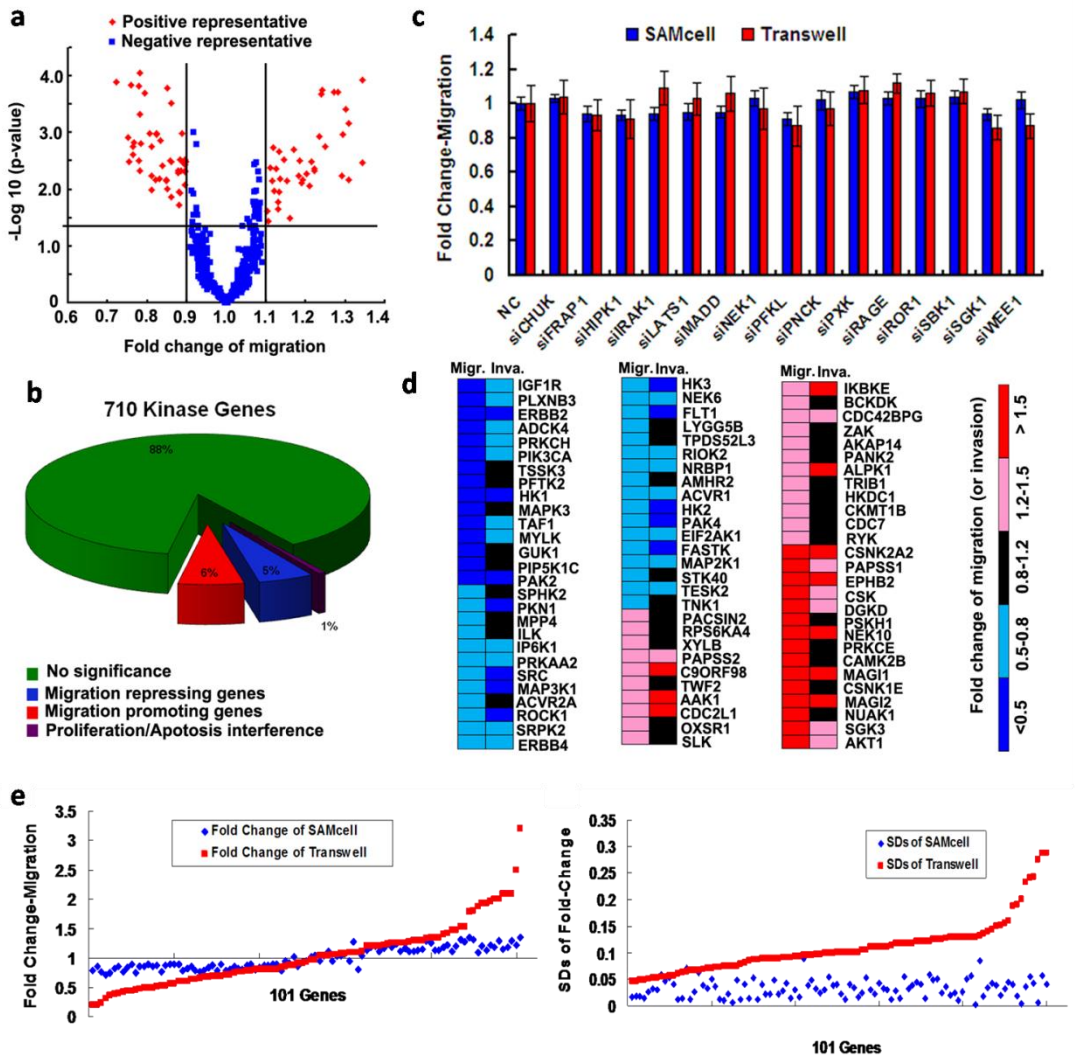


Figure 4.3 Functional screening for kinase genes regulating migration and invasion.

a. Volcano plot for distribution of 710 kinase siRNAs by primary screen. 4 replicate for each siRNA. Fold change is normalized to negative control (Scrambled siRNAs). The positive ones are marked in red, fold change >1.1 or <0.9 & adjusted P value <0.05 ($\log_{10}$).

b. Pie diagram for the distribution of the migratory kinase genes. Hela was used in the primary screen and validation. The interference is due to proliferation and apoptosis effects.

c. Fifteen NR genes were confirmed to have no effect on the migration in Hela cells. n=5.

d. Heat map showed Migration/Invasion activities of 81 migration-significant genes treated with siRNAs in Hela via Transwell assay. Red, blue or black means

up-regulated activity, down-regulated activity, or no significant change, respectively. n=5.

e. Distribution of fold change (left panel) or sample deviation (right panel) detected by both SAMcell and Transwell migration assays. The data points were ordered by red ones, data from Transwell assay.

Table 4.3A List of entire 710 kinase genes screened

AAK1	CDK6	ETNK2	LY6G5B	NME1	PLXND1	STAP1
AATK	CDK7	FASTK	LYN	NME2	PMVK	STAP2
ABL1	CDK8	FER	MADD	NME3	PNCK	STK10
ABL2	CDK9	FES	MAGI1	NME4	PNKP	STK11
ACVR1	CDKL1	FGFR1	MAGI2	NME5	PRKAA1	STK11IP
ACVR1B	CDKL2	FGFR2	MAGI3	NME6	PRKAA2	STK16
ACVR1C	CDKL3	FGFR3	MAK	NME7	PRKACA	STK17A
ACVR2A	CDKL4	FGFR4	MAP2K1	NRBP1	PRKACB	STK17B
ACVR2B	CDKL5	FGFRL1	MAP2K1IP1	NRBP2	PRKACG	STK19
ACVRL1	CERK	FGGY	MAP2K2	NRGN	PRKCA	STK24
ADCK2	CERKL	FGR	MAP2K3	NRK	PRKCB1	STK25
ADCK4	CHEK1	FLJ25006	MAP2K4	NTRK1	PRKCD	STK3
ADCK5	CHEK2	FLT1	MAP2K5	NTRK2	PRKCDBP	STK32A
ADK	CHKA	FLT3	MAP2K6	NTRK3	PRKCE	STK32B
ADPGK	CHUK	FLT4	MAP2K7	NUAK1	PRKCG	STK32C
ADRBK1	CIB1	FN3K	MAP3K1	NUAK2	PRKCH	STK33
ADRBK2	CIB4	FN3KRP	MAP3K10	OXSR1	PRKCI	STK35
AGK	CIT	FRAP1	MAP3K11	PACSIN1	PRKCQ	STK36
AK1	CKB	FRK	MAP3K12	PACSIN2	PRKCSH	STK38
AK2	CKM	FUK	MAP3K13	PACSIN3	PRKCZ	STK38L
AK3	CKMT1B	FYN	MAP3K14	PAK1	PRKD1	STK39
AK3L1	CKMT2	GAK	MAP3K15	PAK2	PRKD2	STK4
AK3L2	CLK1	GALK1	MAP3K2	PAK3	PRKD3	STK40
AK5	CLK2	GALK2	MAP3K3	PAK4	PRKDC	STRADA
AK7	CLK3	GCK	MAP3K4	PAK6	PRKG1	STRADB
AKAP12	CLK4	GCKR	MAP3K5	PAK7	PRKG2	STYK1
AKAP14	CMPK1	GK	MAP3K6	PANK1	PRKX	SYK
AKAP7	CNKSR1	GK2	MAP3K7	PANK2	PRKY	TAF1
AKAP8	CNKSR3	GK5	MAP3K7IP2	PANK3	PRPF4B	TAF1L
AKAP8L	COASY	GLYCTK	MAP3K8	PANK4	PRPS1	TAOK1
AKT1	COL4A3BP	GNE	MAP3K9	PAPSS1	PRPS1L1	TAOK2
AKT2	CRIM1	GRIP2	MAP4K1	PAPSS2	PRPS2	TAOK3

Table 4.3A Continued						
AKT3	CRKRS	GRK1	MAP4K2	PASK	PRPSAP1	TBK1
ALDH18A1	CSF1R	GRK4	MAP4K3	PBK	PRPSAP2	tcag7.875
ALK	CSK	GRK5	MAP4K4	PCM1	PSKH1	TEC
ALPK1	CSNK1A1	GRK6	MAP4K5	PCTK1	PSKH2	TEK
ALPK2	CSNK1A1L	GRK7	MAPK1	PCTK2	PTCD2	TESK1
ALPK3	CSNK1D	GSG2	MAPK10	PCTK3	PTK2	TESK2
AMHR2	CSNK1E	GSK3A	MAPK11	PDGFRA	PTK2B	TEX14
ANKK1	CSNK1G1	GSK3B	MAPK12	PDGFRB	PTK6	TGFBR1
ARAF	CSNK1G2	GUK1	MAPK13	PDGFRL	PTK7	TGFBR2
ASB10	CSNK1G3	HCK	MAPK14	PDIK1L	PXK	TIE1
ATM	CSNK2A1	HGS	MAPK15	PDK1	RAF1	TK1
ATMIN	CSNK2A2	HIPK1	MAPK3	PDK2	RAGE	TK2
ATR	DAK	HIPK2	MAPK4	PDK3	RBKS	TLK1
AURKA	DAPK1	HIPK3	MAPK6	PDK4	RET	TLK2
AURKAIP1	DAPK2	HIPK4	MAPK7	PDPK1	RFK	TNIK
AURKB	DAPK3	HK1	MAPK8	PDXK	RIOK1	TNK1
AURKC	DCAKD	HK2	MAPK9	PFKL	RIOK2	TNK2
AXL	DCK	HK3	MAPKAPK2	PFKM	RIOK3	TNNI3K
BCKDK	DCLK1	HKDC1	MAPKAPK3	PFKP	RIPK1	TPD52L3
BLK	DCLK2	HUNK	MAPKAPK5	PFTK1	RIPK2	TPK1
BMP2K	DCLK3	ICK	MARK1	PFTK2	RIPK3	TPR
BMP2KL	DDR1	IGF1R	MARK2	PGK1	RIPK4	TRAF3IP3
BMPR1A	DDR2	IGFN1	MARK3	PGK2	RIPK5	TRIB1
BMPR1B	DGKA	IGSF22	MARK4	PHKG1	RNASEL	TRIB2
BMPR2	DGKB	IHPK3	MAST1	PHKG2	ROCK1	TRIB3
BMX	DGKD	IKBKB	MAST2	PI4K2A	ROCK2	TRRAP
BRAF	DGKE	IKBKE	MAST3	PI4K2B	ROR1	TSKS
BRSK1	DGKG	IKBKG	MASTL	PI4KA	ROR2	TSSK1B
BRSK2	DGKH	ILK	MATK	PI4KB	ROS1	TSSK2
BTK	DGKI	INSR	MELK	PICK1	RP2	TSSK3
BUB1	DGKK	INSRR	MERTK	PIK3AP1	RPS6KA1	TSSK4
BUB1B	DGKQ	IP6K1	MET	PIK3C2A	RPS6KA2	TSSK6
C19orf35	DGKZ	IP6K2	MFHAS1	PIK3C2B	RPS6KA3	TTBK1
C1orf57	DGUOK	IPMK	MGC42105	PIK3C2G	RPS6KA4	TTBK2
C21orf7	DKFZp761P0423	IPPK	MINK1	PIK3C3	RPS6KA5	TTC33
C9orf95	DMPK	IQCH	MKNK1	PIK3CA	RPS6KA6	TTK
C9orf96	DNAJC6	IRAK1	MKNK2	PIK3CB	RPS6KB1	TWF1
C9orf98	DOK1	IRAK2	MLKL	PIK3CD	RPS6KB2	TWF2
CALM1	DTYMK	IRAK3	MORN1	PIK3CG	RPS6KC1	TXK
CALM2	DYRK1A	IRAK4	MOS	PIK3R3	RPS6KL1	TXNDC3
CALM3	DYRK1B	ITGB1BP3	MPP1	PIK3R4	RYK	TXNDC6
CAMK1	DYRK2	ITK	MPP2	PIM1	SBK1	TYK2

Table 4.3A Continued						
CAMK1D	DYRK3	ITPK1	MPP3	PIM2	SCGB2A1	TYRO3
CAMK1G	DYRK4	ITPKA	MPP4	PIM3	SCYL1	UCK1
CAMK2A	EEF2K	ITPKB	MPP5	PINK1	SCYL2	UCK2
CAMK2B	EGFR	ITPKC	MPP6	PIP4K2A	SCYL3	UCKL1
CAMK2D	EIF2AK1	JAK1	MPP7	PIP4K2B	SGK1	UHMK1
CAMK2G	EIF2AK2	JAK2	MRC2	PIP4K2C	SGK2	ULK1
CAMK2N1	EIF2AK3	JAK3	MST1R	PIP5K1A	SGK269	ULK2
CAMK4	EIF2AK4	KDR	MST4	PIP5K1B	SGK3	ULK3
CAMKK1	EPHA1	KHK	MUSK	PIP5K1C	SH3BP4	ULK4
CAMKK2	EPHA10	KIAA0999	MVK	PIP5KL1	SH3BP5	VRK1
CAMKV	EPHA2	KIAA1804	MYLK	PKLR	SH3BP5L	VRK2
CASK	EPHA3	KIT	MYLK2	PKM2	SHPK	VRK3
CCRK	EPHA4	KSR1	MYLK3	PKMYT1	SIK1	WEE1
CD2	EPHA5	KSR2	MYLK4	PKN1	SIK2	WNK1
CDC2	EPHA6	LATS1	NADK	PKN2	SKAP1	WNK2
CDC2L1	EPHA7	LATS2	NAGK	PKN3	SKP2	WNK3
CDC2L5	EPHA8	LCK	NEK1	PLK1	SLAMF6	WNK4
CDC2L6	EPHB1	LIMK1	NEK10	PLK2	SLK	XYLB
CDC42BPA	EPHB2	LIMK2	NEK11	PLK3	SMG1	YES1
CDC42BPB	EPHB3	LMTK2	NEK2	PLK4	SNRK	YSK4
CDC42BPG	EPHB4	LMTK3	NEK3	PLXNA1	SNX16	ZAK
CDC42SE2	EPHB6	LOC375133	NEK4	PLXNA2	SPHK1	ZAP70
CDC7	ERBB2	LOC390877	NEK5	PLXNA3	SPHK2	ZC3HC1
CDK10	ERBB3	LOC54103	NEK6	PLXNA4	SRC	ZMYND8
CDK2	ERBB4	LRPPRC	NEK7	PLXNB1	SRMS	
CDK3	ERN1	LRRK1	NEK8	PLXNB2	SRPK1	
CDK4	ERN2	LRRK2	NEK9	PLXNB3	SRPK2	
CDK5	ETNK1	LTK	NLK	PLXNC1	SRPK3	

Table 4.3B List of PR genes validated by Transwell

siRNA Targeting Genes	Fold-Migration of SAMcell	Fold-Migration of Transwell
AAK1	1.24	1.25
ACVR1	0.80	0.77
ACVR2A	0.86	0.63
ADCK4	0.72	0.31
AKAP14	1.29	1.33
ALPK1	1.13	1.35
AMHR2	0.82	0.76
ATK1	1.34	3.20
BCKDK	1.14	1.29
BMP2KL	1.11	1.04

Table 4.3B Continued		
C9ORF98	1.17	1.22
CAMK2B	1.29	2.00
CDC2L1	1.19	1.25
CDC42BPG	1.12	1.29
CDC7	1.11	1.48
CKMT1B	1.23	1.43
SRC	0.82	0.61
CSNK1E	1.30	2.10
CSNK2A2	1.31	1.53
DGKD	1.10	1.87
EIF2AK1	0.88	0.80
EPHB2	1.34	1.78
ERBB2	0.76	0.23
ERBB4	0.78	0.67
FASTK	0.90	0.80
FLT1	0.78	0.70
GUK1	0.85	0.49
HK1	0.83	0.43
HK2	0.88	0.78
HK3	0.84	0.68
HKDC1	1.19	1.40
IGF1R	0.78	0.20
IKBKE	1.12	1.28
ILK	0.89	0.56
IP6K1	0.90	0.56
LY6G5B	0.89	0.70
MAGI1	1.19	2.00
MAGI2	1.18	2.10
MAP2K1	0.90	0.80
MAP3K1	0.79	0.61
MAPK3	0.90	0.44
MPP4	0.88	0.53
MYLK	0.86	0.48
NEK10	1.13	1.94
NEK6	0.88	0.69
NRBP1	0.86	0.74
NUAK1	1.15	2.10
OXSR1	1.16	1.26
PACSIN2	1.13	1.20
PAK2	0.85	0.50
PAK4	0.89	0.79
PANK2	1.25	1.34

Table 4.3B Continued		
PAPSS1	1.28	1.54
PAPSS2	1.12	1.21
PFTK2	0.76	0.42
PGK2	0.81	1.10
PIK3CA	0.82	0.38
PIP5K1C	0.90	0.49
PKN1	0.76	0.52
PLK1	0.78	0.84
PLXNB3	0.85	0.20
PRKAA2	0.90	0.60
PRKCDBP	1.27	1.09
PRKCE	1.22	1.96
PRKCH	0.75	0.36
PSKH1	1.20	1.93
RIOK2	0.81	0.72
ROCK1	0.78	0.64
RPS6KA4	1.21	1.20
RYK	1.21	1.48
SGK3	1.22	2.50
SLK	1.11	1.26
SPHK2	0.88	0.51
CSK	1.31	1.81
SRPK2	0.77	0.65
STK40	0.83	0.80
TAF1	0.75	0.45
TESK2	0.88	0.80
TLK2	0.85	0.91
TNK1	0.81	0.80
TPD52L3	0.78	0.71
TRIB1	1.13	1.35
TSSK3	0.86	0.41
TWF2	1.24	1.24
XYLB	1.18	1.20
ZAK	1.13	1.29

Note: Cut-off for SAMcell assay: Fold change<0.9 or >1.1; Cut-off for Transwell assay: Fold change <0.8 or >1.2; non-concordant behaviour in validation was highlight in green.

Table 4.3C List of discordant genes interfered by proliferation or apoptosis

siRNA Targeting Genes	Fold-Proliferation	Fold-Apoptosis

Table 4.3C Continued		
BMP2KL	1.23	1.03
PGK2	0.91	1.27
PLK1	0.52	5.61
PRKCDBP	1.21	0.92
TLK2	0.72	1.04

Note: Cut-off for both assays: Fold change<0.8 or >1.2.

Table 4.3D List of invasion screening results

Table 4.3D Continued		
siRNA Targeting Genes	**Fold-Migration**	**Fold-Invasion**
IGF1R	0.20	0.77
PLXNB3	0.20	0.57
ERBB2	0.23	0.41
ADCK4	0.31	0.63
PRKCH	0.36	0.57
PIK3CA	0.38	0.80
TSSK3	0.41	1.16
PFTK2	0.42	0.83
HK1	0.43	0.33
MAPK3	0.44	1.13
TAF1	0.45	0.55
MYLK	0.48	0.59
GUK1	0.49	0.83
PIP5K1C	0.49	1.07
PAK2	0.50	0.41
SPHK2	0.51	1.11
PKN1	0.52	0.43
MPP4	0.53	1.13
ILK	0.56	1.18
IP6K1	0.56	0.71
PRKAA2	0.60	0.80
SRC	0.61	0.31
MAP3K1	0.61	0.45
ACVR2A	0.63	1.01
ROCK1	0.64	0.44
SRPK2	0.65	0.70
ERBB4	0.67	0.51
HK3	0.68	0.31
NEK6	0.69	0.54

Table 4.3D Continued		
FLT1	0.70	0.41
LY6G5B	0.70	1.07
TPD52L3	0.71	1.11
RIOK2	0.72	0.65
NRBP1	0.74	0.61
AMHR2	0.76	1.14
ACVR1	0.77	0.76
HK2	0.78	0.45
PAK4	0.79	0.41
EIF2AK1	0.80	0.78
FASTK	0.80	0.45
MAP2K1	0.80	0.72
STK40	0.80	1.05
TESK2	0.80	0.76
TNK1	0.80	1.14
PACSIN2	1.20	0.82
RPS6KA4	1.20	0.91
XYLB	1.20	0.87
PAPSS2	1.21	1.29
C9ORF98	1.22	1.56
TWF2	1.24	0.82
AAK1	1.25	1.63
CDC2L1	1.25	1.99
OXSR1	1.26	0.83
SLK	1.26	0.84
IKBKE	1.28	1.96
BCKDK	1.29	0.87
CDC42BPG	1.29	1.31
ZAK	1.29	1.13
AKAP14	1.33	1.1
PANK2	1.34	1.03
ALPK1	1.35	1.65
TRIB1	1.35	1.06
HKDC1	1.40	0.81
CKMT1B	1.43	1.13
CDC7	1.48	1.03
RYK	1.48	1.11
CSNK2A2	1.53	1.93
PAPSS1	1.54	1.33
EPHB2	1.78	1.83
CSK	1.81	1.46
DGKD	1.87	1.48

Table 4.3D Continued		
PSKH1	1.93	0.89
NEK10	1.94	1.67
PRKCE	1.96	0.94
CAMK2B	2.00	1.03
MAGI1	2.00	1.51
CSNK1E	2.10	0.84
MAGI2	2.10	1.67
NUAK1	2.10	1.04
SGK3	2.50	1.44
AKT1	3.20	1.41

Note: Cut-off for both assays: Fold change<0.8 or >1.2.

4.4 Bioinformatics analysis to identify important genes

After functional screening, we also performed bioinformatics analysis as complement. The first step of bioinformatics analysis was gene expression analysis, another powerful tool in understanding the mechanism of cancer metastasis. Such bioinformatics analysis helps identifying clinically important genes in terms of differential expression between normal and cancer samples. We downloaded publicly available microarray dataset (accession number: GSE6791) that studied the expression profile of 28 cervical samples (20 cancer samples and 8 normal samples)[115]. We took use of Rank Products[121], which is a simple, yet powerful, recently popular method to detect significant DEGs (differentially expressed genes). We computed all of the probe sets (more than 54,000) targeting the whole genome, using FDR (false discovery rate) of 0.05 and permutation of 100. Then we picked up the probe sets targeting the 47 migration- and invasion-significant genes and found 17 significant DEGs were identified out of 47 migration- and invasion-related genes from the aforementioned functional screening step (Table 4.4A). Among 17 significant DEGs, *PAPSS1* has the discordant behavior, which represses migration and invasion activity[122] but results in higher expression in cancer samples. Finally, we discovered 16 genes that are important for cancer metastasis from both functional screening and differential expression analysis (Table 4.4B).

The second step of bioinformatics analysis was gene ontology (GO) analysis. We added annotation of biological processes and signalling pathways associated with cancer metastasis for each gene by use of AmiGO[116] and KEGG[117] database. Consequently, 7 out of 16 DEGs had no GO terms related to cancer metastasis (Table 4.4C).

The final step of bioinformatics analysis was PPIs (protein-protein interactions) analysis, which is important for the elucidation of cell regulatory mechanisms. We adopted prePPI database[118], which combines structural and non-structural interaction clues, to investigate the PPIs between these 16 genes. Consequently, 6 of them not only had no GO terms related to cancer metastasis, but also had no high-confidence interactions (Figure 4.4 & Table 4.4D). For high-confidence interactions, LR (likelihood ratio) should be more than 600 based on Bayesian classifier[123]. These 6 genes remained as the prospective metastasis-related genes, which required experimental validation. Importantly, the influences of the other 10 well-characterized genes on cancer were all proved to be consistent with previous literatures (Table 4.4B).

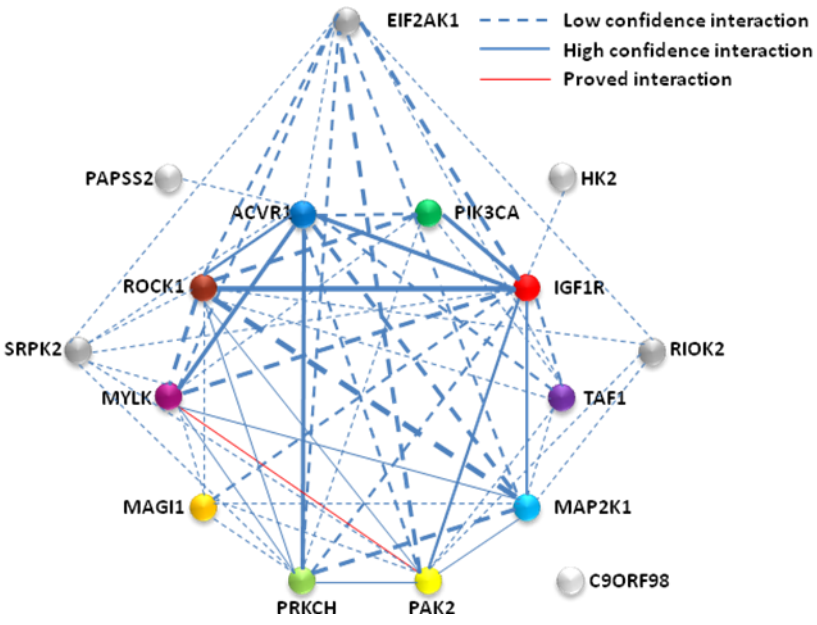


Figure 4.4 Bioinformatics analysis for kinase genes associated with cancer metastasis. Gray balls indicate the prospective metastasis-related kinase genes, chromatic balls indicate the well-characterized genes. Stronger associations are represented by thicker lines. Dash line means low confidence interaction. Solid line means high confidence interaction. The line highlighted in red means the proved interaction[124]. The definite data of LRs between every two genes are represented in Table 4.4D.

Table 4.4A List of genes with significantly different expression levels in clinical samples

Probe Set Number	Gene Symbol	FDR	Relative Expression (Normal/Cancer)
203935_at	ACVR1	7.37E-06	0.306343
230976_at	C9ORF98	0.006698	1.917705
1557666_s_at	C9ORF98	0.008121	1.860398
217736_s_at	EIF2AK1	8.73E-05	0.337994
202934_at	HK2	0	0.26695
225330_at	IGF1R	0.033183	0.573851
232859_s_at	MAGI1	0.049932	1.741719
202670_at	MAP2K1	0	0.201356
224823_at	MYLK	0.007406	0.559965
208875_s_at	PAK2	0	0.207309
208877_at	PAK2	0	0.201757
1559052_s_at	PAK2	0.000392	0.401636
208878_s_at	PAK2	0.002724	0.450724
208876_s_at	PAK2	0.024318	0.556326
209043_at	PAPSS1	0.001268	0.41439
203060_s_at	PAPSS2	5.41E-05	2.292106
203058_s_at	PAPSS2	0.000732	2.050034
204369_at	PIK3CA	0	0.18829
235980_at	PIK3CA	0.002534	0.450777
218764_at	PRKCH	0.005392	0.471105
218535_s_at	RIOK2	0.005106	0.461254
213044_at	ROCK1	0.007998	0.47862
214578_s_at	ROCK1	0.018071	0.510435
1558254_s_at	SRPK2	7.75E-06	0.283112
203182_s_at	SRPK2	0.000147	0.348618

Table 4.4A Continued			
227205_at	TAF1	0.005749	0.477768

Note: Cut-off for FDR: <0.05; non-concordant expression pattern with the gene's function was highlighted in green.

Table 4.4B List of DEGs significant in both functional screenings and expression analysis

siRNA Targeting Genes	Relative Expression (Normal/Cancer)	Fold-Migration	Fold-Invasion	Reference
Metastasis-promoting Genes				
MAP2K1	0.20	0.80	0.72	[125]
HK2	0.27	0.78	0.45	
ACVR1	0.31	0.77	0.76	[126]
PIK3CA	0.32	0.38	0.80	[127]
SRPK2	0.32	0.65	0.70	
EIF2AK1	0.34	0.80	0.78	
PAK2	0.36	0.50	0.41	[128]
RIOK2	0.46	0.72	0.65	
PRKCH	0.47	0.36	0.57	[129]
TAF1	0.48	0.45	0.55	[130]
ROCK1	0.50	0.64	0.44	[95]
MYLK	0.56	0.48	0.59	[131]
IGF1R	0.57	0.20	0.77	[132]
Metastasis-repressing Genes				
MAGI1	1.67	2.00	1.51	[133]
C9ORF98	1.89	1.22	1.56	
PAPSS2	2.02	1.21	1.29	

Note: Prospective metastasis-related genes were highlighted in green.

Table 4.4C List of 81 migratory kinase genes surveyed through gene ontology

Gene Symbol	RefSeq Accession Number	Full Gene Name	Gene Ontology	
			Biological Process	Regulation Pathway
Migration-promoting Genes				
ACVR1	NM_001105	activin A receptor, type I	patterning of blood vessels	TGFB receptor signaling pathway; SMAD signaling pathway
ACVR2A	NM_001616	activin A receptor, type IIA		BMP signaling pathway
ADCK4	NM_024876	aarF domain containing kinase 4		
AMHR2	NM_020547	anti-Mullerian hormone receptor, type II		TGFB receptor signaling pathway
EIF2AK1	NM_014413	eukaryotic translation initiation factor 2-alpha kinase 1		
ERBB2	NM_001005862	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	apoptotic process; cell proliferation	MAPK cascade; Ras protein signal transduction; PI3K cascade; Rho GTPase signalling pathway
ERBB4	NM_001042599	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	cell migration; epithelial cell proliferation	

Table 4.4C Continued				
FASTK	NM_006712	Fas-activated serine/threonine kinase		
FLT1	NM_002019	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	angiogenesis; cell migration; cell proliferation	MAPK cascade; PI3K cascade
GUK1	NM_000858	guanylate kinase 1		
HK1	NM_000188	hexokinase 1		
HK2	NM_000189	hexokinase 2		
HK3	NM_002115	hexokinase 3 (white cell)		
IGF1R	NM_000875	insulin-like growth factor 1 receptor	establishment of cell polarity; apoptotic process; cell migration; cell proliferation	MAPK cascade
ILK	NM_001014794	integrin-linked kinase	cell-matrix adhesion; focal adhesion; establishment of cell polarity;actin cytoskeleton organization; substrate adhesion-dependent cell spreading; cell-cell junction; cell migration; cell proliferation;cell cycle arrest;	integrin-mediated signaling pathway; MAPK cascade; BMP signaling pathway;
IP6K1	NM_001006115	inositol hexakisphosphate kinase 1		
LY6G5B	NM_021221	lymphocyte antigen 6 complex, locus G5B		
MAP2K1	NM_002755	mitogen-activated protein kinase kinase 1	cell cycle arrest; cell motility; cell proliferation; cell migration	MAPK cascade; EGFR signaling pathway; Ras GTPase pathway

Table 4.4C Continued				
MAP3K1	XM_042066	mitogen-activated protein kinase kinase kinase 1	cell migration; cell apoptosis; wound healing	MAPK cascade; TGFB receptor signaling pathway; JUN kinase pathway
MAPK3	NM_001040056	mitogen-activated protein kinase 3	cell cycle	MAPK cascade; BMP signaling pathway; EGFR signaling pathway; FGFR signaling pathway; ERK1 and ERK2 cascade; Ras protein signal transduction
MPP4	NM_033066	membrane protein, palmitoylated 4 (MAGUK p55 subfamily member 4)		
MYLK	NM_053025	myosin light chain kinase	cell migration; actin binding	
NEK6	NM_014397	NIMA (never in mitosis gene a)-related kinase 6		
NRBP1	NM_013392	nuclear receptor binding protein 1		
PAK2	NM_002577	p21 (CDKN1A)-activated kinase 2	apoptotic process; regulation of growth	ERK cascade

Table 4.4C Continued				
PAK4	NM_001014831	p21 protein (Cdc42/Rac)-activated kinase 4	apoptotic process; regulation of growth; cell cycle; cell migration; cell proliferation	
PFTK2	NM_139158	PFTAIRE protein kinase 2		cyclin-dependent protein kinase pathway
PIK3CA	NM_006218	phosphoinositide-3-kinase, catalytic, alpha polypeptide	angiogenesis; anoikis	EGFR signaling pathway; FGFR signaling pathway
PIP5K1C	NM_012398	phosphatidylinositol-4-phosphate 5-kinase, type I, gamma	actin cytoskeleton organization; adherens junction assembly; cell-cell adhesion	
PKN1	NM_002741	protein kinase N1	epithelial cell migration; regulation of cell motility	JUN kinase pathway; Rac GTPase pathway
PLXNB3	NM_005393	plexin B3		
PRKAA2	NM_006252	protein kinase, AMP-activated, alpha 2 catalytic subunit	autophagy; cell cycle arrest; apoptotic process	TOR signaling cascade; Wnt receptor signaling pathway; AMP-activated protein kinase pathway
PRKCH	NM_006255	protein kinase C, eta		
RIOK2	NM_018343	RIO kinase 2 (yeast)		
ROCK1	NM_005406	Rho-associated, coiled-coil containing protein kinase 1	actin cytoskeleton organization; angiogenesis; cell adhesion; cell motility	Rho protein signal transduction
SPHK2	NM_020126	sphingosine kinase 2	blood vessel development; cell proliferation; apoptotic process	Ras GTPase pathway

Table 4.4C Continued				
SRC	NM_005417	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	cell adhesion; cell cycle; anoikis; cell apoptosis; integrin activation	EGFR signaling pathway; FGFR signaling pathway; Wnt receptor signaling pathway; Ras protein signal transduction
SRPK2	NM_182691	SFRS protein kinase 2		
STK40	NM_032017	serine/threonine kinase 40		
TAF1	NM_004606	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250kDa	cell cycle	P53 pathway
TESK2	NM_007170	testis-specific kinase 2	actin cytoskeleton organization; focal adhesion assembly	
TNK1	NM_003985	tyrosine kinase, non-receptor, 1		
TPD52L3	NM_001001874	tumor protein D52-like 3		
TSSK3	NM_052841	testis-specific serine kinase 3		
Migration-repressing Genes				
AAK1	NM_014911	AP2 associated kinase 1		
AKAP14	NM_001008534	A kinase (PRKA) anchor protein 14		
ALPK1	NM_025144	alpha-kinase 1		

Table 4.4C Continued				
AKT1	NM_080871	ankyrin repeat and SOCS box-containing 10	cell cycle; microtubule-based movement	
BCKDK	NM_005881	branched chain ketoacid dehydrogenase kinase		
C9ORF98	NM_152572	chromosome 9 open reading frame 98		
CAMK2B	NM_001220	calcium/calmodulin-dependent protein kinase (CaM kinase) II beta		
CDC2L1	NM_033486	cell division cycle 2-like 1 (PITSLRE proteins)		
CDC42BPG	NM_017525	CDC42 binding protein kinase gamma (DMPK-like)	actin cytoskeleton reorganization; intracellular signal transduction; cell leading edge	
CDC7	NM_003503	cell division cycle 7 homolog (S. cerevisiae)	cell division	
CKMT1B	NM_020990	creatine kinase, mitochondrial 1B		
CSK	NM_004383	c-src tyrosine kinase	cell proliferation, cell-cell junction	
CSNK1E	NM_001894	casein kinase 1, epsilon		Wnt signaling pathway
CSNK2A2	NM_001896	casein kinase 2, alpha prime polypeptide	cell cycle	
DGKD	NM_003648	diacylglycerol kinase, delta 130kDa	intracellular signal transduction	
EPHB2	NM_004442	EPH receptor B2		

Table 4.4C Continued				
HKDC1	NM_025130	hexokinase domain containing 1		
IKBKE	NM_014002	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon		I-kappaB kinase/NF-kappaB cascade
MAGI1	NM_001033057	membrane associated guanylate kinase, WW and PDZ domain containing 1	cell adhesion; cell-cell junction	PTEN pathway
MAGI2	NM_012301	membrane associated guanylate kinase, WW and PDZ domain containing 2	cell migration; cell proliferation	SMAD protein signal transduction; PTEN pathway
NEK10	NM_001031741	NIMA (never in mitosis gene a)-related kinase 10		
NUAK1	NM_014840	NUAK family, SNF1-like kinase, 1	cell adhesion; cell proliferation; cellular senescence	P53 pathway
OXSR1	NM_005109	oxidative-stress responsive 1		
PACSIN2	NM_007229	protein kinase C and casein kinase substrate in neurons 2		
PANK2	NM_024960	pantothenate kinase 2	cell death	
PAPSS1	NM_005443	3'-phosphoadenosine 5'-phosphosulfate synthase 1		
PAPSS2	NM_001015880	3'-phosphoadenosine 5'-phosphosulfate synthase 2		

Table 4.4C Continued				
PRKCE	NM_005400	protein kinase C, epsilon	cell adhesion; cell cycle; cell division; cell apoptosis; epithelial cell migration; wound healing	EGFR signaling pathway; FGFR signaling pathway; nerve growth factor receptor signaling pathway; I-kappaB kinase/NF-kappaB cascade; MAPK cascade
PSKH1	NM_006742	protein serine kinase H1		
RPS6KA4	NM_001006944	ribosomal protein S6 kinase, 90kDa, polypeptide 4		MAPK cascade
RYK	NM_001005861	RYK receptor-like tyrosine kinase		Wnt receptor signaling pathway
SGK3	NM_001033578	serum/glucocorticoid regulated kinase family, member 3	cell growth; cell migration; cell proliferation	
SLK	NM_014720	STE20-like kinase (yeast)	apoptotic process	
TRIB1	NM_025195	tribbles homolog 1 (Drosophila)		
TWF2	NM_007284	twinfilin, actin-binding protein, homolog 2 (Drosophila)	actin binding; intracellular	
XYLB	NM_005108	xylulokinase homolog (H. influenzae)		

Table 4.4C Continued				
ZAK	NM_016653	sterile alpha motif and leucine zipper containing kinase AZK		MAPK cascade

Note: 16 important DEGs significant in both functional screenings and expression analysis are highlighted in red.

Table 4.4D List of PPI analysis results

	C9ORF98	EIF2AK1	HK2	IGF1R	MAGI1	MAP2K1	MYLK	PAK2	PAPSS2	PIK3CA	PRKCH	RIOK2	ROCK1	SRPK2	TAF1
ACVR1	-	22	-	2693	-	239	2574	183	15	128	3667	-	1108	11	24
C9ORF98		-	-	-	-	-	-	-	-	-	-	-	-	-	-
EIF2AK1			-	339	-	32	176	378	-	-	14	14	29	21	17
HK2				14	-	-	-	-	-	-	-	-	-	-	-
IGF1R					35	812	282	1429	-	2101	90	-	22622	61	25
MAGI1						71	24	16	-	-	31	-	15	-	-
MAP2K1							649	901	-	-	207	13	581	97	38

Table 4.4D Continued															
MYLK								575	-	20	1229	-	205	62	-
PAK2									-	-	1365	11	667	59	17
PAPSS2										-	-	-	-	-	-
PIK3CA											51	-	364	-	17
PRKCH												-	1172	17	-
RIOK2													12	-	-
ROCK1														140	79
SRPK2															-

Note: LR (likelihood ratio) scores over 600 are considered as high-confident interactions. "-" means no result in this database. Red means the proved interaction[124].

4.5 Experimental validation of prospective metastasis-related genes

Besides migration and invasion, the influence of the 6 prospective genes on the cancer cells is also required to be studied in the context of proliferation, apoptosis and anoikis activities, considering their involvement in the process of metastasis as well.

When silencing prospective metastasis-promoting genes, including HK2, SRPK2, RIOK2 and EIF2AK1, cell motility (migration and invasion activities) and cell growth (proliferation activity) were down-regulated; whereas anti-survival traits (apoptosis, necrosis and anoikis activities) were up-regulated (Figure 4.5a). When silencing prospective metastasis-repressing genes, including C9ORF98 and PAPSS2, cell motility and cell growth were roughly up-regulated; whereas anti-survival traits were roughly down-regulated (Figure 4.5b). These functional validations confirm that the 6 prospective metastasis-related kinase genes play important roles in cancer regulation.

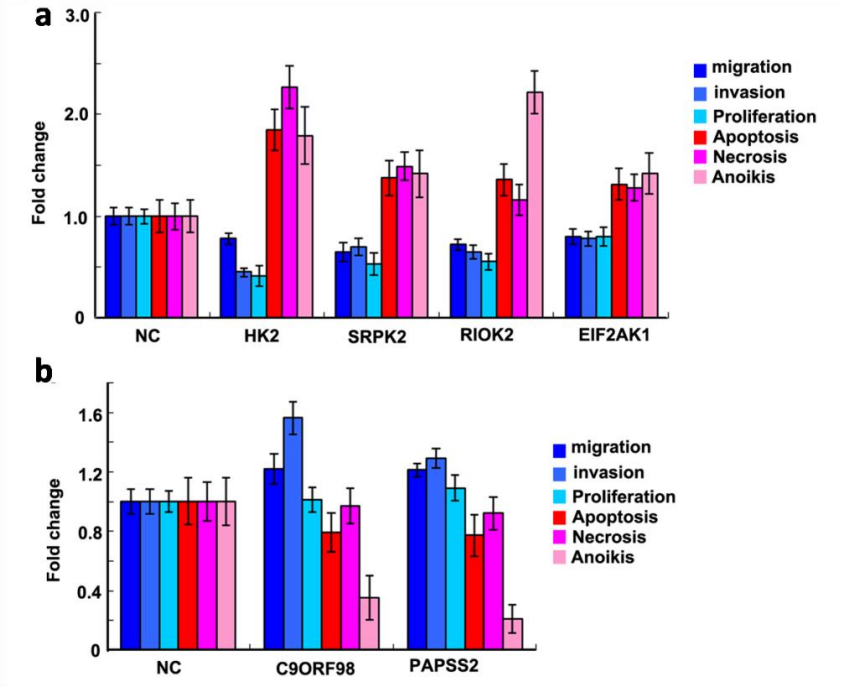


Figure 4.5 Functional validation of prospective metastasis-related genes. Histograms showed functional activities of metastasis traits after silencing metastasis-promoting genes (a) or metastasis-repressing genes (b) in Hela. n=5.

4.6 Summary of kinase screening

We undertook a systematic and stringent screening approach that identified 11% of genes as regulators of migration and 5% of genes suppressing the cellular motility

potential. This proportion is higher than that of a whole genome screening[45], possibly owing to the focused nature of our siRNA library. This observation also supports the claim that the kinase genes play more important roles than the average level in cancer metastasis. Given that our results overlap less than 40% with the screening results of a breast epithelial normal cell line[44], mechanisms of regulating migration should be distinguishable between normal and cancer cells or between different types of tissues.

Further implementation of invasion assay resulted in the identification of 47 genes capable of regulating cancer cell invasion, among which more than half of them have been characterized. These results suggest that more kinase genes might influence migration and invasion behaviors synergistically.

Such functional analysis is more convincing than the conventional differential expression strategy, in which a candidate is chosen mainly based on its distinct expression level in disease samples relative to normal ones. However, we also used the differential expression strategy as complement to our screening results.

Finally, we identified sixteen important genes for cancer metastasis, many of which, such as *MAP2K1*, *PIK3CA*, *IGF1R*, *ROCK1* and *MAGI1*, are already successfully applied to clinical cancer therapy[96, 97, 133-135]. We also discovered six prospective metastasis-related genes. Through experimental validation, all of them are able to regulate cell growth and anti-survival capabilities besides cell motility. We believe that these findings provide new insight into the biological and therapeutical importance of kinases in cancer.

CHAPTER 5

CONCLUSION

First of all, we developed a novel on-chip method, SAMcell (self-assembled cell microarray), and set up a HTS platform to investigate functional genes regulating cancer metastasis on large scale. This method attracted attentions since it was published because of its unique features, such as low cost, simple operation, open system, and no cross-contamination.

Secondly, we adopted this method to screen human genome miRNAs regulating cancer metastasis. We found 20% migratory miRNAs, which can help make an overall judgment of migratory miRNA distribution. We hypothesized a general regulation of miRNAs on cell migration and this seems to be another distinctive feature of miRNAs. After triple-round screenings for three phenotypes, including migration, invasion and apoptosis, we picked up the powerful gene miR-23b with the highest score in three rounds of screenings. miR-23b not only represses metastasis traits, but also has lower expression in cancer samples, suggesting that it has potential in cancer therapy.

Thirdly, we adopted this method to screen human genome kinase genes regulating cancer metastasis. We found 11% migratory genes, suggesting that kinase genes contribute more to cancer metastasis than the average level. Our strategy was a good example for integration of functional screening and bioinformatics analysis, which makes the determination of important genes and therapeutical targets more convictive. Finally, we discovered and validated 6 prospective metastasis-related kinase genes, which can be new potential targets in cancer therapy.

APPENDIX A

SEQUENCE OF PRIMERS AND BINDING SITES

3’ UTR to be cloned	Sequence of primers
ADAMTS5	GCTCTAGACTCACTTACATAACTAAGCACT TGGAATTCCACTATTGGTCATGACAGATT
BNIP2	GCTCTAGA CAGCAAGTACAGATGCAAGCGCAT TGGAATTCGTAAAGGTACTACCAAATGAAACAGT
CHUK	GCTCTAGAGAGTCATTTGTTTAAACAATCTTTCATGT TGGAATTCCCATTTAATACACAAAGTGAAAAACTAT
CNN2	GCTCTAGAGTACAAGGAAGTGAAATTCTGAGTTGT TGGAATTCCTTCGTACAAACAGAGGTTTTATTGCGT
EGR3	GCTCTAGA GTAGAAGGAGAGAGAAGAAGATGAAGT TGGAATTCGGTGAAGAATTTCACCTTTTCACAAT
ERBB4	GCTCTAGACTCTCAAACATTCATCTTATACAT TGGAATTCGGTGCATGATCCTTCATCAAT
FDE4B	GCTCTAGA CCTGTCCAAACTTCTACACAAT TGGAATTCCAACTTGGACCAATGATCAG
FGF2	GCTCTAGAGCATCTGCTGTTACCCAGTGAAGCT CAGAATTCAGGTCCGTCCTGAGTATTCGGC
FMR1	GCTCTAGAGTAACTGCTCTTGGGCAATATTCT TGGAATTCGATAAACAATCTTCAGATTTTTGAAACTTTA
FZD7	GCTCTAGAGTGATTCTGGAGTTCTTTGAAATGT TGGAATTCGTGATCTGTACATGTGATAAAGTGG
HOXA3	CTCTAGAGCCTTTACCTTCTCCACT GGAATTCCTGACTGTTCACCAGCAT
LAMP1	CTCTAGAGGGCTTAGGGTCCTGTCG GGAATTCCACCAGAACCCTAAACTC
LPP	GCTCTAGACTCTGCAGCTCAAAGATGTGGGT TGGAATTCGCCTTTCCAAGCCAAGAGGAATGGT
MAP3K1	GCTCTAGACTCAAAGACTCAGGATAAACT TGGAATTCCACGATACAATTTCCAGTCCAGCT
MAP4K4	GCTCTAGACCATCAGGTGCTATAAGTGTTTG GGAATTCCATATGCTGAGGCAGTGCTAATACTGT
MEF2C	GCTCTAGACATGTCCTGCAAATATGGCCCT TGGAATTCGCCACCCAGCGGCAGCCT

Table Continued	
MET	GCTCTAGACATCATCAGGACTTGAAGCCAAG TGGAATTCCAAGATGTTGCATCACTTTACTTT
OLA1	CTCTAGAGCACCTCAACAACCGAAG GGAATTCCTGTAATCAGCAACCACC
PAK2	CTCTAGAGAATGGCAGATTGGAGTT GGAATTCCGAACTTACTACCACGAA
PRDM1	CTCTAGAGAAGGGTGACAGGAAGGC GGAATTCCTGCCCTTTCATCTTCCA
RAP2B	CTCTAGAGCAGCCTCGTCAACCAGC GGAATTCCAGGGAGAAACGTACATG
REPS2	GCTCTAGA CCTTAGAGCTAAAAACATTTGTT TGGAATTCCTGACTGCTTTAGGGCATGCA
RRAS2	CTCTAGAGGGAATCATACTGCCTAC GGAATTCCTCCCATTCCTACCCAAG
SEMA6D	GGAATTCCATATGCACAGAAACAACCTAGGACAAT TGGAATTCGACTGAGTAGTGTTTATCATTGGT
STAT5B	CTCTAGAGTTCACCAGAGGAATCAC GGAATTCCTCCCATTCCTACCCAAG
TGFBR2	CTCTAGAGAGGATACTGTGGCTTGT GGAATTCCAGGAATGGGAACAGGAG
PLAU	CTCTAGAGTGGGCTGTGAGTGTAAGTG GGAATTCCAATCACATTTTATTGATCACT
YES1	CTCTAGAGCAGCATTAGTGGTTTGA GGAATTCCTGCCTTACTTTCCTGAT
Real-time PCR	
UPA	CTGTGAGATCACTGGCTTTG TTGGAGGGAACAGACGAG
TGFBR2	CCGCTGCACATCGTCCTGTG AGTGGATGGGCAGTCCTATTACA
MAP3K1	GGATGCACTCTTGCCATTTT TGGGCATGGTGATCTACAAA
FZD7	TAGGCACGTCCTTCTTGCTG CGTCGTGTTTCATGATGGTG
RRAS2	TCTGAACAAGTGTCGGAGGC CTACGAATGGGCTTTGATGG
PAK2	GGCTATCTGAACTCTATGCATTCG AGGCCATGAGCGTGCGGGTCATA
SGEF	AGCTGAGCAGTTCACTTC TACTGGGAGAACCATACA

Table Continued	
RHOG	GCGGTCATACTCCTCCTG TCATCTGCTACACAACTAACG
PTEN	TGCAATCCTCAGTTTGTGGTCTGCCA GAAGTTGAACTGCTAGCCTCTGGATTTGA
ERBB2	AGCCGCGAGCACCCAAGT TTGGTGGGCAGGTAGGTGAGTT
ROCK1	CTGCAACTGGAACTCAACCAAGAA TTAGCACGCAATTGCTCAATATCAC
AKT1	GCAGCACGTGTACGAGAAGA GGTGTCAGTCTCCGACGTG
GAPDH	GCACCGTCAAGGCTGAGAAC GCCTTCTCCATGGTGGTGAA
cDNA for over-expression	
FZD7	CAAGCTTGAACCGCCTCGTCGCACTC CGGATCCGCCAAACCGCTTTCCACCC
RRAS2	CGGCTAGCAGTCAGGCTGGGCTCTGT CGGAATTCGAGAAAGAGAAGATGAGGGCT
MAP3K1	CGGAATTCTCTTTCTTTCGGAAGGTG GCTCTAGATCGTAAACCAGGAGACAAA
PAK2	CGGGATCCTCATGTCTGATAACGGAGA GCTCTAGAAAGAGTAATAATTTTCATATACTAA
TGFBR2	CGGAATTCCGGTCTATGACGAGCAGC GCTCTAGACATAGAATGCTCTATGTCACCC
UPA	GGGGTACCGAGCCGCCGTCTAGCG GCTCTAGAGTGGGTGGCACAGGCAAATC

Gene	Wild type binding site	Mutant binding site
FZD7	GGCG**ATGTGAA**TCGTC	GGCG**GCACAGG**TCGTC
MAP3K1	TTTTT**ATGTGAA**TATGTT	TTTTT**GCACAGG**TATGTT
PAK2	AACA**AATGTGAA**AAAG	AACA**GGCACAGG**AAAG
RRAS2	TTTTT**AATGTGA**ATTTTT	TTTTT**GGCACAG**ATTTTT
TGFBR2	ACAA**AATGTGAA**GGTG	ACAA**GGCACAGG**GGTG
UPA	ATAA**AATGTGA**TTTTTC	ATAA**GGCACAG**TTTTTC

APPENDIX B

METHODS AND MATERIAL

Cell culture, miRNA mimics and siRNA duplex

Cell lines including Hela, HepG2, U2OS, MCF-7, PANC-1, and HCT 116 were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin under humidified conditions in 95% air and 5% CO_2 at 37°C. HUVECs, which are donated from Professor Xingyu Jiang at National Center for Nanoscience and Technology, China, were cultured for three passages in ECM medium (ScienCell) containing 1% ECGS, 5% FBS, and 1% P/S. Transfection experiments were followed Lipofectamine™ 2000 (Invitrogen) transfection reagent protocol.

All human miRNA mimics were obtained from the GenePharma (Shanghai, P.R. China). The Select Human Kinase siRNA Library (Ambion, Life Tech) was used and the repression efficiency was guaranteed by the provider.

Transwell migration and invasion assay

For migration assays, Hela cells were seeded into the upper chamber of a Transwell insert (pore size, 8μm; Costar) in 100μL serum-free medium per well. 600μL medium containing 10% serum was placed in the lower chamber to act as a chemoattractant. Non-migratory cells were removed from the upper chamber by scraping with a cotton bud. The cells remaining on the lower surface of the insert were fixed with 2% formaldehyde (Sigma) and stained by DAPI (Roche). For invasion assays, cells were seeded in a Matrigel (Bio-Rad)-coated chamber and were incubated at 37℃.

Proliferation assay

Hela transfected cells were seeded in a 12-well plate. After 48h of incubation, the

cells were trypsinized, and counted by hemocytometer measurement.

Apoptosis and necrosis assay

We used Annexin V-FITC Kit (Biosea Biotechnology Co. LTD) to detect apoptosis and necrosis of cells. Cells were identified by double labeling with Annexin V and PI assay. Cells are incubated with Annexin V solution for 30 minutes, followed by PI solution for 10 minutes. Cells at early apoptotic stage are stained by only Annexin V, and cells at late apoptotic stage are stained by both Annexin V and PI. Cells with necrosis were stained by only PI.

Anoikis assay

Cells with anoikis was detected by seeding 5.0×10^4 cells in ultralow attachment plates (Corning). After 24 hr culture, cells were resuspended in 0.4% trypan blue (Sigma) and cell viability was assessed.

Immunofluorescence

Cells were seeded onto sterile cover slides and allowed to attach overnight. Cells were then fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked in 2% BSA for 1 h at room temperature. The expression of E-cadherin or Vimentin was examined using their antibodies and visualized using Anti-Rabbit IgG (H+L), F (ab')2 Fragment (Alexa Fluor® 488 Conjugate) (Cell Signalling Tech.). Immunofluorescence was examined using an Olympus IX70 Confocal microscope. The concentrations of the antibodies were 1:200 diluted.

Luciferase assay

4.0×10^4 293T cells were co-transfected with 200 ng of the indicated pGL3 firefly luciferase construct and 20ng of a pGL3 Renilla luciferase normalization control. In the meantime, the indicated miRNA expression plasmid or mimics was transfected.

The media was changed after 6 h, and luciferase activity was measured after 48 h using the dual luciferase reporter assay system (Promega, US).

Immunoblotting

Lysates were resolved by electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with antibodies against MAP3K1 (Assay Designs), FZD7 (Abcam), TGFBR2 (CST), RRAS2 (Santa Cruz), PAK2 (CST), and GAPDH (Santa Cruz). The concentrations of the antibodies were 1:1000 diluted.

Statistical analysis

All results were expressed as means and n= derives from independent experiments. To determine whether data sets come from normal distribution, Shapiro-Wilk W-test and Kolmogorov–Smirnov Z-test were used (JMP9.0 Software). When comparing two groups, unpaired Student's t-tests (two-tailed) were used. For all tests, a P value <0.05 was considered significant. Benjamini and Hochberg false discovery rate was used as a correction for multiple testing. * indicated $P<0.05$; ** indicated $P<0.01$; *** indicated $P<0.001$. Error bars represented SDs of at least three independent experiments.

REFERENCES

[1] A. C. Chiang and J. Massague, "Molecular basis of metastasis," *N Engl J Med*, vol. 359, pp. 2814-23, Dec 25 2008.

[2] G. P. Gupta and J. Massague, "Cancer metastasis: building a framework," *Cell*, vol. 127, pp. 679-95, Nov 17 2006.

[3] R. Onuki-Nagasaki, *et al.*, "On-chip screening method for cell migration genes based on a transfection microarray," *Lab Chip*, vol. 8, pp. 1502-6, Sep 2008.

[4] C. A. Klein, "Cancer. The metastasis cascade," *Science*, vol. 321, pp. 1785-7, Sep 26 2008.

[5] K. Podsypanina, *et al.*, "Seeding and propagation of untransformed mouse mammary cells in the lung," *Science*, vol. 321, pp. 1841-4, Sep 26 2008.

[6] I. J. Fidler, "The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited," *Nat Rev Cancer*, vol. 3, pp. 453-8, Jun 2003.

[7] F. Huber, *et al.*, "Emergent complexity of the cytoskeleton: from single filaments to tissue," *Advances in Physics*, vol. 62, pp. 1-112, Feb 2013.

[8] A. S. Meyer, *et al.*, "2D protrusion but not motility predicts growth factor-induced cancer cell migration in 3D collagen," *J Cell Biol*, vol. 197, pp. 721-9, Jun 11 2012.

[9] M. Parri and P. Chiarugi, "Rac and Rho GTPases in cancer cell motility control," *Cell Commun Signal*, vol. 8, p. 23, 2010.

[10] V. Sanz-Moreno, *et al.*, "Rac activation and inactivation control plasticity of tumor cell movement," *Cell*, vol. 135, pp. 510-23, Oct 31 2008.

[11] A. Fire, *et al.*, "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans," *Nature*, vol. 391, pp. 806-11, Feb 19 1998.

[12] F. Crick, "Central dogma of molecular biology," *Nature*, vol. 227, pp. 561-3, Aug 8 1970.

[13] P. Ahlquist, "RNA-dependent RNA polymerases, viruses, and RNA silencing,"

Science, vol. 296, pp. 1270-3, May 17 2002.

[14] X. Cai, *et al.*, "Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs," *RNA,* vol. 10, pp. 1957-66, Dec 2004.

[15] Y. Lee, *et al.*, "MicroRNA genes are transcribed by RNA polymerase II," *EMBO J,* vol. 23, pp. 4051-60, Oct 13 2004.

[16] G. M. Borchert, *et al.*, "RNA polymerase III transcribes human microRNAs," *Nat Struct Mol Biol,* vol. 13, pp. 1097-101, Dec 2006.

[17] Y. Hayashita, *et al.*, "A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation," *Cancer Res,* vol. 65, pp. 9628-32, Nov 1 2005.

[18] A. Rodriguez, *et al.*, "Identification of mammalian microRNA host genes and transcription units," *Genome Res,* vol. 14, pp. 1902-10, Oct 2004.

[19] Y. Saito, *et al.*, "Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells," *Cancer Cell,* vol. 9, pp. 435-43, Jun 2006.

[20] L. He, *et al.*, "A microRNA component of the p53 tumour suppressor network," *Nature,* vol. 447, pp. 1130-4, Jun 28 2007.

[21] K. A. O'Donnell, *et al.*, "c-Myc-regulated microRNAs modulate E2F1 expression," *Nature,* vol. 435, pp. 839-43, Jun 9 2005.

[22] Y. Lee, *et al.*, "The nuclear RNase III Drosha initiates microRNA processing," *Nature,* vol. 425, pp. 415-9, Sep 25 2003.

[23] A. M. Denli, *et al.*, "Processing of primary microRNAs by the Microprocessor complex," *Nature,* vol. 432, pp. 231-5, Nov 11 2004.

[24] R. I. Gregory, *et al.*, "The Microprocessor complex mediates the genesis of microRNAs," *Nature,* vol. 432, pp. 235-40, Nov 11 2004.

[25] J. Han, *et al.*, "The Drosha-DGCR8 complex in primary microRNA processing," *Genes Dev,* vol. 18, pp. 3016-27, Dec 15 2004.

[26] M. Landthaler, *et al.*, "The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis," *Curr*

Biol, vol. 14, pp. 2162-7, Dec 14 2004.

[27] J. Han, *et al.*, "Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex," *Cell,* vol. 125, pp. 887-901, Jun 2 2006.

[28] Y. Zeng and B. R. Cullen, "Sequence requirements for micro RNA processing and function in human cells," *RNA,* vol. 9, pp. 112-23, Jan 2003.

[29] Y. Zeng and B. R. Cullen, "Efficient processing of primary microRNA hairpins by Drosha requires flanking nonstructured RNA sequences," *J Biol Chem,* vol. 280, pp. 27595-603, Jul 29 2005.

[30] R. Yi, *et al.*, "Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs," *Genes Dev,* vol. 17, pp. 3011-6, Dec 15 2003.

[31] E. Bernstein, *et al.*, "Role for a bidentate ribonuclease in the initiation step of RNA interference," *Nature,* vol. 409, pp. 363-6, Jan 18 2001.

[32] A. Grishok, *et al.*, "Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing," *Cell,* vol. 106, pp. 23-34, Jul 13 2001.

[33] G. Hutvagner, *et al.*, "A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA," *Science,* vol. 293, pp. 834-8, Aug 3 2001.

[34] R. F. Ketting, *et al.*, "Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans," *Genes Dev,* vol. 15, pp. 2654-9, Oct 15 2001.

[35] E. Ma, *et al.*, "Autoinhibition of human dicer by its internal helicase domain," *J Mol Biol,* vol. 380, pp. 237-43, Jun 27 2008.

[36] J. E. Babiarz, *et al.*, "Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs," *Genes Dev,* vol. 22, pp. 2773-85, Oct 15 2008.

[37] W. J. Chung, *et al.*, "Computational and experimental identification of mirtrons in Drosophila melanogaster and Caenorhabditis elegans," *Genome Res,* vol. 21, pp. 286-300, Feb 2011.

[38] K. Okamura, *et al.*, "The mirtron pathway generates microRNA-class

regulatory RNAs in Drosophila," *Cell,* vol. 130, pp. 89-100, Jul 13 2007.

[39] J. G. Ruby, *et al.*, "Intronic microRNA precursors that bypass Drosha processing," *Nature,* vol. 448, pp. 83-6, Jul 5 2007.

[40] S. Cheloufi, *et al.*, "A dicer-independent miRNA biogenesis pathway that requires Ago catalysis," *Nature,* vol. 465, pp. 584-9, Jun 3 2010.

[41] D. Cifuentes, *et al.*, "A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity," *Science,* vol. 328, pp. 1694-8, Jun 25 2010.

[42] J. M. Wild and N. O. Krutzfeldt, "Neocortical-like organization of avian auditory 'cortex'. Commentary on Wang Y, Brzozowska-Prechtl A, Karten HJ (2010): Laminar and columnar auditory cortex in avian brain. Proc Natl Acad Sci USA 107:12676-12681," *Brain Behav Evol,* vol. 76, pp. 89-92, 2010.

[43] S. M. Hammond, *et al.*, "An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells," *Nature,* vol. 404, pp. 293-6, Mar 16 2000.

[44] K. J. Simpson, *et al.*, "Identification of genes that regulate epithelial cell migration using an siRNA screening approach," *Nat Cell Biol,* vol. 10, pp. 1027-38, Sep 2008.

[45] A. W. Whitehurst, *et al.*, "Synthetic lethal screen identification of chemosensitizer loci in cancer cells," *Nature,* vol. 446, pp. 815-9, Apr 12 2007.

[46] M. RS, in *Applying genomic and proteomic microarray technology in drug discovery*, ed: CRC Press, 2005.

[47] Z. XHD, " Optimal High-Throughput Screening: Practical Experimental Design and Data Analysis for Genome-scale RNAi Research," ed: Cambridge University Press, 2011.

[48] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell,* vol. 136, pp. 215-33, Jan 23 2009.

[49] B. Kusenda, *et al.*, "MicroRNA biogenesis, functionality and cancer relevance," *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub,* vol.

150, pp. 205-15, Nov 2006.

[50] I. Bentwich, *et al.*, "Identification of hundreds of conserved and nonconserved human microRNAs," *Nat Genet,* vol. 37, pp. 766-70, Jul 2005.

[51] R. C. Friedman, *et al.*, "Most mammalian mRNAs are conserved targets of microRNAs," *Genome Res,* vol. 19, pp. 92-105, Jan 2009.

[52] B. P. Lewis, *et al.*, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell,* vol. 120, pp. 15-20, Jan 14 2005.

[53] L. P. Lim, *et al.*, "The microRNAs of Caenorhabditis elegans," *Genes Dev,* vol. 17, pp. 991-1008, Apr 15 2003.

[54] G. Hutvágner and P. D. Zamore, "A microRNA in a multiple-turnover RNAi enzyme complex," *Science,* vol. 297, pp. 2056-60, 2002.

[55] E. Wienholds, *et al.*, "MicroRNA expression in zebrafish embryonic development," *Science,* vol. 309, pp. 310-1, 2005.

[56] A. S. Flynt and E. C. Lai, "Biological principles of microRNA-mediated regulation: shared themes amid diversity," *Nat Rev Genet.* , vol. 9, pp. 831-42, 2008.

[57] H. I. Suzuki, *et al.*, "Modulation of microRNA processing by p53," *Nature,* vol. 460, pp. 529-33, 2009

[58] S. M. Johnson, *et al.*, "RAS is regulated by the let-7 microRNA family," *Cell,* vol. 120, pp. 635-47, Mar 11 2005.

[59] J. Lu, *et al.*, "MicroRNA expression profiles classify human cancers," *Nature,* vol. 435, pp. 834-8, Jun 9 2005.

[60] C. M. Croce, "Causes and consequences of microRNA dysregulation in cancer," *Nat Rev Genet.*, vol. 10, pp. 704-14, 2009

[61] Q. Huang, *et al.*, "The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis," *Nat Cell Biol,* vol. 10, pp. 202-10, Feb 2008.

[62] L. Ma, *et al.*, "Tumour invasion and metastasis initiated by microRNA-10b in breast cancer," *Nature,* vol. 449, pp. 682-8, Oct 11 2007.

[63] S. F. Tavazoie, *et al.*, "Endogenous human microRNAs that suppress breast

cancer metastasis," *Nature,* vol. 451, pp. 147-52, Jan 10 2008.

[64] M. S. Nicoloso, *et al.*, "MicroRNAs--the micro steering wheel of tumour metastases," *Nat Rev Cancer,* vol. 9, pp. 293-302, Apr 2009.

[65] G. A. Calin and C. M. Croce, "MicroRNA signatures in human cancers," *Nat Rev Cancer,* vol. 6, pp. 857-66, Nov 2006.

[66] J. Yang, *et al.*, "Exploring a new twist on tumor metastasis," *Cancer Res,* vol. 66, pp. 4549-52, May 1 2006.

[67] L. Ma, *et al.*, "miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis," *Nat Cell Biol,* vol. 12, pp. 247-56, Mar 2010.

[68] M. V. Iorio, *et al.*, "MicroRNA gene expression deregulation in human breast cancer," *Cancer Res,* vol. 65, pp. 7065-70, Aug 15 2005.

[69] Y. Sun, *et al.*, "Expression profile of microRNAs in c-Myc induced mouse mammary tumors," *Breast Cancer Res Treat,* vol. 118, pp. 185-96, Nov 2009.

[70] R. Reeves, "Molecular biology of HMGA proteins: hubs of nuclear function," *Gene,* vol. 277, pp. 63-81, Oct 17 2001.

[71] X. Zhou, *et al.*, "Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C," *Nature,* vol. 376, pp. 771-4, Aug 31 1995.

[72] Y. S. Lee and A. Dutta, "The tumor suppressor microRNA let-7 represses the HMGA2 oncogene," *Genes Dev,* vol. 21, pp. 1025-30, May 1 2007.

[73] S. Valastyan, *et al.*, "A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis," *Cell,* vol. 137, pp. 1032-46, Jun 12 2009.

[74] G. A. Calin, *et al.*, "Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers," *Proc Natl Acad Sci U S A,* vol. 101, pp. 2999-3004, Mar 2 2004.

[75] L. X. Yan, *et al.*, "MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis," *RNA,* vol. 14, pp. 2348-60, Nov 2008.

[76] P. S. Steeg, "Metastasis suppressors alter the signal transduction of cancer

cells," *Nat Rev Cancer,* vol. 3, pp. 55-63, Jan 2003.

[77] A. I. McClatchey, "Merlin and ERM proteins: unappreciated roles in cancer development?," *Nat Rev Cancer,* vol. 3, pp. 877-83, Nov 2003.

[78] E. Sahai and C. J. Marshall, "RHO-GTPases and cancer," *Nat Rev Cancer,* vol. 2, pp. 133-42, Feb 2002.

[79] M. Sanchez-Carbayo, *et al.*, "Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays," *J Clin Oncol,* vol. 24, pp. 778-89, Feb 10 2006.

[80] Y. Husemann, *et al.*, "Systemic spread is an early step in breast cancer," *Cancer Cell,* vol. 13, pp. 58-68, Jan 2008.

[81] J. P. Thiery and J. P. Sleeman, "Complex networks orchestrate epithelial-mesenchymal transitions," *Nat Rev Mol Cell Biol,* vol. 7, pp. 131-42, Feb 2006.

[82] G. Berx, *et al.*, "Pre-EMTing metastasis? Recapitulation of morphogenetic processes in cancer," *Clin Exp Metastasis,* vol. 24, pp. 587-97, 2007.

[83] T. Brabletz, *et al.*, "Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment," *Proc Natl Acad Sci U S A,* vol. 98, pp. 10356-61, Aug 28 2001.

[84] A. Barrallo-Gimeno and M. A. Nieto, "The Snail genes as inducers of cell movement and survival: implications in development and cancer," *Development,* vol. 132, pp. 3151-61, Jul 2005.

[85] H. Hugo, *et al.*, "Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression," *J Cell Physiol,* vol. 213, pp. 374-83, Nov 2007.

[86] U. Burk, *et al.*, "A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells," *EMBO Rep,* vol. 9, pp. 582-9, Jun 2008.

[87] P. A. Gregory, *et al.*, "MicroRNAs as regulators of epithelial-mesenchymal transition," *Cell Cycle,* vol. 7, pp. 3112-8, Oct 2008.

[88] G. Manning, *et al.*, "The protein kinase complement of the human genome,"

Science, vol. 298, pp. 1912-34, Dec 6 2002.

[89] G. G. Choudhury, *et al.*, "PI-3-kinase and MAPK regulate mesangial cell proliferation and migration in response to PDGF," *Am J Physiol,* vol. 273, pp. F931-8, Dec 1997.

[90] D. J. Sieg, *et al.*, "Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration," *J Cell Sci,* vol. 112 (Pt 16), pp. 2677-91, Aug 1999.

[91] C. Thibault, *et al.*, "HER2 status for prognosis and prediction of treatment efficacy in adenocarcinomas: A review," *Crit Rev Oncol Hematol,* Apr 5 2013.

[92] A. Goldhirsch, *et al.*, "Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007," *Ann Oncol,* vol. 18, pp. 1133-44, Jul 2007.

[93] D. J. Slamon, *et al.*, "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene," *Science,* vol. 235, pp. 177-82, Jan 9 1987.

[94] U. Wilking, *et al.*, "HER2 status in a population-derived breast cancer cohort: discordances during tumor progression," *Breast Cancer Res Treat,* vol. 125, pp. 553-61, Jan 2011.

[95] D. Vigil, *et al.*, "ROCK1 and ROCK2 are required for non-small cell lung cancer anchorage-independent growth and invasion," *Cancer Res,* vol. 72, pp. 5338-47, Oct 15 2012.

[96] R. A. Patel, *et al.*, "Identification of novel ROCK inhibitors with anti-migratory and anti-invasive activities," *Oncogene,* Feb 11 2013.

[97] P. Cohen, "Protein kinases--the major drug targets of the twenty-first century?," *Nat Rev Drug Discov,* vol. 1, pp. 309-15, Apr 2002.

[98] G. J. Todaro, *et al.*, "The initiation of cell division in a contact-inhibited mammalian cell line," *J Cell Physiol,* vol. 66, pp. 325-33, Dec 1965.

[99] C. C. Liang, *et al.*, "In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro," *Nat Protoc,* vol. 2, pp. 329-33, 2007.

[100] H. C. Chen, "Boyden chamber assay," *Methods Mol Biol,* vol. 294, pp. 15-22, 2005.

[101] H. Erfle, *et al.*, "Reverse transfection on cell arrays for high content screening microscopy," *Nat Protoc,* vol. 2, pp. 392-9, 2007.

[102] J. Xi, *et al.*, "Self-assembled microdevices driven by muscle," *Nat Mater.* , vol. 4, pp. 180-4., 2005.

[103] H. Katoh, *et al.*, "Activation of Rac1 by RhoG regulates cell migration," *J Cell Sci,* vol. 119, pp. 56-65, Jan 1 2006.

[104] Z. Yu, *et al.*, "PTEN associates with the vault particles in HeLa cells," *J Biol Chem,* vol. 277, pp. 40247-52, Oct 25 2002.

[105] J. Krutzfeldt, *et al.*, "Silencing of microRNAs in vivo with 'antagomirs'," *Nature,* vol. 438, pp. 685-9, Dec 1 2005.

[106] S. M. Frisch and R. A. Screaton, "Anoikis mechanisms," *Curr Opin Cell Biol,* vol. 13, pp. 555-62, Oct 2001.

[107] B. Yao, *et al.*, "Quantitative analysis of zeptomole microRNAs based on isothermal ramification amplification," *RNA,* vol. 15, pp. 1787-94, Sep 2009.

[108] A. Krek, *et al.*, "Combinatorial microRNA target predictions," *Nat Genet.*, vol. 37, pp. 495-500, 2005

[109] A. Grimson, *et al.*, "MicroRNA targeting specificity in mammals: determinants beyond seed pairing," *Mol Cell.* , vol. 27, pp. 91-105, 2007.

[110] X. Wang, "miRDB: a microRNA target prediction and functional annotation database with a wiki interface," *Rna,* vol. 14, pp. 1012-7, Jun 2008.

[111] J. Kota, *et al.*, "Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model," *Cell,* vol. 137, pp. 1005-17, Jun 12 2009.

[112] S. Zhu, *et al.*, "MicroRNA-21 targets tumor suppressor genes in invasion and metastasis," *Cell Res.*, vol. 18, pp. 350-9., 2008.

[113] S. Varambally, *et al.*, "Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer," *Science,* vol. 322, pp. 1695-9, 2008

[114] H. Zhang, *et al.*, "Genome-wide functional screening of miR-23b as a

pleiotropic modulator suppressing cancer metastasis," *Nat Commun,* vol. 2, p. 554, 2011.

[115] D. Pyeon, *et al.*, "Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus-negative head/neck and cervical cancers," *Cancer Res,* vol. 67, pp. 4605-19, May 15 2007.

[116] S. Carbon, *et al.*, "AmiGO: online access to ontology and annotation data," *Bioinformatics,* vol. 25, pp. 288-9, Jan 15 2009.

[117] M. Kanehisa, *et al.*, "KEGG for integration and interpretation of large-scale molecular data sets," *Nucleic Acids Res,* vol. 40, pp. D109-14, Jan 2012.

[118] Q. C. Zhang, *et al.*, "Structure-based prediction of protein-protein interactions on a genome-wide scale," *Nature,* vol. 490, pp. 556-60, Oct 25 2012.

[119] M. Higuchi, *et al.*, "Akt1 promotes focal adhesion disassembly and cell motility through phosphorylation of FAK in growth factor-stimulated cells," *J Cell Sci,* Dec 21 2012.

[120] K. Asrani, *et al.*, "The HER2- and Heregulin beta1 (HRG)-inducible TNFR Superfamily Member Fn14 Promotes HRG-driven Cell Migration, Invasion and MMP9 Expression," *Mol Cancer Res,* Feb 1 2013.

[121] R. Breitling, *et al.*, "Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments," *FEBS Lett,* vol. 573, pp. 83-92, Aug 27 2004.

[122] Y. Xu, *et al.*, "Effect of estrogen sulfation by SULT1E1 and PAPSS on the development of estrogen-dependent cancers," *Cancer Sci,* vol. 103, pp. 1000-9, Jun 2012.

[123] R. Jansen, *et al.*, "A Bayesian networks approach for predicting protein-protein interactions from genomic data," *Science,* vol. 302, pp. 449-53, Oct 17 2003.

[124] Z. M. Goeckeler, *et al.*, "Phosphorylation of myosin light chain kinase by p21-activated kinase PAK2," *J Biol Chem,* vol. 275, pp. 18366-74, Jun 16 2000.

[125] M. D. Amatangelo, *et al.*, "c-Myc expression and MEK1-induced Erk2 nuclear localization are required for TGF-beta induced epithelial-mesenchymal

transition and invasion in prostate cancer," *Carcinogenesis,* vol. 33, pp. 1965-75, Oct 2012.

[126] L. A. Wiley, *et al.*, "The tumor suppressor gene Trp53 protects the mouse lens against posterior subcapsular cataracts and the BMP receptor Acvr1 acts as a tumor suppressor in the lens," *Dis Model Mech,* vol. 4, pp. 484-95, Jul 2011.

[127] G. A. Weiss, *et al.*, "Evaluation of phosphatidylinositol-3-kinase catalytic subunit (PIK3CA) and epidermal growth factor receptor (EGFR) gene mutations in pancreaticobiliary adenocarcinoma," *J Gastrointest Oncol,* vol. 4, pp. 20-9, Mar 2013.

[128] R. Kosoff, *et al.*, "Pak2 kinase restrains mast cell FcepsilonRI receptor signaling through modulation of Rho protein guanine nucleotide exchange factor (GEF) activity," *J Biol Chem,* vol. 288, pp. 974-83, Jan 11 2013.

[129] S. D. Smith, *et al.*, "Protein kinase Calpha (PKCalpha) regulates p53 localization and melanoma cell survival downstream of integrin alphav in three-dimensional collagen and in vivo," *J Biol Chem,* vol. 287, pp. 29336-47, Aug 24 2012.

[130] H. H. Li, *et al.*, "Phosphorylation on Thr-55 by TAF1 mediates degradation of p53: a role for TAF1 in cell G1 progression," *Mol Cell,* vol. 13, pp. 867-78, Mar 26 2004.

[131] Y. J. Han, *et al.*, "A transcribed pseudogene of MYLK promotes cell proliferation," *FASEB J,* vol. 25, pp. 2305-12, Jul 2011.

[132] C. Bitelman, *et al.*, "IGF1R-directed targeted therapy enhances the cytotoxic effect of chemotherapy in endometrial cancer," *Cancer Lett,* Feb 10 2013.

[133] J. Zaric, *et al.*, "Identification of MAGI1 as a tumor-suppressor protein induced by cyclooxygenase-2 inhibitors in colorectal cancer cells," *Oncogene,* vol. 31, pp. 48-59, Jan 5 2012.

[134] A. Levitzki, "Protein kinase inhibitors as a therapeutic modality," *Acc Chem Res,* vol. 36, pp. 462-9, Jun 2003.

[135] D. Fabbro, *et al.*, "Protein kinases as targets for anticancer agents: from inhibitors to useful drugs," *Pharmacol Ther,* vol. 93, pp. 79-98, Feb-Mar 2002.

PUBLICATIONS

Hanshuo Zhang, Yang Hao, Junyu Yang, Ying Zhou, Juan Li, Shenyi Yin, Changhong Sun, Ming Ma, Yanyi Huang and Jianzhong Jeff Xi. Genome-wide Functional Screening of miR-23b as a Pleiotropic Modulator Suppressing Cancer Metastasis. *Nature Communication*, vol. 2, pp. 554, 2011.

Hanshuo Zhang, Po-Yen Wu, Ming Ma, Yanzheng Ye, Yang Hao, Junyu Yang, Shenyi Yin, Changhong Sun, John H Phan, May Wang, and Jianzhong Xi. An Integrative Approach for the Large-scale Identification of Human Genome Kinases Regulating Cancer Metastasis. *Nanomedicine: NBM*, 2013. (Accepted; Joint paper)

Hanshuo Zhang, Juan Li, Gancheng Wang, Sha Hou, Mingjun Jiang, Ming Ma, Xiongbing Hu, Fengfeng Zhuang, Junbiao Dai and Jianzhong Jeff Xi. Engineered TAL Effector (TALE) Modulators for the Large-scale Gain-of-Function Screening. *Nucleic Acids Res*, 2013. (In revision)

Zhao Wang, Huang Huang, Hanshuo Zhang, Changhong Sun, Yang Hao, Junyu Yang, Yu Fan and Jianzhong Xi. A Magnetic Bead-Integrated Chip for the Large Scale Manufacture of Normalized esiRNAs. *PLoS ONE*, vol. 7, pp. 39419, 2012.

Yueguang Rong, Mei Liu, Liang Ma, Wanqing Du, Hanshuo Zhang, Yuan Tian, Zhen Cao, Ying Li, He Ren, Chuanmao Zhang, Lin Li, She Chen, Jianzhong Xi and Li Yu. Clathrin and Phosphatidylinositol 4,5-bisphosphate Regulate Autophagic Lysosome Reformation. *Nature Cell Biology*, vol. 9, pp. 924, 2012.

Zhao Wang, Juan Li, Huang Huang, Gancheng Wang, Mingjun Jiang, Shenyi Yin,

Changhong Sun, Hanshuo Zhang, Fengfeng Zhuang, and Jianzhong Jeff Xi. An Integrated Chip for the High-Throughput Synthesis of Transcription Activator-like Effectors. *Angew. Chem. Int. Ed.*, vol. 51, pp. 8505, 2012.

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