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## MicroRNA Regulation of Prostate Cancer Stem/Progenitor Cells and Prostate Cancer Development

Can Liu

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**MICRORNA REGULATION OF PROSTATE CANCER STEM/PROGENITOR CELLS  
AND PROSTATE CANCER DEVELOPMENT/METASTASIS**

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**MICRORNA REGULATION OF PROSTATE CANCER STEM/PROGENITOR CELLS  
AND PROSTATE CANCER DEVELOPMENT**

**A DISSERTATION**

Presented to the Faculty of

The University of Texas

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in Partial Fulfillment

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for the Degree of

**DOCTOR OF PHILOSOPHY**

by

**Can Liu, B.S.**

Smithville, Texas

**May, 2012**

## **Dedication**

*To my parents, for their endless love, support and encouragement*

*To my husband, Chaoming Zhang, for being my true love and inspiration for life*

## **ACKNOWLEDGMENT**

It is with sincerest gratitude that I thank my advisor Prof. Dean Tang for the challenging and exciting research topics he suggests, and for countless insightful comments and guidance he gives me. He has always been a constant source of inspiration and support throughout my Ph.D study. I have learned from him the characteristics of a real scientists, the passion about science and the perseverance during difficult times.

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Finally, I am grateful to my parents and my husband for their patience, love and support. Without them this work would never have come into existence.

# **MicroRNA Regulation of Prostate Cancer Stem/Progenitor Cells and Prostate Cancer Development**

Publication No. \_\_\_\_\_

Can Liu, B.S.

Supervisory Professor: Dean G. Tang, M.D., Ph.D.

Most human tumors contain a population of cells with stem cell properties, called cancer stem cells (CSCs), which are believed to be responsible for tumor establishment, metastasis, and resistance to clinical therapy. It's crucial to understand the regulatory mechanisms unique to CSCs, so that we may design CSC-specific therapeutics. Recent discoveries of microRNA (miRNA) have provided a new avenue in understanding the regulatory mechanisms of cancer. However, how miRNAs may regulate CSCs is still poorly understood. Here, we present miRNA expression profiling in six populations of prostate cancer (PCa) stem/progenitor cells that possess distinct tumorigenic properties. Six miRNAs were identified to be commonly and differentially expressed, namely, four miRNAs (miR-34a, let-7b, miR-106a and miR-141) were under-expressed, and two miRNAs (miR-301 and miR-452) were over-expressed in the tumorigenic subsets compared to the corresponding marker-negative subpopulations. Among them, the expression patterns of miR-34a, let-7b, miR-141 and miR-301 were further confirmed in the CD44<sup>+</sup> human primary prostate cancer (HPCa) samples. We then showed that miR-34a functioned as a critical negative regulator in prostate CSCs and PCa development and metastasis. Over-expression of miR-34a in either bulk or CD44<sup>+</sup> PCa cells significantly suppressed clonal expansion, tumor development and metastasis. Systemic delivery of miR-34a in tumor-bearing mice demonstrated a potent

therapeutic effect again tumor progression and metastasis, leading to extended animal survival. Of great interest, we identified CD44 itself as a direct and relevant downstream target of miR-34a in mediating its tumor-inhibitory effects. Like miR-34a, let-7 manifests similar tumor suppressive effects in PCa cells. In addition, we observed differential mechanisms between let-7 and miR-34a on cell cycle, with miR-34a mainly inducing G1 cell-cycle arrest followed by cell senescence and let-7 inducing G2/M arrest. MiR-301, on the other hand, exerted a cell type dependent effect in regulating prostate CSC properties and PCa development. In summary, our work reveals that the prostate CSC populations display unique miRNA expression signatures and different miRNAs distinctively and coordinately regulate various aspects of CSC properties. Altogether, our results lay a scientific foundation for developing miRNA-based anti-cancer therapy.

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## List of Abbreviations

μL	microliter
μM	micromolar
μg	microgram
ADT	Androgen deprivation treatment
ALDH	Aldehyde dehydrogenase
AR	Androgen receptor
BCSC	Breast cancer stem cell
CARNs	Castration Resistant Nkx3.1 expressing
C elegans	<i>Caenorhabditis elegans</i>
ceRNAs	competing endogenous RNAs
CLL	chronic lymphocytic leukemia
CSC	cancer stem cell
DAPI	4',6-diamidino-2-phenylindole
DDR1	discoidin domain receptor 1 tyrosine kinase
Dlx5	distal-less homeobox 5
DP	dorsal prostate
EMT	epithelial-mesenchymal transition
ESC	embryonic stem cell
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FFPE	formalin-fixed, paraffin-embedded
HPCa	human primary prostate cancer
IHC	immunohistochemical
iPSC	induced pluripotent stem cell
ISH	in situ hybridization

ITGB3	integrin $\beta$ 3
LSCs	leukemic stem cells
LRC	label retaining cells
MACS	magnetic activated cell sorting
MDR	multidrug resistant proteins
miRNA	microRNA
MKK4	mitogen-activated protein kinase kinase 4
NC	negative control
NHP	normal human prostate
NS	non silencing
nt	nucleotide
PCa	prostate cancer
PD	population doubling
PrEBM	prostate epithelial basal medium
PFA	paraformaldehyde
PSA	prostate specific antigen
qPCR	quantitative real-time PCR
RNP	ribonucleoproteins
SA- $\beta$ gal	senescence-associated $\beta$ -gal
SC	stem cell
shRNA	short hairpin RNA
SP	Side population
SIP1	Smad Interacting Protein 1
UBC9	ubiquitin-conjugating enzyme 9
ULA	ultra low attachment
UTR	untranslated region

TSA	Trichostatin A
wt	wild type
ZEB1	zinc-finger E-box binding homeobox 1

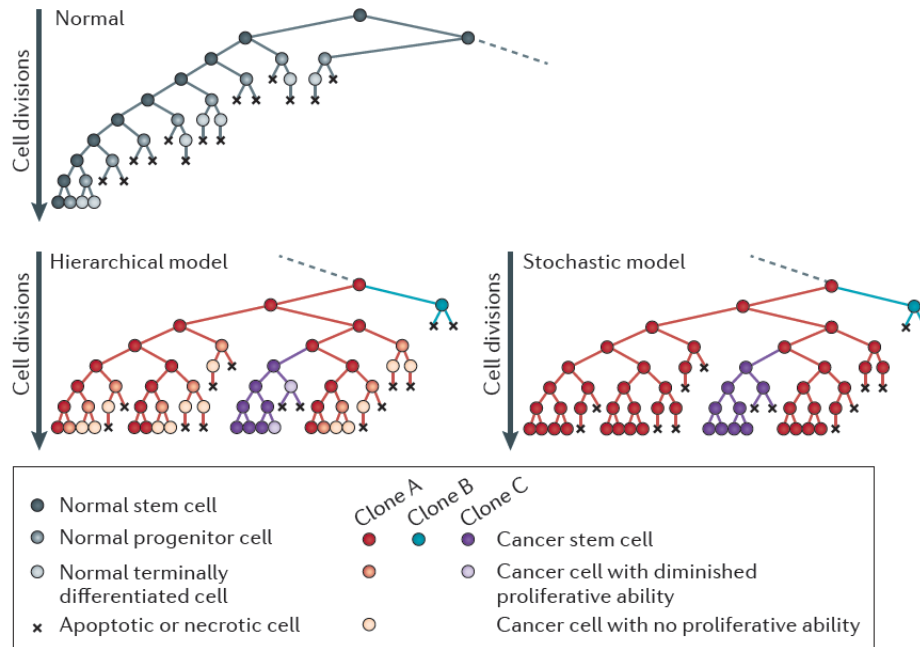
## **Chapter 1: Introduction**

Prostate cancer is the most common cancer among men in America, and it is the second leading cause of death following only lung cancer (American Cancer Society, 2012). Despite the extensive studies on its cause, early detection, and therapy, there are still many uncertainties/questions about the origin of the cancer, about the molecular mechanisms driving the uncontrolled expansion of cancer cells, as well as tumor recurrence and metastasis due to the failure to clinical therapies including surgical and chemical treatment such as androgen deprivation treatment (ADT). There is emerging evidence that a small population of cells with stem cell properties called cancer stem cells (CSCs) driving tumor initiation, progression, metastasis and response to the clinical treatments (1-4). The CSC hypothesis has provided significant implications for better understanding of the biology of cancer cells and to ultimately achieve cures to cancer.

### **1.1 CSCs: definition, identification, and characterization**

CSCs are operationally defined as a subset of tumorigenic cells that are endowed with enormous proliferative, clonogenic, self-renewal, and multi-lineage differentiation potentials *in vitro* and tumor-initiating ability in immune-deficient animals such as NOD/SCID mice (1-4). The CSC hypothesis proposes that cancer cells are organized in a hierarchical fashion, where CSCs occupy the apex and the majority of the differentiated cells lie at the bottom (Figure 1-1). CSCs, like their normal counterparts, possess two important and fundamental properties, which are self-renewal and differentiation. Self-renewal is the ability for CSCs to undergo symmetric division and replenish the cancer stem cell pool. During differentiation, CSCs may undergo asymmetric or symmetric division, therefore giving rise to the multi-lineage of differentiated cells that comprise the heterogeneous populations of cancer cells. Besides sharing those fundamental properties with the normal adult stem cells,

Figure 1-1



**Figure 1-1. Two possible models for CSC hypothesis.** The hierarchical model (bottom left) assumes that only a small population of biologically distinct cells is cancer stem cells (CSCs) within the total heterogeneous tumor cell populations. While the stochastic model (bottom right) assumes that every cell within the tumor have the same potential to be a CSC. Reprinted by permission from Nguyen *et al.*, Nature Reviews Cancer (5) copyright 2012.

CSCs are also believed to be responsible for metastasis and resistance to clinical treatment and relapse.

The most important criteria for defining CSCs is their ability to regenerate serially transplantable tumors that histologically recapitulate the cellular and phenotypic heterogeneity of parental tumors. CSCs were first identified in leukemia (6). Leukemic stem cells (LSCs), although constituting a minority (~0.1%), are the only cells that can transfer the disease to NOD/SCID mice. Since 2003, putative CSCs have been reported for many human solid tumors including brain tumor, breast cancer, colon cancer, pancreatic cancer and prostate cancer (7-13).

CSC populations may be phenotypically purified based on the expression of cell surface markers. For example, CD44 is one of the markers that has been widely used for enriching the stem cell populations in cancers of the breast (9), pancreas (13), head and neck (14), and prostate (15, 16). Other markers such as CD34, CD133, epithelial cell adhesion molecule (EpCAM) and many others have also been identified as markers of CSC populations (6, 11, 17). There are other methods that have also been widely used to identify CSCs in a specific tumor type or among many tumors. These include the side population (SP) phenotype, based on stem cells expressing multidrug resistant proteins (MDR) that can efflux the Hoechst dye 33342 (18, 19) and label-retaining cells (LRC), based on the properties that stem cells are generally more 'quiescent' than the fast-dividing tumor progenitor cells (20, 21). It is now clear that using cell surface markers or the above-mentioned techniques alone to define a stem cell is not sufficient, identification of the cancer stem cell population should always be put in an operational setting by performing the 'gold standard' tumor-regeneration assay.

Interestingly, CSC populations purified by the above-mentioned markers or techniques are phenotypically and functionally heterogeneous in regards to their intrinsic

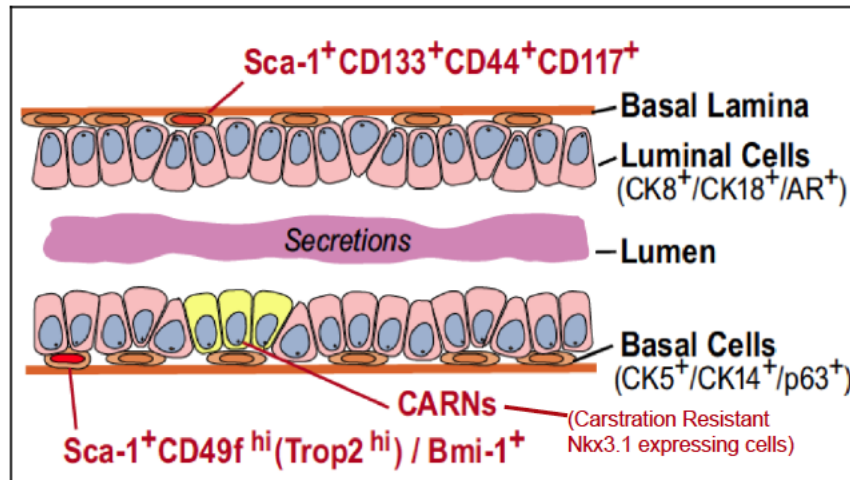
molecular traits and tumor regeneration abilities (22-24). For example, CD44(9), SP(25, 26), Aldehyde dehydrogenase (ALDH) activity (27, 28), and the PKH26 dye retaining (23) may all be used to enrich breast CSC; however, their expression level varied greatly, and their tumor regeneration abilities were significantly different. When there is overlap between different marker expression populations, it is possible that more defined CSC populations may be further enriched by combining these markers (27, 29).

## **1.2 Normal prostate epithelial stem/progenitor cells**

The prostate is a hormone-regulated glandular organ whose growth accelerates at sexual maturity. Existence of stem cells (SCs) in adult rodent prostate is supported by observations that the organ can undergo multiple rounds of castration-induced regression and testosterone-induced regeneration. Phenotypically, mouse prostate stem/progenitor cells in the proximal regions preferentially express surface markers Sca-1 and CD49f (30, 31). Recently, the Gao lab reported murine prostate SCs bearing the Lin<sup>-</sup> Sca1<sup>+</sup>CD133<sup>+</sup>CD44<sup>+</sup>CD117<sup>+</sup> phenotype that can regenerate secretion-producing prostatic glands (32). Another group reported that CARNs (Castration Resistant Nkx3.1 expressing) cells localized in the luminal layer can generate the glandular structure of mouse prostate (33) (Figure 1-2). Whether adult human prostate contains definitive SCs is less certain although several candidate populations of stem/progenitor cells have been reported (2, 34, 35). Work from our lab on normal human prostate (NHP) progenitor cells indicates that they express CD44 and  $\alpha 2\beta 1$  as well as some other stem cell markers including p63 and hTERT although CD133 could not be detected (36, 37).



Figure 1-2



**Figure 1-2. Illustration of a longitudinal view of prostatic duct from the proximal region and putative prostatic stem cells are indicated.** Adapted from Laffin B and Tang D, Cell Stem Cell, (38).

### 1.3 Hierarchical organization of Prostate Cancer cells

Studies on Prostate Cancer (PCa) stem/progenitor cells lag behind those of many other tumors such as breast and brain cancers and melanoma. Although both ABCG2 (39) and CD133 (15, 40) have been proposed to mark potential prostate CSCs, in these studies, the key experiments that define CSCs, i.e., to demonstrate enhanced tumor-initiating potential and self-renewal capacity in the marker-positive cell populations were not reported. Using tumor regeneration in NOD/SCID mice as the main yardstick combined with serial tumor transplantation, several PCa cell populations with enhanced tumor-initiating capacities that fit the definitions of CSCs have been identified (16, 19, 41-43) (Figure 1-3).

Firstly, putative prostate CSCs have been isolated using single or combinations of cell surface markers. For example, the CD44<sup>+</sup>CD24<sup>-</sup> population purified from patient prostate cancer samples likely harbors cancer stem-like cells with greater *in vitro* clonal expansion abilities(15). However, this study lacks *in vivo* tumor transplantation assay. Other prostate CSC-enriched populations such as CD44<sup>+</sup>/α2β1<sup>hi</sup>/CD133<sup>+</sup> Du145 cells (44), and CD44<sup>+</sup>CD133<sup>+</sup> cells from PC3 and Du145 cell lines (45) were identified based on their higher ability in clonal assay *in vitro* and tumor regeneration assay in the mice. Work from our lab has also identified that CD44<sup>+</sup> cells purified from three prostate xenograft models (LAPC9, LAPC4 and DU145) possess much higher clonal, clonogenic, serial sphere-forming, and, more importantly, tumorigenic potential than the isogenic CD44<sup>-</sup> PCa cells. Furthermore, the CD44<sup>+</sup> PCa cells express higher levels of 'stemness' genes including Oct-3/4, Bmi-1, β-catenin, and Smoothened (16). Clonal analysis indicates that a small subset of the CD44<sup>+</sup> cells can undergo asymmetric cell division, with only one of the daughter cells retaining CD44 expression (16).

Figure 1-3

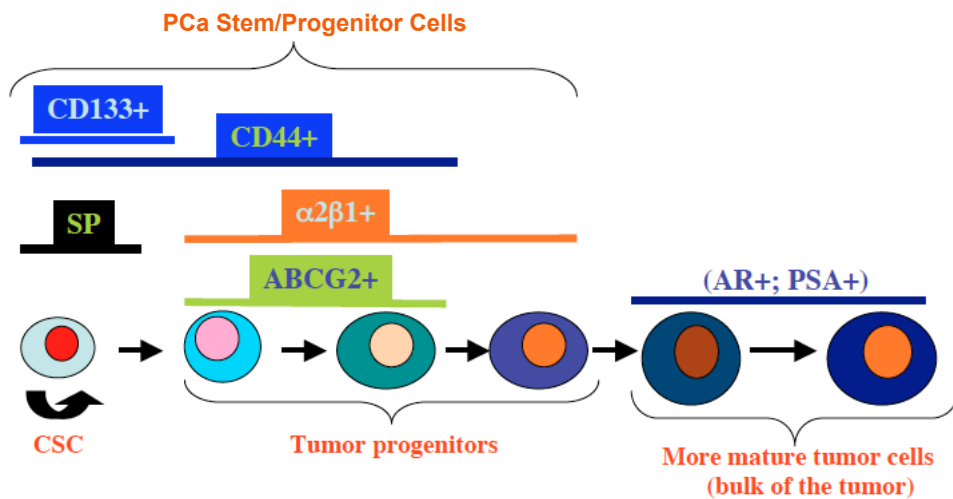


Figure 1-3. A model of hierarchical organization of heterogeneous PCa cells.

Adapted from Li *et al.*, Methods Mol. Biol., 2009.

Secondly, using the marker-independent method, several other CSC populations have been identified. The side population (SP) of PCa cells also contains tumorigenic cells (19, 46). The SP cells are highly tumorigenic with 1-100 cells being able to initiate serially transplantable tumors. The SP cells over-express 'stemness' genes such as  $\beta$ -catenin, Notch-1 and can self-renew regenerating SP and non-SP cells (19). Interestingly, the CD44<sup>+</sup> and SP PCa cells significantly overlap with ~97% of the SP cells being CD44<sup>+</sup>. Remarkably, essentially all metastatic activity resides in the CD44<sup>+</sup> (16) or the SP (43) cell population, suggesting that prostate CSCs may be the cells that mediate metastasis.

Thirdly, in contrast to the SP and CD44<sup>+</sup> cells, integrin  $\alpha 2\beta 1$ <sup>+</sup> (or  $\alpha 2\beta 1^{\text{hi}}$ ) and ABCG2<sup>+</sup> PCa cells identify fast-proliferating tumor progenitors (19, 41). Both ABCG2<sup>+</sup> (19) and  $\alpha 2\beta 1$ <sup>+</sup> (41) PCa cells, although highly proliferative *in vitro*, possess tumor-initiating capacities similar to the corresponding marker-negative cells.

Interestingly, PCa cell holoclones also contain self-renewing, highly tumorigenic cells that preferentially express CD44,  $\alpha 2\beta 1$ , and  $\beta$ -catenin whereas meroclone/paraclones are devoid of tumor-initiating cells (42).

Most of the aforementioned studies were performed in PCa cell lines or xenograft models. Direct evidence of the existence of CSCs in primary human prostate cancer is still lacking. In the past 4 years, our lab has worked on more than 100 fresh primary human PCa (HPCa) samples and has made critical progress by establishing a novel tumor-reconstitution protocol that allows purified HPCa cells to reliably regenerate tumors in NOD/SCID- $\gamma$  mice. These studies in HPCa samples will not only solidify our observations in xenografts but also provide a powerful tool to unveil the mystery of the human prostate cancer biology.

#### **1.4 MicroRNA: A brief introduction**

MicroRNAs (miRNA) are evolutionally conserved, small non-coding RNAs that are about 19-22 nucleotides (nt) long. MiRNAs modulate gene expression by repressing their targets' translation or inducing mRNA degradation through binding to the complementary sequence in target messenger RNA. Although tiny, miRNAs have been shown to play important roles in regulating developmental, physiological and oncogenic processes. So far, there are 1921 potential mature miRNAs have been reported in human (miRbase, v18, [www.mirbase.org](http://www.mirbase.org)), and they are predicated to regulate more than 60% of the human protein coding genes.

#### **1.4.1 miRNA biogenesis pathway**

The miRNA genes are first transcribed by RNA polymerase II into primary transcripts (pri-miRNA) in the nucleus; these pri-miRNAs are about hundreds to thousands of nt long and contain a characteristic hairpin stem-loop structure. Then a micro-processing complex including an RNase III enzyme, Drosha, and a double-stranded RNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8), cuts the hairpin stem-loop structure and process the species into precursor miRNA (pre-miRNA). The 70-100 nt long pre-miRNA is then actively exported into cytoplasm via Exportin 5, where it is cleaved by another RNase III enzyme, Dicer, resulting a miRNA:miRNA\* duplex. Then the mature miRNA is selected and assembled into a ribonucleoprotein complex, called miRNA-induced silencing complex (RISC), which contains an Argonaute family member, that directs the identification of target mRNA (47).

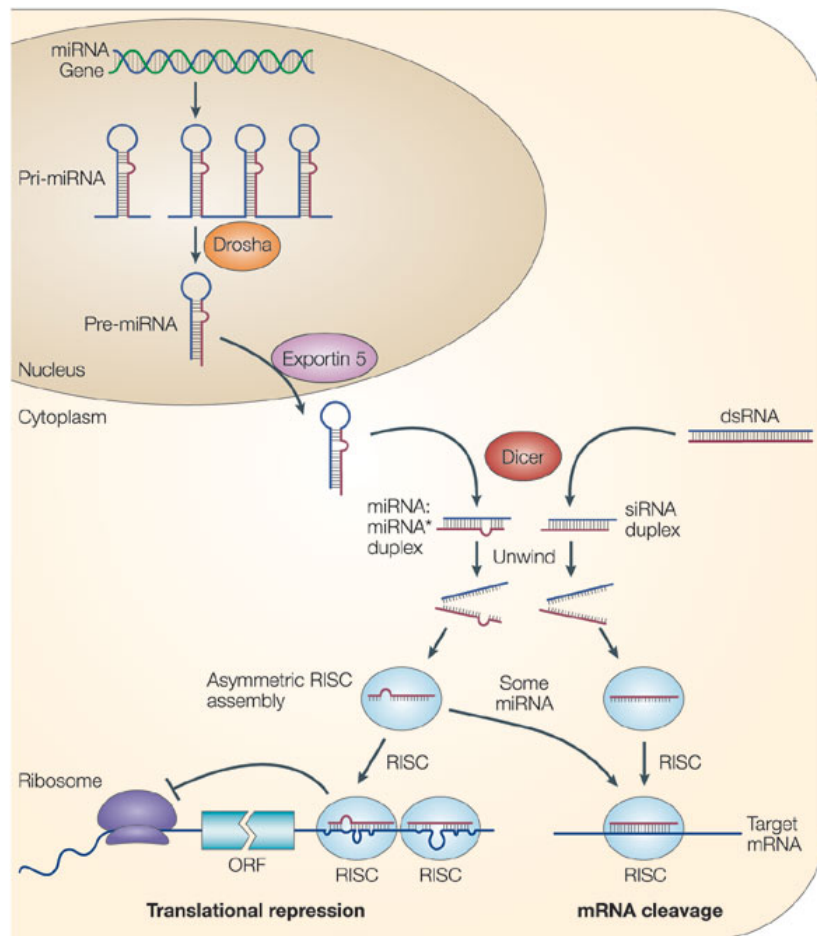
The miRNA target recognition is mediated through the sequence complementary between the 2-8 nt at the 5' end of miRNA (seed sequence) and the 3'-untranslated region (UTR) of target mRNA. The mechanisms of miRNA post-transcription regulation on its targets include blocking its translation and/or inducing mRNA degradation by deadenylation

and decapping. No matter which mechanism predominates, the overall output is the reduction of amount of protein encoded by the target messenger (48) (Figure 1-4).

Nevertheless, recent advances in miRNA studies have indicated that besides the above mentioned “canonical” mechanisms of gene regulations by miRNAs; there exist other alternative mechanisms of miRNA regulation. First, besides the recognition of its targets through 3'-UTR, there is evidence that the coding region or 5'-UTR of a mRNA might be recognized and involved in the gene regulation by miRNA (49). Secondly, miRNA may also activate gene expression rather than repress it. For example, several miRNAs were found to be enriched in the nucleus, and to activate gene transcription by binding to the promoter of the target genes (50). MiRNAs have also been shown to bind to other ribonucleoproteins (RNP) such as hnRNP E2, and exert a “decoy effect” by preventing its original function in RNA binding (51). Moreover, miRNAs may also involve in another layer of RNA transcript regulation called “competing endogenous RNAs (ceRNAs)” in which protein coding transcript and non-protein coding transcripts such as pseudogenes of an ancestral protein coding gene sharing sequence homology compete for the miRNA binding, thereby affecting protein coding gene expression (52, 53).

Each miRNA is predicted to target hundreds of mRNAs, and one mRNA can be regulated by multiple miRNAs simultaneously. Therefore it is estimated that more than 60% of the whole protein coding genome is regulated by the 1921 miRNAs currently reported. miRNA regulation is involved in almost all physiological processes, including stem cell self-renewal, differentiation, proliferation, metabolism, survival and death pathways. miRNAs have also been shown to regulate many aspects of oncogenesis.

Figure 1-4



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**Figure 1-4. miRNA biogenesis pathways and function in gene regulation.**

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#### **1.4.2 miRNA and stem cells**

The discovery of the first miRNA (lin-4) was in association with stem cell (SC) development in *Caenorhabditis elegans*. This miRNA was required for the transition from the L1 to L2 stage (55, 56). Let-7-associated family members (miR-48, miR-84 and miR-241) were later shown to be involved in the transition from L2 to L3 (57, 58), further implicating miRNAs in the maintenance and progression of SCs. Let-7 itself is involved in the transition from L4 to adult phenotypes (57). In the absence of lin-4 and members of the let-7 family, the SC lineage fails to differentiate and continues a proliferative cycle. The fact that lin-4 and let-7 are conserved over a number of species suggests that their developmental regulatory functions may also be conserved (59). There are several additional examples of miRNAs being key regulators of cell proliferation and death (60), cell differentiation (61), skeletal and cardiac muscle development (62-64) and brain and neural development (65-67). In addition, ongoing research is building solid evidence for the role of miRNAs in regulating SC division (68-74) and other SC properties (75-77).

#### **1.4.3 miRNA in cancer**

The first study of miRNA in cancer discovered that miR-15a/16-1 is often lost in chronic lymphocytic leukemia (CLL) (78). The advances in profiling techniques such as high-throughput screening and miRNA microarray in the past few years has facilitated investigator to identify aberrant expression of miRNAs in lung cancer, breast cancer, glioblastomas, pancreatic tumors and prostate cancers (79). Differential miRNA expression profiles were also observed between primary tumors and metastatic tumors. These differential expression profiles of miRNA are informative for diagnosis of the cancer origin and for prognosis prediction (80).



The mechanisms involved in the differential expression of miRNA include genetic location of the miRNA at cancer related regions (81), epigenetic regulation of miRNA expression (82), or abnormalities in genes and proteins involved in miRNA maturation processes such as Dicer, Exportin 5 or Ago family members (82). miRNA can be regulated by important oncogenes or tumor suppressor genes with transcription activities such as, p53, Myc and lin-28 (83-85).

#### **1.4.4 miRNA may function as oncogene or tumor suppressor gene**

The aberrant expression of miRNAs in cancer suggests that these miRNAs might be involved in pathological process of cancer development. Indeed, more and more studies showed that miRNAs may function as either oncogene or tumor suppressor gene. For example, miR-21, which is upregulated in cancers of the breast, lung, prostate, colon and glioblastomas, has been shown to be self-sufficient in promoting proliferation and can evade apoptosis by targeting several tumor suppressors like PTEN and PDCD4 (86, 87). Moreover, transgenic over-expression of miR-21 result in B cell lymphoma and that depends on the presence of miR-21 (“oncomiR addiction”) (88). Many other miRNAs that display oncogenic functions includes the miR-17-92 cluster, which is transactivated by oncogene c-Myc and promotes tumor growth in a lymphoma mouse model (89, 90).

On the other hand, many miRNAs that are under-expressed in cancers might exert tumor suppressor functions. For example, loss of miR-15 and miR-16 is often found in CLL patients. It has been shown that miR-15/16 cluster resides in chromosome 13q14, which is a genomic region often deleted in various cancers including CLL, prostate cancer, and pancreatic cancer, and loss of miR-15/16 negatively correlates with the level of anti-apoptotic protein BCL2 and is therefore associated with evasion of apoptosis (78, 81). Other important tumor suppressive miRNAs include the let-7 family, which is widely under-

expressed miRNAs in various tumors, and can suppress tumor development by targeting multiple oncogenic factors involved in tumor initiation and progression such as RAS, MYC and HMGA2 (91-93).

#### **1.4.5 miRNA in cancer therapeutics**

Given that miRNAs are differentially expressed in cancer compared to normal tissue and that miRNAs are involved in various aspects of cancer development, investigators have begun to develop miRNA based anti-cancer diagnostics and therapeutics (94).

First of all, miRNA expression profiles can be used as informative biomarkers in cancer diagnosis and prognosis. In several recent studies using miRNA expression profiles in classifying cancer origin, miRNA profiles surprisingly give a better correlation with the tissue origin of the cancer and metastatic status than the traditional mRNA profiles (79, 80). Also, miRNA levels in serum and other body fluids have been observed to associate with cancer progression and are being developed as powerful biomarkers for cancer diagnosis (95, 96).

Researchers have also been developing new techniques to either replace tumor suppressive miRNAs or target oncogenic miRNAs so as to negatively regulate tumor development. The advantage of using miRNAs as therapeutic targets is understandable. Each miRNA may target multiple factors in an oncogenic pathway therefore may exert a synergistic effects compared to the traditional therapeutic methods, which generally target a single molecule.

The miRNA mimic oligonucleotides (oligos), which are synthetic double-stranded RNA oligos that mimic the mature endogenous miRNAs, are often used in restoring tumor

suppressive miRNAs. For example, let-7 mimics delivered systemically through tail vein inhibited tumor development in a KRAS model of non-small cell lung cancer (97).

On the other hand, anti-sense oligos against miRNAs (antagomiRs) have been developed to block the oncogenic activities of the over-expressed miRNAs. For example, systemic delivery of antagomir miR-10b, which has been shown to promote metastasis in breast cancer, has successfully suppressed the metastasis of breast cancer cells to the lung (98).

### **1.5 Outline for my Ph.D thesis research: Understanding miRNA regulation of prostate cancer stem cells**

Based on these previous observations, we hypothesize that miRNAs are critical regulators of PCa stem/progenitor cells and PCa development. We were the first to carry out miRNA expression profiling in prostate CSC populations. In Chapter 2, we present the experimental process of miRNA expression profiling in several PCa stem/progenitor cell populations, and further discuss the prostate CSC-specific miRNA signature as well as several commonly differentially expressed miRNAs in the CSC populations. In Chapter 3, we focus on one of the commonly under-expressed miRNAs, i.e., miR-34a, and perform *in vivo* and *in vitro* functional assays regarding the stem cell related properties. Our results show that miR-34a is a critical negative regulator in prostate CSCs and PCa metastasis by targeting CD44. In Chapter 4, we adopt similar experimental design to investigate the effect of let-7 in PCa cells. We demonstrate that like miR-34a, let-7 also negatively regulates the prostate tumor development, but with different mechanisms of action on cell cycle. In Chapter 5, we present our pilot study on the role of miR-141 in regulating PCa development and propose several experiments to further elucidate its mechanisms of action.

## **Chapter 2: miRNA expression profiling in prostate cancer stem/progenitor cells**

### **2.1 Introduction**

#### **2.1.1 miRNA regulation of development and embryonic stem cells (ESCs)**

Lin-4 and let-7 are the first two miRNAs to be identified. Interestingly, both of them were discovered during *Caenorhabditis elegans* (*C. elegans*) development. Lin-4 controls the larval stage transition from L1 to L2, by repressing lin-14, a factor required for proper developmental timing (55, 99). Let-7 controls the *C. elegans* larval stage transition from L4 to adulthood by repressing lin-41 (58). Since then, evidence from several system has implicated that miRNA may be involved in regulating embryonic stem cell (ESC) and embryonic development.

The global functions of miRNA have been evaluated by the consequences of Dicer and DGCR8 mutants in human and mouse ESCs. Specifically, complete blockage of miRNA maturation by deletion of Dicer in mouse caused embryonic lethality (100). Both dicer-deficient as well as DGCR8-deficient mouse ESCs exhibited defect in differentiation and G1 cell-cycle arrest (101, 102).

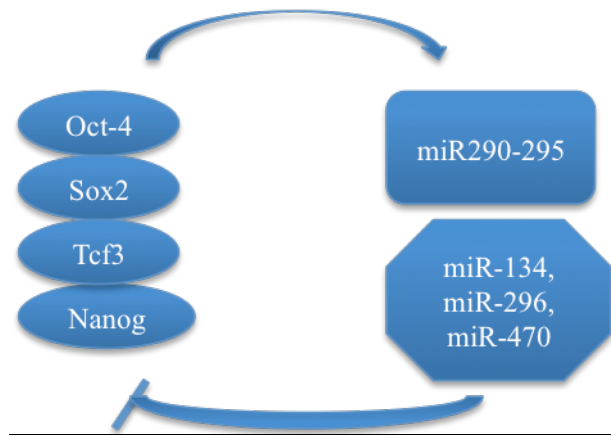
In addition to the overall involvement of the miRNA pathway in ESCs, other researchers have also revealed the specific miRNA expression and functions in ESCs. For example, a set of 32 miRNA differentially expressed in human ESCs was reported (103). In addition, miR-290-295 cluster, miR-296, miR-302, miR-17-92 cluster and miR-15b-16 cluster are over-expressed in ESCs but decreased during differentiation and absent in adult tissue, whereas miR-21 and 22 are found abundantly expressed in differentiated and adult tissue (68, 104).

Importantly, several regulatory circuits between miRNAs and the pluripotent genes required for maintaining the stemness have been reported, revealing an amazing network of stem cell regulation. For example, the core transcription factors of stem cells pluripotency, OCT-4, NANOG, SOX2, TCF3, occupy the promoter regions of a set of miRNAs that are preferentially expressed in mouse ESCs such as miR-290-295 cluster and transcriptionally activate their expression (49, 105). On the other hand, some of these pluripotent genes are regulated by miRNAs at the post-transcriptional level. MiR-134, miR-296, and miR-470, for instance, have been shown to suppress the expression of the core transcription factors NANOG, OCT4 and SOX2 by binding to their coding regions (49) (Figure 2.1)

### **2.1.2 miRNA regulation of cancer stem cells**

Increasing evidence has suggested that miRNAs might also be involved in regulating CSC properties. First of all, miRNA expression signature specific for CSC populations has been reported in several cancers. In breast cancer, Yu *et al.* reported that let-7 as well as a number of other miRNAs including miR-16, miR-107, miR-128 and miR-20b were significantly reduced in breast CSC (BCSC) enriched by consecutively passaging breast cancer cell line SKBR3 in mice treated with chemotherapy (106). Clarke's group used the cell surface marker CD44 and CD24 expression to enrich BCSC, and identified a set of 37 miRNAs to be differentially expression in CD44<sup>+</sup>/CD24<sup>-/lo</sup> BCSC population, in which three clusters, miR-200c-141, miR-200b-200a-429, and miR-183-96-182 were significantly down-regulated (107). MiRNA deregulation has also been reported in glioblastoma and other brain CSCs (108-110). For example, by comparing miRNA expression in CD133<sup>+</sup> glioblastoma stem cells with the CD133<sup>-</sup> population, Gal *et al.* showed that miR-451 as well as miR-486, miR-425, miR-16, miR-107 and miR-185 level were increased in the CD133<sup>-</sup> population (108). In hepatic CSC identified by EpCAM<sup>+</sup>AFP<sup>+</sup> profile, researchers also discovered a

Figure 2-1



**Figure 2-1. Regulatory circuit of miRNA and core “pluripotent” genes.**

unique miRNA signature in which miR-181 family and several miR-17-92 cluster members were up-regulated in the CSC population (111).

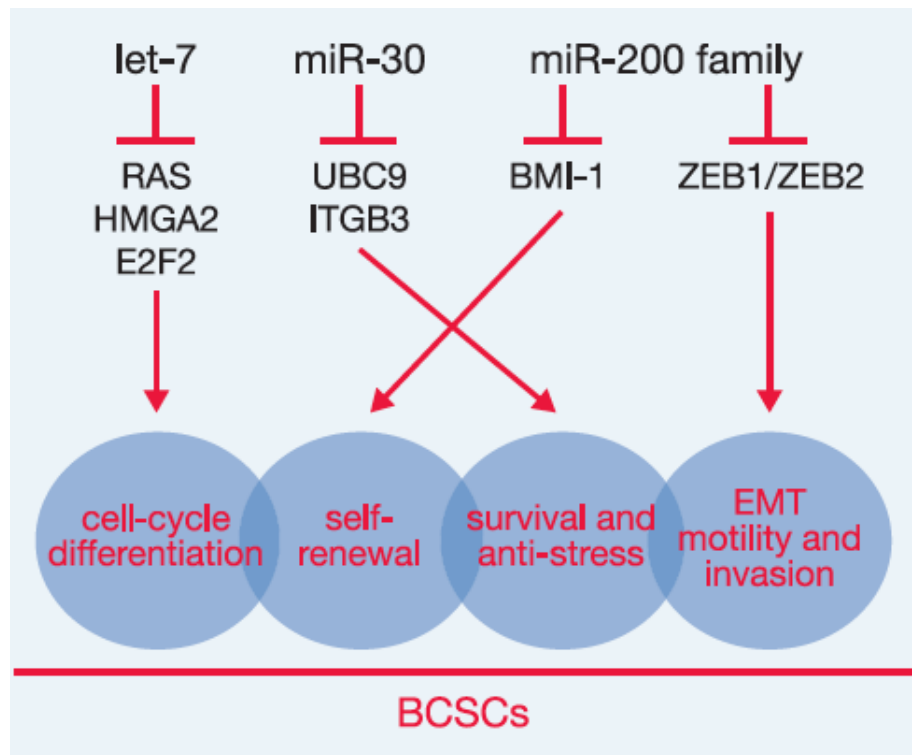
Furthermore, miRNAs have been shown to regulate various CSCs properties, including self-renewal and differentiation, tumorigenesis, metastasis and chemoresistance. For instance, under-expression of let-7 in BCSCs seems to be important for maintaining the stem cell properties (106). Thus, overexpression of let-7 with let-7-lentivirus inhibited proliferation, mammosphere formation, and the proportion of undifferentiated cells *in vitro* and tumor formation and metastasis in NOD/SCID mice (106). In contrast, antagonizing let-7 enhanced the *in vitro* self-renewal of non-CSC (106).

Interestingly, over-expression of miR-30, another miRNA that was also markedly reduced in BCSCs, not only inhibited their ability of self-renewal, but also inhibited anoikis resistance and increased apoptosis by directly targeting UBC9 (ubiquitin-conjugating enzyme 9) and ITGB3 (integrin  $\beta$ 3) (112). Importantly, a more complete inhibition of self-renewal and mammosphere formation in BCSC was observed when introducing both let-7 and miR-30 at the same time (112). This synergistic inhibitory effect of let-7 and miR-30 on self-renewal indicated that multiple miRNAs distinctively and concertedly regulate CSC properties (Figure 2.2).

Several other miRNAs such as miR-200c that is under-expressed in BCSCs and normal mammary stem cells, and miR-128 that was under-expressed in glioma stem cells may regulate the clonogenic and tumor-initiating activities by targeting the stem cell factor BMI-1 (107, 109).

miR-34, a p53 downstream target, has also been shown to negatively regulate the stem cell properties of pancreatic and gastric CSCs; thus, over-expression of miR-34 inhibited sphere formation *in vitro* and tumor formation *in vivo* via modulating downstream targets Bcl-2, NOTCH, HMGA2 (113, 114).

Figure 2-2



**Figure 2-2. miRNAs distinctively and coordinately regulate key properties of CSCs** Reprinted by permission from Liu C and Tang D, Cancer Research (115), copyright 2011.



miRNAs may also regulate metastasis, which might be mediated by CSC. Several studies have reported that miR-205 and miR-200 family members are involved in one most critical step in the metastatic cascade, epithelial-mesenchymal transition (EMT). For instance, miR-205 and miR-200 family members were found significantly down-regulated in cells that undergone EMT and in metastatic breast cancer specimens (116, 117). Over-expression of miR-200 negatively regulates the expression of EMT activator ZEB1 and ZEB2. Interestingly, ZEB1/ZEB2 also transcriptionally repress the expression of miR-200 by binding to its promoter and strongly activate EMT. These findings establish a negative feedback loop between miR-200 family and ZEB1/ZEB2 that regulates important biological processes in cancer metastasis. (116, 117).

### **2.1.3 Rationale for the experiments in this chapter**

Deregulation of miRNAs has been observed in a large variety of human tumors (78, 79, 118). In PCa, several groups have performed miRNA expression profiling studies using either miRNA microarray (119-123) or whole-genome deep sequencing (124) in PCa cell lines, xenografts or patient samples, and have reported PCa-specific miRNA alterations. These studies, although shedding light on differential miRNA expression in PCa (relative to benign tissues), have all been performed in bulk tumor cells and thus fail to address alterations of miRNA expression and functions specifically in tumorigenic PCa cell subsets. To explore the miRNA expression signature in the CSC populations in PCa, we took 6 marker-sorted prostate CSCs enriched populations and carried out miRNA expression profiling by quantitative real-time PCR (qPCR). From the profiling, we identified CSC specific miRNA expression signatures and several miRNAs that are commonly and differentially expressed in PCa stem/progenitor cell populations.

## **2.2 Materials and Methods**

### **Cells, xenografts, and animals**

Human xenograft prostate tumors, LAPC9 (bone metastasis; AR<sup>+</sup> and PSA<sup>+</sup>), LAPC4 (lymph node metastasis; AR<sup>+</sup> and PSA<sup>+</sup>), and Du145 (brain metastasis; AR<sup>-</sup> and PSA<sup>-</sup>) were maintained in NOD/SCID mice. NOD/SCID mice were produced mostly from our own breeding colonies and purchased occasionally from the Jackson Laboratories (Bar Harbor) and maintained in standard conditions according to the Institutional guidelines. All animal experiments were approved by our institutional IACCUC.

### **PCa cell purification**

Human PCa cells were purified out of xenografts by first depleting murine cells. CD44<sup>+</sup> and CD44<sup>-</sup> cells from LAPC9, LAPC4, and Du145 xenografts were further purified using fluorescence-activated cell sorting (FACS) with the purities of both populations being >98% (16, 41). CD133<sup>+</sup> and CD133<sup>-</sup> LAPC4 cells were purified using biotinylated monoclonal antibody to CD133 (AC133) and the magnetic activated cell sorting (MACS) by following the manufacturer's instructions (Miltenyi Biotech). Post-sort analysis revealed purities of both populations being >95%. Side population (SP) of LAPC9 cells were purified by FACS as previously described (19).

Human primary prostate tumors (HPCa) samples were obtained with the patients' consent from robotic surgery. All work with HPCa samples was approved by the M.D. Anderson Cancer Center Institutional Review Board (IRB LAB04-0498). We purified epithelial HPCa cells through a multi-step enzyme digestion process and by depleting

lineage-positive hematopoietic, stromal, and endothelial cells (43, 125). Lin<sup>-</sup>CD44<sup>+</sup> and CD44<sup>-</sup> HPCa cells were further purified by using MACS or FACS.

### **Quantitative RT-PCR**

MiRNA levels were quantified using TaqMan MicroRNA Assays (Applied Biosystems). Briefly, total RNA was isolated from unsorted LAPC9, LAPC4 and Du145 xenograft cells using the mirVANA PARIS miRNA Isolation Kit (Ambion), and was used to measure the levels of a library of 310 sequence-validated human miRNAs. Then 136 miRNAs were further measured in purified marker-positive and corresponding marker-negative cell populations, i.e. CD44<sup>+</sup> and <sup>-</sup> populations from LAPC9, LAPC4 and Du145, CD133<sup>+</sup> and <sup>-</sup> cells from LAPC4 tumor and α2β1<sup>+</sup> and α2β1<sup>-</sup> cells from Du145 tumor. Finally, 57 miRNAs were measured in the SP and non-SP LAPC9 cells due to limited numbers of cells. Quantitative miRNA expression data were normalized to internal 'housekeeping' miRNAs, i.e. miR-24 and miR-103, and difference between the positive population and that of the negative population, i.e., ddCt values, for each of the miRNAs were converted to percentage of expression using the formula  $2^{-ddCt}$ .

## **2.3 Results**

### **2.3.1 miRNA expression profiling in PCa stem/progenitor cell populations**

To determine the expression profiles of miRNA in PCa stem/progenitor cell populations, we first measured the expression levels of a library of 310 mature human miRNAs by qPCR in unsorted PCa cells purified from 3 xenografts, i.e., LAPC9, LAPC4, and Du145 (Figure 2-3, step I). The average Ct value of each miRNA in all 3 xenograft PCa cells

ranged from 22 up to 50. Among the 310 miRNAs, we chose 136 miRNAs, which included 120 miRNAs that were expressed at reliably detectable levels (Ct values less than 33) and 16 miRNAs of interests that were less abundant (Ct values between 35 to 50). We subsequently measured these 136 miRNAs in 5 pairs of marker sorted populations, i.e., CD44<sup>+</sup> and CD44<sup>-</sup> cells purified from LAPC9, LAPC4 and Du145 tumors; CD133<sup>+</sup> and CD133<sup>-</sup> cells from LAPC4 tumors, and  $\alpha 2\beta 1^{+}$  and  $\alpha 2\beta 1^{-}$  cells from Du145 tumors (Figure 2-3, step II). Our lab has previously demonstrated that in these xenograft models the CD44<sup>+</sup> PCa cells are highly enriched in both tumor- and metastasis-initiating cells (16, 41) whereas the  $\alpha 2\beta 1^{+}$  PCa cells mostly represent fast proliferating tumor progenitors (41). Collins *et al* have shown that the CD133<sup>+</sup>(CD44<sup>+</sup>  $\alpha 2\beta 1^{hi}$ ) cells purified from primary PCa (i.e., HPCa) samples are highly clonogenic (15) and we have also found that the CD133<sup>+</sup> LAPC4 cells possess higher clonogenic potential than the corresponding CD133<sup>-</sup> cells. In addition, we also purified side population (SP) from the LAPC9 tumor, which harbors great tumor-regenerative activity (19). Since the SP represents <0.1% of the total population in LAPC9 tumor (19), we manually curated 57 miRNAs that could be reliably detected in PCa cells and compared their expression levels in the SP vs. non-SP cells (Figure 2-3, step III). Comparisons of 6 marker-positive and the corresponding marker-negative populations revealed informative differences in miRNA expression patterns.

### **2.3.2 Unique miRNA expression patterns in different marker sorted populations**

By comparing the miRNA expression levels in marker positive populations to their corresponding marker negative populations, we observed that each marker-sorted population exerted its unique miRNA expression patterns. Overall, there were significantly more under-expressed miRNAs with much higher magnitude than the over-expressed ones in the CD44<sup>+</sup> LAPC4 (Figure 2-4) and LAPC9 (Figure 2-5) tumor cell compared to the

corresponding CD44<sup>-</sup> cells as well as in LAPC9 SP compared to the non-SP cells (Figure 2-6). In contrast, the CD44<sup>+</sup> and CD44<sup>-</sup> Du145 cells had roughly similar numbers of over-expressed and under-expressed miRNAs (Figure 2-7). On the other hand, CD133<sup>+</sup> LAPC4 (Figure 2-8) and  $\alpha$ 2 $\beta$ 1<sup>+</sup> Du145 (Figure 2-9) cell populations showed more over-expressed miRNAs than the under-expressed ones.

In CD44<sup>+</sup> LAPC4 cells, miR-10b was one of the top over-expressed miRNAs (Figure 2-4). MiR-10b is upregulated in metastatic breast cancer cells and positively regulates breast cancer cell migration and invasion (126).

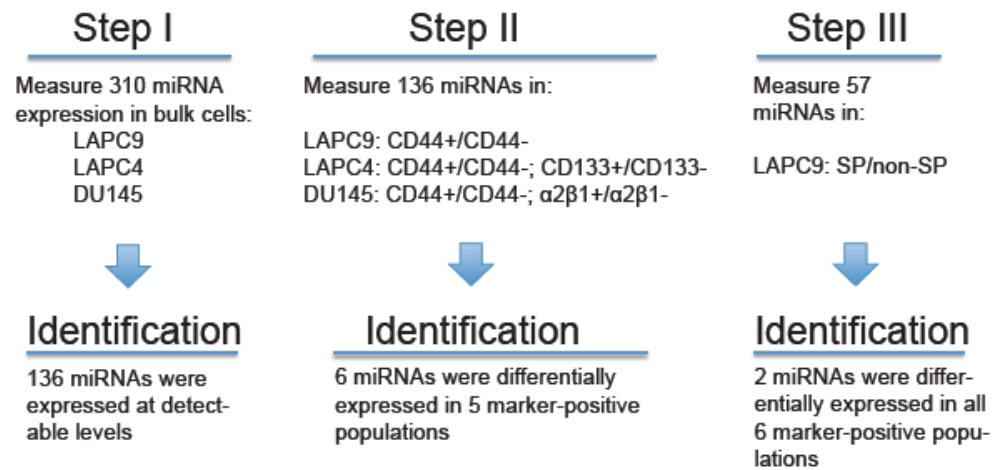
In LAPC9 SP cells, among the top over-expressed miRNAs was miR-451 (Figure 2-6), which was recently shown to regulate the self-renewal and tumorigenicity in glioblastoma (108) and colorectal cancer stem cells (127). Among the top 10 under-expressed miRNAs in the SP were miR-15a/15b and several tumor suppressive miRNAs.

Interestingly, both miR-21 and miR-451 were turned up as top over-expressed miRNAs in CD44<sup>+</sup> Du145 cells (Figure 2-7).

Among the top over-expressed miRNAs in CD133<sup>+</sup> LAPC4 cells was miR-21, one of the best-characterized oncomiRs in human cancers (128). Among the top 10 down-regulated miRNAs were several well-known tumor-suppressive miRNAs including miR-133a, miR-126, miR-15a and miR-200a (Figure 2-8).

Surprisingly, among the top 10 upregulated miRNAs in  $\alpha$ 2 $\beta$ 1<sup>+</sup> Du145 cells were miR-30a-5p, let-7a, and miR-196a (Figure 2-9), which were under-expressed in CD44<sup>+</sup> PCa cells. These results are, nevertheless, consistent with our earlier conclusions that the  $\alpha$ 2 $\beta$ 1<sup>+</sup> PCa cells mark fast proliferating progenitor cells (rather than slow-cycling CSCs), and that the  $\alpha$ 2 $\beta$ 1<sup>-</sup> cell population contains CD44<sup>+</sup> PCa cells (41).

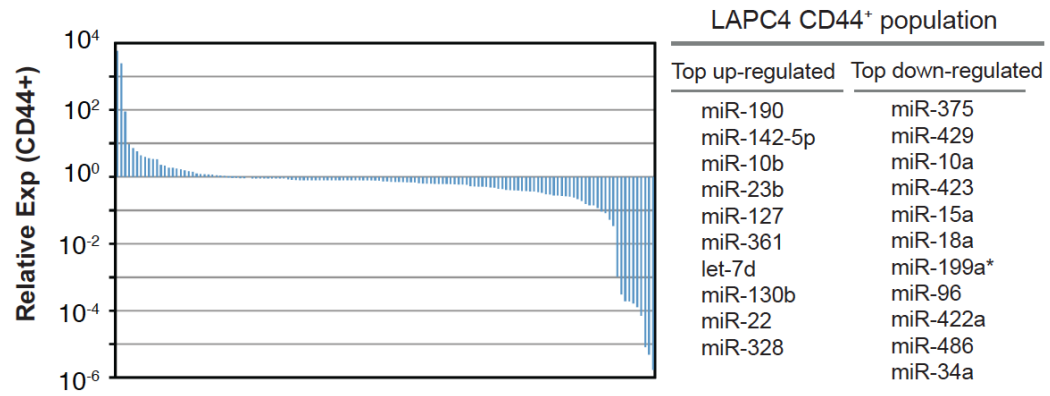
Figure 2-3



**Figure 2-3. miRNA expression profiling scheme.**

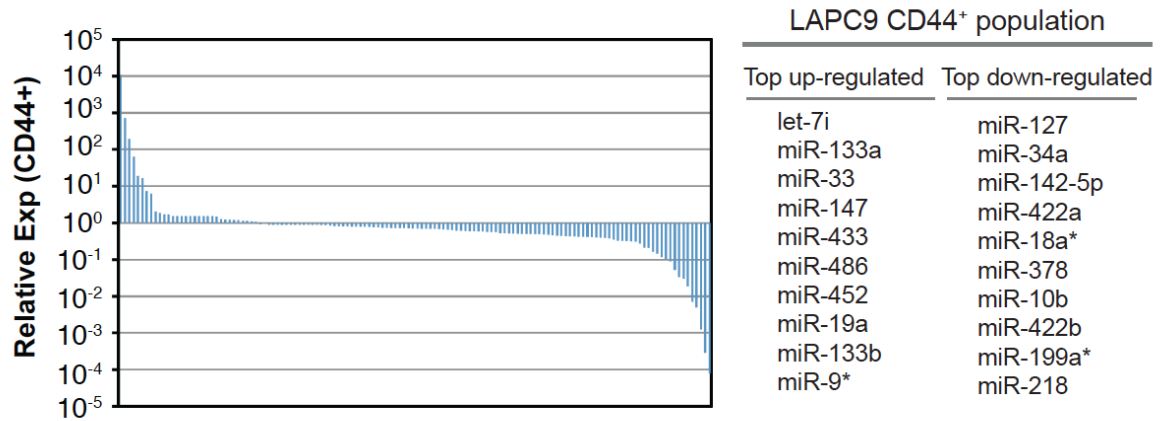
Three major steps were indicated.

Figure 2-4



**Figure 2-4. miRNA expression profiles in CD44<sup>+</sup> LAPC4 cells.** Shown are the relative miRNA expression levels in CD44<sup>+</sup> compared to CD44<sup>-</sup> LAPC4 cells. The top 10 up- or down- regulated miRNAs are listed on the right.

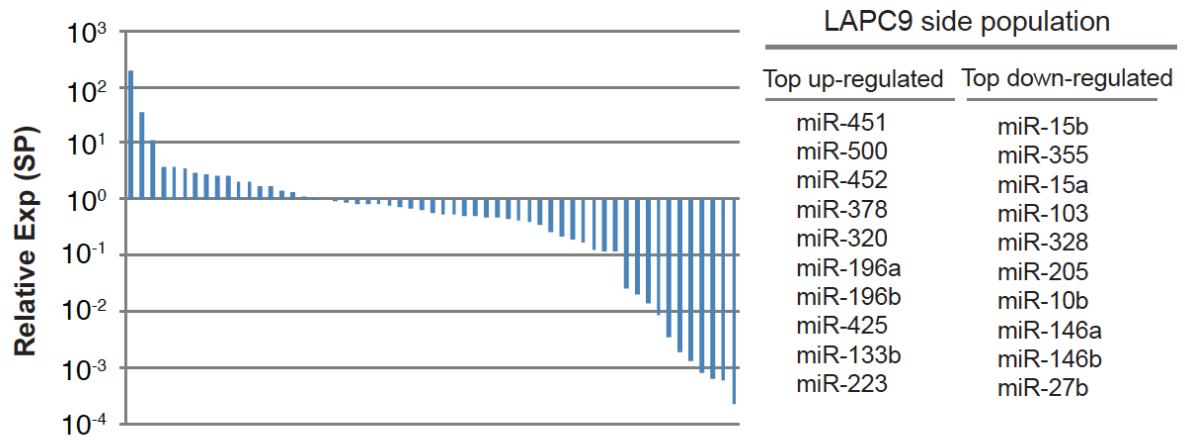
Figure 2-5



**Figure 2-5. miRNA expression profiles in CD44<sup>+</sup> LAPC9 cells.** Shown are the relative miRNA expression levels in CD44<sup>+</sup> compared to CD44<sup>-</sup> LAPC9 cells. The top 10 up- or down- regulated miRNAs are listed on the right.

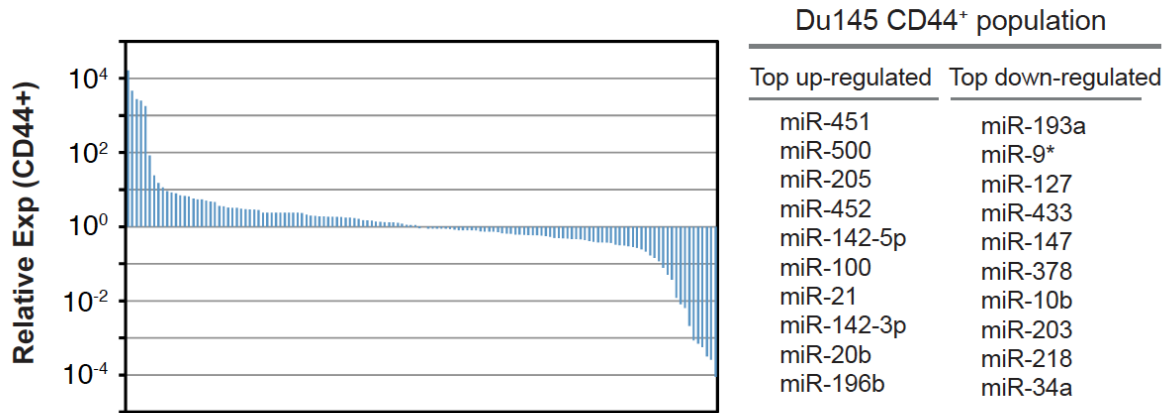


Figure 2-6



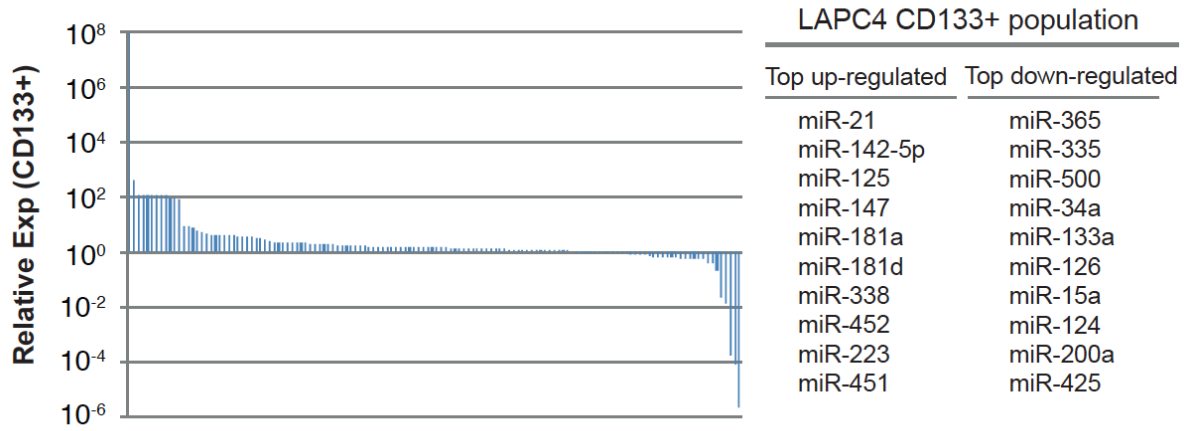
**Figure 2-6. miRNA expression profiles in the LAPC9 side population.** Shown are the relative miRNA expression levels in SP compared to non-SP LAPC9 cells. The top 10 up- or down- regulated miRNAs are listed on the right.

Figure 2-7



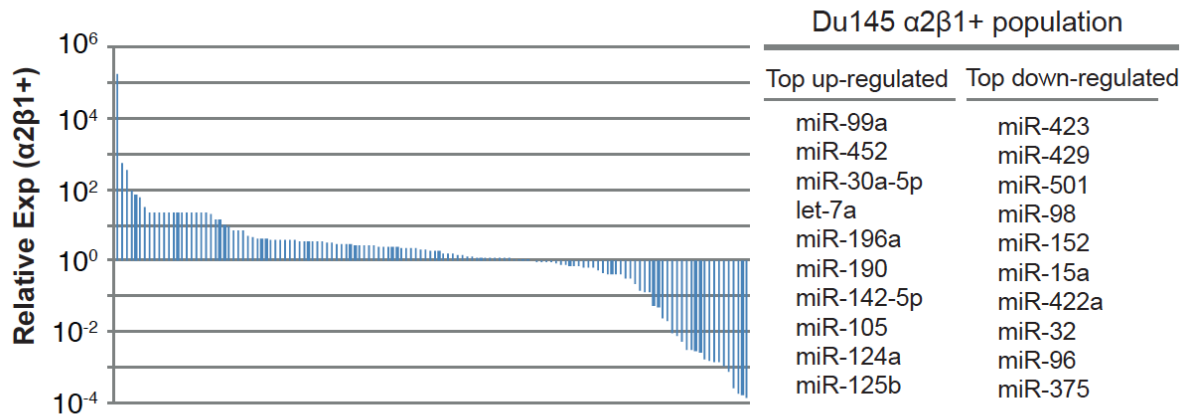
**Figure 2-7. miRNA expression profiles in the CD44<sup>+</sup> Du145 cells.** Shown are the relative miRNA expression levels in CD44<sup>+</sup> compared to CD44<sup>-</sup> Du145 cells. The top 10 up- or down- regulated miRNAs are listed on the right.

Figure 2-8



**Figure 2-8. miRNA expression profiles in the CD133<sup>+</sup> LAPC4 cells.** Shown are the relative miRNA expression levels in CD133<sup>+</sup> compared to CD133<sup>-</sup> LAPC4 cells. The top 10 up- or down- regulated miRNAs are listed on the right.

Figure 2-9



**Figure 2-9. miRNA expression profiles in  $\alpha 2\beta 1^+$  Du145 populations.** Shown are the relative miRNA expression levels in  $\alpha 2\beta 1^+$  compared to  $\alpha 2\beta 1^-$  Du145 cells. The top 10 up- or down- regulated miRNAs are listed on the right.

### **2.3.3 Cell type specific miRNA expression patterns**

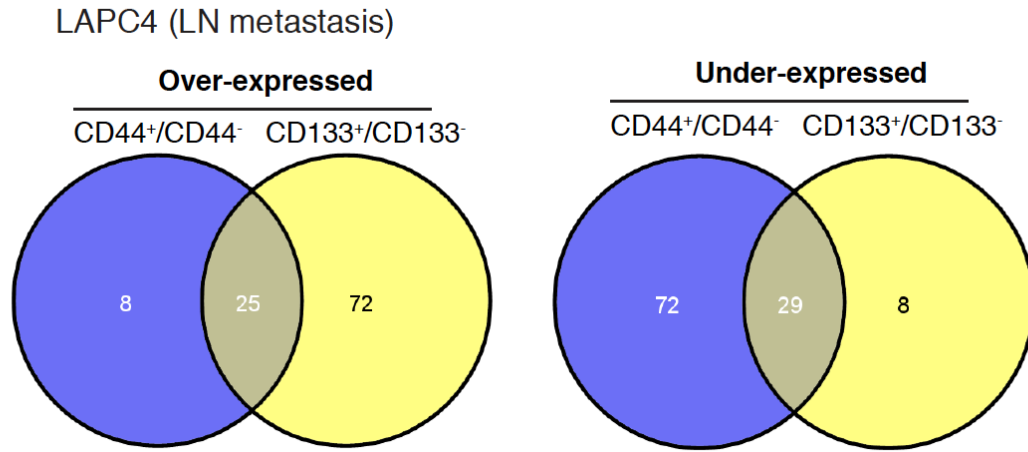
We then compared the expression profiles between different marker-sorted cell populations in each cell type and identified miRNAs that were commonly under- or over-expressed specific to each cell type.

When we compared the 136 miRNA expressions in CD133<sup>+</sup> vs. CD44<sup>+</sup> LAPC4 cells, we observed 23 commonly over-expressed and 29 commonly under-expressed miRNAs (Figure 2-10). Four let-7 miRNA family members (let-7b, let-7f, let-7g and let-7i) were commonly under-expressed, three miR-200 family members were also under-expressed including miR-200a, miR-200a\* and miR-141, whereas miR-10b was the top commonly over-expressed miRNAs in both CD133<sup>+</sup> and CD44<sup>+</sup> LAPC4 cells (Table 2-1).

We observed 6 commonly over-expressed and 31 commonly under-expressed miRNAs in the SP and CD44<sup>+</sup> LAPC9 cells (Figure 2-11). Of interest, the miR-15/16 cluster members including miR-15a, miR-15b and miR-16, and two miR-200 family members, miR-200a and miR-200c were commonly under-expressed in both SP and CD44<sup>+</sup> LAPC9 cells (Table 2-1).

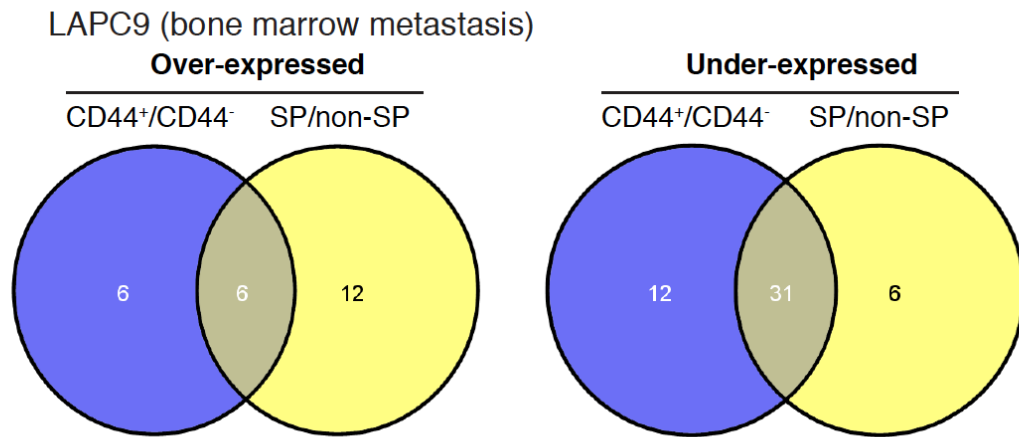
Finally, we observed 43 commonly over-expressed and 22 commonly under-expressed miRNAs in the  $\alpha 2\beta 1$ <sup>+</sup> and CD44<sup>+</sup> Du145 cells (Figure 2-12).

Figure 2-10



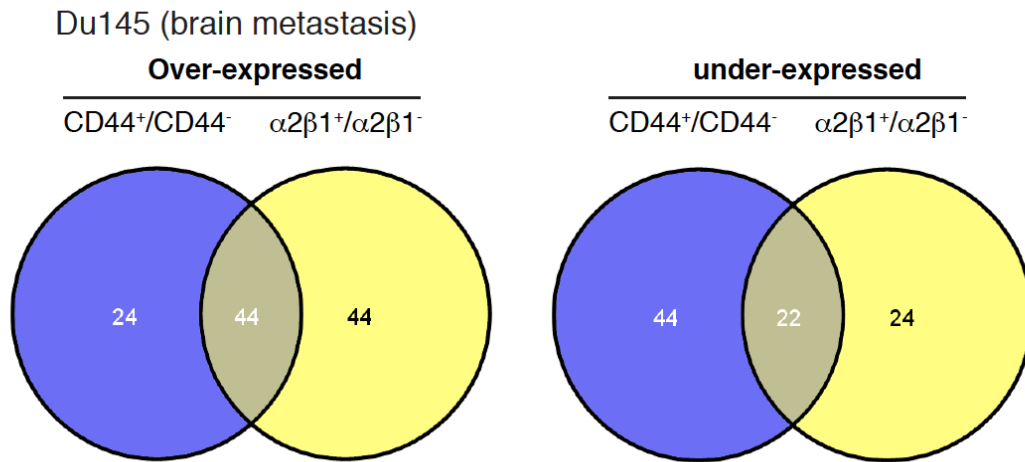
**Figure 2-10 Venn diagrams showing unique and common miRNAs in LAPC4 xenograft model.** Shown are the unique and shared miRNAs that are over-expressed (left panels) or under-expressed (right panels) in the indicated cell populations.

Figure 2-11



**Figure 2-11 Venn diagrams showing unique and common miRNAs in LAPC9 xenograft model.** Shown are the unique and shared miRNAs that are over-expressed (left panels) or under-expressed (right panels) in the indicated cell populations.

Figure 2-12



**Figure 2-12 Venn diagrams showing unique and common miRNAs in Du145 xenograft model.** Shown are the unique and shared miRNAs that are over-expressed (left panels) or under-expressed (right panels) in the indicated cell populations.



Table 2-1

LAPC4 (CD133 <sup>+</sup> vs CD44 <sup>+</sup> )		LAPC9 (SP vs CD44 <sup>+</sup> )		Du145 ( $\alpha$ 2 $\beta$ 1 <sup>+</sup> vs CD44 <sup>+</sup> )	
Up (23)	Down (29)	Up (6)	Down (31)	Up (43)	Down (22)
miR-10b	Let-7b	miR-133b	Let-7c	Let-7c	Let-7b
miR-125a	Let-7f	miR-221	miR-10b	Let-7g	Let-7e
miR-127	let-7g	miR-33	miR-126	miR-100	Let-7i
miR-142-5p	Let-7i	miR-425	miR-146b	miR-105	miR-106a
miR-148a	miR-106a	miR-451	miR-15a	miR-106b	miR-10b
miR-15b	miR-124	miR-452	miR-15b	miR-124a	miR-141
miR-18a*	miR-133a		miR-16	miR-125a	miR-148a
miR-190	miR-141		miR-181b	miR-125b	miR-183
miR-19a	miR-149		miR-182	miR-133b	miR-203
miR-23b	miR-152		miR-183	miR-135a	miR-218
miR-27b	miR-15a		miR-18a	miR-142-3p	miR-221
miR-28	miR-186		miR-191	miR-142-5p	miR-25
miR-301	miR-18a		miR-195	miR-146a	miR-30e-5p
miR-30e-3p	miR-196b		miR-200a	miR-146b	miR-32
miR-30e-5p	miR-200a		miR-200c	miR-149	miR-342
miR-32	miR-200a*		miR-203	miR-16	miR-34a
miR-324-3p	miR-20a		miR-21	miR-17-5p	miR-378
miR-328	miR-210		miR-218	miR-181d	miR-422a
miR-331	miR-320		miR-27b	miR-186	miR-422b
miR-345	miR-335		miR-29b	miR-18a	miR-425
miR-361	miR-340		miR-29c	miR-195	miR-433
miR-374	miR-34a		miR-30a-3p	miR-200a*	miR-7
miR-452	miR-362		miR-30a-5p	miR-200c	
	miR-365		miR-335	miR-205	
	miR-375		miR-34a	miR-20b	
	miR-500		miR-365	miR-21	
	miR-92		miR-375	miR-210	
	miR-93		miR-422a	miR-26	
	miR-98		miR-422b	miR-27b	
			miR-423	miR-29a	
			miR-429	miR-29b	
				miR-29c	
				miR-301	
				miR-30d	
				miR-30e-3p	
				miR-33	
				miR-338	
				miR-339	
				miR-362	
				miR-451	
				miR-452	
				miR-99a	

Table 2-1. Commonly up- and down- regulated miRNAs in each xenograft models.

### **2.3.4 Common under-expression of multiple tumor-suppressive miRNAs in CD44<sup>+</sup> PCa cells**

When we analyzed the miRNA expression patterns common to all 3 populations of CD44<sup>+</sup> PCa cells, we found that 3 miRNAs, i.e., miR-452, miR-19a, and miR-301, were commonly over-expressed and, remarkably, 37 miRNAs were commonly under-expressed (Table 2-2). Among the 37 under-expressed miRNAs, miR-34a was the most dramatically down-regulated miRNA, representing 2% of the level in the CD44<sup>-</sup> cell populations. In addition to miR-34a, four let-7 family members (let-7a, let-7b, let-7e and let-7f) were under-expressed in the 3 CD44<sup>+</sup> populations (Table 2-2). Moreover, miR-141, a miR-200 family member, was also expressed at lower levels in CD44<sup>+</sup> than in CD44<sup>-</sup> PCa cells (Table 2-2). miR-34, let-7, and miR-200 families of miRNAs are well-established tumor-suppressive miRNAs (97, 106, 129).

miR-199a\*, which is down-regulated in many cancers (in particular, hepatocellular carcinoma) and possesses tumor-suppressive functions by targeting oncogenic molecules such as c-MET, IKK $\beta$ , DDR1 (discoidin domain receptor 1 tyrosine kinase), versican, PAK4 (a serine/threonine-protein kinase), Brm (a component of the SWI/SNF complex, Axl (a receptor tyrosine kinase), mTOR, and AKT (130-132), was expressed in the CD44<sup>+</sup> PCa cells at only ~4% levels of the CD44<sup>-</sup> cells (Table 2-2). Strikingly, in other cancer cells, miR-199a\* has been demonstrated to target CD44 leading to its deficiency in CD44<sup>+</sup> cancer cells (131, 132). Of interest, miR-214 is in a cluster with miR-199a\* (~6 kb apart) within human dynamin-3 gene intron (DNM3os) and was co-downregulated with miR-199a in CD44<sup>+</sup> cells (Table 2-2). Similarly, miR-10a and miR-196a are embedded in the HoxB gene cluster and both were under-expressed in CD44<sup>+</sup> PCa cells (Table 2-2). Several other clusters of miRNAs, including miR-183/miR-182 (7q31-34), miR106a/19b/92a (in the Chr-X *mir-106a-363* cluster), and miR-193b/miR-365 (16p13.12; 36), were also coordinately down-regulated

in the CD44<sup>+</sup> PCa cells (Table 2-2). miR-193b targets multiple oncogenic molecules including uPA, cyclin D1, 14-3-3 $\zeta$ , c-Kit, and Mcl-1. Many other miRNAs under-expressed in CD44<sup>+</sup> PCa cells (Table 2-2), including miR-218 (133), miR-148a (134), miR-181b(135), miR-203 (136), miR-183 (107), and miR-335 (137) have all been shown to possess tumor or metastasis inhibitory functions. Together, our profiling results indicate that multiple tumor-suppressive miRNAs are coordinately under-expressed in tumorigenic CD44<sup>+</sup> PCa cells.

We employed the online database Diana mir-Path (138) to probe the potential signaling pathways that might be engaged by miRNAs differentially expressed in the CD44<sup>+</sup> PCa cells (Table 2-2). The software performs an enrichment analysis of multiple microRNA target genes in all known KEGG pathways. When we input the set of miRNAs commonly under-expressed in CD44<sup>+</sup> cells, the top hits were TGF $\beta$ , Wnt, and MAPK signaling pathways (not shown).

Table 2-2

Over-expressed		Under-expressed			
miRNA	fold change	miRNA	fold change	miRNA	fold change
miR-452	832.77	miR-34a	0.02	miR-183	0.59
miR-19a	2.99	miR-199a*	0.04	miR-132	0.60
miR-301	1.84	miR-218	0.06	let-7e	0.62
		miR-422b	0.24	miR-340	0.62
		miR-422a	0.27	miR-30a-3p	0.64
		miR-378	0.27	miR-30a-5p	0.64
		miR-196a	0.28	miR-324-5p	0.64
		miR-10a	0.33	miR-365	0.66
		let-7b	0.35	miR-193b	0.67
		miR-214	0.39	miR-24	0.67
		miR-148a	0.41	miR-335	0.67
		miR-203	0.43	miR-191	0.68
		miR-181b	0.43	miR-92	0.70
		let-7a	0.44	miR-182	0.76
		miR-141	0.47	miR-99b	0.80
		miR-222	0.48	miR-30c	0.80
		miR-342	0.52	miR-106a	0.83
		let-7f	0.53	miR-19b	0.85
		miR-151	0.57		

**Table 2-2. miRNAs commonly over- or under-expressed in CD44<sup>+</sup> PCa cells.**

Presented are the miRNAs that are commonly over- or under-expressed in the purified CD44<sup>+</sup> Du145, LAPC9, and LAPC4 cells compared to the corresponding CD44<sup>-</sup> cells. The fold changes represent the mean values of the miRNAs in three xenograft models.

### **2.3.5 Distinctive and common miRNA expression profiles in different PCa stem/progenitor cell populations**

Subsequently, by comparing the miRNA expression profiles in different tumorigenic populations, we identified the miRNAs that were commonly changed in different marker-sorted stem/progenitor cell populations. We first compared the common CD44 profiles (Table 2-2) with the profiles generated from the LAPC4 CD133<sup>+</sup> or Du145  $\alpha 2\beta 1^{+}$  populations, and uncovered the miRNAs that were commonly over- or under-expressed in the 4 tumorigenic (i.e., 3 CD44<sup>+</sup> and CD133<sup>+</sup> or  $\alpha 2\beta 1^{+}$ ) cell populations (Table 2-3). When we combined the 5 tumorigenic populations (i.e., 3 CD44<sup>+</sup> together with CD133<sup>+</sup> and  $\alpha 2\beta 1^{+}$ ), only 4 miRNAs (i.e., let-7b, miR-106a, miR-141 and miR-34a) were commonly under-expressed and 2 miRNAs (i.e., miR-301 and miR-452) were commonly over-expressed (Fig. 2-13; Table 2-3). When we further combined the expression profile from the LAPC9 SP, only one miRNA, i.e., miR-34a was commonly under-expressed and one miRNA, miR-452, was commonly over-expressed in all 6 CSC populations (Table 2-3).

### **2.3.6 Validation of commonly changed miRNAs in primary tumor (HPCa) samples**

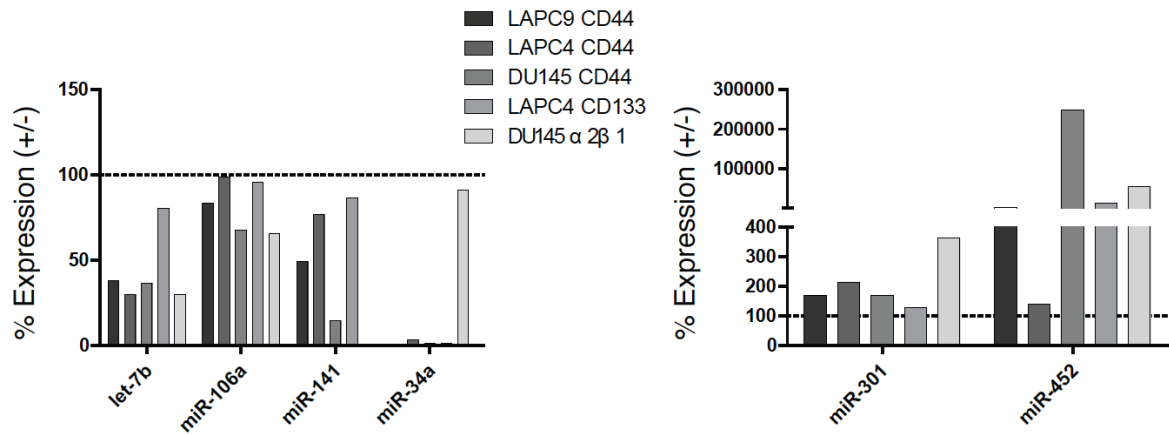
To establish potential clinical relevance and to further validate the expression in clinical prostate cancer samples, we obtained 21 prostatectomy human primary prostate tumors (HPCa), and first purified epithelial cancer cells. CD44<sup>+</sup> and CD44<sup>-</sup> cells from each samples were further purified out using either MACS (magnetic affinity-based cell sorting) or FACS (Table 2-4), and measured the levels of 4 commonly under-expressed (miR-34a, let-7b, miR-141, and miR-106a) and 2 commonly over-expressed (miR-301 and miR-452) miRNAs. We were able to verify miR-34a under-expression in all the HPCa-purified CD44<sup>+</sup> PCa cells (Figure 2-14). Let-7b also showed under-expression in the majority (18 out the 21)

Table 2-3

4 populations (a)		4 populations (b)		5 populations (c)		6 populations (d)	
over-expressed	under-expressed	over-expressed	under-expressed	over-expressed	under-expressed	over-expressed	under-expressed
miR-19a	miR-34a	miR-301	miR-34a	miR-301	miR-34a	miR-452	miR-34a
miR-301	let-7b	miR-452	let-7b	miR-452	let-7b		
miR-452	miR-106a		miR-106a		miR-106a		
	miR-141		miR-141		miR-141		
	let-7f		let-7e				
	miR-335		miR-183				
	miR-340		miR-203				
	miR-365		miR-218				
	miR-92		miR-342				
			miR-378				
			miR-422a				
			miR-422b				

**Table 2-3. Commonly over- and under-expressed miRNAs in tumorigenic PCa cell populations.** a. These 4 populations refer to the 3 CD44<sup>+</sup> populations from LAPC9, LAPC4 and Du145 plus the CD133<sup>+</sup> populations from LAPC4. b. These 4 populations refer to the 3 CD44<sup>+</sup> populations from LAPC9, LAPC4 and Du145 plus the  $\alpha 2\beta 1$ <sup>+</sup> populations from Du145. c. Refer to the 3 CD44<sup>+</sup> populations from LAPC9, LAPC4 and Du145 plus the LAPC4 CD133<sup>+</sup> and the Du145  $\alpha 2\beta 1$ <sup>+</sup> cell populations. d. The 6 populations include the 5 populations in c plus the LAPC9 SP.

Figure 2-13



**Figure 2-13. Commonly and differentially expressed miRNAs in 5 marker positive populations.** Relative expression levels of 4 commonly under-expressed (left panel) and 2 commonly over-expressed (right panel) miRNAs in marker-positive populations compared to the corresponding marker-negative populations.

of samples in the CD44<sup>+</sup> HPCa cells (Figure 2-15). Likewise, miR-141 was also detected at much lower levels in CD44<sup>+</sup> than in CD44<sup>-</sup> cells derived from most HPCa samples (Figure 2-16). In contrast, although miR-106a was under-expressed, to varying degrees, in all 5 xenograft-derived PCa cells, we detected its under-expression in only ~50% of 21 HPCa-derived CD44<sup>+</sup> PCa cells (Figure 2-17). In the 2 commonly over-expressed miRNAs, we detected the over-representation of miR-301 in the CD44<sup>+</sup> cells in 18 of 21 HPCa samples (Figure 2-18). Strikingly, although miR-452 was dramatically upregulated in 4 of the 5 xenograft-derived PCa cell populations, it was actually downregulated in the majority of CD44<sup>+</sup> HPCa cell populations (Figure 2-19). In summary, these experiments validate the under-expression of miR-34a, let-7b, and miR-141 and over-expression of miR-301 in the CD44<sup>+</sup> HPCa cells.

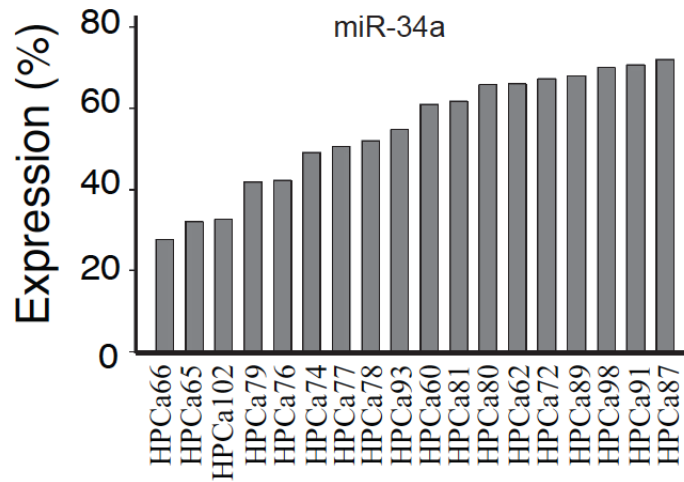


Table 2-4

HPCa Samples	Age	Gleason
HPCa57*	53	7 (3+4)
HPCa60	54	8 (3+5)
HPCa61	56	6 (3+3)
HPCa62	59	7 (4+3)
HPCa65	59	7 (4+3)
HPCa66	58	6 (3+3)
HPCa72	58	7 (3+4)
HPCa74	59	7 (3+4)
HPCa76	64	7 (4+3)
HPCa77	46	6 (3+3)
HPCa78	64	7 (3+4)
HPCa79	67	7 (4+3)
HPCa80	65	9 (5+4)
HPCa80*	65	9 (5+4)
HPCa81	54	7 (3+4)
HPCa87*	57	9 (4+5)
HPCa89	55	9 (4+5)
HPCa91*	60	8 (3+5)
HPCa93	58	7 (4+3)
HPCa98	64	8 (4+4)
HPCa102	55	7 (3+4)

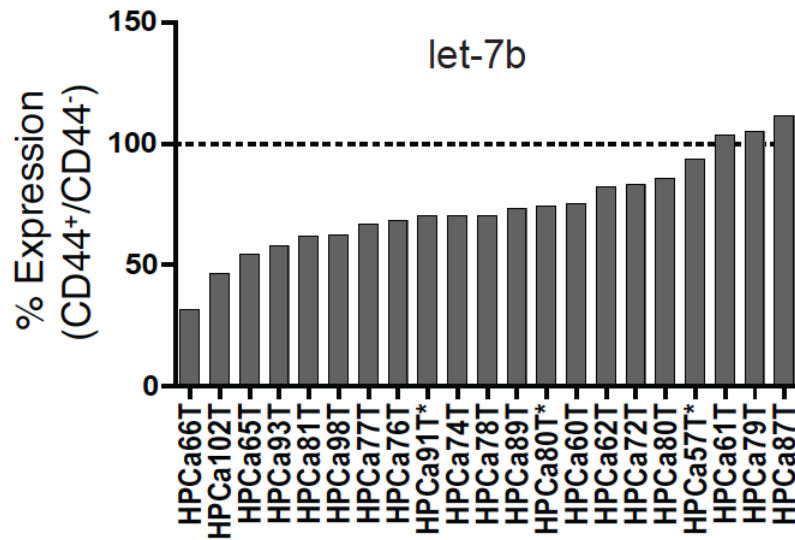
**Table 2-4. HPCa sample information.** HPCa samples were patient tumors obtained from the robotic (Da Vinci) surgery. The patient age and Gleason score of each tumor were indicated. \*These were first-generation xenograft tumors established in our lab and used to purify the CD44<sup>+</sup>/CD44<sup>-</sup> cells

Figure 2-14



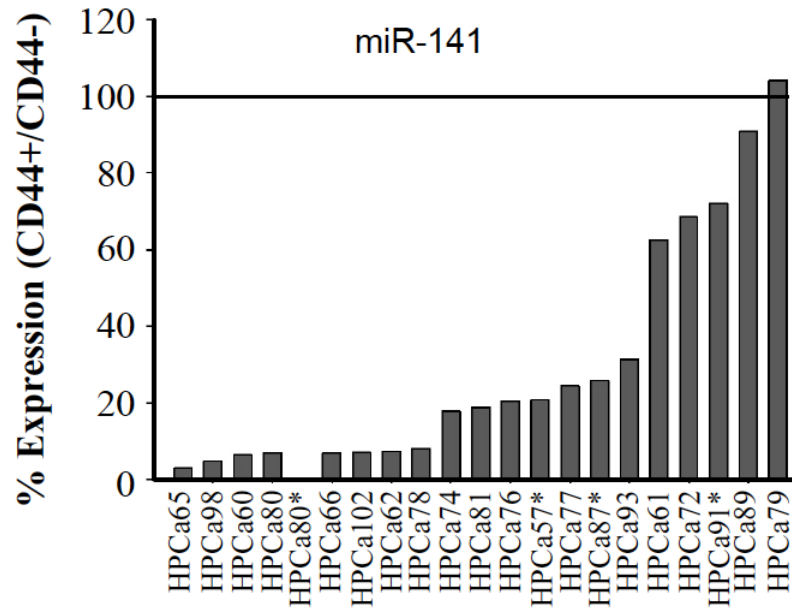
**Figure 2-14 Validation of miR-34a under-expression in HPCa CD44<sup>+</sup> cells.** Bar graph showing the relative expression of miR-34a in the CD44<sup>+</sup> populations compared to the corresponding CD44<sup>-</sup> populations in HPCa samples.

Figure 2-15



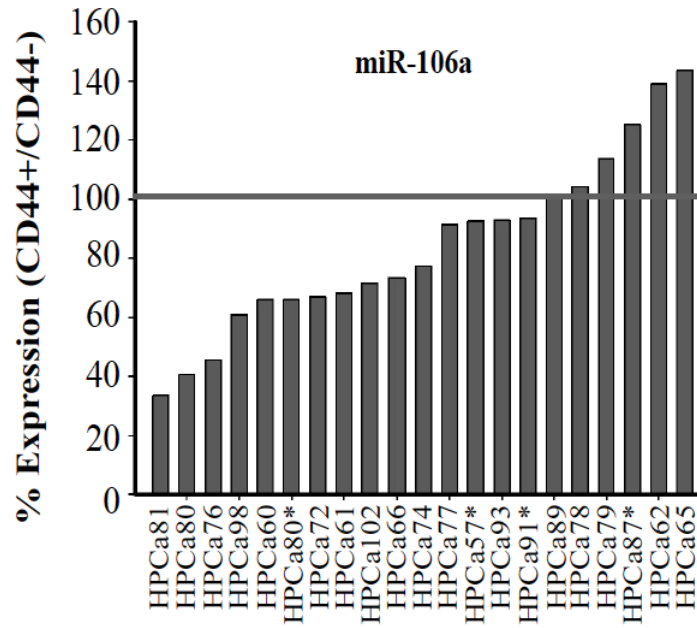
**Figure 2-15. Validation of let-7b under-expression in HPCa CD44<sup>+</sup> cells.** Bar graph showing the relative expression of let-7b in the CD44<sup>+</sup> populations compared to the corresponding CD44<sup>-</sup> populations in HPCa samples.

Figure 2-16



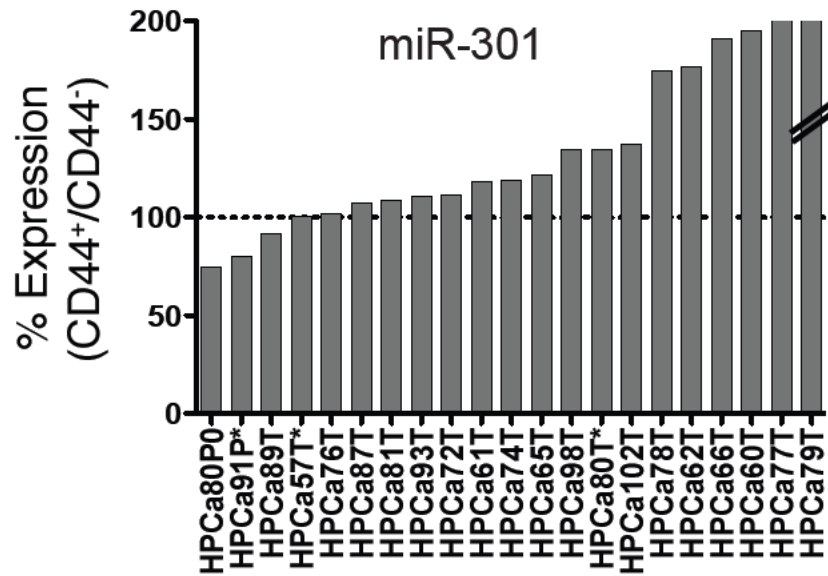
**Figure 2-16. Validation of miR-141 under-expression in HPCa CD44<sup>+</sup> cells.** Bar graph showing the relative expression of miR-141 in the CD44<sup>+</sup> populations compared to the corresponding CD44<sup>-</sup> populations in HPCa samples.

Figure 2-17



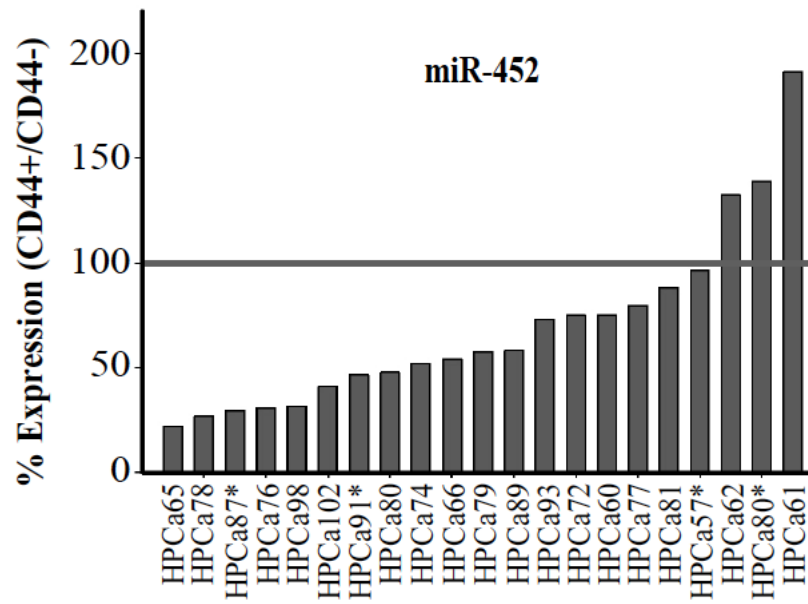
**Figure 2-17. Validation of miR-106a under-expression in HPCa CD44<sup>+</sup> cells.** Bar graph showing the relative expression of miR-106a in the CD44<sup>+</sup> populations compared to the corresponding CD44<sup>-</sup> populations in HPCa samples.

Figure 2-18



**Figure 2-18. Validation of miR-301 over-expression in HPCa CD44<sup>+</sup> cells.** Bar graph showing the relative expression of miR-301 in the CD44<sup>+</sup> populations compared to the corresponding CD44<sup>-</sup> populations in HPCa samples.

Figure 2-19



**Figure 2-19. Validation of miR-452 over-expression in HPCa CD44<sup>+</sup> cells.** Bar graph showing the relative expression of miR-452 in the CD44<sup>+</sup> populations compared to the corresponding CD44<sup>-</sup> populations in HPCa samples.

## 2.4 Discussion

In this part of my Ph.D research work, I have presented the miRNA expression profiles in tumorigenic PCa populations. Our results show that these CSC-enriched cell populations display different miRNA expressions compared to the non-tumorigenic populations. By combining miRNA expression profiles from different CSC populations, we identified miRNAs commonly and differentially expressed in several different tumorigenic PCa cells, among which are miR-34a, let-7b, miR-106a, miR-301 and miR-452. This is the first study to profile miRNA expression in purified PCa stem/progenitor cells, and in an unbiased way, we have identified several miRNAs that might be important in regulating PCa stem cell properties.

Many studies have reported miRNA expression profiles in various cancers compared to corresponding normal tissues. These “cancer-specific” miRNA signatures are certainly very informative for diagnostic and prognostic purposes (79, 80). These studies reveal that there is a global down-regulation of miRNAs in cancers and microRNA profiles can be used to classify different origin and differentiation status of tumors. Our study of miRNA expression profiles in CSC populations in PCa provides support to these observations. For example, we find that there are more miRNAs (37 miRNAs) that are under-expressed in the CD44<sup>+</sup> populations than the over-expressed miRNAs (3 miRNAs). Some miRNAs that turned up in our profiling have also been implicated in other tumor systems. miR-199a\* and miR-214, are drastically under-expressed in three CD44<sup>+</sup> populations, and this cluster is under-expressed in the Type I/CD44<sup>+</sup> epithelial ovarian cancer cells and targets CD44 and Met in hepatocellular carcinoma cell lines (131, 132). MiR-21, an oncomiR over-expressed in many cancers (79) and an androgen-receptor regulated miRNA that regulates androgen dependent and independent PCa cell growth (128), is also one of the top over-expressed



miRNA in the CD133<sup>+</sup> PCa cells, suggesting that a connection might exist between AR with PCSC.

The miRNA expression profiles in different marker-sorted PCa cell populations may also be distinct. Some miRNA may be over-expressed in one population but not in the other. This can be explained because CSCs are heterogeneous and frequently one marker, and, even a combination of markers, can only partially enrich CSC (3, 139).

To make the results more clinical relevant, we have made efforts to validate some of these commonly and differentially expressed miRNAs in primary prostate cancer (HPCa) samples that are freshly removed from the patients. We find that some miRNAs such as miR-34a, let-7b, miR-141 and miR-301, share similar expression patterns (either under-expression or over-expression) in tumorigenic populations in both xenograft and HPCa samples. However, some miRNAs such as miR-452 and miR-106a are found to be differentially expressed only in the xenograft-derived PCa stem/progenitor cells but not in CD44<sup>+</sup> HPCa. Whether this is because of the difference between xenograft and primary samples, or the differences in tumor subtypes such as Gleason grade, tumor involvement, and CD44<sup>+</sup> cell abundance, etc, needs further characterization.

In summary, we have demonstrated miRNA expression profiles in several tumorigenic PCa cell populations, and identified several miRNAs that are commonly up- or down- regulated. These miRNA expression profiles may be of importance for further study of miRNA functions in prostate and perhaps other types of CSCs.

## **Chapter 3 The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44**

### **3.1 Introduction**

In the preceding chapter of the dissertation, I presented the miRNA expression profiling in several prostate CSC populations, and identified several commonly and differentially expressed miRNAs including miR-34a, let-7b, miR-106a, miR-141, miR-301 and miR-452. We then asked whether these commonly and differentially expressed miRNA might be involved in regulating CSC properties or PCa development. The current chapter focuses on miR-34a.

miR-34 is an evolutionarily conserved miRNA family that consist of three members, miR-34a, miR-34b and miR-34c. The miR-34a gene located at Chr. 1p.36, whereas miR-34b and miR-34a arise from one genomic locus at Chr. 11q23. MiR-34 has recently emerged as one of the critical downstream effectors of tumor suppressor gene p53 and p53 activation leads to upregulation of miR-34a and miR34b/c, which in turn causes apoptosis, cell-cycle arrest or senescence depending on particular cell types (83, 140-143). In some PCa cells, miR-34a mediates p53-induced, AR-dependent apoptosis (144). These observations, together with its somatic loss in neuroblastoma (143, 145), suggest that miR-34a has tumor-suppressive functions. In support, the miR-34a gene appears to be silenced in multiple cancers due to promoter CpG hypermethylation (146) and reduced miR-34a is associated with chemoresistance in leukemia and PCa cell lines (147). Also, miR-34a is down-regulated in a rat hepatocellular carcinogenesis model (148). Moreover, restoration of miR-34a in PCa (149) and other cancer cells (113, 150, 151) induces cell-cycle arrest and apoptosis or inhibits invasion.

## **3.2 Materials and methods**

### **Transient transfection with oligonucleotides**

PCa cells were transfected with 30 nM of miR-34a microRNA mimics or non-targeting negative control miRNA (miR-NC) oligos (Ambion) using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. In some experiments, antisense oligonucleotides (i.e., antagomirs) against miR-34a (anti-miR-34a) or scramble control anti-miR-NC (anti-NC) oligos (Ambion) were introduced into PCa using the same conditions. After culturing for overnight to 48 h, transfected cells were harvested for *in vitro* and *in vivo* studies.

### **Retroviral and lenti-viral mediated overexpression of miR-34a**

The MSCV retroviral vector over-expressing of miR-34a (MSCV-34a) and the empty control vector (MSCV-PIG) were kindly provided by Dr. G. Hannon. PCa cells were infected with the retroviral supernatant for 48 h in the presence of 8 µg/ml polybrene. Two days after infection, puromycin was added to the media at 3 µg/ml, and cell populations were selected for 2 weeks. A lenti-viral vector encoding miR-34a (lenti-miR-34a) and a control lenti-viral vector (lenti-ctl) were obtained from Systems Biosciences (SBI; Mountain View, CA). Lentivirus was produced in 293FT packaging cells and titers were determined for the GFP-tagged viruses using HT1080 cells. PCa cells were infected at an MOI of 10 - 20 and harvested for *in vitro* or *in vivo* studies 48-72 h post-infection.

### **Tumor transplantation experiments**

Basic procedures for subcutaneous (s.c) and orthotopic (i.e., dorsal prostate or DP) tumor transplantations can be found in our earlier publications (43). PCa cells from maintenance tumors were harvested and transfected with oligos or infected with lenti-viral vectors. 24 - 48 h later, cells were harvested and implanted, in 50% Matrigel, s.c or in the DP of intact male NOD/SCID mice.

### **Therapeutic experiments**

PCa cells LAPC9 or PC3 were implanted into the dorsal prostate (DP) of NOD/SCID mice. On day 22, animals were randomly assigned to miR-34a or NC group, injected through tail vein with the oligos and terminated when they became moribund. Tumors and lungs as well as several other organs such as pancreas, spleen, lymph nodes and liver were harvested. GFP<sup>+</sup> metastatic foci in each organ were examined and quantified under a Nikon SMZ1500 whole-mount fluorescence microscope.

### **Clonal, and sphere-formation assays**

For clonal assays, cultured PCa cells were plated at a clonal density (i.e., 100 cells/well) in a 6-well dish. The number of holoclones was enumerated at several days to 2 weeks after plating. For sphere-formation assays, cells were plated (5,000–20,000 cells/well) in serum-free prostate epithelial basal medium (PrEBM) supplemented with 4 µg/ml insulin, B27 (Invitrogen), and 20 ng/ml EGF and bFGF in ultra-low attachment (ULA) plates. Floating spheres that arose in 1–2 weeks were counted. For all these experiments, a minimum of triplicate wells was run for each condition and repeat experiments were performed when necessary and feasible.

### **miR-34a binding site, site-specific mutation and luciferase experiments**

We used RNA22 to compute putative binding site for miR-34a. 3'UTR of CD44 from LNCaP genomic DNA were cloned into pGEM-T vector (Promega). For site-specific mutagenesis, we mutated the region that is complementary to miR-34a seed sequence using the Quick change II site-directed mutagenesis kit (Stratagene). For luciferase assay, we cloned wild-type or mutated 3'UTR of CD44 into pmiR-REPORTER miRNA expression reporter vector (Ambion). PCa cells were seeded in 24-well plates ( $3 \times 10^4$  cells per well) and co-transfected with 1  $\mu$ g reporters with 24 pmol miR-34a or miR-NC together with Renilla luciferase internal normalization plasmid (phRL-CMV). The ratio of firefly to Renilla luciferase activity was determined with a dual luciferase assay (Promega) 48 h later.

### **Migration and invasion assays**

Invasion assays in CD44<sup>+</sup> and CD44<sup>-</sup> Du145 cells were performed using Matrigel Invasion Chamber (8- $\mu$ m pore size, BD). We carried out migration assays in a similar way but without the Matrigel. For the rescue experiments, we infected CD44<sup>+</sup> Du145 cells with pBabe-puro or pBabe-CD44 (Addgene) retroviruses in the presence of 8  $\mu$ g/ml polybrene. After 24 h, we transfected cells with miR-34a oligos before invasion assays. In these experiments, the percentage of invaded cells was converted into an invasion index, which was considered as one in all control groups.

### **shRNA lenti-viral systems**

CD44 knockdown experiments were performed using pGIPz-CD44shRNA (CD44-sh) or pGIPz-NS (non-silencing) lentiviruses (Open Biosystems) at a multiplicity of infection (MOI) of 20.

### **BrdU incorporation assays and SA- $\beta$ gal staining**

Cells grown on coverslips were pulsed for 4 h with 10  $\mu$ M BrdU, fixed in 4% PFA (paraformaldehyde) and denatured in 2 M hydrochloric acid (HCl) in 1% Triton at room temperature (RT) for 15 min followed by neutralization with 0.1 M sodium borate ( $\text{Na}_2\text{B}_4\text{O}_7$ ) at RT for 10 min. After blocking with 20% goat whole serum, cells were stained for nuclear BrdU using anti-BrdU antibody (i.e., the Bu20a hybridoma) followed by FITC- or Texas Red-conjugated secondary antibodies. DAPI (4',6-diamidino-2-phenylindole) was used for 10 min at RT for nuclear counterstaining.

For senescence  $\beta$ -gal staining, PPC-1 cells were transfected with miRNA-mimic oligos for 24 h, after which cells were fixed with 0.5% glutaraldehyde in PBS for 15 min at RT. After being washed with PBS, cells were stained in X-Gal staining solution (1 mg/ml X-Gal, 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferricyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ], 5 mM potassium ferrocyanide [ $\text{K}_4\text{Fe}(\text{CN})_6$ ], 150 mM NaCl, 2 mM  $\text{MgCl}_2$ ) at 37°C for 12 h. Cells with blue staining were counted.

### **Statistical analyses**

In general, unpaired two-tailed Student's *t*-test was used to compare differences in cell numbers, cumulative PDs, percentages of CD44<sup>+</sup>, % BrdU<sup>+</sup> cells, % cell-cycle phases, cloning and sphere-formation efficiency, and tumor weights. Fisher's Exact Test and  $\chi^2$  test were used to compare incidence and latency. In all these analyses, a *P* < 0.05 was considered statistically significant.

### **3.3 Results**

#### **3.3.1 Enforced expression of miR-34a inhibited PCa development.**

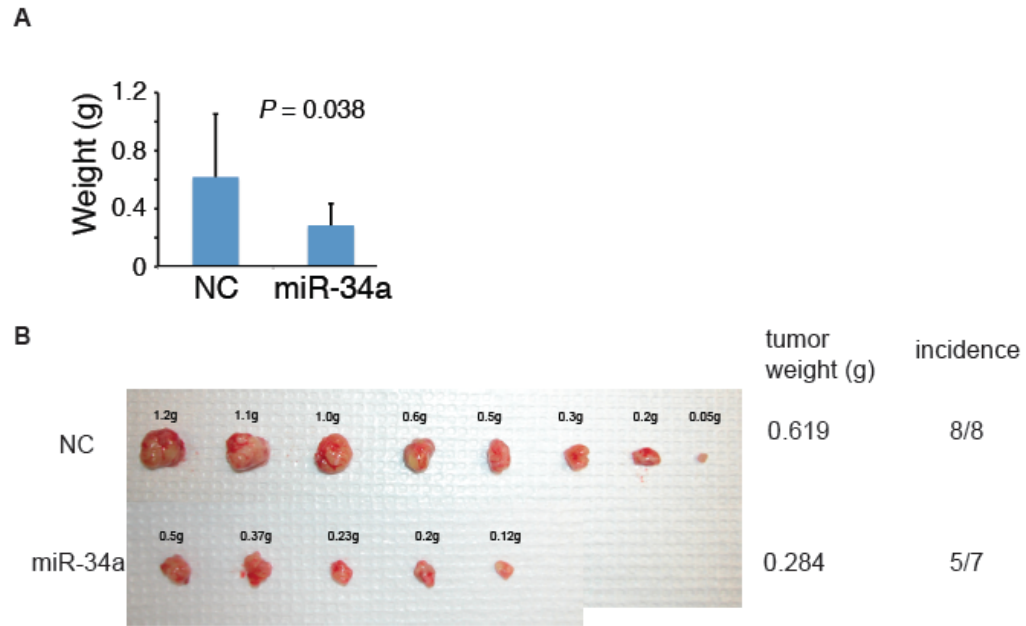
We transfected AR-positive (LAPC9 and LAPC4) or AR-negative (Du145 and PPC1) xenograft-derived PCa cells with pre-miR-34a oligonucleotides or pre-miR-NC (negative control). The pre-miRNA oligonucleotides are double-stranded RNA that mimics the endogenous mature miRNA, which will not need further processing through Dicer, and the NC oligos are double-stranded RNA that mimics miRNA but with scramble sequences that are tested to have no biological effect in cells. As shown in Figure 3-1, over-expression of miR-34a significantly inhibited LAPC9 tumor growth in the orthotopic site (i.e. dorsal prostate, or DP) evidenced by smaller average tumor weights and lower tumor incidences. Similar tumor suppressive effects of miR-34a were also observed in LAPC4 and Du145 models (Figure 3-2).

Over-expression of miR-34a in HPCa58 cells, derived from the xenograft of a primary human PCa sample, produced significantly smaller tumors than the same cells transfected with NC oligos (Figure 3-3). Importantly, miR-34a also inhibited the secondary transplantation of HPCa58 cells (Figure 3-3), indicating that miR-34a might be inhibiting the CSC populations in the HPCa58 tumors.

Enforced expression of miR-34a also significantly halted PPC-1 tumor growth when we electroporated pre-miR-34a oligos into PPC-1 cell, and then reintroduce the oligos intratumorally every 7 days (Figure 3-4).

We also utilized a lentiviral vector (lenti-34a) and a retroviral vector (MSCV-34a) to over-express miR-34a in PCa cells. Tumor regenerations of LAPC4, Du145 and LAPC9 cells were inhibited by the viral vector-mediated over-expression of miR-34a (Figure 3-5).

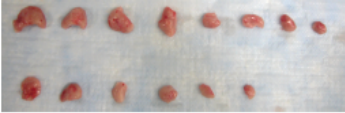

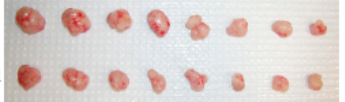

Figure 3-1



**Figure 3-1. Over-expression of miR-34a in LAPC9 significantly inhibited orthotopic tumor growth.** A. Shown is the mean tumor weight. P value of mean tumor weight is indicated. B. Shown are the tumor images, mean tumor weight and incidence (tumor outgrowth/number of injections) are listed on the right.

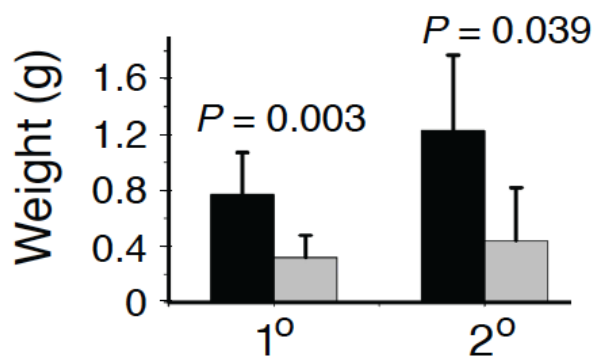


Figure 3-2

		<i>Incidence</i>	<i>Harvest</i>	<i>Weight (g)</i>	<i>P value</i>
	LAPC4 (100,000 cells/s.c inj.)				
miR-NC		8/8 (100%)	10 d (2/03/08 - 2/13/08)	0.56 ± 0.27	
miR-34a		6/8 (75%)	10 d	0.29 ± 0.16	0.02
	Du145 (100,000 cells/s.c inj.)				
miR-NC		8/8 (100%)	62 d (1/31/09 - 4/03/09)	0.82 ± 0.39	
miR-34a		8/8 (100%)	62 d	0.5 ± 0.18	0.03

**Figure 3-2. Over-expression of miR-34a in LAPC4 and Du145 cells significantly inhibited subcutaneous tumor growth.** Shown are the representative tumor images, mean tumor weight, harvest date, incidence (tumor outgrowth / number of injections) and the P values for mean tumor weight are listed on the right.

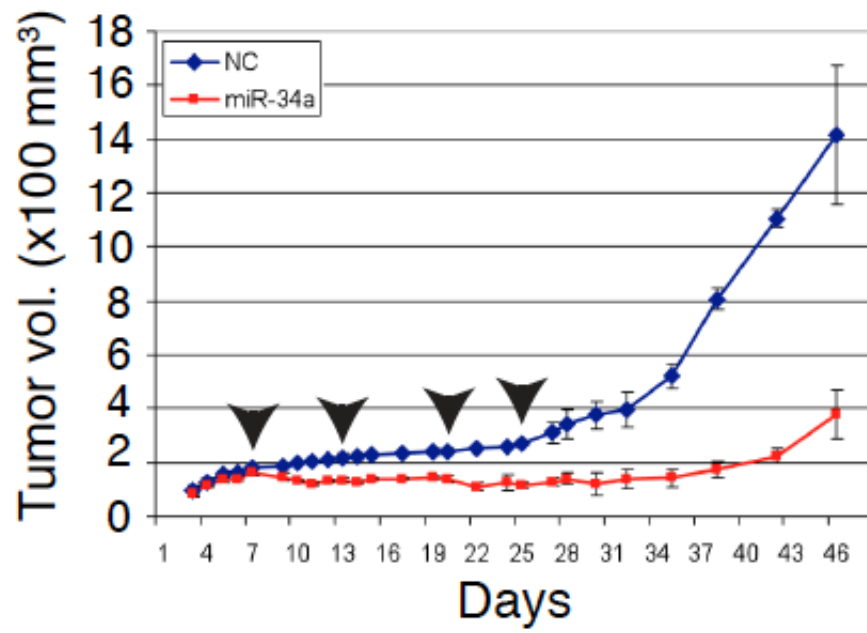
Figure 3-3



**Figure 3-3. miR-34a over-expression inhibited HPCa58 tumor regeneration.**

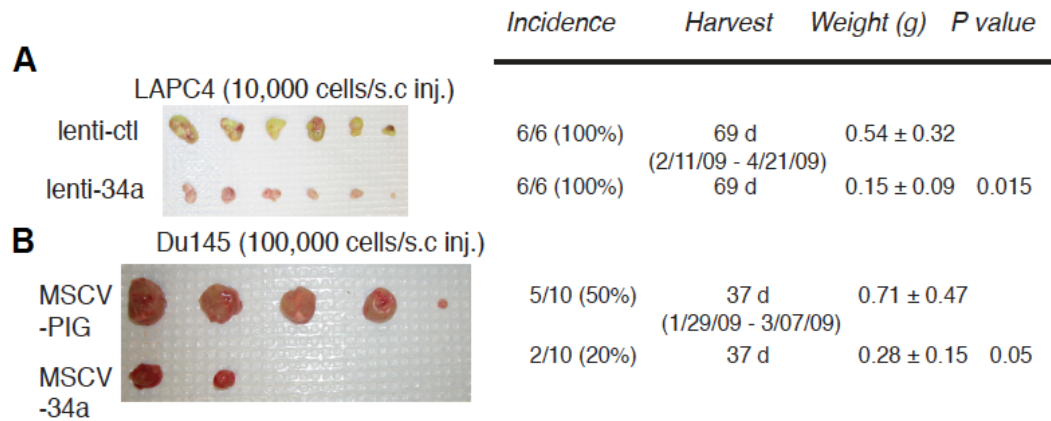
Shown are bar graphs for the mean tumor weight for the first and second generations. P value for tumor weight in each generation is indicated. Black, lenti-control; grey, lenti-34a.

Figure 3-4



**Figure 3-4. Repeat delivery of miR-34a halted PPC-1 tumor growth.** Arrowheads indicate delivery of miR-34a or NC oligo intratumorally.

Figure 3-5



**Figure 3-5. miR-34a over-expression by a lentiviral or a retroviral vectors infection inhibits PCa regeneration.** MiR-34a over-expression by lentiviral infection inhibits LAPC4 tumor growth (A). miR-34a over-expression by retroviral infection inhibits Du145 tumor regeneration (B). Shown are the representative tumor images, mean tumor weight, harvest date, incidence (tumor outgrowth/number of injections) and P values for mean tumor weight are indicated on the right.

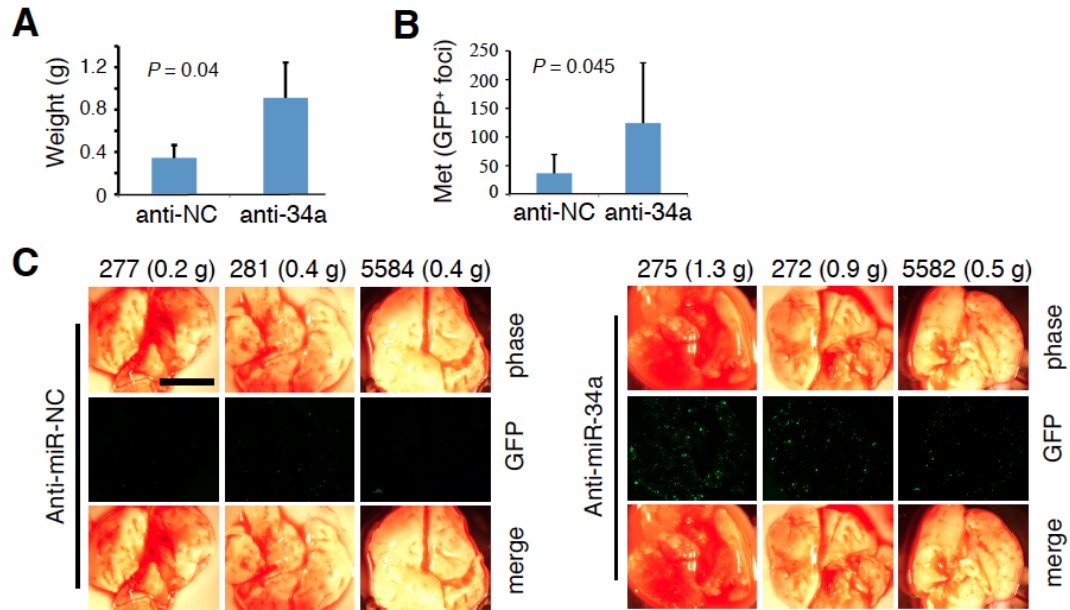
When anti-sense oligos of miR-34a (i.e., anti-miR-34a) that were specifically designed to bind to and inhibit endogenous mature miR-34a were introduced into LAPC9 cells, they promoted LAPC9 tumor growth with tumors twice as big as in control group (Figure 3-6A). Anti-miR-34a also promoted lung metastasis (Figure 3-6B).

We also made efforts in validating and characterizing miR-34a levels in PCa cells (or in tumors) when miR-34a levels were manipulated (Figure 3-7). As expected, miR-34a transfected PCa cells showed miR-34a levels at several orders of magnitude higher than cells transfected with miR-NC. In contrast to freshly transfected cells, the residual tumors showed only a marginal or no increase in miR-34a levels, which could explain why miR-34a-overexpressed PCa cells still regenerated some tumors. As validations of the specificity of anti-34a, the antagomir-transfected LAPC9 cells showed reduced endogenous miR-34a (Figure 3-7).

### **3.3.2 miR-34a inhibited tumor regeneration of CD44<sup>+</sup> PCa cells whereas anti-miR-34a promoted tumor regeneration of CD44<sup>-</sup> PCa cells.**

Since miR-34a was shown to be significantly under-expressed in prostate CSCs, we decided to evaluate whether miR-34a may have a direct effect on at least one prostate CSC population. We therefore performed tumor growth experiments using purified CD44<sup>+</sup> or CD44<sup>-</sup> PCa cells that had been subjected to manipulation of miR-34a levels. Over-expression of miR-34a in CD44<sup>+</sup> Du145 cells completely blocked their tumor initiation ability (Figure 3-8A). Similarly, CD44<sup>+</sup> LAPC9 cells transfected with miR-34a oligos, generate fewer and smaller tumors compared to the NC group (Figure 3-8B).

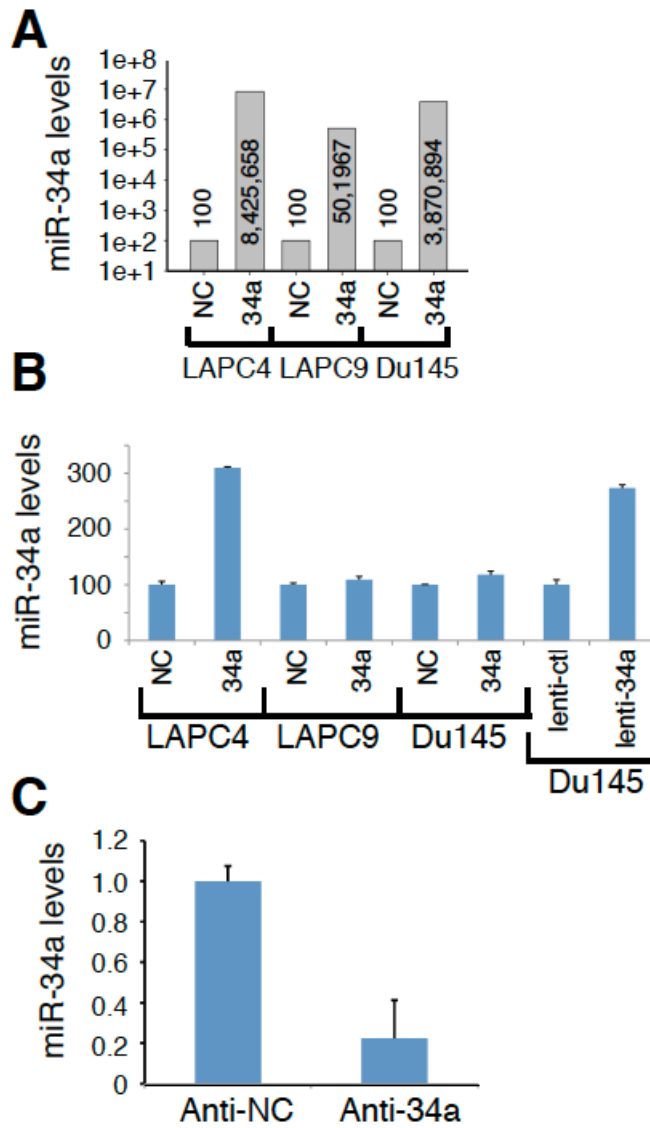
Figure 3-6



**Figure 3-6. Anti-miR-34a enhances LAPC9 tumor growth and lung metastasis.**

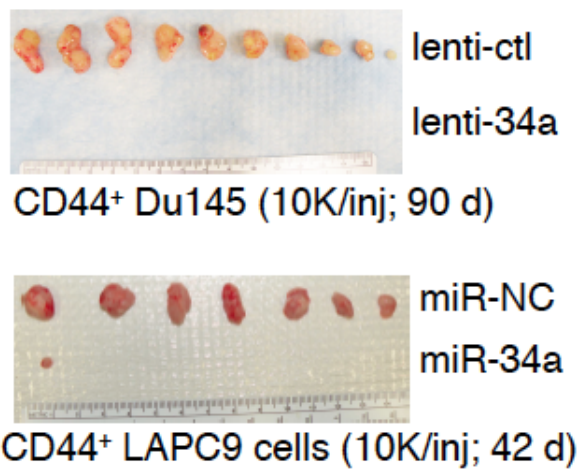
A. Anti-miR-34a promotes orthotopic LAPC9 tumor growth. B & C. Anti-miR-34a promotes LAPC9 lung metastasis. Shown in B is the quantification of GFP<sup>+</sup> foci/lung and in C are representative phase and GFP images of 3 lungs from each group (scale bar, 100  $\mu$ m).

Figure 3-7



**Figure 3-7. Validations of miRNA level in freshly transfected cells and residue tumors.** Shown are the mean miR-34a levels (in log scale; n = 3) in miR-34a transfected cells (A) relative to those in the miR-NC transfected cells (actual mean values indicated in the bars), in residual tumors (B) and in anti-sense oligo transfected LAPC9 cells (C).

Figure 3-8

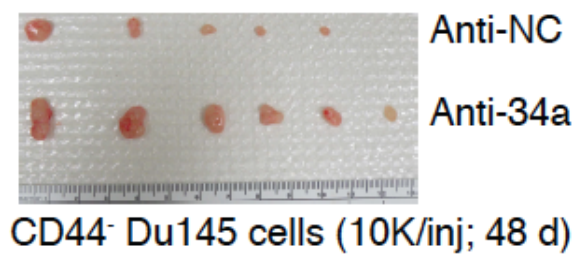


**Figure 3-8. Over-expression of miR-34a in CD44<sup>+</sup> PCa cells significantly suppressed tumor regeneration.** Shown are the representative tumor images.



We also performed the knockdown experiments by introducing an antisense inhibitor of miR-34a (anti-34a) into purified CD44<sup>-</sup> Du145 or LAPC9 cells, which are less tumorigenic than the corresponding CD44<sup>+</sup> cells. Knocking down endogenous miR-34a in CD44<sup>-</sup> Du145 cells developed larger tumors than those with anti-NC oligos (Figure 3-9). Likewise, anti-34a also promoted tumor growth in purified CD44<sup>-</sup> LAPC9 cells. Notably, we observed an increase of lung metastasis in anti-34a mice when we quantified the GFP-bright foci ( $\geq 1$  mm<sup>3</sup>) in the mouse lungs. Taken together, these in vivo experiments in purified PCa cells suggest that miR-34a negatively regulates the tumor-initiating capacity of prostate CSCs.

Figure 3-9

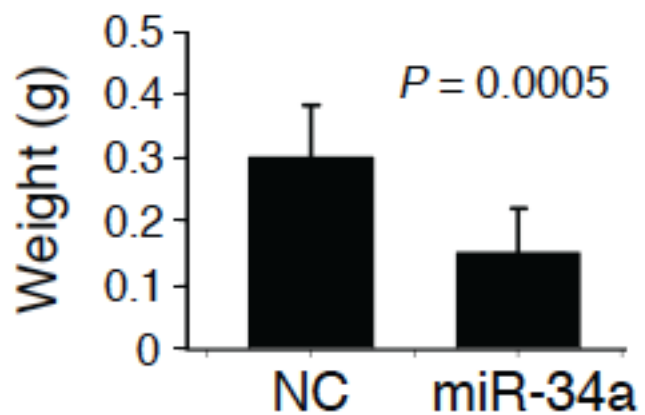


**Figure 3-9. Knocking down miR-34a by antisense oligos promoted tumor regeneration of CD44<sup>+</sup> Du145 cells.** Shown are the representative images of tumor when we knocked down miR-34a by antagomir into Du145 CD44<sup>+</sup> cells.

### **3.3.3 Therapeutic effects of miR-34a in PCa xenograft models**

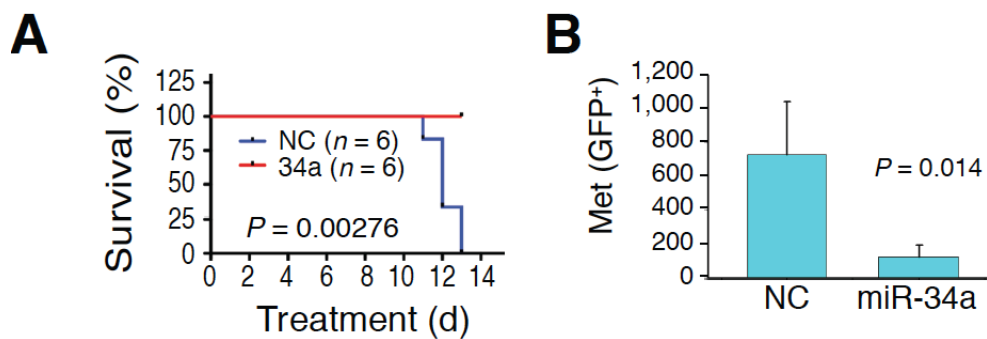
The above observations that enforced expression of miR-34a significantly inhibited tumor development and our pilot experiment in which repeated intratumoral injections of miR-34a into subcutaneous PPC-1 tumors halted tumor growth (Figure 3-5) suggest that miR-34a may exert therapeutic efficacy in PCa. To directly test this possibility, we designed experiments to mimic clinical situations in which we delivered the miR-34a oligos systemically through tail vein injection into the mice bearing a pre-established orthotopic prostate tumor that was implanted 3 weeks earlier. As shown in Figure 3-10, miR-34a treatment significantly inhibited the growth of PC3 tumors. By the time we terminated the mice, the average weight of miR-34a tumors was only half of the control tumors. In another set of therapeutic experiments in LAPC9 model, we decided to focus on the effect of miR-34a on tumor metastasis and animal survival. MiR-34a treatment extended the survival of mice bearing orthotopic LAPC9 tumors (Figure 3-11), likely attributed to the inhibitory effects of miR-34a on lung metastasis as supported by quantification of GFP-bright foci (Figure 3-12). Tumor cell dissemination to some other organs such as pancreas was also reduced by miR-34a treatment (not shown).

Figure 3-10



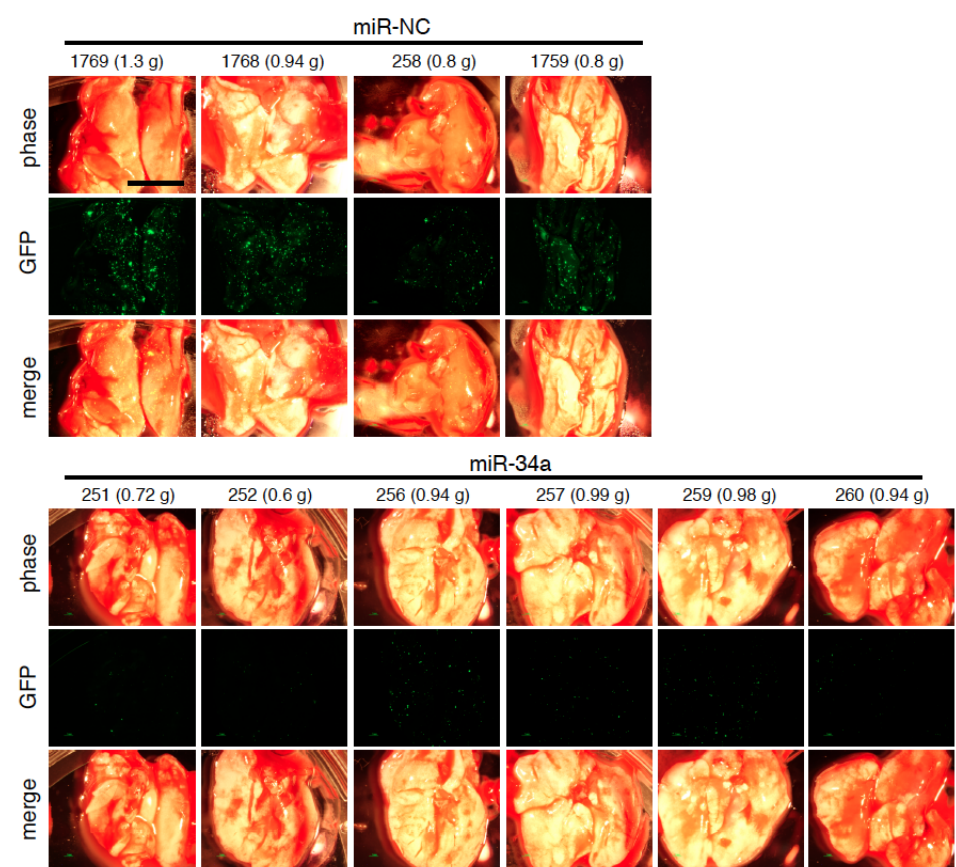
**Figure 3-10. Therapeutic effects of miR-34a on PC3 tumor growth.** miR-34a oligo and NC oligo were delivered through tail vein injection every other day. Shown is the bar graph of mean tumor weight (g) and the P value.

Figure 3-11



**Figure 3-11 Therapeutic effects of miR-34a in LAPC9 tumor-bearing mice.** Systemically delivery of miR-34a in LAPC9 tumor bearing mouse significant inhibited lung metastasis and extended the mice survival. Shown in A is the mouse survival curve (Kaplan-Meier analysis) and in B the quantification of lung metastasis (averaged GFP<sup>+</sup> foci per lung).

Figure 3-12

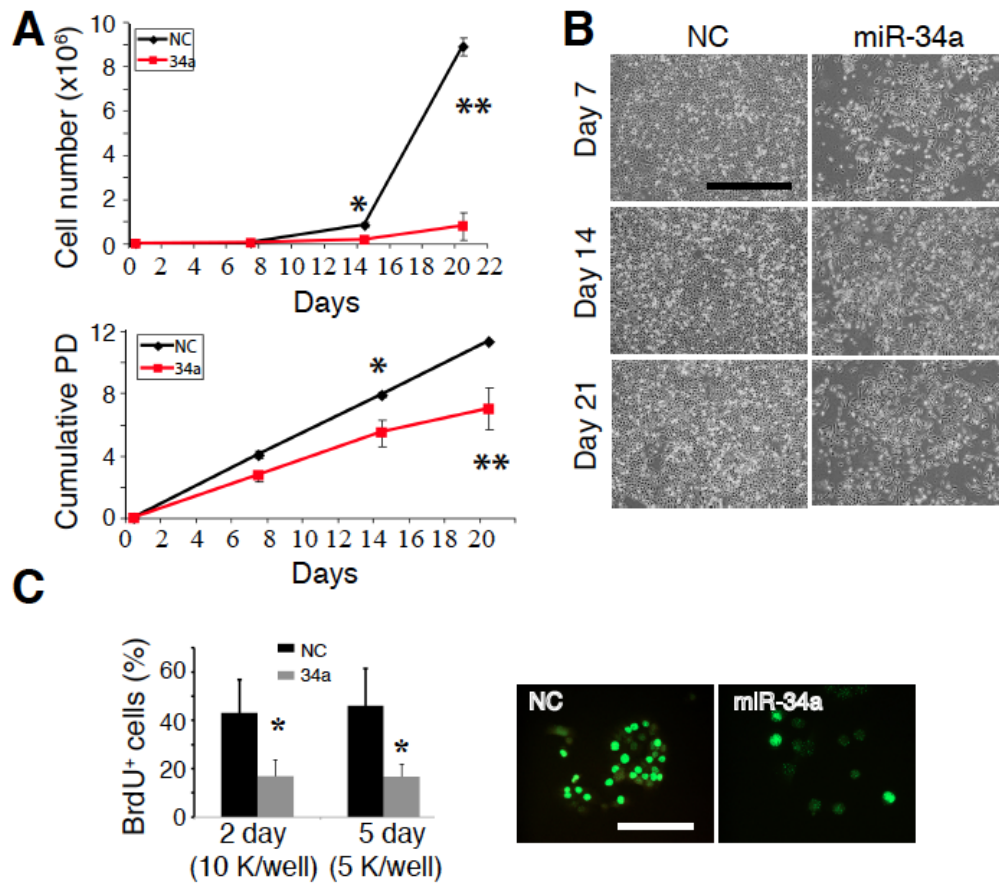


**Figure 3-12. Systemic miR-34a inhibited LAPC9 lung metastasis.** Shown are representative lung images illustrating dramatically reduced lung metastases in the miR-34a treated group compared to the miR-NC group. Animal tag number and tumor weight are indicated. Scale bar, 100  $\mu$ m.

#### **3.3.4 Biological effects of miR-34a in PCa cells**

Consistent with the reported effects of miR-34a on proliferation, apoptosis and senescence, miR-34a also exerted a cell type specific inhibitory effect in PCa cells. Overexpression of miR-34a in Du145 inhibited proliferation by inducing cell cycle arrest as indicated by BrdU staining (Figure 3-13). PC-3 cells with enforced expression of miR-34a showed a suppressed proliferation and activation of apoptosis (Figure 3-14). Increased cell senescence cells as assessed by senescence-associated  $\beta$ -gal (SA- $\beta$ gal) staining was observed in PPC-1 cells transfected with miR-34a, which partly explained the inhibition of proliferation in these cells (Figure 3-15).

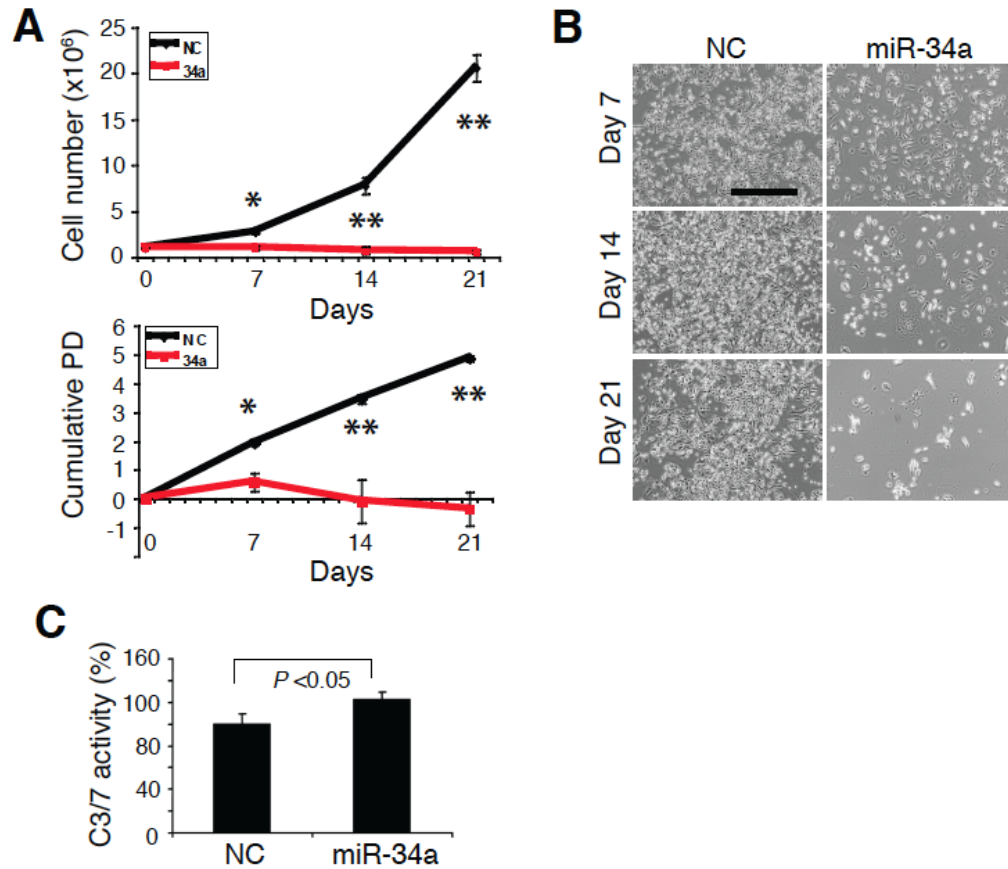
Figure 3-13



**Figure 3-13. miR-34a inhibited Du145 cell proliferation.** Shown in A are the cumulative cell numbers or population doublings (PDs) as a function of time and bars represent the mean  $\pm$  S.D (\* $P < 0.05$ ; \*\* $P < 0.001$ ). Shown in B are representative microphotographs (scale bar, 10  $\mu$ m). Shown in C are the results of BrdU incorporation assays. Presented in the bar graph is the mean % of BrdU-positive cells (\* $P < 0.001$ ) and representative images (scale bar, 10  $\mu$ m) of BrdU staining.

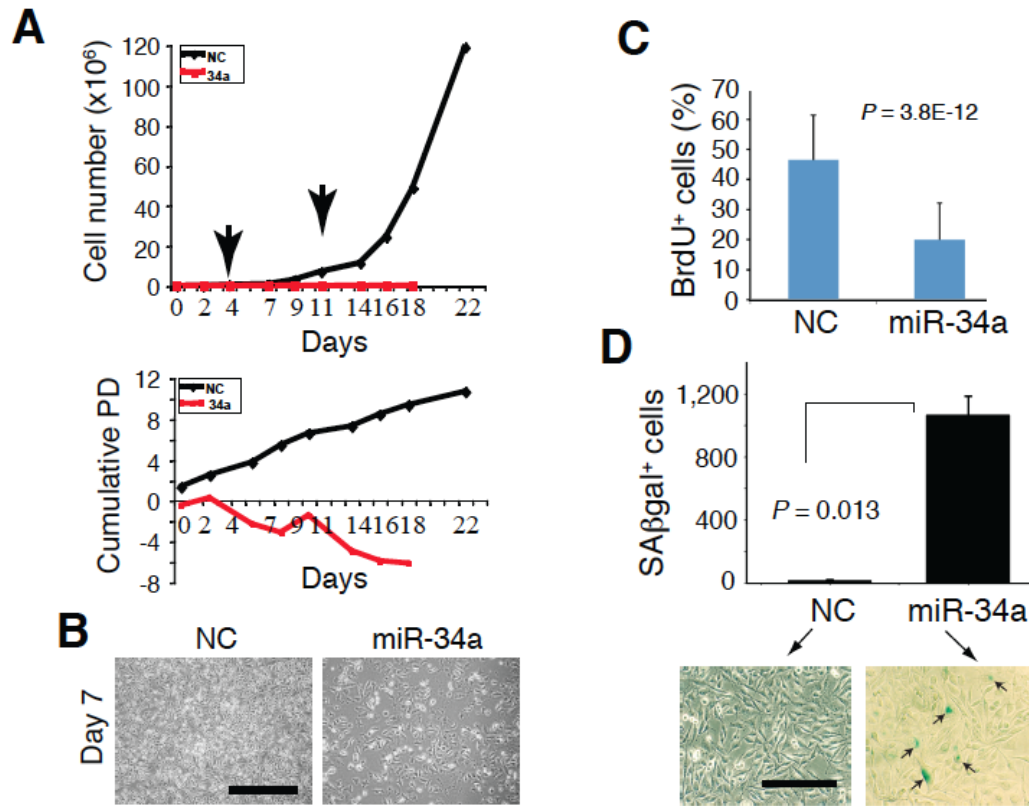


Figure 3-14



**Figure 3-14. Effects of miR-34a over-expression on PC3 cells.** Shown in A are the cumulative cell numbers or population doublings (PDs) as a function of time and bars represent the mean  $\pm$  S.D (\* $P < 0.05$ ; \*\* $P < 0.001$ ). Shown in B are representative microphotographs (scale bar, 10  $\mu$ m). C. miR-34a induces apoptosis in PC3 cells. PC3 cells transfected with miR-34a were harvested and used in DEVDase assays, which measure caspase-3 or 7 (C3/7) activities.

Figure 3-15

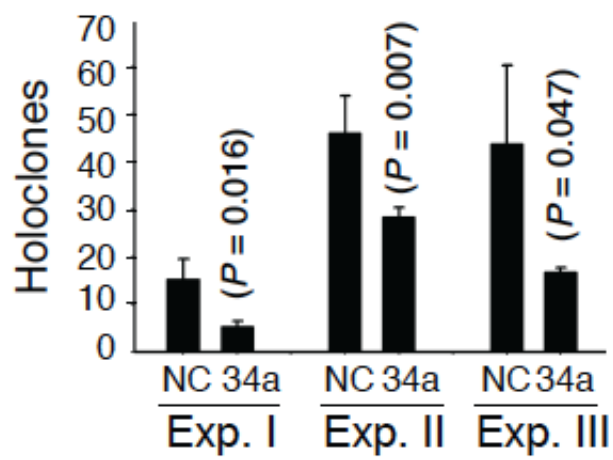


**Figure 3-15. Effects of miR-34a over-expression on PPC-1 cells.** Shown in A are the cumulative cell numbers or population doublings (PDs) as a function of time and bars represent the mean  $\pm$  S.D (\* $P < 0.05$ ; \*\* $P < 0.001$ ). Shown in B are representative microphotographs (scale bar, 10  $\mu$ m). Presented in C is the % of BrdU<sup>+</sup> cells (mean  $\pm$  S.D;  $n = 3$ ). Shown in D are total number of SA- $\beta$ gal<sup>+</sup> cells ( $n = 3$ ) and representative microphotographs (below; scale bar, 10  $\mu$ m).

### 3.3.5 miR-34a regulates the stem cell properties of PCa cells

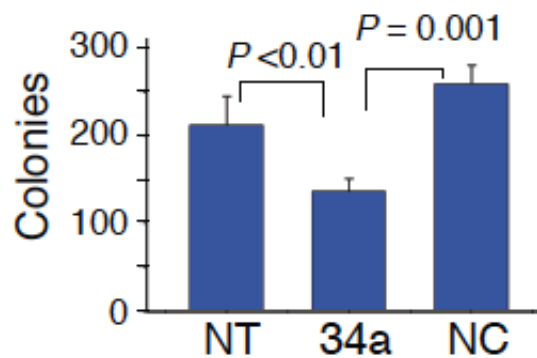
To further investigate the miR-34a effects on CSC properties, we performed *in vitro* clonal, clonogenic and sphere formation assays. Over-expressing miR-34a significantly inhibited the clonal formation of Du145 cells (Figure 3-16). Control oligo transfected Du145 cells formed large compact clones, whereas miR-34a oligo transfected cells generally formed fewer, smaller and less compact, scattered clones. Previous work from our lab has shown that large compact clones (holoclones) contain self-renewing CSCs than the loosely compacted clones (meroclones or paraclones) (42). Similarly, miR-34a also greatly inhibited clonogenic sphere formation of Du145 (Figure 3-17) and LAPC4 (Figure 3-18) cells plated in Matrigel, under which conditions essentially all spheres were found to be of a clonal origin as revealed by our mixing (i.e., mixing unlabeled cell at 1:1 ratio with GFP-labeled cells) experiments (data not shown). In addition, miR-34a inhibited sphere formation in primary HPCa cells. HPCa cells over-expressing miR-34a formed tiny or differentiated spheres (Figure 3-19). Notably, over-expression of miR-34a abrogated secondary sphere establishment in HPCa cells. When introduced into purified CD44<sup>+</sup> HPCa116 cells, over-expression of miR-34a significantly inhibited its sphere formation (Figure 3-20A), whereas anti-34a transfected CD44<sup>-</sup> HPCa116 cells increased the inherently low sphere-forming capacity by almost 4 fold (Figure 3-20B). All together, these observations strongly suggest that miR-34a negatively regulates prostate CSC properties.

Figure 3-16



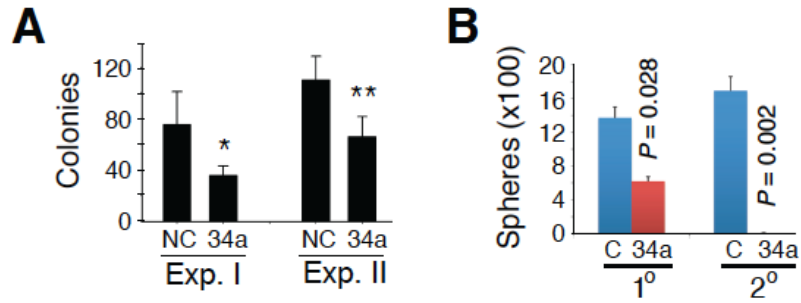
**Figure 3-16. miR-34a inhibited clonal formation in Du145 cells.** Shown are the average counts of holoclones (n=3) in three independent experiments. P values are indicated.

Figure 3-17



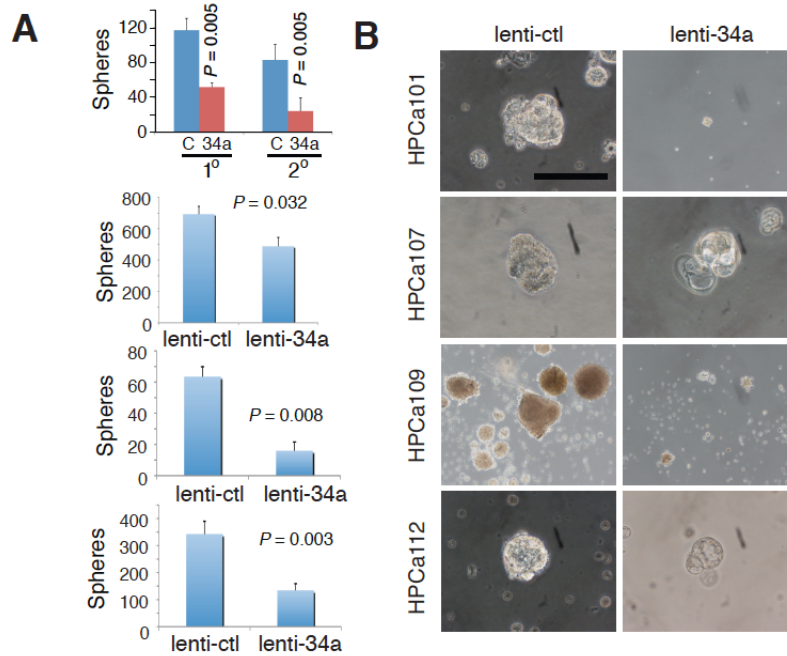
**Figure 3-17. miR-34a inhibited colony formation in Du145 cells.** Du145 cells transfected with miR-34a or NC oligo together with non-transfected cells (NT) were plated for clonogenic assays in Matrigel. Shown are the average counts of colonies generated (n=3) in each group. P values are indicated.

Figure 3-18



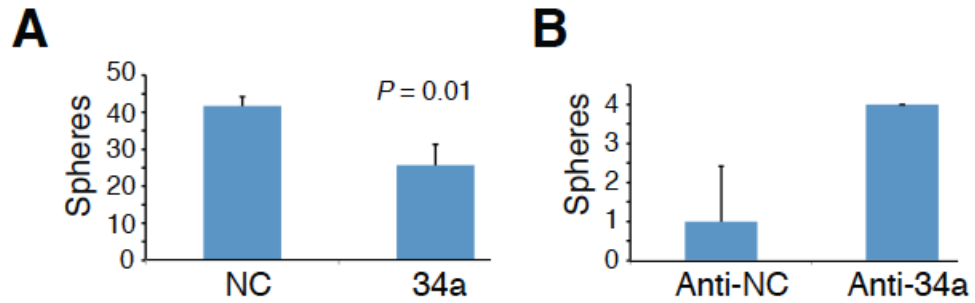
**Figure 3-18. miR-34a inhibited LAPC4 cells colony formation of in the Matrigel and floating sphere formation in ultra low attachment plates.** Shown are the bar graphs of colonies (A) and spheres (B). P values are indicated.

Figure 3-19



**Figure 3-19. miR-34a inhibited prostasphere formation in HPCa cells.** HPCa101 (Gleason 9), HPCa107 (Gleason 7), HPCa109 (Gleason 7), and HPCa112 (Gleason 6), were plated in triplicate in ULA plates in serum-free medium containing B27, EGF, and bFGF and spheres enumerated (A). Shown in B are representative images of spheres formed by primary HPCa cells. Scale bar, 20  $\mu$ m.

Figure 3-20



**Figure 3-20. Effects of miR-34a and anti-34a on sphere formation in purified CD44<sup>+</sup> or CD44<sup>-</sup> HPCa116 cells.** HPCa116 CD44<sup>+</sup> cells were transfected with miR-34a or NC oligo (A) whereas CD44<sup>-</sup> cells were transfected with the antisense oligo (B). Cells were plated for sphere formation assays. Shown are the bar graphs and the P value is indicated.



### **3.3.6 Identification of direct downstream targets of miR-34a**

Based on the observations that miR-34a seems to restrict some stem cell properties of PCa cells, we hypothesized that miR-34a might directly regulate some stem cell related molecules. Among more than 1,000 targets predicted by online search programs, two genes, i.e., CD44 and NANOG are of great interest to us because our lab has been studying both molecules.

#### **3.3.6.1 Identification of CD44 as a direct target of miR-34a**

Using the target predicting program RNA22 (152), we observed 2 putative binding sites in 3'-UTR of human CD44 mRNA (Figure 3-21). Together with our observations that miR-34a is significantly down-regulated in CD44<sup>+</sup> PCa stem/progenitor populations, we hypothesize that CD44 may be a direct downstream target of miR-34a.

Consistent with our hypothesis, the residual tumors from miR-34a transfected Du145 and PC3 cells showed a significant reduction in CD44 protein level (Figure 3-22A). We then performed Western blotting of CD44 in miR-34a over-expression Du145 cells and we observed a 60% reduction in CD44 protein level upon transfection of miR-34a (Figure 3-22B).

To test whether the regulation was direct, we performed luciferase reporter assay. 3'-UTR of CD44 mRNA were constructed into a luciferase reporter construct (pMIR-Reporter, Ambion, TX). Co-transfection of miR-34a but not NC specifically reduced luciferase activity of the wild type reporter (Figure 3-23). Moreover, mutations in the seed sequence abolished the miR-34a –repressed luciferase activity (Figure 3-23).

To determine whether CD44 is a functionally important target of miR-34a in the context of PCa development, we knocked down CD44 expression using a lenti-viral vector carrying a

short hairpin RNA (shRNA) against CD44 in LAPC4, PC3 and Du145 cells. Knockdown of CD44 inhibited both orthotopic tumor formation and lung metastasis in LAPC4 cells (Figure 3-24) as well as in PC3 cells (Figure 3-25). These results not only show that CD44 has a key role in determining the tumorigenic and metastatic capacity of PCa cells but also indicate that knockdown of CD44 phenocopies the anti-PCa effects of miR-34a. Mechanistically, the CD44<sup>+</sup> PCa cells showed higher migratory and invasive capacities than CD44<sup>-</sup> cells, and these capacities were partially inhibited by miR-34a (Figure 3-26A). Rescue experiments wherein CD44 was over-expressed using a cDNA that lacked the 3'- UTR containing the miR-34a binding sites abrogated miR-34a-mediated inhibition of invasion of CD44<sup>+</sup> Du145 cells (Figure 3-26B), reinforcing the idea that CD44 is a direct and functional target of miR-34a.

Figure 3-21

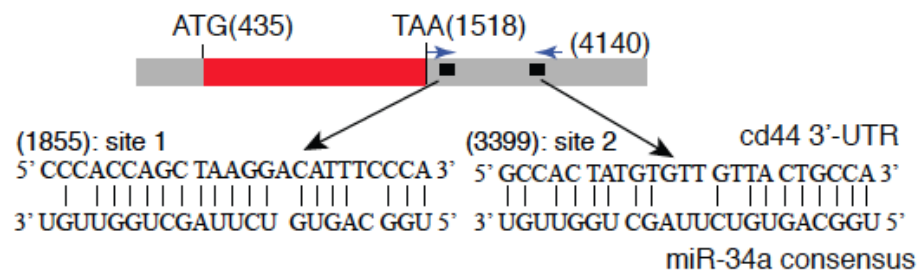
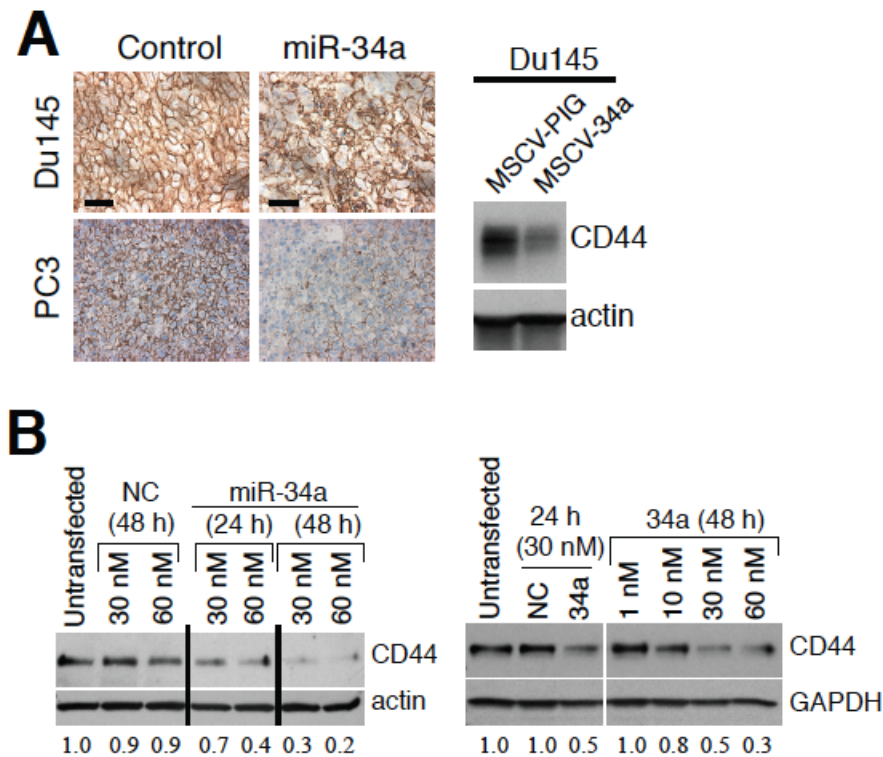


Figure 3-21. In silico analysis using RNA22 revealed putative miR-34a binding sites in 3'-UTR of CD44.

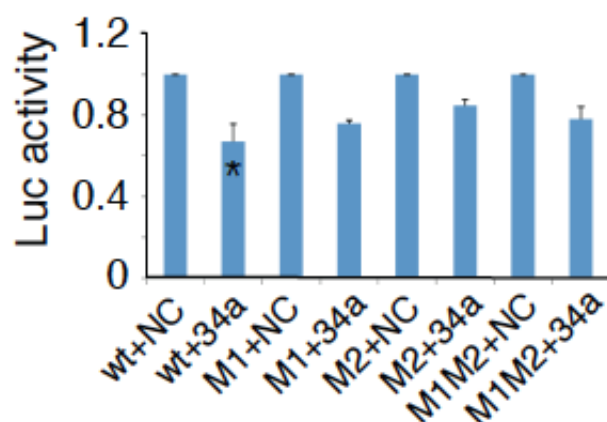
Figure 3-22



**Figure 3-22. CD44 protein level was reduced by over-expressing miR-34a.**

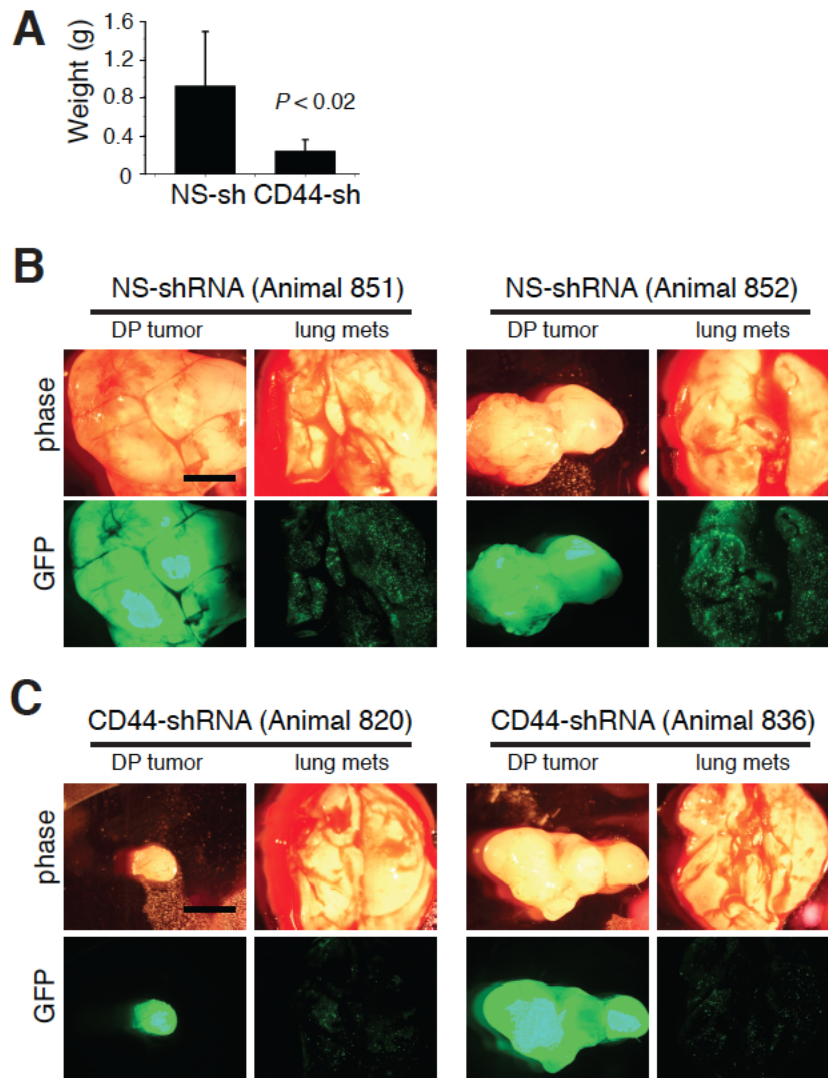
Shown in A are the IHC staining images of CD44 in tumors from miR-34a or control transfected Du145 and PC3 cells (left) and Western blotting of CD44 in Du145 tumors from MSCV-34a over-expression (right). Shown in B are the Western blotting of Du145 (left) and PPC-1 (right) cells freshly transfected with miR-34a oligos. Relative CD44 protein levels were indicated at the bottom.

Figure 3-23



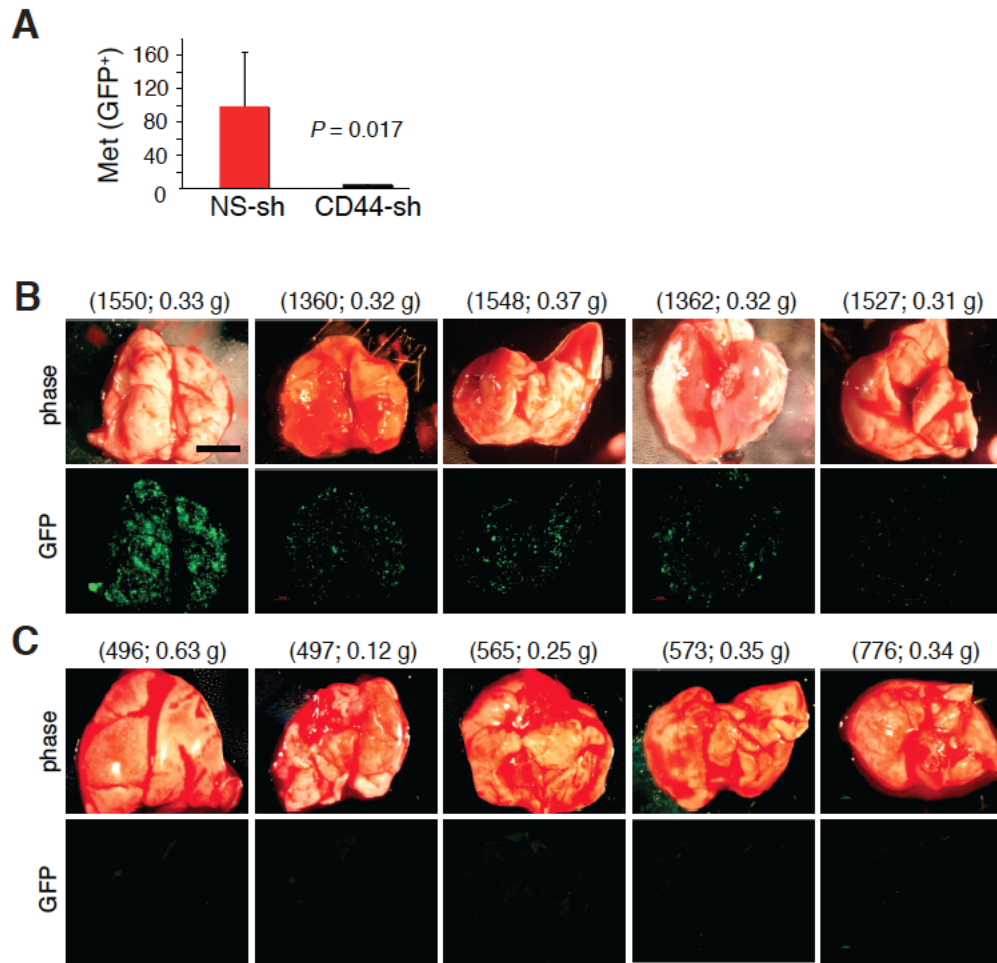
**Figure 3-23. Luciferase reporter assays in Du145 cells.** Luciferase reporter vector harboring wild type (wt) or mutated (M1, mutated at the putative binding site 1, M2, Mutated at the putative binding site 2) were co-transfected with NC or miR-34a oligo. Relative luciferase activities are presented. \*P<0.01.

Figure 3-24



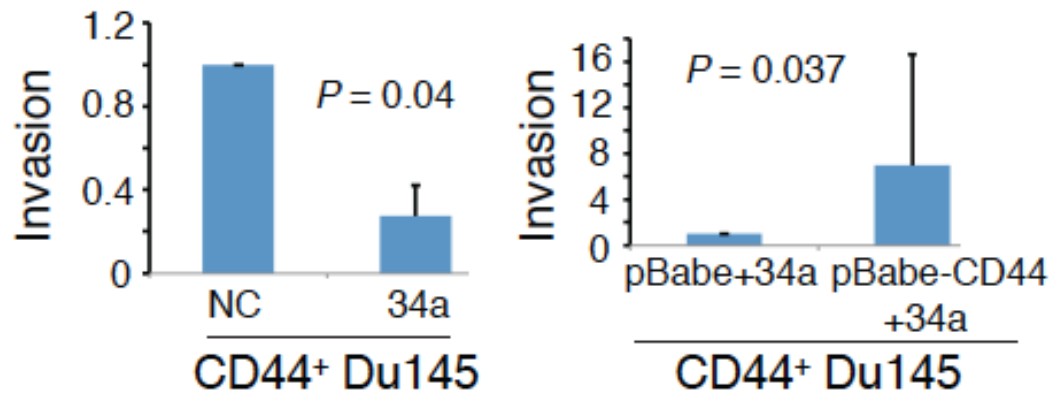
**Figure 3-24. CD44 knockdown inhibits LAPC4 tumor growth and lung metastasis.** Purified LAPC4 cells were infected with either non-silencing (NS) pGIPz control lenti-viral vector or pGIPz-CD44shRNA. Tumors were harvested and weighed (A) and lungs were harvested to image and quantify GFP<sup>+</sup> pulmonary metastases (B&C). Shown are two representative animals from each group (n = 7). Scale bar, 100  $\mu$ m.

Figure 3-25



**Figure 3-25 CD44 knockdown inhibits PC3 cell lung metastasis.** PC3 cells were infected with pGIPz control (a) or pGIPz-CD44shRNA (b) lenti-viral vectors. Tumors were harvested (tumor weights showed no difference between the two groups) and lungs were imaged and quantified for GFP<sup>+</sup> pulmonary metastases. The CD44-shRNA animals showed much less lung metastasis than in NS-shRNA animals. Shown in A is the bar graph for averaged count of GFP<sup>+</sup> foci in the lung, and shown in B and C are representative images of 5 lungs for each group (animal number and tumor weight indicated). Scale bar, 100  $\mu$ m.

Figure 3-26



**Figure 3-26. Rescue experiment in CD44<sup>+</sup> Du145 cells by invasion assay.** MiR-34a inhibited CD44<sup>+</sup> Du145 cell invasion (left), and CD44 over-expression by a vector that lack 3'-UTR partially rescue the inhibition (right).



### 3.3.6.2 Identification of Nanog as a direct target of miR-34a

Nanog, a homeobox transcription factor play an important role in regulate embryonic stem cell (ESC) self-renewal and pluripotency, recently have been reported to also regulate CSC activity and tumor development (153). Noteworthy, knocking down Nanog using shRNA phenocopied tumor-inhibitory effects of overexpression of miR-34a (153). In silico analysis using the target prediction software RNA22 revealed that mRNA of NANOG and NANOG pseudogene 8 (NANOG P8, a splicing variant predominantly expressed in cancer cells) indeed contained 2 putative binding site of miR-34a (Figure 3-27). Based on these observations, we proposed that Nanog might be another important direct target of miR-34a

We first performed Western blotting to test whether over-expression of miR-34a would repress Nanog protein levels. Nanog, which is normally expressed at very low level in Du145 cells, increased by  $\gamma$ -irradiation in a dose-dependent manner (unpublished observation). Transfection of Du145 cells with miR-34a, but not NC, completely blocked irradiation-induced upregulation of Nanog (Figure 3-28). Pilot luciferase experiments indicated that the candidate miR-34a binding sites at the 3'-UTRs of Nanog/NanogP8 are functional. These preliminary data suggests that miR-34a might directly target Nanog. In further support, Choi *et al.* recently reported that miR-34a directly targets Nanog, Sox2 and Mycn and consequently regulates somatic reprogramming and iPSC (induced pluripotent stem cell) differentiation (154).

Figure 3-27

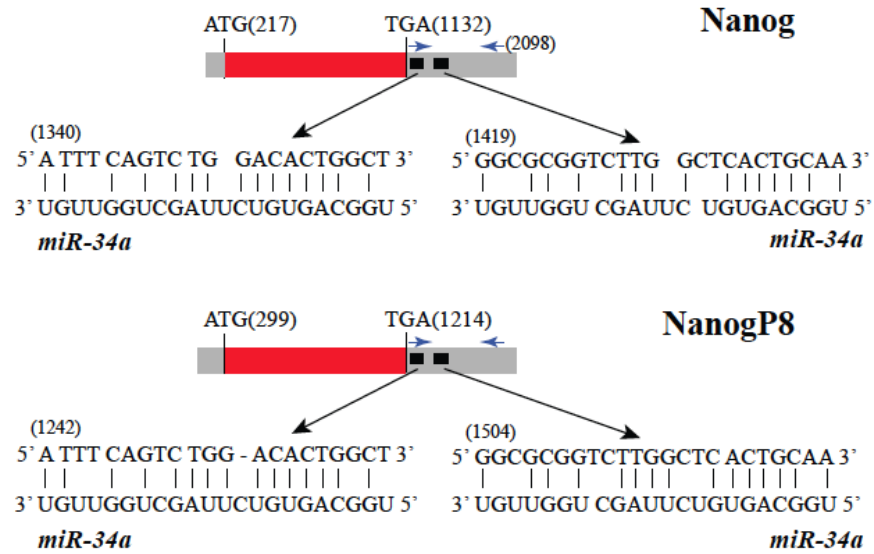
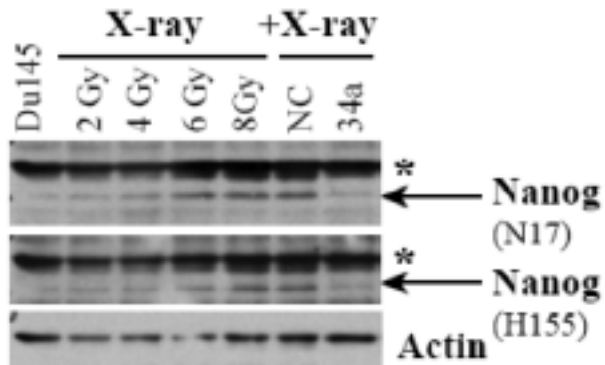


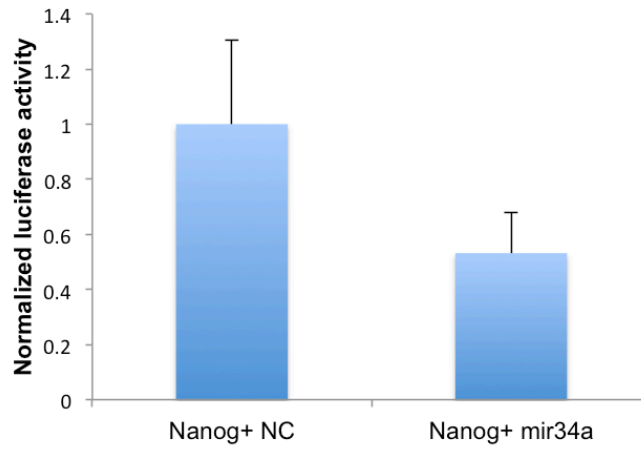
Figure 3-27. In silico analysis using RNA22 revealed putative miR-34a binding sites in 3'-UTR of Nanog and NanogP8.

Figure 3-28



**Figure 3-28. Western blotting of Nanog protein in Du145 cells.** Du145 cells were either  $\gamma$ -irradiated alone at 2-8Gy or irradiated at 8Gy after transfection of miR-34a or NC, cells were harvested and used for western blotting with two different antibodies, N17 (goat pAb against N-terminus 17aa) and H155 (Rb pAb against C-terminus 155 aa). The Nanog proteins were indicated by arrow, asterisk indicated non-specific bands. Actin was included as loading control.

Figure 3-29

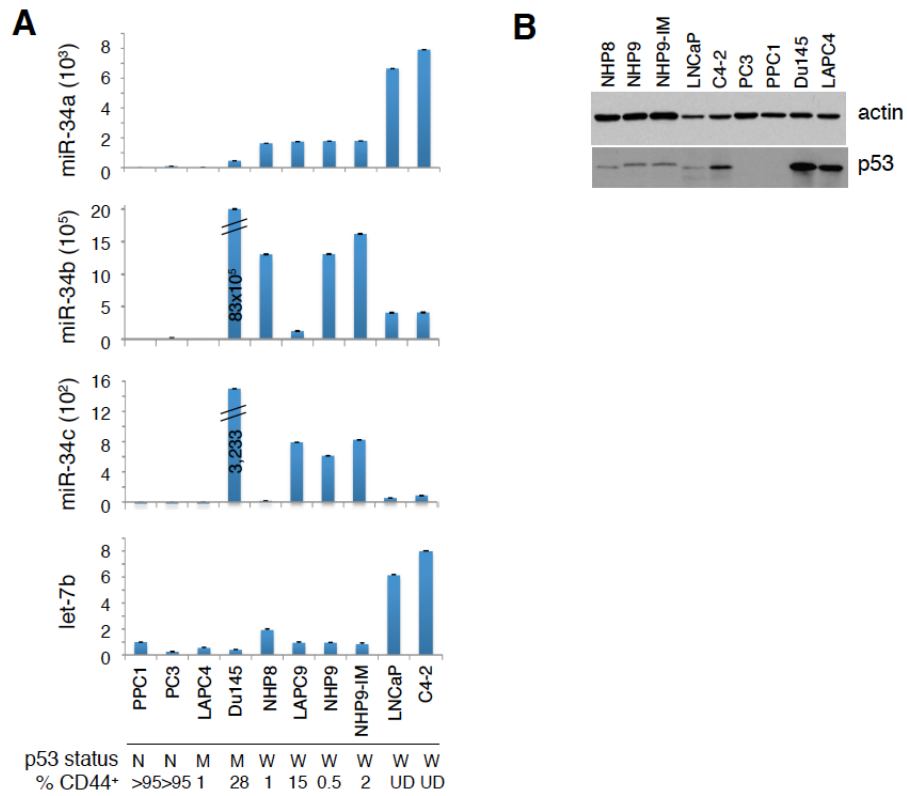


**Figure 3-29. Pilot luciferase reporter assay of Nanog 3'-UTR.** Co-transfection of miR-34a with the luciferase vector harboring wild type Nanog 3'-UTR significantly reduced its relative luciferase activity, compared to co-transfection with NC.

### 3.3.7 miR-34a upstream regulations

We then tried to understand how miR-34a might be under-expressed in PCa stem/progenitor cell populations. We first employed qRT-PCR analysis to correlate the expression levels of endogenous miR-34a and, for comparisons, miR-34b, miR-34c, and let-7b, with the p53 status (Figure 3-30A) in 10 prostate (cancer) cell types, which included two normal human prostate epithelial cell strain (NHP) NHP8, NHP9, and an immortalized NHP9 (NHP9-IM) cells, all expressing wild-type (wt) p53, and LNCaP, LNCaP subline C4-2, PC3, PPC-1, and Du145 cells, as well as LAPC4 and LAPC9 cells freshly purified from xenograft tumors. The two LNCaP lines express wt p53. LAPC9 cells also expressed wt p53 as revealed by our genomic DNA sequencing of exons 5-8. PC3 and PPC-1 cells were p53 null whereas Du145 and LAPC4 cells harbor mutant p53. We observed that the four PCa cell types harboring mutant (Du145 and LAPC4) or null (PC3 and PPC-1) p53 had much lower levels of miR-34a than the six cell types with wild-type (wt) p53 (Figure 3-30B), suggesting that p53 may also regulate baseline miR-34a expression in prostate (cancer) cells. In NHP8, NHP9, NHP9-IM, and LAPC9 cells with wt p53, miR-34a was expressed at similar levels but, interestingly, p53-wt LNCaP and C4-2 cells expressed much higher levels of miR-34a (Figure 3-30A). miR-34b and miR-34c, in contrast, showed similar expression patterns but were not strictly correlated with the p53 status (Figure 3-30A). For example, in p53 null or mutant cells, PPC-1, PC3, and LAPC4 cells displayed low level of miR-34b and miR-34c as expected, but Du145 cells showed extremely high levels of both miRNAs, suggesting miR-34b/c might have some p53-independent regulations in certain PCa cells. Similarly, miR-34b and miR-34c levels showed wide variations in the six p53-wt cell types (Figure 3-30A).

Figure 3-30

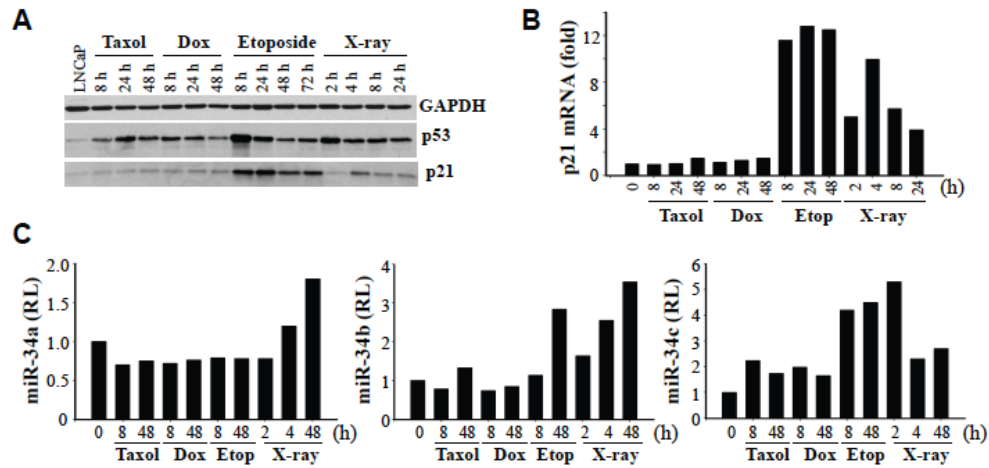


**Figure 3-30. Correlation of miR-34a, miR-34b, miR-34c and let-7 levels with the p53 status in 10 prostate and PCa cell lines.** Shown in A are the relative miRNAs levels analyzed by qPCR (miRNA levels in PPC-1 were designated as 1). Shown in B is the Western blotting of p53 in cell lines indicated. Actin is included as loading control.

We further tried to activate p53 to see whether the miR-34a level would increase. We treated LNCaP cells with paclitaxel and three DNA-damaging agents, i.e., doxorubicin, etoposide, and  $\gamma$ -irradiation (X-ray). All 4 treatments activated p53 as evidenced by increased p53 levels (Figure 3-31A). Etoposide and X-ray treatments induced increased mRNA levels of p53 target gene p21 (Figure 3-31B), whereas etoposide and taxol, to a less degree, induced apoptosis evidenced by activated caspase-3. These results suggest that LNCaP cells respond to p53 activation with different outcomes. When miR-34a and miR-34b/c levels were measured in treated LNCaP cells, we observed that the miR-34a levels did not show any significant changes except ~2-fold increase in cells 48 h after irradiation (Figure 3-31C). In contrast, both etoposide and X-ray increased miR-34b and miR-34c levels by several fold (Figure 3-28C). This result suggests that miR-34a expression may not be restricted to p53 regulation.

In further support, when we tried to correlate the endogenous mRNA levels of miR-34a with those of p53 and c-Myc, the latter of which has been reported to repress miR-34a expression (85), in CD44<sup>+</sup>/CD44<sup>-</sup> HPCa cells purified from 14 patient tumors, the results did not reveal differential expression of p53 and c-Myc in CD44<sup>+</sup> vs. CD44<sup>-</sup> HPCa cells that could explain reduced miR-34a in CD44<sup>+</sup> cells (Figure 3-32).

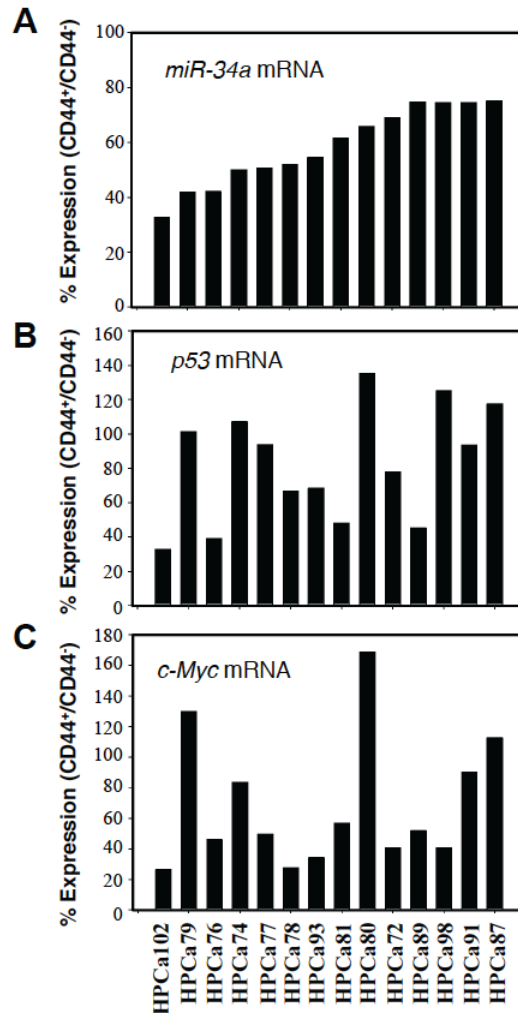
Figure 3-31



**Figure 3-31. p53 activation in LNCaP cells differentially affects miR-34 family members.** **A.** Western blotting for p53, p21, or GAPDH (loading control) in LNCaP cells treated with paclitaxel (Taxol, 25 nM), doxorubicin (Dox, 10 ng/ml), etoposide (Etop, 50 nM), or  $\gamma$ -irradiation (10 Gy). **B.** Verification by qRT-PCR of upregulation of p21 mRNA in LNCaP cells treated with etoposide (Etop) and  $\gamma$ -irradiation (X-ray). **C.** qPCR of miR-34a (left), miR-34b (middle) and miR-34c (right) levels in treated LNCaP cells.



Figure 3-32



**Figure 3-32. Correlation of miR-34a expression with p53 and Myc level in CD44<sup>+</sup> HPCa samples.** Shown are the relative expression levels of mature miR-34a (A), p53 (B), and c-Myc (C) mRNA by qPCR analysis in CD44<sup>+</sup>/CD44<sup>-</sup> cell fractions purified from 14 HPCa samples. The results were relative expression levels in CD44<sup>+</sup> over the corresponding CD44<sup>-</sup> HPCa cells.

### 3.4 Discussion

miRNAs play an essential role in regulating both normal and cancer stem cell properties and deregulation of miRNA expression has been implicated in tumorigenesis. Several PCa stem/progenitor cell populations have been reported but it remains unclear whether those differentially expressed miRNAs may regulate their biological and tumorigenic properties. In last section, we presented data showing that, by screening a library of 310 miRNAs, miR-34a, a recently identified tumor-suppressive miRNA and a direct p53 target, was significantly under-expressed in PCa stem/progenitor cell-enriched cells, in particular, in the CD44<sup>+</sup> PCa cells in both xenograft and primary patient tumors. In this chapter, we have focused on the biological functions of miR-34a in PCa cells.

Enforced expression of miR-34a in bulk PCa cells inhibits PCa cell expansion in vitro and tumor development in vivo, whereas knocking down endogenous miR-34a by antagomir promotes tumor regeneration and metastasis. More importantly, over-expression of miR-34a in purified CD44<sup>+</sup> PCa cells completely abrogates tumor regeneration. Replating assays and serial tumor transplantation assays demonstrate that miR-34a negatively regulates CSC-associated properties in long-term cultured PCa cells as well as in fresh HPCa cells. Of great significance, we have obtained evidence that miR-34a may directly target CD44 itself, and knocking down of CD44 in multiple PCa models phenocopies the miR-34a effects.

Previous work from our lab and others' has shown that the CD44<sup>+</sup> PCa cell population is enriched in highly tumorigenic and metastatic cells. Furthermore, CD44 is intimately involved in development and metastasis of multiple tumor types and CD44, as a cell surface adhesion molecule, has been utilized, either individually or in combination with other molecules, as a marker of cancer stem cells in numerous tumor systems. Importantly, anti-CD44 mAbs have been demonstrated to possess potent therapeutic effects against CSCs and are in clinical trials. Our study also reveals that systemically delivered miR-34a

possesses therapeutic efficacy against pre-established PCa, extending the survival of tumor-bearing animals as a result of inhibiting metastasis. These observations may establish a strong rationale for developing miR-34a as a novel PCa therapeutic.

To understand the potential upstream regulators of miR-34a in PCa cells, we have first surveyed whether the genomic locus where miR-34a is located (i.e., chr.1p36) is frequently mutated or deleted in PCa. We find that although the genomic region chr.1p36 is frequently lost in neuroblastoma and pancreatic cancer, it's not a mutation hotspot for PCa. Interestingly, one of the enzymes involved in the miRNA maturation, Dicer, has been reported to be upregulated in PCa cells, which would be inconsistent with our and others' observations that cancers including PCa generally show a global reduction of miRNAs compared to normal or benign cells. Whether Dicer is differentially expressed in the CSC compartment is not clear and will be our future pursuit. miR-34a can be epigenetically silenced by DNA methylation in multiple cancers including prostate and pancreatic cancer cell lines. However, when we treated Du145 cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine and histone deacetylation inhibitor Trichostatin A (TSA), we were unable to observe the restoration of miR-34a level (data not shown). Finally, since miR-34a represents a direct downstream target of p53, we then focus our attention on the p53 regulation of miR-34a expression in PCa cells. We found that, indeed, the basal level of the miR-34a and miR-34b/c correlated with the p53 status in 10 prostate and PCa cell lines. In LNCaP cells, the miR-34 family members seem to respond differently to the p53 activation, in which miR-34b/c level increased several fold whereas miR-34a levels do not. These results may be explained by the fact that LNCaP cells endogenously express high levels of miR-34a and consequently, p53 activation would not further upregulate miR-34a levels. In contrast, LNCaP cells express hardly detectable endogenous miR-34b/c and therefore p53 activation induces their upregulation. Disappointingly, correlating the miR-34a levels with

those of p53 or Myc mRNA in purified CD44<sup>+</sup> HPCa cells does not reveal any positive correlation. Further investigations will be needed to understand how miR-34a is under-expressed in the CSC enriched PCa cell populations.

## Chapter 4 Functional studies of let-7 and miR-301 in PCa cells

### 4.1 Introduction

In previous sections, I presented our miRNA expression profiling in prostate CSC populations, and reported several miRNAs that are commonly and differentially expressed, including four under-expressed miRNAs, namely, miR-34a, let-7b, miR-106a and miR-141, and two over-expressed miRNAs, namely, miR-301 and miR-452. We then carried out functional investigations on miR-34a and showed that the miR-34a is a critical negative regulator of PCa development and metastasis by regulating prostate CSCs and directly repressing CD44. In this chapter, I focus on two other miRNAs, one commonly under-expressed, let-7b, and one commonly over-expressed, miR-301, for the following reasons. First, both let-7b and miR-301 are commonly and differentially expressed in xenograft – derived PCa stem/progenitor cells. In fact, four let-7 miRNA members (i.e., let-7a, let-7b, let-7e and let-7f) are under-expressed in all three CD44<sup>+</sup> PCa cell populations. We speculate that under-expression of let-7 or over-expression of miR-301 may also be causally involved in regulating prostate CSC properties and PCa development. Secondly, both the under-expression of let-7b and over-expression of miR-301 are validated in the CD44<sup>+</sup> HPCa cells establishing a potential clinical relevance in PCa patients.

Let-7 miRNA family is evolutionarily conserved miRNA, first discovered in controlling the developmental timing in *C. elegans* (57). In human, let-7 miRNA family consists of 12 members sharing the same seed sequence including let-7a (from three precursors, let-7a-1, let-7a-2, and let-7a-3), let-7b, let-7c, let-7d, let-7e, let-7f (from two precursors, let-7f-1 and let-7f-2), let-7g, let-7i and miR-98. Extensive profiling of miRNA expression in human tumor samples and cancer cell lines has revealed that the let-7 miRNA family is under-expressed in various human cancers, most notably, in breast cancer and lung cancer indicating their

tumor suppressor functions (97, 106, 155-157). As observed in the original study in *C. elegans*, let-7 act as critical regulators of proliferation in human cancer cells. Let-7 miRNAs target several cell cycle related genes including cyclin D2, cyclin A2, CDK6, CDK25, E2F5 (93, 156). As tumor suppressors, let-7 miRNAs also target oncogenic genes such as Ras, Myc, BCL-2, and HMGA2 (156, 158). Most relevant to my studies, let-7 miRNA family has also been shown to be under-expressed in breast tumor initiating cells (BT-IC) and negatively regulate BT-ICs (106). Based on these studies, and our observations that let-7b is commonly under-expressed in several prostate CSC-enriched PCa cell populations, we speculate the let-7b may also negatively regulate prostate CSCs and prostate cancer development.

MiR-301, on the other hand, is a less studied miRNA with respect to its function in cancer and its downstream targets. Genomically, miR-301 is an intronic miRNA localized in the host gene *ska2* whose function is not clear (159). miR-301 is differentially expressed in pancreatic cancer, hepatocellular carcinoma and small cell lung cancer (160-162). Recently, miR-301 has been shown to promote breast cancer cell proliferation and invasion (163).

To investigate the functions of let-7 and miR-301 in regulating PCa development, we take similar experimental strategies to those when we explored the miR-34a functions, by performing *in vivo* tumor regeneration and *in vitro* proliferation, clonal and sphere formation assays.

## **4.2 Materials and methods**

### **Cells, xenografts, and animals**

PPC-1, PC3, and Du145 cells were obtained from ATCC and cultured in RPMI-1640 plus 7% fetal bovine serum (FBS). Human xenograft prostate tumors, LAPC9 (bone

metastasis; AR<sup>+</sup> and PSA<sup>+</sup>), LAPC4 (lymph node metastasis; AR<sup>+</sup> and PSA<sup>+</sup>), and Du145 (brain metastasis; AR<sup>-</sup> and PSA<sup>-</sup>) were maintained in NOD/SCID mice that were produced mostly from our own breeding colonies maintained in standard conditions according to the Institutional guidelines.

### **Transient transfection with oligonucleotides**

PCa cells were transfected with 30 nM of let-7a, let-7b, or miR-301 mirVana mimics or non-targeting negative control miRNA (miR-NC) oligos (Ambion) using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions (22). mirVana miRNA inhibitors, chemically modified antisense oligos against let-7b, miR-301, or Negative Control (NC) (Ambion) were introduced into PCa using the same conditions. Transfected cells were harvested for *in vitro* and *in vivo* studies after culturing for overnight to 48 h.

### **Lentiviral-mediated overexpression of let-7a**

pLL3.7-let-7a and pLL3.7 control vector were kindly provided by Dr. J. Lieberman (Harvard University). Lentiviruses were produced in 293FT packaging cells and titers determined for GFP using HT1080 cells. PCa cells were infected with the lenti-viral supernatant (MOI 5-10) in the presence of 8 µg/ml polybrene and harvested 48–72 h post-infection for experiments.

### **Tumor transplantation experiments**

Basic procedures for subcutaneous (s.c) and orthotopic (DP) tumor transplantations were described in the last chapter. Basically, LAPC9 and Du145 cells, from the maintenance

tumors were harvested and transfected with let-7a, let-7b mimic and negative control (NC) oligonucleotide or infected with pII3.7-let-7a and pII3.7 control lentivirus as mentioned above. 48 h later, cells were harvested and 100,000 cells each mixed with 50% Matrigel were injected into the subcutaneous or orthotopic site of intact male NOD-SCID mice.

### **Clonal, and sphere-formation assays**

For clonal assays, cultured PCa cells were plated at a clonal density (*i.e.*, 100 cells/well) in a 6-well dish. The number of holoclones was counted 7-14 days later. For sphere-formation assays, cells were plated (5,000–20,000 cells/well) in serum-free prostate epithelial basal medium (PrEBM) supplemented with 4  $\mu\text{g ml}^{-1}$  insulin, B27 (Invitrogen), and 20  $\text{ng ml}^{-1}$  EGF and bFGF in ultra-low attachment (ULA) plate. Floating spheres that arose in 1–2 weeks were counted. For all these experiments, a minimum of triplicate wells was run for each condition and repeat experiments were performed when necessary and feasible.

### **Statistical analyses**

In general, unpaired two-tailed Student's *t*-test was used to compare differences in cell numbers, cumulative PDs, percentages of CD44<sup>+</sup>, % BrdU<sup>+</sup> cells, % cell-cycle phases, cloning and sphere-formation efficiency, and tumor weights. Fisher's Exact Test and  $\chi^2$  test were used to compare incidence and latency. In all these analyses, a  $P < 0.05$  was considered statistically significant.



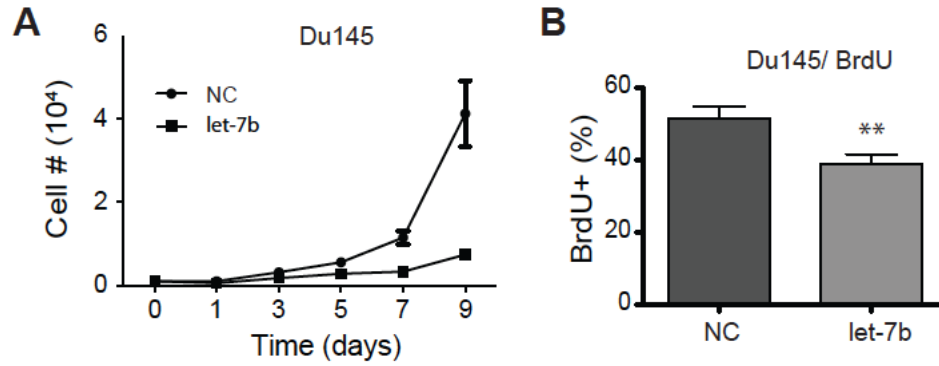
### 4.3 Results

#### 4.3.1 Let-7 inhibited clonal and sphere formation in PCa cells

To understand how let-7 may regulate biological activities of PCa cells, we first performed *in vitro* cell proliferation, clonal, and sphere formation assays. Over-expression of let-7b by transfection of a let-7b mimic oligo into Du145 cells inhibited cell proliferation and BrdU incorporation (Figure 4-1). Over-expression of let-7b in Du145 and PPC-1 cells also suppressed clonal expansion, especially the formation of holoclones that contain the self-renewing tumor initiating cells (42) (Figure 4-2 A-C). In addition, when Du145 cells were transfected with let-7b oligos and plated for sphere formation assays, we observed much fewer spheres formed from the let-7b treated cells (Figure 4-2D). In another PCa cell type, PPC-1 cells, we observed similar inhibitory effect of let-7b on clonal and sphere formation abilities (Figure 4-3).

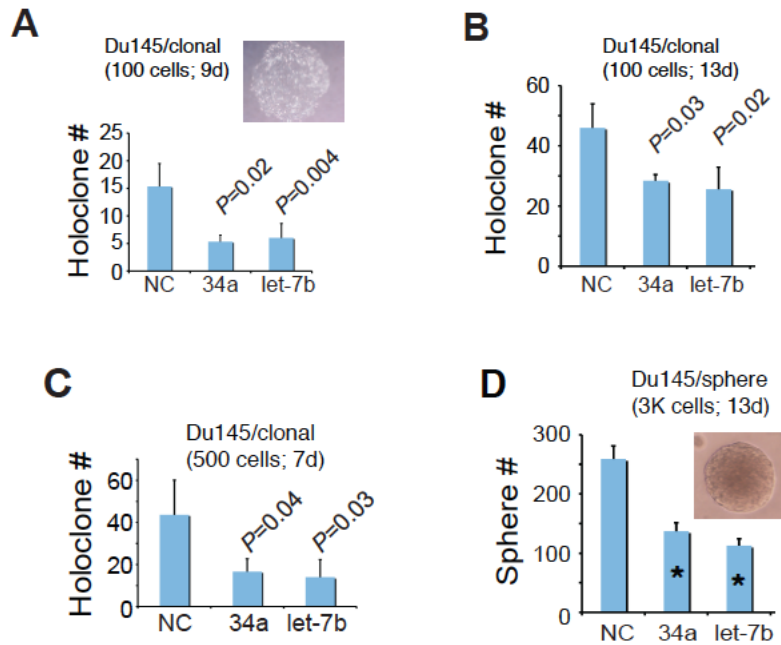
In the above-mentioned experiments, we also included miR-34a as a positive control since we have shown that miR-34a over-expression inhibits clonal and sphere formation in PCa cells. Overall, let-7b over-expression demonstrated similar inhibitory effects as miR-34a over-expression. However, we also observed a differential effect between let-7b and miR-34a on cell cycle progression in PCa cells. When we treated PPC-1 cells with miR-34a or let-7b oligos and analyzed cell-cycle profiles, we found that miR-34a mainly caused G1 cell-cycle arrest whereas let-7b led to prominent G2/M phase arrest (Figure 4-4). Consistent with this observation, over-expression of miR-34a significantly increased cell senescence revealed by senescence associated- $\beta$ gal (SA- $\beta$ gal) staining. In contrast, let-7b oligos did not induce senescence in PPC-1 cells (Figure 4-4 C&D).

Figure 4-1



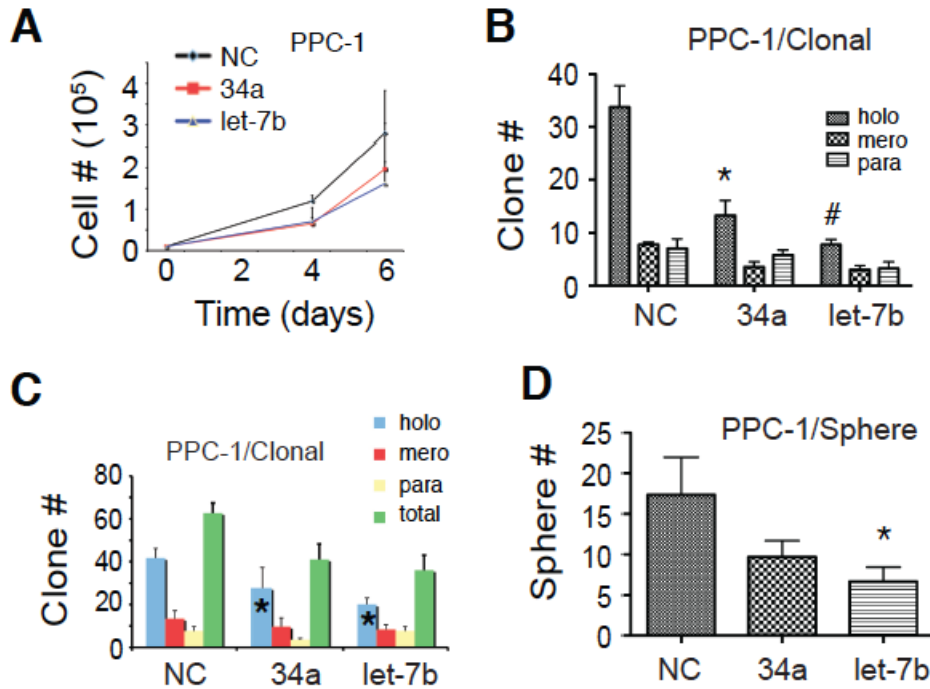
**Figure 4-1. Over-expression of let-7b inhibited Du145 cell proliferation.** Shown in A is the cell number of let-7b or NC transfected Du145 cells at the indicated time point. Presented in B is the mean % of BrdU-positive cells counted from a total of 800–1,000 cells.

Figure 4-2



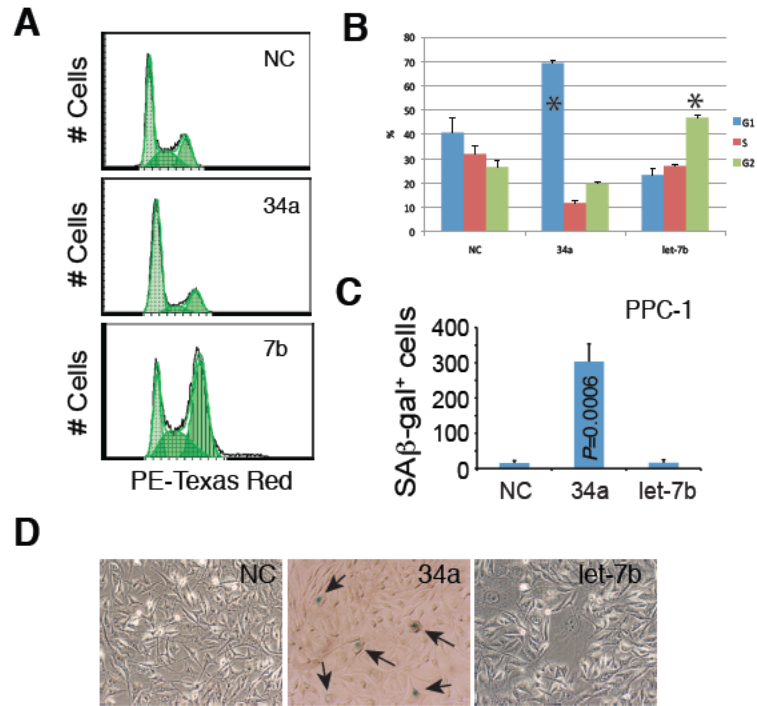
**Figure 4-2. Over-expression of let-7b inhibited clonal and sphere formation in Du145 cells.** A-C. Holoclone assays in Du145 cells. The plating cell numbers and the days when the holoclones (a representative picture shown in the inset, A) were enumerated are indicated. D. Sphere formation assays in Du145 cells. A representative sphere is shown in the inset. In these experiments, miR-34a was included as control.

Figure 4-3



**Figure 4-3. Biological effects of let-7b in PPC-1 cells.** Shown in A is the proliferation assay in PPC-1. The cell number was counted and plotted at the indicated time points. B-C. Two sets of clonal assays in PPC-1 cells. The holoclone, meroclone, paraclone and the total colony numbers were enumerated and plotted. D. Sphere formation assay in PPC-1 cells. miR-34a were included as control.

Figure 4-4



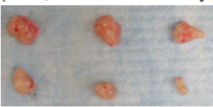
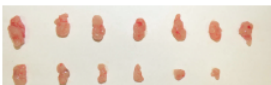
**Figure 4-4. Differential effects of let-7b and miR-34a on cell cycle.** Shown in A and B are the cell cycle analysis (A) and quantification of percentage of cells in the G1, S or G2 phase. C-D. Over-expression of miR-34a but not let-7b induced cell senescence. Shown in C is the quantification of senescence-associated  $\beta$ -gal (SA- $\beta$ gal) positive cells and in D the representative images.

#### **4.3.2 Let-7 inhibited tumor formation in PCa cells**

Interestingly, when we carried out tumor regeneration experiments with let-7 over-expressing PCa cells, we didn't observe a consistent tumor inhibitory effect of let-7 as we expected. We first carried out a positive control experiments by implanting a non-small cell lung cancer cell line A549 transfected with let-7b or NC oligo into subcutaneous (s.c) site in NOD/SCID mice. Similar as previous reports (97), let-7b over-expression suppressed A549 tumor growth (Figure 4-5). However, when we performed the similar tumor experiments in Du145 or LAPC9 (Figure 4-6) cells, let-7b oligos did not show any obvious tumor-inhibitory effects when cells were implanted either s.c or in the dorsal prostate. Intriguingly, in one LAPC9 tumor experiment, we also included miR-34a as a positive control, and we observed a strong inhibition of tumor development by miR-34a but not let-7b or let-7a (Figure 4-7).

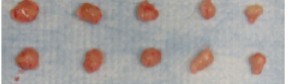
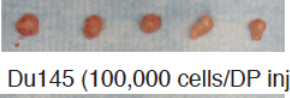
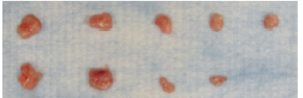

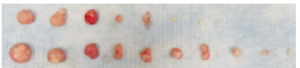

We suspected that this might be the result of rapid turnover of transiently transfected let-7 oligos. Indeed, when we examined the residual let-7 levels in the endpoint tumors, there was no difference between NC and let-7 groups (Figure 4-8A), whereas in freshly transfected cells there were hundreds of times more let-7b 48 hours after transfection (Figure 4-8 B & C). Surprisingly, both miR-34a transfected PCa cells and let-7b transfected A549 cells showed much more prominent accumulation of oligo with thousands of folds of increases in the respective mature miRNA levels shortly after transfection (Figure 4-8C). These observations help explain why we observed significant tumor inhibitory effect in miR-34a oligo transfected PCa cells and let-7b oligo transfected A549 cells, but not in the let-7 oligo transfected PCa cells (Figure 4-5, Figure 4-6 and Figure 4-7). However, mechanisms underlying these interesting observations await further investigation.

Figure 4-5

		<i>Incidence</i>	<i>Harvest</i>	<i>Weight (g)</i>	<i>P value</i>
A549 (500,000 cells/s.c inj.)					
	NC	3/3 (100%)	52 d	0.37 ± 0.14	0.05
	let-7b	3/3 (100%)	52 d	0.16 ± 0.09	
A549 (1,000,000 cells/s.c inj.)					
	NC	7/7 (100%)	42 d	0.29 ± 0.05	0.007
	let-7b	6/7 (86%)	42 d	0.12 ± 0.33	

**Figure 4-5. Over-expression of let-7b in A549 cells significantly inhibited tumor regeneration.** Shown are the tumor images with tumor incidence, harvest time, mean tumor weight and the P values.

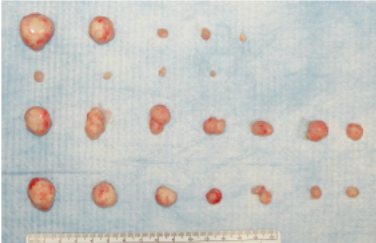
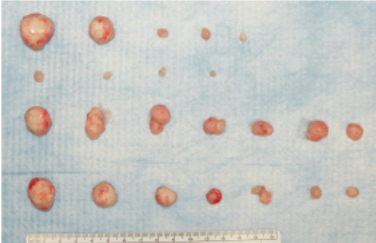
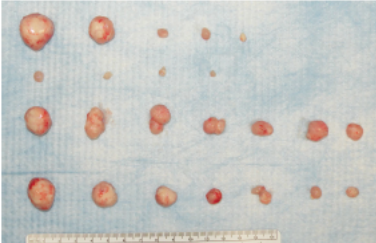
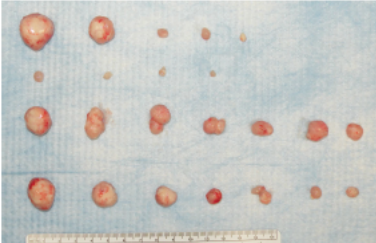
Figure 4-6

		<i>Incidence</i>	<i>Harvest</i>	<i>Weight (g)</i>	<i>P value</i>
Du145 (100,000 cells/s.c inj.)					
	NC	5/6 (83.3%)	46 d	0.60 ± 0.26	0.49
	let-7b	5/6 (83.3%)	46 d	0.60 ± 0.1	
Du145 (100,000 cells/DP inj.)					
	NC	5/6 (83.3%)	46 d	0.35 ± 0.16	0.4
	let-7b	4/6 (66.7%)	46 d	0.41 ± 0.32	
LAPC9 (1,000,000 cells/s.c inj.)					
	NC	7/10 (70%)	33 d	0.42 ± 0.17	0.29
	let-7b	10/10 (100%)	33 d	0.56 ± 0.18	

**Figure 4-6. Over-expression of let-7b by oligo transfection didn't suppress tumor development in Du145 or LAPC9 cells.** Shown are the tumor images with tumor incidence, harvest time, mean tumor weight and the P value.

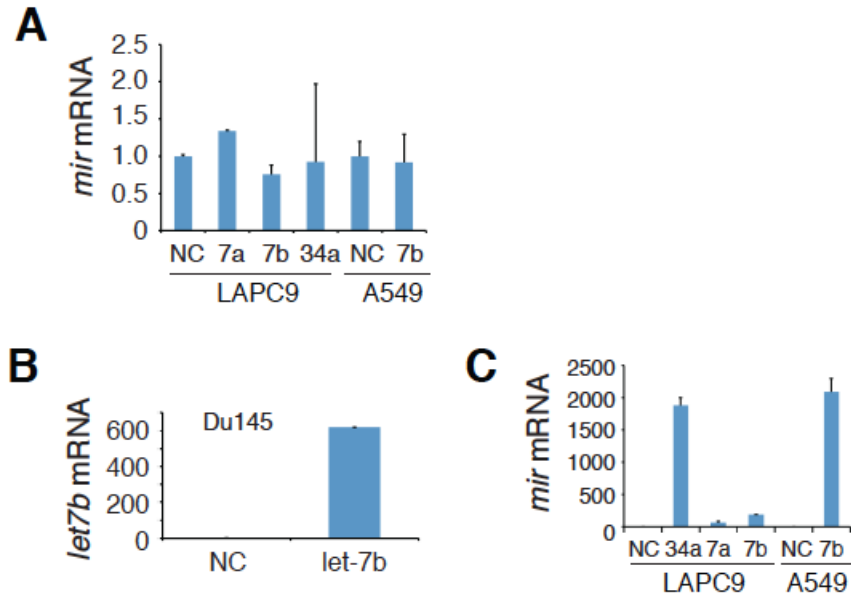


Figure 4-7

LAPC9 (100,000 cells/s.c inj./44 d		Mean weight	Incidence
NC		0.98g	5/7
34a		0.04g	4/7
let-7b		0.91g	7/7
let-7a		0.73g	7/7

**Figure 4-7. Over-expression by oligo transfection of miR-34a, but not let-7, inhibited the LAPC9 tumor regeneration.** Shown are the tumor images with mean tumor weight and incidence indicated on the right.

Figure 4-8



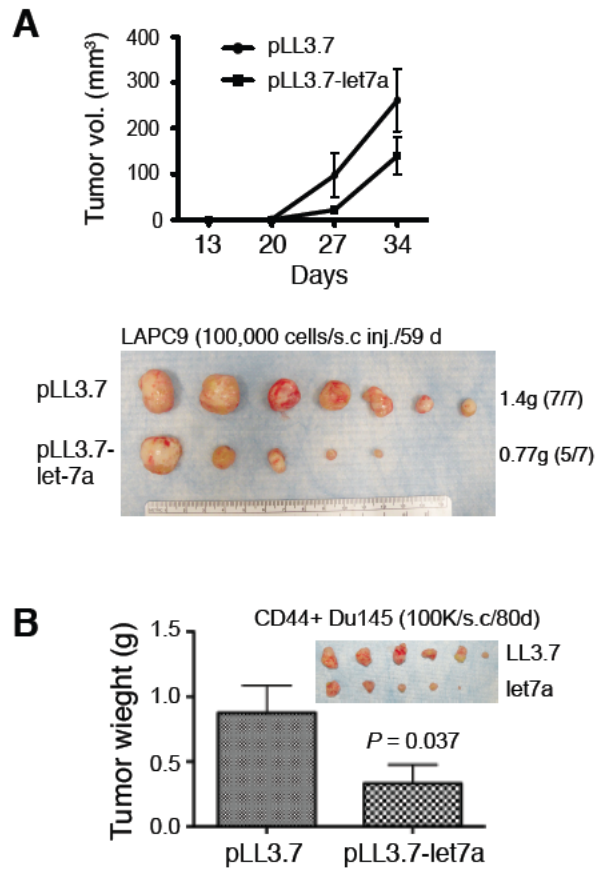
**Figure 4-8. Measurement of let-7 and miR-34a in residual endpoint tumors and freshly transfected cells.** Shown are the relative miRNA levels in residual tumors (A) or freshly transfected Du145 (B) and LAPC9 and A549 cells (C).

To circumvent the fast turnover of let-7, we employed a lentiviral vector encoding let-7a to perform the in vivo tumor experiments. Let-7a and let-7b recognize the same seed sequence in the targets. Over-expressing let-7a by a lentiviral vector suppressed LAPC9 tumor development in NOD/SCID mice (Figure 4-9A). Impressively, pLL3.7-let-7a also inhibited tumor regeneration from purified CD44<sup>+</sup> Du145 cells (Figure 4-9B).

#### **4.3.3 Validation of downstream targets of let-7**

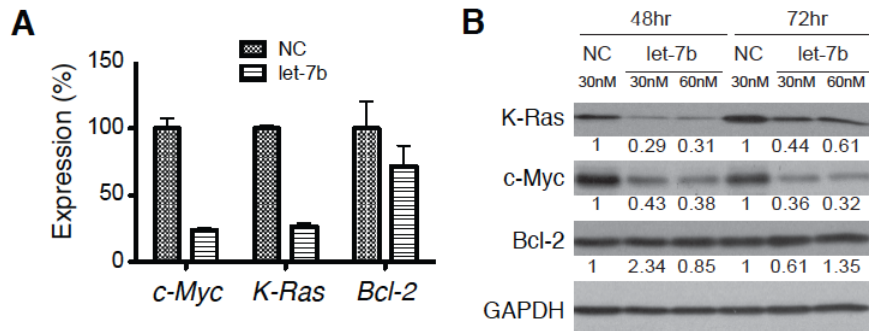
To identify downstream targets that involved in regulating PCa development, we chose to focus on a few critical oncogenic molecules including K-Ras, c-Myc, and Bcl-2, which have been previously shown to be targeted by let-7 in other system, such as lung cancer (97), breast cancer(106). Cells freshly transfected with let-7a or let-7b oligos were collected for Western blotting and qRT-PCR to measure the protein and RNA levels of these targets, respectively. The results revealed that let-7 reduced the mRNA and protein levels of K-Ras and c-Myc but not Bcl-2 (Figure 4-10).

Figure 4-9



**Figure 4-9. Lentiviral mediated over-expression of let-7a significantly inhibited LAPC9 and CD44<sup>+</sup> Du145 tumor growth.** Shown in A are the tumor growth curve, and tumor images. Shown in B is the bar graph for average tumor weight from pLL3.7 and pLL3.7-let7a infected CD44<sup>+</sup> Du145 cells, with tumor image shown in the inset.

Figure 4-10

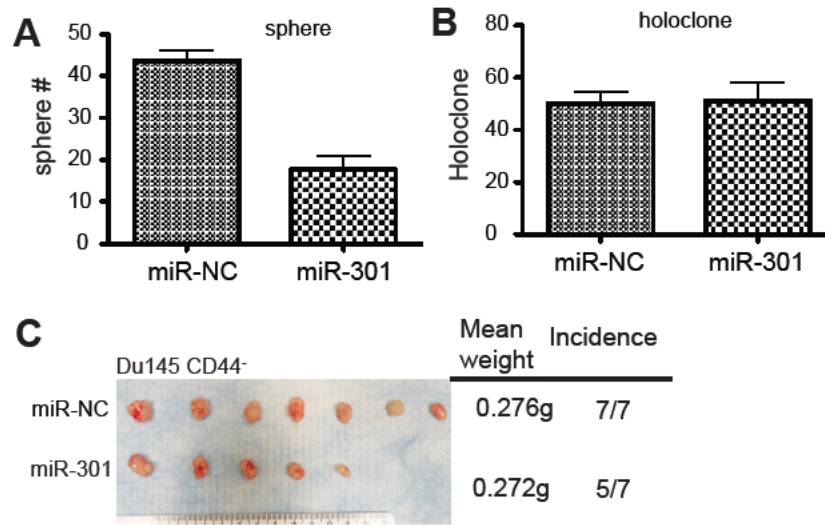


**Figure 4-10. let-7 targets K-Ras and c-Myc but not Bcl-2 in Du145 cells.** Shown in A are the mRNA levels of C-Myc, K-Ras and Bcl-2 in NC or let-7b transfected Du145 cells. Shown in B are the Western blotting results of K-Ras, C-Myc, and Bcl-2 proteins in NC or let-7b transfected Du145 cells. GAPDH was used as loading control. Densitometry values relative to GAPDH are given below.

#### **4.3.4 Cell type dependent effects of miR-301 in PCa cells**

Unlike the obvious effects we observed with the under-expressed miRNAs such as miR-34a and let-7, manipulating the intracellular levels of miR-301, one of the over-expressed miRNAs in tumorigenic populations, manifested differential effects in different PCa cells. In the CD44<sup>-</sup> and CD44<sup>+</sup> Du145 cells, transfection of miR-301 mimicking or antisense oligos, respectively, did not affect tumor growth in vivo or clonal expansion in vitro. We over-expressed miR-301 in CD44<sup>-</sup> Du145 cells which had lower endogenous miR-301. We plated transfected cells for clonal and sphere formation assay in vitro and tumor development assays in NOD/SCID mice. However, enforced expression of miR-301 did not promote sphere formation (Figure 4-11A) or clonal expansion (Figure 4-11B) in vitro, nor tumor development in vivo (Figure 4-11C). Meanwhile, we suppressed the endogenous miR-301 in CD44<sup>+</sup> Du145 cells by transfecting with anti-sense oligos. Cells were again plated for sphere and clonal assays and tumor development experiments in NOD/SCID mice. Unfortunately, we did not observe any inhibitory effects of anti-301 on sphere formation (Figure 4-12A), clonal expansion (Figure 4-12B), or tumor regeneration (Figure 4-12C). Similar results were obtained when we knocked down miR-301 in PC3 cells that are 100% CD44<sup>+</sup> (data not shown). We then utilized another PCa xenograft model LAPC9, which is different from Du145 in that it contains a lower percentage of CD44<sup>+</sup> cells and it harbors a differentiated (AR<sup>+</sup>/PSA<sup>+</sup>) cellular compartment. Interestingly, over-expression of miR-301 in LAPC9 cells promoted the clonal and clonogenic growth (Figure 4-13). These results, altogether, suggest that miR-301 may exert its 'oncogenic' effects in PCa cells in a cell type-dependent manner.

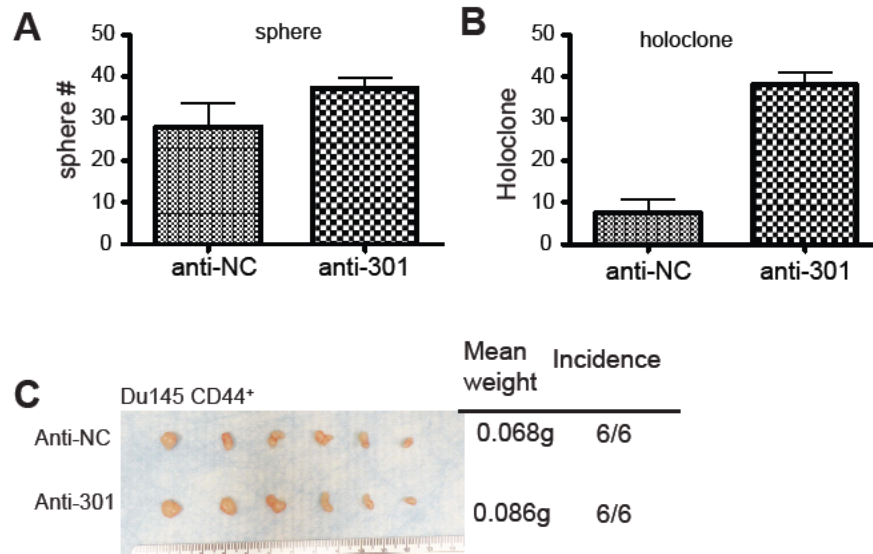
Figure 4-11



**Figure 4-11. Biological effects of over-expressing miR-301 in CD44<sup>+</sup> Du145 cells.**

FACS-purified CD44<sup>+</sup> Du145 cells were transfected with miR-NC or miR-301 oligos (30 nM each) and sphere formation (A), clonal (B) experiments and in vivo tumor regeneration (C) were performed. Shown are the average sphere numbers (A), holoclone number (B) and tumor images with mean tumor weight and incidence indicated (C).

Figure 4-12

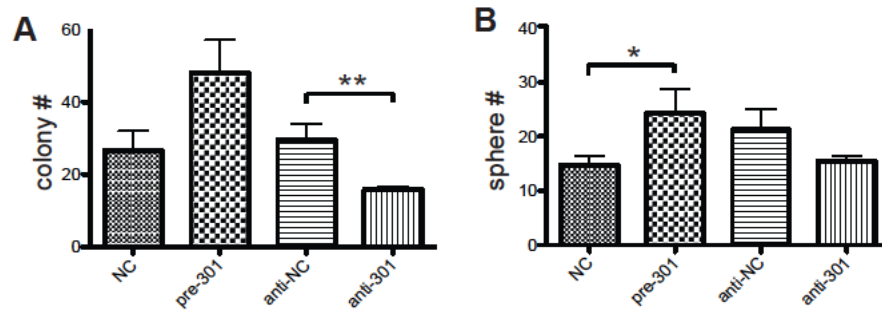


**Figure 4-12. Biological effects of knocking down miR-301 in CD44<sup>+</sup> Du145 cells.**

FACS-purified CD44<sup>+</sup> Du145 cells were transfected with antisense NC or anti-miR-301 oligos (30 nM each) and sphere formation (A), clonal (B) experiments and in vivo tumor regeneration (C) were performed. Shown are the average sphere numbers (A), holoclone number (B) and tumor images with mean tumor weight and incidence indicated (C).



Figure 4-13



**Figure 4-13. miR-301 effects on clonal and sphere formation abilities in LAPC9 cells.** A. Clonal assay in LAPC9 cells transfected with miR-NC or miR-301, or anti-miR-NC (anti-NC) or anti-miR-301 (anti-301), and plated on the Swiss 3T3 feeder layer. Colonies were counted. \* $p<0.05$ , \*\*  $p<0.01$ . Shown in A is average colony number. B. Sphere assays in LAPC9 cells transfected with miR-NC or miR-301, or anti-miR-NC (anti-NC) or anti-miR-301 (anti-301). 10,000 cells per well were plated in the 6- well ultra low attachment plate.

#### 4.4 Discussion

In this chapter, we utilized similar experimental strategies that we used in studying miR-34a to explore the biological function of two other commonly and differentially expressed miRNAs, let-7b and miR-301. Consistent with previous studies showing that let-7 is a 'universal' tumor suppressor miRNA in various cancers (129), and is under-expressed in breast tumor-initiating cells (106), let-7 also exhibits tumor suppressive function in PCa cells. In vitro functional analysis of let-7 using oligo transfection demonstrates that over-expression of let-7b significantly inhibits proliferation, and clonal and sphere formation. Since these in vitro experiments generally take only about 1-2 weeks; transient transfection of let-7 oligos is sufficient to manifest its effects. However, in tumor development experiments, which usually take months, we have observed a discrepancy between oligo- and lentiviral- mediated let-7 over-expression in modulating prostate tumor growth. Let-7 oligo transfection in several PCa models does not show obvious tumor-inhibitory effects whereas the lentiviral mediated over-expression of let-7 clearly suppresses tumor regeneration. Furthermore, under similar transfection conditions using oligos, miR-34a but not let-7 oligos cause clear clear-cut tumor inhibition. Surprisingly, transfected let-7a and let-7b oligos seem degraded faster than miR-34a oligos and faster in PCa cells compared to A549. Our observations suggested that 1) let-7 miRNAs might exert differential effects on lung (A549) vs. prostate (Du145 and LAPC9) cancer cells; 2) transfected let-7 oligos might be turned over faster in PCa cells compared to lung cancer cells; 3) let-7 and miR-34a might exert divergent regulatory roles in PCa cells; and/or 4) let-7 oligos might become degraded or turned over faster than miR-34a oligos in PCa cells. Future experiments will be directed to addressing these suggestions.

Another interesting observation is that let-7 and miR-34a possess mechanistic differences in regulating PCa cell cycle: miR-34a, as previously reported to be a mediator of

p53 effect in cell cycle progression, induces G1 cell cycle arrest followed by cell senescence in PCa cells, whereas let-7 causes a prominent G2/M phase cell cycle arrest without significantly inducing cell senescence. Furthermore, miR-34a, but not let-7, induces apoptosis in some PCa cells. In addition, let-7 over-expression does not affect the levels of pro-survival molecule Bcl-2 in Du145 cells.

In contrast to the consistent PCa-inhibitory effects of miR-34a and let-7, miR-301, which is one of the commonly over-expressed miRNAs in prostate CSCs and recently shown to promote breast cancer cell proliferation and invasion(163), seems to exhibit different effects in different cell types. Although manipulation of miR-301 levels does not affect Du145 and PC3 cells, its over-expression promotes whereas its knockdown inhibits the clonogenic properties of LAPC9 cells. Suggesting that miR-301 may exert its influences on PCa cells in a cell type-dependent fashion. It is also plausible to speculate that the effects of miR-301 alone are subtle, but together with other over-expressed oncomiRs (e.g., miR-452), it contributes to the tumorigenic properties of PCSCs.

## **Chapter 5 Potential biological functions of miR-141 in PCa cells**

### **5.1 Introduction**

In the final section of my thesis, I would like to discuss some preliminary studies aimed at understanding miR-141 functions in prostate CSCs and prostate cancer development, and propose several aims that I would like to pursue in the near future. As mentioned in chapter 2, miR-141 is one of the commonly under-expressed miRNAs in CSC populations from xenograft-purified PCa cells (Figure 2-13). Remarkably, in CD44<sup>+</sup> HPCa cells purified from HPCa samples, 20 of 21 samples showed drastic under-expression of mir-141 (Figure 2-16). These observations of miR-141 under-expression in prostate CSC populations in both xenograft and primary patient samples lead to our hypothesis that miR-141 may be causally involved in regulation prostate CSC.

miR-141, together with four other miRNAs, i.e., miR-200a, miR-200b, miR-200c, and miR-429, consist the miR-200 family. The expression pattern of miR-200 family including miR-141 in cancer has been complex, since both up-regulation (164), and down-regulation (116, 165, 166) have been reported. In PCa, the study of miR-141 expression levels has also been equivocal. Several studies have reported that miR-141 is over-expressed in PCa cells compared to normal or benign tissue (167), and the miR-141 levels are elevated in the serum of PCa patients and correlate with tumor progression (96, 168), providing a potentially novel biomarker for PCa patient diagnosis and prognosis. On the other hand, others have shown that miR-141/miR-200c is expressed at reduced levels in metastatic PCa cells such as PC3 (169). The expression of miR-141 and its biological functions in prostate CSCs has not been reported.

There is strong evidence that the miR-141/miR-200 miRNAs are involved in epithelial-mesenchymal transition (EMT) and cancer metastasis (170). EMT occurs during embryonic development, wound healing, and cancer progression. During EMT, tumor cells lose adhesion, and change from a differentiated state resembling epithelial cells to a more de-differentiated state like mesenchymal cells with increased motility. EMT is characterized by cell morphological change, with repression of E-cadherin expression and activation of vimentin expression. Cancer metastasis shares a lot of similarities with EMT. Among the reported miR-141 targets are TGF $\beta$ 2, ZEB1 (zinc-finger E-box binding homeobox 1), SIP1 (Smad Interacting Protein 1; also called ZEB2), Dlx5 (distal-less homeobox 5), MKK4 (mitogen-activated protein kinase kinase 4), and SEMA6A (116, 171-174), most of which are well-known EMT mediators. In addition, one recent study is of great interest to us, which shows that the miR-200 family is under-expressed in and regulates the CD44<sup>+</sup> BCSC properties by directly targeting a stem cell factor BMI-1 (107).

The question we aim to address in this still ongoing research is whether the under-expression of miR-141 in CSC populations observed in both prostate xenograft models and primary PCa patient samples possesses any biological consequence. To start, we first explored the miR-141 functions in regulating EMT by performing the migration and Boyden Chamber invasion assays, and also carried out in vitro clonal and sphere assays.

## **5.2 Materials and Method**

**Basic procedures were as previously described.**

### **Transient transfection of with oligos**

Du145 cells were transfected with 30 nM of miR-141 microRNA mimics or non-targeting negative control miRNA (miR-NC) oligos (Ambion) using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. After culturing for overnight to 48 h, transfected cells were harvested for further studies.

### **Invasion assay**

Cell invasion assays were performed using Matrigel Invasion Chamber (8  $\mu$ m pore size, BD). Du145 cells were transfected with miR-NC and miR-141 mimic oligonucleotides.  $5 \times 10^4$  cells were seeded in the upper chamber of the insert. Medium with 20% FBS in the lower chamber served as chemoattractant. After 22hr, non-invading cells were removed by a cotton swab. Invading cells were stained with HEMA3 stain (Fisher) and counted under a microscope.

### **Clonal, and sphere-formation assays**

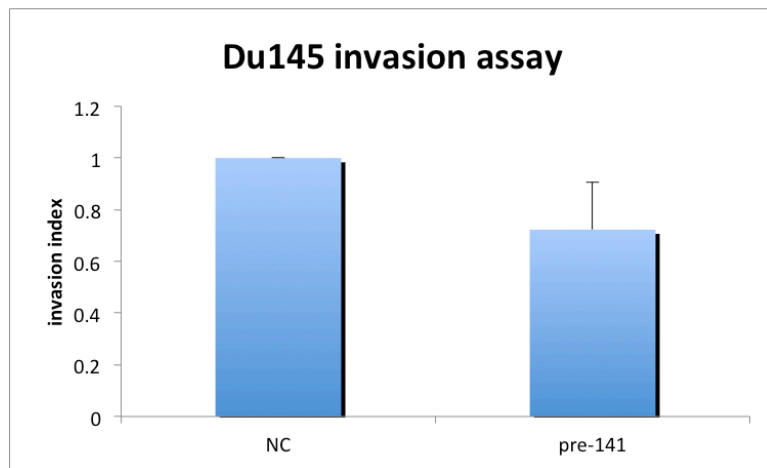
For holoclone assays, transfected Du145 cells were plated at a clonal density (i.e., 100 cells/well) in a 6-well dish. The number of holoclones was counted after 7-14 days. For sphere-formation assays, cells were suspended in serum free epithelial basal medium (PrEBM) supplemented with 4 $\mu$ g/ml Insulin, B27 (Invitrogen), 20ng/ml EGF and 20ng/ml bFGF.

Single-cell suspension was plated (1,000 cells/well) in Ultra-Low Attachment (ULA) plate and floating spheres that arose in 1 - 2 weeks were counted. For all these experiments, a minimum of triplicate wells was performed for each condition.

### **5.3 Preliminary results**

We observed that over-expressing miR-141 by oligo transfection in Du145 cells inhibited cell invasion in Boyden Chambers (Figure 5-1). We then tried to determine whether miR-141 would affect the stem cell related properties such as clonal expansion and sphere formation. We took miR-141 transfected Du145 cells and plated for in vitro clonal and sphere formation assays. Enforced expression of miR-141 indeed inhibited both clonal expansion (Figure 5-2) as well as sphere formation (Figure 5-3) in Du145 cells, suggesting that miR-141 may restrict the prostate CSC properties.

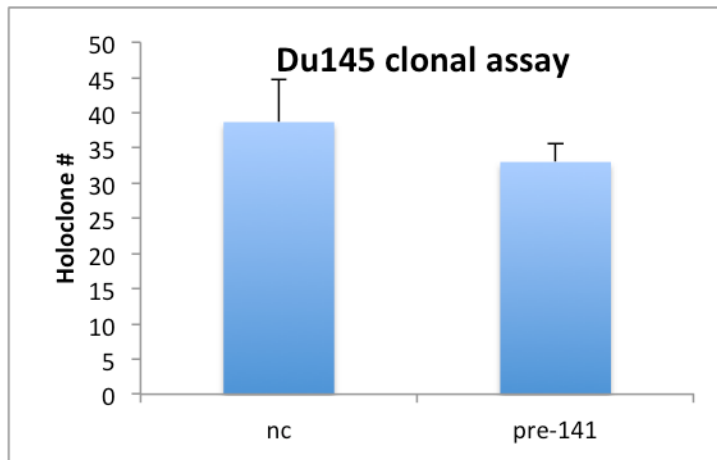
Figure 5-1



**Figure 5-1. Over-expression of miR-141 inhibited Du145 cell invasion in Boyden Chamber assays.** Shown is the relative invasion index of NC or miR-141 transfected Du145 cells.

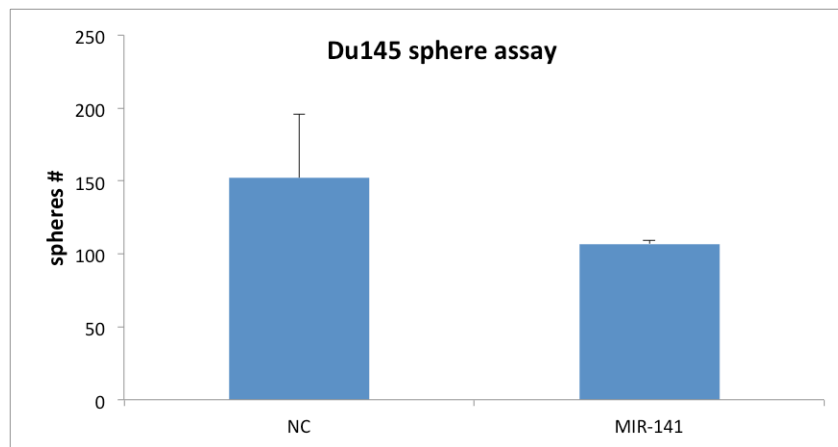


Figure 5-2



**Figure 5-2. Enforced expression of miR-141 inhibited clonal expansion of Du145 cells.** Shown are the average holoclone numbers counted in NC and miR-141 transfected Du145 cells.

Figure 5-3



**Figure 5-3. Enforced expression of miR-141 inhibited sphere formation of Du145 cells.** Shown is the average sphere number in NC and miR-141 transfected Du145 cells.

#### **5.4 Future research plan on miR-141**

To further elucidate the role of miR-141 in regulating prostate CSCs and PCa development/metastasis, I plan to pursue the following Specific Aims:

1. To further map miR-141 expression in PCa cells and prostate CSCs using the “miRNA sensor” technique and in situ hybridization;
2. To further explore the biological functions of miR-141 in PCa cells using miRNA sensor and inducible miR-141 expression systems;
3. To identify novel direct targets that mediate the biological functions of miR-141 in PCa cells by whole-genome RNA sequencing.

**Specific aim 1: To further map miR-141 expression in PCa cells and prostate CSCs using the “miRNA sensor” technique and in situ hybridization;**

Previous work from our lab has examined the miR-141 expression levels in purified CD44<sup>+</sup> and other marker-sorted CSC population in both xenograft models and primary patient samples. The results, although providing the foundation for our hypothesis, lack the resolution to show the precise localization of this miRNA within the heterogeneous cancer cell populations. In this specific aim, we plan to use in situ hybridization and miRNA sensor technique to pinpoint the miR-141 expression in cancer cells and, importantly, to visually connect miR-141 expression and prostate CSCs.

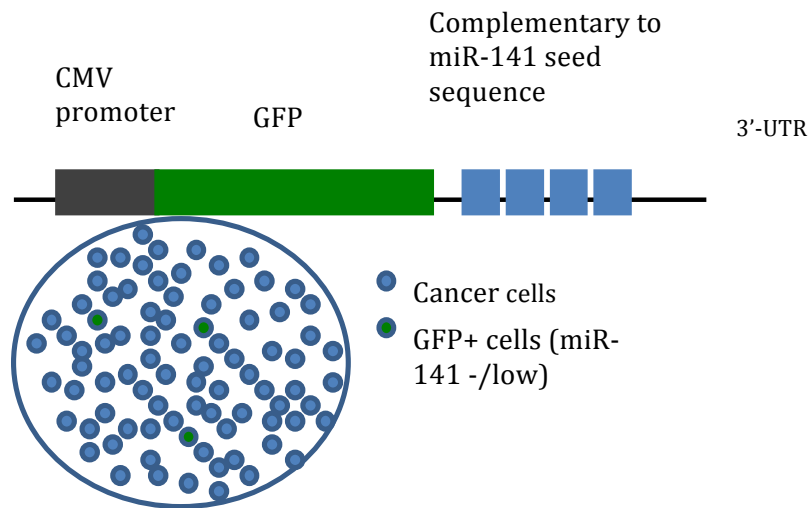
1). In situ hybridization: To further confirm that miR-141 is under-expressed in prostate CSC populations, we will utilize a combined fluorescence based in situ hybridization (FISH) and immunohistochemical (IHC) assay (175) to visualize miR-141 accumulation and the protein marker expression in individual cancer cells. Basically, we will use locked-nucleic acid modified oligonucleotide probes (Exiqon), which has been used

successfully in visualizing miRNAs in whole-mount zebrafish, mouse embryos, as well as formalin-fixed, paraffin-embedded (FFPE) brain, breast and colon tissue sections, to detect the miR-141 expression in readily available FFPE PCa sections. The advantage of the combined assay is that, on the same slide, it will allow us to do the routine IHC to detect the protein marker. For example, we can perform the IHC staining for the stem cell makers like CD44, CD133, MYC and BMI-1, prostate differentiation markers such as AR, and PSA as well as some known miR-141 targets like ZEB1, to reveal the specific subcellular localization of miR-141 in relation to these protein markers. We expect to see: 1) under-expression of miR-141 in PCa cells compared to normal/benign prostate tissue; 2) an inverse correlation between the staining of miR-141 and the stem cell markers such as CD44; 3) A good correlation between miR-141 and differentiation markers. Since miR-141 has been shown to regulate the EMT in many cancers, we expect to see weaker or lack of staining in the invasive front of cancer.

2). miR-141 sensor: To detect the miR-141 expression in situ, we will also generate the miR-141 sensor construct (176-178) (Figure 5-4). The miR-141 sensor contains a constitutively expressed reporter (GFP) under a ubiquitously expressed promoter, followed by 2 to 4 repeated sequences complementary to miR-141 (seed) sequence in the 3'-UTR region of the GFP. The construct will then be transfected into PCa cells Du145, LAPC9, and LAPC4. In cells that lack endogenous miR-141, the construct is stable and allows the GFP to express so we will see green cells. In contrast, in cells that express high level of miR-141, its perfect complementarity to the seed sequences in the 3'-UTR will result in an inhibition of GFP expression. A drug selection construct can also be cotransfected into the cells to allow us to eliminate the cells that did not harbor the introduced construct. With this powerful tool, we can sort out the GFP<sup>+</sup> cells and GFP<sup>-</sup> cells representing their endogenous miR-141 levels. Based on our earlier observations that miR-141 is lower in CSC populations, we shall

take the GFP<sup>+</sup> and <sup>-</sup> cells and perform staining of several CSC markers such as CD44, CD133, MYC and BMI-1 as well as some differentiation markers like AR and PSA. We expect to observe an inverse correlation between the miR-141 and CSC markers, and a positive correlation between miR-141 and differentiation markers. The advantage of using cells from the miRNA sensor experiment is that it represents the endogenous level of the miRNA, and circumvents the complexity of ectopically over-expressing of a miRNA mimic.

Figure 5-4



**Figure 5-4. Illustration of the miRNA sensor construct.**

**Specific aim 2: To further explore the biological functions of miR-141 in PCa cells using miRNA sensor and inducible miR-141 expression systems;**

1). Using miRNA sensor

Cells obtained from the miRNA sensor experiments will also allow us to perform further *in vitro* and *in vivo* assays to further characterize the biological functions of miR-141 high and low expressing cells. To determine whether cells with different miR-141 levels are intrinsically different in their stem cell properties, we shall sort out the GFP<sup>+</sup> (miR-141 low) and GFP<sup>-</sup> (miR-141 high) PCa cells and perform clonal and clonogenic assays. We will also inject miR-141 low and high cells into immuno-deficient mice to monitor their tumor regeneration ability. Since miR-141 is deficient in CSC populations, we expect to see that miR-141 low cells will give rise to more and bigger tumors. We will also transfect the miR-141 sensor construct into an RFP labeled PCa cells (such as LAPC9-RFP), and sort out the miR-141 low and high cells according to their GFP expression. These cells will then be injected into the orthotopic site (dorsal prostate) of immuno-deficient mice. This will allow us not only to compare the tumor regeneration but also metastasis. We expect to see more metastasis from GFP<sup>+</sup> cell-derived tumors.

2). pTRPIZ-miR-141 inducible vector

We will also use the Tet-on pTRIPZ-miR-141 over-expression vector (Figure 5-5), in which miR-141 expression together with a RFP reporter is induced by doxycycline. We will first apply it to xenograft cell lines such as Du145 and LAPC9. Cells bearing this vector will be injected into NOD/SCID mice subcutaneously or orthotopically. We will let the tumor to develop into certain size and then induce the miR-141 expression by adding doxycycline food/water. We expect to observe decreases in tumor size or lung metastasis, compared with tumors not induced with doxycycline.

Figure 5-5

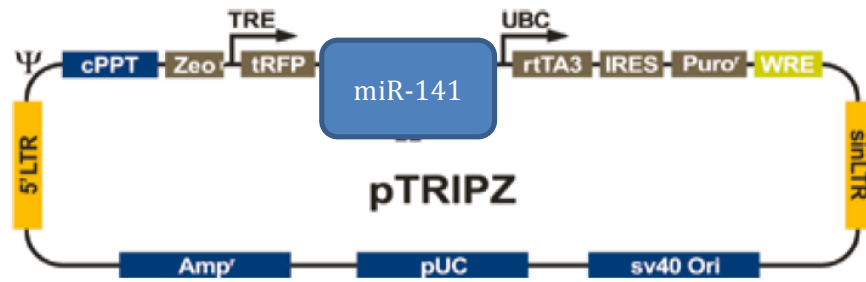


Figure 5-5. Illustration of pTRIPZ-miR-141 inducible vector



**Specific aim 3: To identify novel direct targets that mediate the biological functions of miR-141 in PCa cells by whole-genome RNA sequencing.**

From the available miRNA target prediction databases based on computational algorithms, there are thousands of putative targets of miR-141 involving various biological pathways. Although it's a powerful and useful way to identify potential targets, it's not clear whether the predicted targets are real targets and it cannot tell the most relevant targets apart from the rest. To identify the authentic direct targets of miR-141 that may regulate the stem cell properties of prostate CSCs, we will combine computational approaches with other experimental approaches. Firstly, we shall utilize GFP<sup>+</sup> and GFP<sup>-</sup> cells sorted from the sensor experiments and perform RNA sequencing on these cells. By comparing the mRNA levels between the GFP positive and negative cells, we will identify the transcripts whose expressions are differential; especially those miRNAs whose levels are lower in the GFP negative cells. Those candidate targets will be further processed using the Ingenuity Pathways software to examine the interactions. Those highly connected nodes from the interactome are of great biological importance and therefore will be our focus for the downstream targets (179). Then we will continue to validate those targets of interests by the reporter assays in which we will incorporate the 3'-UTR of the target mRNA into a luciferase expression plasmid. Reduced expression of the luciferase activity by over-expression of miR-141 will support the direct regulation of miR-141 on those targets.

## **Chapter 6 Summary and future directions**

### **6.1 Summary**

In my Ph.D. thesis research, I performed the miRNA expression profiling in several prostate CSC populations, and further unraveled the roles of three miRNAs that were on the top of the commonly and differentially expressed miRNA list, namely, miR-34a, let-7b, and miR-301 in regulating prostate CSCs and PCa development. This work presented the first study in the literature on the miRNA expression patterns in not the bulk but a small and important subpopulation within heterogeneous cancer cells. Our work also provided novel mechanisms of miRNAs that involve various aspects of PCa development such as tumor progression and cancer metastasis by directly regulating CSCs. Importantly, our work establishes strong rationales for developing novel miRNA-based therapeutic for prostate cancer patients.

I started out by presenting our initial miRNA expression profiling in six prostate CSC populations from three xenograft models. I characterized the distinct and common miRNA expression patterns in these CSC populations. I also validated several miRNAs that were commonly and differentially expressed in these CSC populations in human primary PCa samples. Four of the six commonly and differentially expressed miRNAs from the xenograft models showed similar expression pattern in the majority of HPCa sample-derived CD44<sup>+</sup> cells, including under-expression of miR-34a, let-7b and miR-141, as well as over-expression of miR-301. I then went on to show the roles of three of these miRNA, miR-34a, let-7b and miR-301 in regulating PCa development and prostate CSCs. For miR-34a, I found that over-expression of miR-34a significantly inhibited PCa growth and metastasis to the lung by regulating the stem cell properties and directly suppressing CD44. More importantly, systemically delivered miR-34a suppressed the tumor growth and extended the

survival of tumor-bearing mice due to the inhibition of metastasis. In the study of let-7, I showed that over-expression of let-7 also manifested tumor inhibitory effects by regulating the CSCs, although the tumor-inhibitory effect is not as potent as miR-34a. Surprisingly, for miR-301, we observed a cell type dependent response to manipulating miR-301 levels. These observations, taken together, suggest that different miRNAs may regulate different aspects of tumor development and distinct CSC properties, and together, they coordinately control the tumor progression.

## **6.2 Future directions**

Besides the miR-141 related studies that I proposed in the previous chapter, there are a few other unanswered questions that I would also like to pursue in the near future.

### **6.2.1 The mechanisms of miR-34a under-expression in the CSC populations.**

As mentioned in chapter 3, we attempted to explore the miR-34a upstream regulation and understand the mechanisms of miR-34a under-expression in prostate CSC populations. We first tried to correlate the miR-34a expression with p53 in PCa cells. In silico promoter analysis revealed two p53 binding sites in human miR-34a -2kb promoter region. In support, we have showed that the basal miR-34a levels correlate well with the p53 status in prostate and PCa cells. However, it would not explain differential miR-34a expression in isogenic CD44<sup>+</sup> and CD44<sup>-</sup> cells. qPCR analysis of p53 and miR-34a did not reveal any correlations in CD44<sup>+</sup> and CD44<sup>-</sup> HPCa cells purified from 22 patient tumors. Correlation of miR-34a and c-Myc, another transcription factor that has been reported to suppress miR-34a, in the purified CD44<sup>+</sup> HPCa cells did not reveal any obvious relationship, either.

Other mechanisms that could explain the under-expression of miR-34a in PCa stem/progenitor cell populations include genetic mutation or deletion, deregulation of miRNA

machinery and epigenetic regulations, all of which merit further exploration. To test the possibility of genetic mutation or deletion of miR-34a in PCa stem/progenitor populations, we will need to purify the CSC-enriched populations such as CD44<sup>+</sup> LAPC9, LAPC4 and Du145 cells and then analyze the genomic loci where miR-34a resides. Another possibility is the deregulation of miRNA maturation mechanism in PCa stem/progenitor cell populations. To test that possibility, we will check the status of Dicer, DGCR8 and other molecules that are involved in miRNA processing in the stem/progenitor populations of PCa cells to see if there is any differential expression between tumorigenic PCa cells and non-tumorigenic cells. Thirdly, DNA methylation as well as histone modification status will also be examined to see whether epigenetic regulation play a role in regulating the miR-34a expression in the different subset of PCa cells. Finally, in silico analysis of the transcription factor binding elements in the upstream of miR-34a will help to identify novel regulators of miR-34a expression. Understanding the upstream regulatory mechanisms of miR-34a under-expression in the CSC populations will advance our understanding of CSC regulation by specific miRNAs.

### **6.2.2 The mechanism of let-7 fast turnover in PCa cells.**

In my studies of let-7b in regulating PCa, I made an unexpected observation, that is, let-7b seems degraded faster than miR-34a in PCa cells and let-7b also seems turned over faster in PCa cells compared to other cancer cell lines such as A549. This could be a very interesting phenomenon as it might represent yet another mechanism by which cancer cells (in this case, PCa cells) lose the expression of tumor-suppressive MiRNA let-7. Firstly, we shall confirm the fast turnover of let-7 by examining the let-7 levels at different time points after transfection with oligos. Shorter time intervals within 72 hours will be included in order to observe the dynamic accumulation of let-7 and its level decrease in PCa cells and in lung

cancer cell line A549. Secondly, several post-transcriptional regulations of let-7 have been proposed in the literature including lin-28 that blocks the maturation of precursor-let-7 to the single stranded mature let-7 miRNA. To determine whether this post-transcriptional regulation might be involved in the fast turnover of let-7 in PCa cells, we could compare the lin-28 levels between PCa cells and lung cancer cells

Another possible explanation for the fast turnover of let-7 miRNA might be different target pool between miR-34a and let-7 in PCa cells, as well as between PCa versus lung cancer cells. The “competing endogenous RNA” (ceRNA) hypothesis suggests that mRNA transcripts might regulate miRNA levels. In other words, the abundance mRNA transcripts in the cells could affect the levels of miRNA that bind to these mRNAs. We suspect that in PCa cells, there might be more over-expressed targets that could take up let-7 miRNA than those for miR-34a, and there might be more over-expressed targets for let-7 in PCa cells than in lung cancer cell lines. To test these possibilities, we could compare the available mRNA targets for let-7 and miR-34a in PCa, and between PCa and lung cancer cell lines from public available microarray data to see whether the abundance of over-expressed target transcripts correlates with depletion of miRNA. If possible, we could also compare the whole transcriptome generated by deep sequencing in let-7 or miR-34a over-expressing PCa cells, as well as between let-7 over-expressing PCa cells and lung cancer cell lines. It will be of general interest to unveil the molecular mechanisms responsible for differential turnover between different miRNAs and between different cancer cell types.

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## **Vita**

Can Liu was born in Jingzhou, Hubei, China on September 26, 1981, the Daughter of Yicheng Liu and Jin Wang. After completing her work at Shashi No.3 High School, Jingzhou, Hubei, China in 2000, she entered Medical School of Wuhan University in Wuhan, Hubei, China. She received the degree of Bachelor of Medicine in July, 2005. During the following year, she worked as a research assistant in Department of Immunology in Wuhan University, China. In August of 2006, Can came to the United States and entered The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. Can pursued her PhD in the laboratory of Dr. Dean Tang at the University of Texas MD Anderson Cancer Center, Science Park Research Division, Department of Carcinogenesis in Smithville, Texas.

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