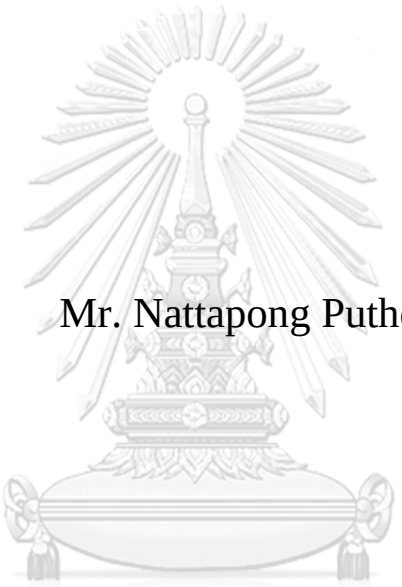
Roles of LIN28 Proteins in Cholangiocarcinoma

Mr. Nattapong Puthdee



A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Medical Biochemistry
Department of Biochemistry
FACULTY OF MEDICINE
Chulalongkorn University
Academic Year 2019

บทบาทของโปรตีนลิน 28 ในมะเร็งท่อน้ำดี

นายณัฐพงษ์ พุทธดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมีทางการแพทย์ ภาควิชาชีวเคมี
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

Thesis Title	Roles of LIN28 Proteins in Cholangiocarcinoma
By	Mr. Nattapong Puthdee
Field of Study	Medical Biochemistry
Thesis Advisor	Assistant Professor NIPAN ISRASENA, M.D. Ph.D.
Thesis Co Advisor	Professor PISIT TANGKIJVANICH, M.D.

Accepted by the FACULTY OF MEDICINE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the FACULTY OF MEDICINE
(Professor SUTTIPONG WACHARASINDHU, M.D.)

DISSERTATION COMMITTEE

..... Chairman
(Professor Sittisak Honsawek, M.D.Ph.D.)
..... Thesis Advisor
(Assistant Professor NIPAN ISRASENA, M.D. Ph.D.)
..... Thesis Co-Advisor
(Professor PISIT TANGKIJVANICH, M.D.)
..... Examiner
(SUTIMA LUANGDILOK, M.D.Ph.D.)
..... Examiner
(NAPHAT CHANTARAVISOOT, Ph.D.)
..... External Examiner
(Associate Professor WATCHARASAK CHOTIYAPUTTA, M.D.)

ณัฐพงษ์ พุทธดี : บทบาทของโปรตีนลิน 28 ในมะเร็งท่อน้ำดี. (Roles of LIN28 Proteins in Cholangiocarcinoma) อ.ที่ปรึกษาหลัก : ผศ. ดร. นพ.นิพัญจน์ อิศรเสนา ณ อยุธยา, อ.ที่ปรึกษาร่วม : ศ. นพ.พิสิฐ ตั้งกิจวานิชย์

RNA binding protein LIN28 มีบทบาทสำคัญในการควบคุมกระบวนการสร้าง microRNA และมีบทบาทสำคัญในกระบวนการต่างๆของเซลล์เช่น การคงสภาพความเป็นเซลล์ต้นกำเนิด กระบวนการเมตาบอลิซึมของเซลล์และเกี่ยวข้องในกระบวนการก่อมะเร็ง ในการศึกษามะเร็งพบว่าโปรตีน LIN28 มีการแสดงออกสูงและสัมพันธ์กับความรุนแรงของโรคซึ่งจากการศึกษาก่อนหน้านี้พบว่า LIN28 ชนิดบี (LIN28B) มีการแสดงออกในช่วงของการพัฒนาโรคมะเร็งท่อน้ำดี อย่างไรก็ตามบทบาทของโปรตีนนี้ในกระบวนการก่อโรคดังกล่าวยังไม่เป็นที่ทราบแน่ชัด โดยในการศึกษานี้พบว่าโปรตีน LIN28B มีการแสดงออกสูงในเนื้อเยื่อผู้ป่วยมะเร็งท่อน้ำดีและจากการเหนี่ยวนำการแสดงออกของยีน LIN28B ในเซลล์เพาะเลี้ยงท่อน้ำดีพบว่าโปรตีน LIN28B ส่งเสริมความสามารถการเพิ่มจำนวนของเซลล์ การสร้างโคโลนีและการเปลี่ยนแปลงรูปร่างของเซลล์จากลักษณะ epithelial ไปเป็นลักษณะคล้ายเซลล์ mesenchymal นอกจากนี้เซลล์เพาะเลี้ยงท่อน้ำดีที่มีการแสดงออกของโปรตีน LIN28B มีการแสดงออกของลักษณะบ่งชี้ความเป็นเซลล์ต้นกำเนิดมะเร็งเช่น ความสามารถในการสร้าง sphere และจากการศึกษากลไกการทำงานอื่น ๆ ที่เกี่ยวข้องพบว่าโปรตีน TGFβI ซึ่งเป็นโปรตีนที่ถูกควบคุมการแสดงออกภายใต้วิถีสัญญาณของ TGF-β มีการแสดงออกเพิ่มมากขึ้นและเมื่อยับยั้งสัญญาณดังกล่าวพบว่าความสามารถในการรุกรานของเซลล์ที่เหนี่ยวนำการแสดงออกของโปรตีน LIN28B ลดลง จากผลการศึกษานี้แสดงให้เห็นว่าการแสดงออกของโปรตีน LIN28B อาจมีบทบาทเกี่ยวข้องในการก่อมะเร็งท่อน้ำดี โดยสร้างเซลล์ที่มีคุณสมบัติคล้ายเซลล์ต้นกำเนิดมะเร็งในเซลล์ท่อน้ำดี ดังนั้นวิถีของโปรตีน LIN28B จึงเป็นที่น่าสนใจในการพัฒนาไปเป็นเป้าหมายใหม่ในการรักษามะเร็งท่อน้ำดีในอนาคต

สาขาวิชา	ชีวเคมีทางการแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5774755430 : MAJOR MEDICAL BIOCHEMISTRY
KEYWORD: LIN28B, Cholangiocarcinoma, Cancer stem cells

Nattapong Puthdee : Roles of LIN28 Proteins in Cholangiocarcinoma. Advisor: Asst. Prof. NIPAN ISRASENA, M.D. Ph.D. Co-advisor: Prof. PISIT TANGKIJVANICH, M.D.

LIN28 is an RNA-binding protein, a known as regulator of microRNAs biogenesis and play a role in multiple cellular functions such as stem cell maintenance, cell metabolism and implicated in oncogenesis. In cancer study, LIN28 protein is frequently expressed and associated with tumor aggressiveness and poor disease outcome. Previous studies have shown that paralog-LIN28B was upregulated in during CCA development. However, the role of LIN28B in cholangiocarcinogenesis remains largely unknown. The present study, we found LIN28B is overexpressed in CCA patients' tissues. Overexpression of LIN28B in cholangiocytes-MMNK-1 cells showed that LIN28B enhanced cell proliferation, clonogenic potential, and promoted epithelial-to mesenchymal transition (EMT). Moreover, we found LIN28B enhanced cancer stem cells marks, sphere forming ability in MMNK-1 cells. Mechanistically, we found that TGFβI protein which is downstream target of TGF-β signaling was upregulated and migration activity of LIN28B-overexpressing MMNK-1 cells was decreased when inhibit the TGF-β/TGFβI cascade. Collectively, our findings suggest that LIN28B may play a role in generating subpopulation of CCA by exhibiting cancer stem-like properties in cholangiocytes via partly activated of TGF-β signaling. Therefore, LIN28B pathway could become an attractive target for new therapeutic intervention of CCA.

Field of Study:	Medical Biochemistry	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest and sincere gratitude to my advisors, Asst. Prof. Nipan Israsena and co-advisor, Prof. Pisit Tangkijvanich for their kindnesses, valuable suggestions, encouragements, and supportive guidance throughout the course of my study. I would like to express my greatest appreciation and sincere gratitude to Dr. Sutheera Ratanasirintrawoot for her invaluable advice, kind comments and suggestions and special thanks to all pathologists, Dr. Anapat Sanpavat, Dr. Krit Suwannaphom, Dr. Fern Chanduayvit, Dr. Shanop Shuangshoti from the Department of Pathology to provide the specimens and analyze the tissue staining. My special thanks are also widening to all staff in Stem Cells Excellent Center and Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, for their friendship and helpfulness throughout my study.

This study was supported by Chulalongkorn University graduate scholarship to commemorate the 72nd anniversary of his majesty King Bhumibol Adulyadej.

Finally, I would like to express my deepest gratitude and greatest appreciation to my dear parents and other members of my family, who gave me a chance to study, being very supportive and encouraging me throughout the study.

Nattapong Puthdee

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I
INTRODUCTION

1. Background and rationale of the study

Cholangiocarcinoma or CCA is a rare malignant tumor that has arisen from bile duct epithelium. It can be classified as a subset of primary liver cancer. CCA is categorized into 2 main types; intrahepatic (ICC) and extrahepatic [1] based on the tumor's anatomical positions. ICC is originated from intrahepatic portion of biliary tree while ECC arises from extrahepatic part of bile ducts [2, 3]. CCA is known as a cancer with very poor prognosis and known to be virtually resistant to chemotherapy. Radiation and surgery are the only effective treatment but it is not frequently appropriate due to late presentation of symptoms and delayed diagnosis. Even when adjunct chemotherapy was added, the outcome of current chemotherapeutic regimen is not appreciable. Therefore, this disease is a real need to find novel treatments or adjuvant therapeutic strategies for CCA that would impact clinical outcome in a significant way. For the development of cancer therapy, our understanding of carcinogenesis and molecular pathway is crucial. Several studies have reported possible molecular mechanisms of CCA. Abnormalities of various signaling cascades and genes mutation involved in cholangiocarcinogenesis include TP53, KRAS, BRAF, Smad and $p16^{INK4a}$ genes, cytokines affected such as IL-6, TGF-β, TNF-α and PDGF and dominant signaling pathways, IL-6/STAT-3, Wnt, TGF-β/Smad, PI3K/AKT, RAF/MEK/MAPK, and Notch [4]. However, upstream signaling or other underlying mechanisms of CCA are still unclear and need to be further explored.

Recently, roles of RNA-binding protein in cancer have become topics of interest. One of them, LIN28 protein, which comprise of two paralogs, LIN28A and LIN28B, has a vigorous role in the cell development, differentiation and pluripotency, glucose metabolism, and cancer. Normally, LIN28 protein is overexpressed in embryonic stage and help regulate the cell development into specific cell types. It is subsequently downregulated in adult tissue. Regarding the mechanism, LIN28 regulates the biogenesis of let-7 miRNAs through inhibiting steps of let-7 maturation. Consequently, the gene targets of let-7 miRNAs which play significant roles in multiple cellular functions such as cell cycle, metabolism, and inflammation will be upregulated

[5]. Additionally, it can also bind target mRNAs to modulate their translation. LIN28 is aberrantly overexpressed in variety of cancers and potentially induces cancer promotion/progression. LIN28 also has an emerging role in the maintenance of cancer stem cells property and it has been advised as a prognostic marker to predict the disease outcome [6]. LIN28A is more relevant in embryonic carcinoma cells and germ cell tumor, whereas LIN28B is extensively study in epithelial cancer cells including liver cancer. LIN28B was reported to promote the liver cancer. An overexpression of LIN28B is sufficient to promote cancer formation and IMP3 was required for maintain disease progression in mouse model [7]. This study provides a strong evidence that LIN28B serves as an oncoprotein that play a role in tumor promotion. From this regulation of LIN28B and IMP3, IMP3 is found to be overexpressed and correlated with poor prognosis of CCA patients [8]. Furthermore, the significant role of LIN28B was reported in CCA where LIN28B was upregulated during CCA development in mouse-induced cholestasis model [9]. These evidences may support the idea that LIN28B has a significant role in the promotion of CCA and it might be a therapeutic target for CCA treatment.

According to such knowledge, it seems that LIN28 is a master protein which regulates diverse cellular functions. In particular, LIN28B is highly expressed in cancer and can promote tumor initiation and tumor progression. Hence, LIN28B is an important factor in tumorigenesis and it can be a candidate targeted for cancer therapy. Yet, the evidence of the functional role of LIN28B in cholangiocarcinoma is still unclear. Therefore, we hypothesize that the association of LIN28B proteins may have a significant role in cholangiocarcinogenesis and aggressive tumor promotion for CCA.

CHAPTER II
LITERATURE REVIEWS

1. Cholangiocarcinoma

CCA is a kind of liver cancer that is arising from bile duct epithelial cells, and has become grave public health issues worldwide. Patients with CCA have a low survival rate, usually presenting with an advanced stage of the disease due to the lack of effective early diagnostic tools and limited cancer treatments [10]. The therapeutic options depend on the severity of the disease. Radical surgery is a gold standard method for CCA treatment. However, due to several limitations including patient fitness, absence of metastatic lesions and the possibility of surgical margin free of cancers, current surgical outcomes were still unsatisfying [11]. Other options including postoperative-chemotherapy, radiotherapy or chemoradiation have been applied to improve the disease-free survival in CCA patients. Several drugs have been tested alone or in combination. The chemotherapeutic drugs that have been extensively studied in CCA treatment are 5- fluorouracil (5-FU) either alone or in combination with other agents such as methotrexate, cisplatin, leucovorin, mitomycin C, etoposide and interferon α and gemcitabine-based combined with platinum-based drugs for example, cisplatin and oxaliplatin. However, the results of these regimens remain ineffective and elicit considerable side effects [12, 13]. CCA incidence rates vary markedly worldwide. The highest incidence rates have been reported in Northeast Thailand, followed by China and some parts of Southeast Asia [14]**.** For the risk factors and etiology of CCA, different incidences in differing geographical areas suggested the distinctive dissemination of local geographic risk factors and genetic variations among various ethnics [12]. However, they are common in inflammatory related nature [15, 16]. Several risk factors related to CCA have been suggested including primary sclerosing cholangitis (PSC), ulcerative colitis, parasitic infection, hepatolithiasis, congenital disorder (such as Caroli' s disease, choledocal cyst and abnormal pancreatobiliary junction), vital infection (such as HCV, HBV), and hepatotoxic compounds. In Asian countries, CCA is closely related to liver flukes infection especially infections with *Clonorchis sinensis*; *C. sinensis,* and *Opisthorchis viverrini; O. viverrini* [17, 18]. *C. sinensis* infection was reported mainly in Hong Kong, South of China, and Korea [19,

20] while endemic area of *O. viverrini* was reported in Southeast Asian Countries including Thailand [17]. Among them, the prevalence of *O. viverrini* infection in North and Northeastern parts of Thailand were the highest, numbering approximately 20% with male around 2.5 fold to female [21, 22].The pathogenesis of CCA is related to the chronic inflammation by which bile duct was exposed to the risk factors, leading to activation of the immune system and recruitment of inflammatory cells to inflamed site, producing inflammatory cytokines as well as oxidative/nitrative products. Generations of excessive oxidative/nitrosative agents from inflammatory cells in response to chronic inflammation might exert direct mutagenic effects and promote cell proliferation and potentiate tumorigenesis of CCA [23].

The typical pathogenesis of CCA is a multiple-step process that originates from induction of chronic inflammation, conducting to biliary epithelial cell damage, impaired DNA and activation of several proto-oncogenic genes resulting in malignant transformation [24]. A number of genetic alteration and aberrant signaling pathways have been reported to explain the molecular mechanisms of CCA. The genetic mutation study in *O. viverrini*-related tumors and matched normal tissue were validated using whole-exome sequencing analysis. The result showed that 206 somatic mutations can be identified in 187 genes. Then they selected 15 genes with top mutations to be further validated in another 46 CCA cases. Known cancer associated genes found are TP53 (mutated in 44.4% of cases), KRAS (16.7%) and Smad4 (16.7%) [25]. Another evidence showed that KRAS and BRAF mutations were associated with high tumor grade and short survival rate. The result suggested that these mutations could be valuable for prognosis and outcome stratification among patients with intrahepatic CCA [26]. Several studies have evaluated the possible roles of genetic/epigenetic alterations in CCA and many potent genes were discovered to exert an influence onto CCA. However, such data are frequently inconclusive due to limited cases and variability of genetic background among CCA patients.

Additionally, multiple molecular pathways were investigated in *in vitro* and *in vivo* experiments to clarify the molecular signaling which have pivotal roles in tumor initiation and promotion of aggressive phenotypes of CCA. During inflammatory processes, cytokines are produced and these secreted cytokines, in effect, were implicated in carcinogenesis. An inflammatory cytokine in the cancer biology of CCA

is interleukin-6 (IL-6) which has a significant role in tumorigenesis and may serve as potential therapeutic targets for CCA [27]. Recently, several studies have linked the signaling of IL-6 to the development of CCA. For example, STAT3/IL-6 axis is crucial in the pathogenesis of CCA [28-30] and the activation of IL-6 was regulated by the p38 MAPK, ERK and PI3- kinase pathway [31, 32]. Another important pathway, Wnt/ β-catenin, is involved in the promotion of CCA through contributing to CCA cell proliferation and high Wnt expression was correlated with poor survival of CCA patients [33]. Moreover, the aberrant expression of Wnt/β-catenin promoted multi-drug resistance in CCA treatment [34]. These molecular aberrations and inhibitors targeting these pathways have been suggested with some currently entering clinical trial. However, the prevalence of these genetic alterations is low in frequency and leads to significant drug toxicity as well as interfere with normal cells [35].

Numerous studies have demonstrated the biological significance of microRNAs in CCA. The differential miRNA expression profile in tumor and normal cholangiocytes tissue was determined. A group of 38 miRNAs could discriminate between cancer and normal tissue. Within this cluster, 18 miRNAs were upregulated including miR-21, miR-106a, miR-25, whereas 20 miRNAs were downregulated such as let-7a, let-7b and miR-320 in cancer [36]. In the recent study, miRNA expression profiles in human CCA were determined using small RNA sequencing. The result showed decreasing of let-7c, miR-99a and miR-125b in CCA tissue. Moreover, these downregulated miRNAs are in the same clusters that were associated with IL-6/STAT3 pathway. Also, the functional study showed that overexpression of let-7c, miR-99a or miR-125b in CCA cell lines could reduce STAT3 activity, suppress tumor growth in *in vivo*, inhibit the cell motility, decrease cancer stem cell-like phenotype of CCA by down-regulating CD133 and CD44 and inhibiting mammosphere formation. The result indicates that let-7c, miR-99a and miR-125b regulate the inflammation and stem-like properties in CCA and provide a new strategy for CCA treatment by developing an miRNA-based targeting the IL-6/STAT3 pathway [37]. Another study reveals that contrasting of miR-124 and miR-200 expression was collaboratively promoted bile duct proliferation via the IL-6/STAT3 signaling pathway. According to miRNA reports in CCA indicate that role of dependent-miRNAs in CCA are interesting mechanism and miRNAs was suggested to be a tool for diagnosis and therapeutic targets for CCA [38].

2. LIN28

2.1 General information of LIN28 and its mechanisms

Abnormal cell lineage protein 28 (LIN28) is an RNA-binding protein with two paralogs, LIN28A and LIN28B. The heterochronic gene lin-28 was initially identified in a mutant of the nematode *Caenorhabditis elegan* which displayed a defect on developmental timing. Mutant lin28 caused a skip in the larval stage resulting in precocious development of *C. elegan* [39].The genomic loci of LIN28A and LIN28B are positioned at chromosome 1p36.11 and 6q21, encoding proteins with 209 and 250 amino acid in length, respectively. In addition, the two paralogs of LIN28 share a structural domain, which contains two nucleic acid interaction domains, a cold shock domain (CSD) and two zinc finger motifs (Cys-Cys-His-Cys; CCHC) [39, 40]. Among them, nucleolar localization signal is present between CSD and first CCHC domain from N-terminal and a nuclear localization signal at the C-terminal of LIN28B [41]. However, other reports demonstrated that subcellular localization of LIN28B can be in the cytoplasm depending on the cell types [40, 42]. Of note, the issue of LIN28B localization in the cells remains to be further elucidated. In 2011, the specific-interaction between LIN28 and let-7 was defined. The LIN28 CSD and two CCHC domains bind to distinct parts of terminal loop of let-7, where CSD binds near the central stem-loop. The two CCHC domains recognize a GGAG motif at the other end of terminal loop. Therefore, this evidence provides a molecular description for LIN28 specificity and how it regulates let-7. The cellular mechanism of LIN28 regulates let-7 processing is demonstrated in Figure 1. Recently studies showed not only LIN28 can bind to let-7 miRNA but others miRNA as well. The regulation of miRNA-125a/b and LIN28 was also identified in hepatoblasts cells. The result indicates that miRNA-125a/b and LIN28B create reciprocal inhibitory loop between them by which LIN28B inhibits maturation of miRNA-125a/b resulting in enhance cell proliferation and maintenance stem cell properties of hepatoblasts. Conversely, miRNA-125a/b can bind to 3'-UTR region of LIN28B mRNA and directly block the translation process of LIN28B mRNA, thereby leading to an inhibition of hepatoblasts proliferation. However, note that hepatoblasts is able to maintain the stem cell properties by positively regulates the expression of LIN28B itself [43].

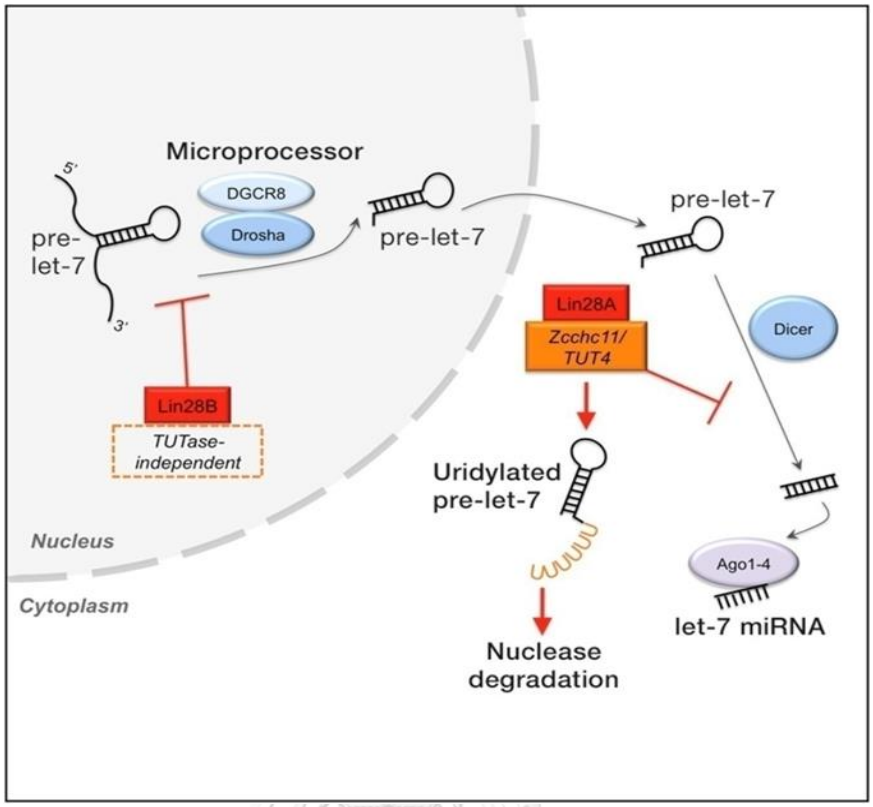


Figure 1. LIN28 inhibits let-7 miRNAs biogenesis.

On the cellular mechanism, LIN28 can block the biogenesis of let-7 miRNA by binding to primary let-7, consequently inhibiting Drosha processing in the nucleus. It can also inhibit the Dicer processing in the cytoplasm leading to suppression of the maturation of let-7 by binding to the terminal loop of precursor let-7 and recruiting the terminal uridyl transferase 4 (TUT4; also known as ZCCHC11) to catalyze the poly-uridylation at the 3' of precursor let-7, leading to precursor let-7 for degradation by 3'–5' exonucleases activity [41].

Moreover, LIN28A can directly suppress miRNA-125b leading to increased cell proliferation of glioblastoma stem cells [44]. The promising role of LIN28 and miRNA-125b in cell differentiation revealed that miRNA-125b was dramatically downregulated and contrasting with highly express LIN28A during differentiation timing of mouse embryonic stem cell, result in impaired endoderm and mesoderm development and defective cardiomyocytes formation. Hence, together LIN28 and miRNA-125b is suggested as important regulators in cell fate decision [45]. Other miRNAs are also

proposed in the interaction with LIN28, miRNA-212 was downregulated in prostate cancer with LIN28B overexpression, whereas miRNA-212 was upregulated in knockdown LIN28B. Moreover, LIN28B mRNA is predicted as a potential target of miRNA-212. Nevertheless, the functional study must be further validated to prove the hypothesis of the regulatory loop between LIN28/miRNA-212 [46]. The leukemogenesis study demonstrated that miRNA-150 maturation is inhibited by MYC/LIN28 axis and subsequently activates the oncogenic signaling pathways. Therefore, induction of leukemic carcinogenesis through inhibition of miRNA-150 by MYC/ LIN28 signaling cascade may provide the optional strategies for anti-leukemia therapy [47].

Moreover, it has been reported that LIN28 not only binds to miRNA but it also directly interacts with multiple mRNAs and influences their translation. LIN28 can binds insulin-like growth factor 2 (IGF-2) and conducts IGF-2 mRNA to polysomes and enhances its translation through the translation initiation machinery [48]. Consequently, a number of studies revealed additional of mRNA targets of LIN28. For example, mRNA targets of LIN28A, H2a (histone 2a), Hmga1, Cyclin A, Cyclin B, Cdk4 and Oct4 were identified in mouse embryonic stem cells. This implicates that LIN28A is associated with enhanced translation process of its mRNA targets, indicating that the maintenance of pluripotency is associated with LIN28A stimulation of the translation of cell cycle genes. [49-51]. Furthermore, a number of genome-wide studies are performed and thousands more mRNAs and ribosomal RNAs were identified. Some coding mRNAs are relevant in the cell cycle regulators, splicing factors and as well as regulators of LIN28 itself. LIN28 binding site within the mRNA was defined where LIN28 binds to GGAGA sequences enriched within the loop of them RNAs [52-54]. These investigations suggest that LIN28 proteins are constitutively expressed in the proliferative cell types by maintaining an auto-feedback loop.

2.2 Cellular functions of LIN28

The expression of LIN28 was extensively studied in the *in vitro* and *in vivo* settings. The temporal and spatial expression patterns of LIN28 varies according to cellular context including cell development, differentiation and pluripotency, glucose metabolism, and cancer.

2.2.1 LIN28 regulates development and differentiation. LIN28 is highly expressed in an embryonic stage, emerging as a powerful regulator of organism development. Several studies have elucidated the developmental time course of LIN28 and let-7 expression. LIN28/ let-7 pathway regulates the development of *C. elegan* during larval stages. First, LIN28 acts during the L2 stage and promotes L2 cell fates. Second, a let-7-dependent step controls L3 cell fates and subsequent differentiation [55]. Upregulation of LIN28 is also pivotal in the maintenance of the properties of embryonic stem cells (ESCs). LIN28 is found to be downregulated in response to differentiation [56]. The experimental study of transgenic mice overexpressing LIN28A showed a delay in the onset of puberty [57]. LIN28 protein is also upregulated in mouse and human primordial germ cells (PDG), where LIN28 was an essential regulator for PDG formation by inducing of let-7 target, Blimp1expression [58]. In addition, LIN28 was defined as one of several factors (others as SOX2, OCT4, and NANOG) which can reprogram somatic cells into induced pluripotent stem cells [59]. LIN28 is shown to be required for cell differentiation to a committed cell lineage. For example, LIN28 is required for efficient differentiation of *Xenopus tropicalis* by which knocking down of LIN28 protein causes abnormalities in the differentiation and the modeling of mesodermal structures at early larval stages, inhibiting of pluripotency cells ability to promotes cell differentiation into mesoderm under growth factor condition [59, 60]. Therefore, LIN28 has an essential role in dedifferentiation and maintenance of pluripotency features [61].

2.2.2 LIN28 regulates glucose metabolism. Another function of LIN28 was described in the regulation of glucose metabolism. In 2011, LIN28/let-7 axis was implicated in glucose regulation and diabetic mice. Mice engineered to overexpress both LIN28A and LIN28B in separate trials showed an impairment of glucose level regulation, resulting in mice resisted to high fat- diet induced obesity and an enhanced glucose tolerance. The experiments revealed that LIN28 induced the insulin-PI3K-mTOR signaling, leading to de-repression of the multiple let-7 targets, including IGF1R, INSR, IRS2, PIK3IP1, AKT2, TSC1 and RICTOR. This evidence suggested that LIN28/let-7 pathway is relevant on glucose metabolism and the fact that those genes under the control of let-7 are associated with type 2 diabetes risk inhuman [62]. Another study demonstrated the functional role of LIN28 in cellular metabolism of

embryonic stem cells. LIN28A can bind to numerous mRNAs that encoded for mitochondrial enzymes in human embryonic stem cells [63]. Overexpression of LIN28A in mouse embryonic stem cells showed high level of mitochondrial metabolites of threonine (Thr) into glycine (Gly) which are generated the intermediates to nucleotide synthesis [64]. As well as cancer stem cells, high expression levels of both LIN28B and glycine decarboxylase (Gldc) in the Thr-Gly pathway are potentially initiated tumor development in lung cancer stem cells [65]. Moreover, LIN28/let-7 can significantly facilitate aerobic glycolysis or the Warburg effect and promote cell proliferation in cancer cells with upregulation of LIN28A and LIN28B. In contrast, let-7 suppression could enhance aerobic glycolysis via targeting pyruvate dehydrogenase kinase 1 in the hypoxic condition [66]. Therefore, LIN28 could potentially regulate growth and metabolism of stem cells, and be involved in normal and cancer cells [67].

2.2.3 Role of LIN28 in cancer. Downregulation of let-7 miRNA family is associated with tumor progression in numerous cancers. The master regulator of let-7 miRNAs, LIN28, is widely expressed in multiple cancers and its role in tumor initiation and malignant progression has been implicated. Several experiments demonstrated the aberrant expression of LIN28 and its significance roles in many types of cancer as shown in Table 1.

Table 1. Oncogenic roles of LIN28B

Cancer	LIN28	Significance of a study	Reference
Malignant germ cell tumors	LIN28A	• LIN28A was highly expressed in tissue of yolk sac tumor, germinoma, and embryonal cancer. • Restoration of let-7 levels was sufficient to suppress cell proliferation and reduce LIN28A expression.	[68]
Testicular germ cell tumors	LIN28A	LIN28A is strongly expressed with highly specificity in primary and metastatic testicular GCTs tissues, and weakly expressed in non-GCTs tissues.	[69]
B cell lymphoma	LIN28B	Myc induced LIN28B expression; knockdown LIN28B cells elevated let-7 miRNA, leading to an inhibition of cell proliferation.	[70]
Prostate cancer	LIN28A	Overexpression of LIN28A enhanced cell proliferation and shown adverse effect in LIN28A knockdown cells.	[71]
Breast cancer	LIN28B	The positive feedback loop of NF-kB/ LIN28B/let-7 pathway driven normal breast cells to transform cells by inducing inflammatory IL-6/STAT3 signaling.	[72]
Colon cancer	LIN28B	• High LIN28B expression was correlated with poor survival rate and increased the tumor recurrence. • An overexpression of LIN28B repressed let-7 expression and promoted colon cancer cells metastasis.	[73]

Cancer	LIN28	Significance of a study	Reference
Ovarian cancer	LIN28A	LIN28A is highly expressed, and suggested as a sensitive marker for germ cell tumor.	[74]
	LIN28B	• High expression of LIN28B and IMP3 were associated with poor survival time. • Knockdown of IMP3 or LIN28B cells decreased cell proliferation, migration/ invasion and increased the sensitivity of platinum-induced cell death.	[75]
Colorectal cancer (CRC)	LIN28A or LIN28B	• An aberrant expression of LIN28A and/or LIN28B was found in 38% of human CRC samples (n = 595). • LIN28 cooperates with Wnt signaling to promote tumor formation and enhance tumor aggressiveness in mice model.	[76]
Esophagus cancer	LIN28A or LIN28B	• High expression of LIN28A and LIN28B was correlated with lymph node metastasis and poor prognosis. • Knocking down LIN28B could inhibit cell proliferation and invasion.	[77]
Hepato cellular carcinoma (HCC)	LIN28B	• The expression of LIN28B but not LIN28A was detected in tumor and correlated with high-grade tumors. • Knockdown of LIN28B inhibited epithelial-mesenchymal transition (EMT).	[78]
	LIN28B	Knockdown of LIN28B decreases the colonies formed ability of HepG2cells	[79]
	LIN28B	High LIN28B expression was found in aggressive subtypes of hepatoblastoma.	[80]

Cancer	LIN28	Significance of a study	Reference
Lung cancer	LIN28A	Restoration of let-7 was found in LIN28A knockdown cells, resulting in inhibited cell cycle.	[81]
	LIN28B	LIN28B induced lung tumorigenesis by promoting EMT, angiogenesis and enriching the number of cancer stem cells via AKT and c-MYC activity in KRAS mutation-driven lung cancer mouse model.	[82]
Neuroblastoma (NB)	LIN28B	• Overexpression of LIN28B was correlated with poor outcome of patient. • DFMO treatment decreased neurosphere formation by inhibiting the expression of LIN28B and MYCN proteins.	[83]
Glioblastoma (GMB)	LIN28A	• The expression of LIN28A was significantly higher in the group of patients with a poor prognosis. • Downregulation of LIN28A caused cell cycle arrest in the G1 phase, increased cell apoptosis, and reduced colonies number of GMB cells.	[84]

The roles of LIN28 in promoting cancer progression involve with its regulation of let-7 targets, MYC, HMGA2, RAS, which are oncogenes playing important roles in aggressive malignance phenotypes [85, 86]. Overexpression of Myc onco-protein promotes the development of cancer by promoting cell growth and angiogenesis, DNA replication, development, and metastasis of tumors [87]. Expression of LIN28 can be increased by MYC at transcriptional level through a positive feedback loop of LIN28 and MYC [70]. A study of non-muscle invasive bladder cancer shows that LIN28A/ let-7a and MYC are aberrantly expressed with significantly higher level in high grade

tumors [88]. Another study showed that highly expressed LIN28B was correlated with short survival time of patient with neuroblastoma, and that their disease progression was induced by LIN28B induction of MYC expression via suppression of let-7, resulting in development of neuroblastoma in murine model [42]. Ras is associated with another signaling pathways including MAPK, PI3K/Akt and Ras/Rho/Rac. Hence, Ras is an effectors which triggers further signaling cascades, and consequently, modulates cellular process including growth, migration, differentiation and survival [89]. The evidence of LIN28 and Ras signaling crosstalk was described in the instance where inhibition of LIN28A, resulting in de-repressedlet-7, attenuated K-Ras expression, and thus enhanced radio-sensitivity of lung carcinoma A549 cells and pancreatic ASPC1cancer cells [90]. HMGA2 oncogene was observed in various cancers and it is associated with cancer metastasis, chemoresistance, and poor survival [91]. It has been suggested that HMGA2 could serve as tumor marker in oral squamous cell carcinoma progression and promote cell motility via modulation of LIN28/let-7 [92, 93]. Strong evidence of LIN28 on driving cancer was reported in liver cancer, HCC. In 2014, Nguyen and colleagues demonstrated that overexpressing LIN28B in transgenic mice with hepatocyte-specific regulatory element led to abnormality of hepatic features. LIN28B overexpression can be observed in MYC-driven hepatoblastoma and deletion of LIN28B gene showed a clearly prolonged survival and a reduced tumor burden. Moreover, they found let-7 miRNA targets, Igf2bp1 (IMP1) and Igf2bp3 (IMP3) proteins to be up-regulated in LIN28B overexpressed mice. A decrease in cell proliferation can be observed when knocking down IMP3 gene in LIN28B overexpressing cell line. Therefore, these results indicate that LIN28B is adequate to drive liver cancer, and that IMP3 is required in the context of LIN28B expression to promote cell growth [7]. Interestingly, elevated IMP3 was also reported in CCA. Gao and colleagues determined IMP3 expression in intrahepatic CCA (ICC) by immunohistochemistry staining, and they found that IMP3 was particularly expressed in bile duct tumor cells (59 cases of 72 ICC patient tissues) but not expressed in the surrounding normal tissue. IMP3 was also positively expressed in lymph node vascular invasion (26/35 cases of lymph node metastasis tissues). Moreover, they indicated that IMP3 is a significantly associated with severity of disease such as tumor size, pathological grade, metastasis, tumor stage and poor survival rate [8]. Another evidence

has also confirmed that IMP3 was frequently expressed in CCA by staining positively in 37/41 (90%) of ICC patient tissues [94]. It seems that IMP3 is upregulated in CCA and may have a function in tumor development and disease progression. However, the functional role of IMP3 in tumorigenesis and tumor aggressiveness of CCA has not yet been explored. As LIN28B regulates IMP3 expression, these knowledges may suggest the possibility of LIN28B playing pivotal role in CCA. Remarkably, LIN28B was suggested to be one of factor that mediate CCA tumorigenesis. In 2011, Yang and colleagues aimed to study the effects of cholestasis-induced chronic inflammation of the liver injury and promoting liver cancer in bile duct ligation mouse model and its possible oncogenic pathways. They see abnormality of hepatic feature and pathological lesions of bile duct at weeks 8, 12, 16, and 28 of experiment. Moreover, elevated LIN28B expression with downregulation of let-7a were observed in the early stage of CCA. Collectively, they mention that regulation LIN28B/ let7 axis may contribute to liver injury response and implicate in the initiation and progression of CCA [9].

Another supporting evidence for LIN28 in tumorigenesis is its emerging role in the maintenance of cancer stem cells (CSCs). CSCs are cancer cells which can be found inside a tumor bulk and defined by their capability such as self-renewal, proliferation, multi-lineage differentiation into tumor diversity. These properties are an essential for tumor initiation, progression and exhibit drug resistance and metastasis [95]. Therefore, cancer researchers aim to explore the CSCs biology to find CSCs markers and develop tools/or drugs for cancer treatment by targeting CSCs. According to the essential role of LIN28 in stem cells, it is a pluripotency factor that can reprogram human somatic cells into induced pluripotent stem cells (iPSCs) and maintain of pluripotency property, and drive cell differentiation/ development in embryonic stage [96-98]. Therefore, it is not surprising that LIN28 would be found in CSCs and function in CSCs property. LIN28 is overexpressed in multiple cancers and associated with poor patient's outcome. More importantly, LIN28 mediates in tumor growth, invasion, and metastasis, which are known to be CSCs characteristics. Hence, currently, a growing body of evidences have directing on the association between LIN28 protein and CSCs biology. An emerging role of LIN28 in CSCs formation, its contribution to cancer initiation capacity, cancer stem cell-like properties, and tumor progression is summarized in Table 2.

Table 2. The aberrantly expressed LIN28 promotes cancer stem cells properties

Cancer	LIN28	Significance of a study	Reference
Prostate cancer	LIN28B	Elevated LIN28B promoted cancer stem cell-like features, EMT, mammosphere-formation, tumor growth *in vivo*, and self-renewal ability.	[99].
	LIN28A and LIN28B	LIN28 was upregulated in CSC subpopulation and require for cellular transformation and self-renewal capacity of prostate CSCs.	[100]
Acute myeloid leukemia (AML)	LIN28B	PRL-3 (PTP4A3), a metastasis-associated phosphatase promoted transformation of AML by promoting stem cell–like properties via activation of LIN28B/let-7.	[101].
Ovarian cancer	LIN28A	The LIN28A expression positively correlated with high ALDH1 positive tumor cells and regulated and ALDH1 expression in ovarian CSC population.	[102]
	LIN28A	• LIN28A is selectively expressed in CSC population, as indicated by positive CD44, CD24, EpCam and negative E-cadherin • LIN28A positive cells are resisted to doxorubicin treatment.	[103]

Cancer	LIN28	Significance of a study	Reference
Oral squamous cell carcinoma (OSCC)	LIN28A	LIN28A is overexpressed in side population (SP) cells and show enrichment for CSC-like cells properties, contributing to enhance cell proliferation, colony-forming ability and cell invasion.	[104]
	LIN28B	LIN28B/let-7 axis promoted CSCs-like property, self-renewal ability in OSCC cells by inducing Oct4 and Sox2 gene expression via regulation of downstream let-7, ARID3B and HMGA2.	[105]
Breast cancer	LIN28A	Double-negative feedback loop of lncRNA H19/let-7/LIN28A promoted breast cancer stem cell properties, cell growth, migration and tumorsphere-forming ability.	[106]
	LIN28A	LIN28A/let-7 regulated by Wnt-β-catenin pathway was significantly implicated in the expansion of breast CSCs.	[107]
HCC	LIN28B	Overexpression of LIN28B induced expression of stemness factors, OCT4, Nanog and SOX2 and enhance tumorsphere-forming ability in HCC cells.	[108]

3. Hypothesis

Pathogenesis of CCA is closely associated with chronic-inflammation which alters the environment of the bile duct and induces genetic aberrations. Numerous oncogenic signaling pathways were reported to play a role in tumor progression and suggested as a therapeutic target for CCA. However, underlying mechanisms driving tumor initiation

process is largely unknown and need to be further explored. Based on previous study of CCA, inductions in mouse model shown that LIN28B/let-7 regulation is involved in CCA formation by which LIN28B was upregulated in pre-cancerous stage of cholestasis inducing chronic inflammation-accelerated CCA. However, the possible function of LIN28B/let-7 mediating in CCA initiation and progression is still unknown. In addition, LIN28B is reactivated in cancer and facilitates tumor initiation and tumor progression by promoting cancer aggressive phenotypes and cancer stem-like properties. Therefore, LIN28B may be an important factor in tumorigenesis of CCA. We hypothesize that LIN28B may promote cancer initiation capacity and cancer progression of CCA.

4. Research questions

1. Does LIN28B express in CCA patient's tissue? If so, what is the frequency of LIN28B expression in CCA?

2. Could LIN28B induces cancer stem-like phenotypes in normal immortalized cholangiocytes MMNK-1 cells?

3. Could LIN28B promotes an aggressive phenotype in CCA cell lines?

4. What is/are molecular signaling pathway(s) which LIN28B induces that lead to cancer stem-like phenotypes of normal cholangiocytes cells and promotion of cancer development?

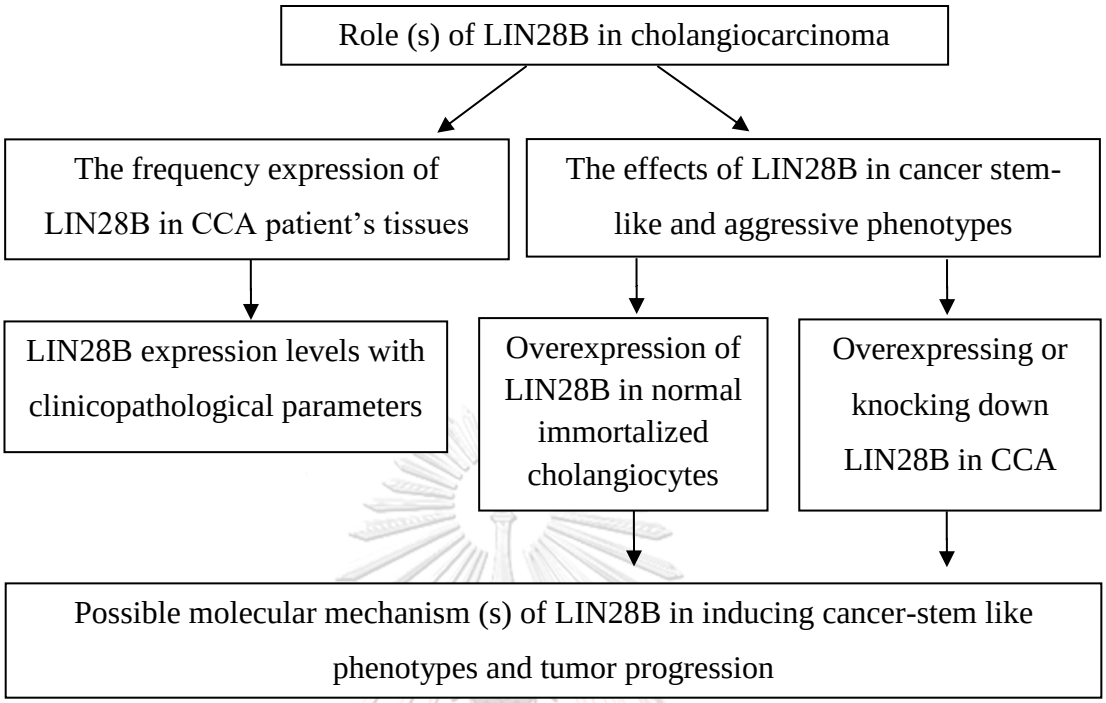
5. Objectives

1. To determine the expression of LIN28B in CCA patient’s tissue, and to analyze the frequency of LIN28B expression in CCA.

2. To determine whether LIN28B could promote cancer stem cell properties in MMNK-1 cells and induce tumor aggressiveness of CCA cells.

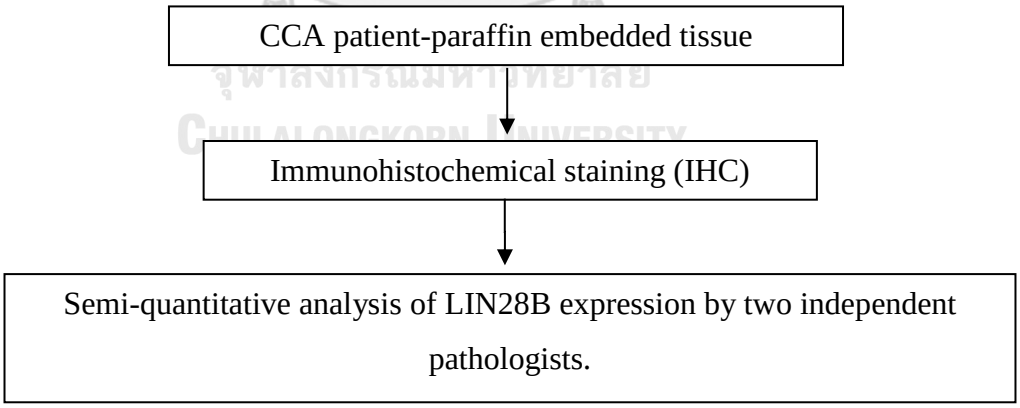
3. To determine the LIN28B-associated signaling pathway(s) that mediate cancer stem cell-like phenotypes in MMNK-1 cells.

6. Conceptual framework

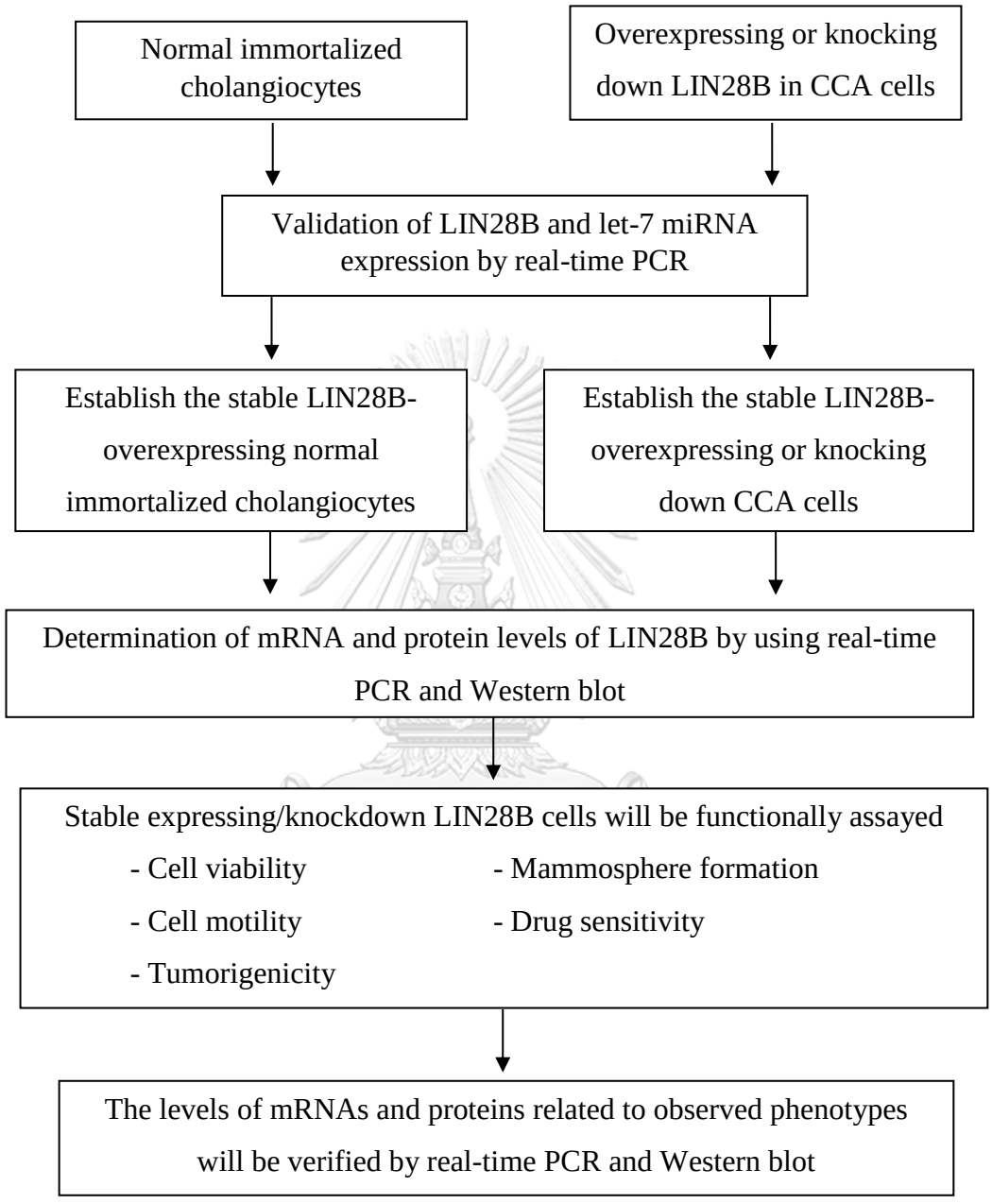


7. Experimental design

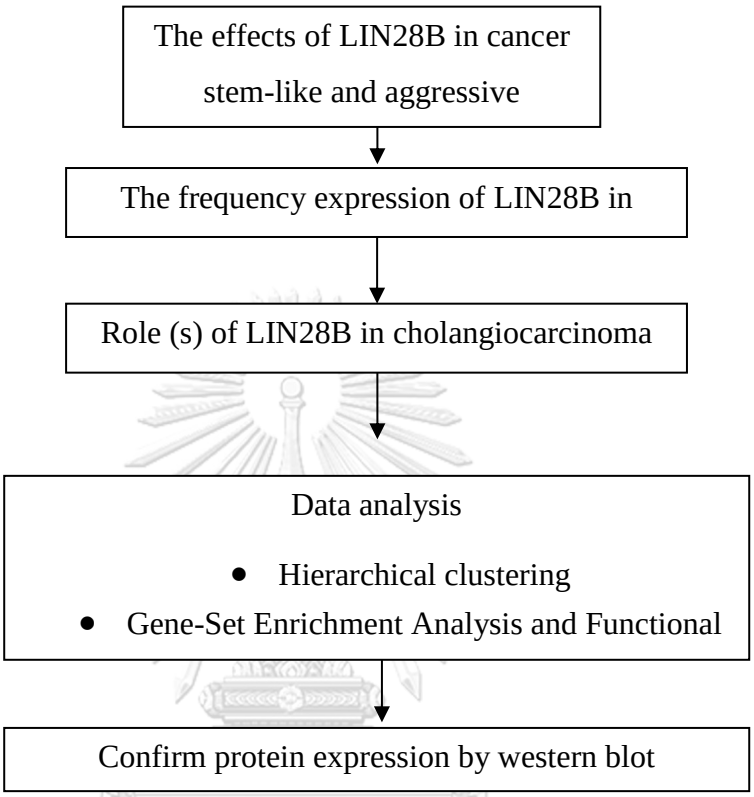
1. To determine the expression of LIN28B in CCA patient's tissue, and to analyze the frequency of LIN28B expression in CCA.



2. To determine whether LIN28B could promote cancer stem cell properties in MMNK-1 cells and induce tumor aggressiveness of CCA cells.



3. To determine the LIN28B-associated signaling pathway(s) that mediate cancer stem cell-like phenotypes in MMNK-1 cells.



CHAPTER III
RESEARCH METHODOLOGY

1. Materials

1.1 Cell lines and cell culture

Immortalized cholangiocyte cell lines; MMNK-1 was obtained from JCRB cell bank, Japan. HEK293gp and HepG2 cells were obtained from Stem Cell and Cell Therapy Research Center, Chulalongkorn University, Thailand. MMNK-1 and HEK293gp were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% Gluta Max and 1% antibiotic-antimycotic reagents. HuCCT1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% Gluta Max and 1% antimycotic-antibiotic reagents. All cells were maintained in a humidified incubator at 37 ºC with 5% CO_2 atmosphere.

1.2 CCA patient's tissue

Total 32 cases of paraffin-embedded tissue with CCA were provided from department of pathology, faculty of Medicine, Chulalongkorn University. All tissues were collected from patients with diagnosed CCA in King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The permission of using human sample for research was approved by Institutional Review Board, Faculty of Medicine, Chulalongkorn University (IRB.No 348/60).

1.3 NSG mice

The protocol of *in vivo* study was approved by Chulalongkorn University animal care and use committee of Chulalongkorn University Laboratory Animal Center (CULAC), and proceeded in accordance with CULAC guidelines.

1.4 Chemicals and reagents

All chemicals and reagents used in this study were listed in Table S1 as shown in the appendices section.

2. Methods

2.1 Viral production and establishment of stable cells

For the retroviral packaging, pBABE-hLin28B and control construct was a gift from George Daley (Addgene plasmid #26358; http://n2t.net/addgene:26358; RRID: Addgene_26358). VSVG plasmid was obtained from Stem Cell and Cell Therapy Research Unit, Chulalongkorn University, Thailand. HEK293gp cells were seeded into 6-well plate and allow cells adhered for 24 h before transfection. The compositions of transfection-complex formation were prepared by which the plasmid encoding LIN28B or control, VSVG and X-tremeGENE HP DNA transfection reagent were diluted with Opti-MEM I medium. HEK293gp cells were transfected with transfection-components and incubated for 72 h. Then, viral supernatant was collected and filtrated through 0.45micron sterile syringe filter. MMNK-1cells were seeded in 6-well plate and allow cells adhered overnight before viral infection procedure. All cells were incubated with 1X polybrene and viral containing LIN28B and control. After 72 h of infection, the transduced cells were selected by replacing with fresh medium supplemented with 1 μg/ml of puromycin. The viable cells were maintained in medium containing 1 mg/ml of puromycin.

2.2 RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent, then followed by converting to cDNA using miScript II RT kit according to manufacturer's direction. cDNA samples were subjected for determine the miRNA and mRNA expression by using SYBR Green PCR master mix and quantitative qRT-PCR analysis. The miScript Primer Assays were used to evaluate the specific miRNAs expression. Relative miRNA and mRNA expression were normalized to internal control U6 and GAPDH, respectively. All primers used in this study are listed in Table S2 as shown in the appendices section.

2.3 Western blotting analysis

2.3.1 Protein preparation. Cells were washed with twice cold PBS and then lysed with RIPA lysis buffer containing 1X Protease inhibitor and 1X Phosphatase inhibitor cocktail. Cells lysate was centrifuged at 12,000 rpm for 10 minutes at 4℃ and supernatant was collected. Protein amount was measured using Pierce™ BCA Protein Assay, as described by the manufacturer. Briefly, diluted protein lysate and

diluted albumin (BSA) standards were mixed with 200 µl of BCA kit (50:1, Reagent A: B) and then incubated at 37 ºC for 30 minutes. The absorbance was read at 562 mm against blank. The concentration of protein sample was estimated from a standard curve of standard BSA.

2.3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Total 20 µg of protein was loaded an equal volume of Laemmli sample buffer and boiled for 5 minutes. Protein sample were loaded into an individual well of 4-20% precast protein gels. Proteins were separated by electrophoresing in a running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS and adjusted pH to 8.3) at a 120 voltage to finish the run for 1 h 30 minutes.

2.3.3 Blotting and imaging and data analysis. Proteins in electrophoresed polyacrylamide gel were transferred onto a nitrocellulose membrane using 1X transfer buffer and transferred by Trans-Blot Turbo blotting system with standard program. Blotting membrane was soaked with blocking buffer for 1 h then replaced with diluted primary antibody (1; 2,000) overnight at 4ºC with gentle agitation. Rinse the membrane 3x5 minutes with TBST buffer (20 mM Tris pH 7.5, 150 mM NaCl and 0.1% Tween 20) and then incubated for 1 h at room temperature with 1; 5,000 secondary antibodies (IRDye® 800CW Goat anti-Rabbit IgG (H + L) diluted in blocking buffer. The membrane was rinsed 3x5 minutes with TBST buffer and fluorescence signal was detected and band intensity analysis using Odyssey® CLx Imaging System.

2.4 Immunohistochemistry staining

The paraffin embedded sections were obtained from department of pathology, faculty of medicine, Chulalongkorn University. Tissue sections were prepared according to the standard protocol, briefly, deparaffinization in xylene and rehydration by submerging in decreasing concentration of ethanol, the sections were heat-induced antigen retrieval with 0.01 M Sodium citrate buffer pH 6.0 using a microwave. The sections were incubated with 0.3% H_2O_2 in methanol for 30 minutes to neutralize endogenous peroxidase. Then non-specific binding was blocked for 20 minutes with diluted of 5% FBS in PBS. The tissue sections were incubated at room temperature for overnight with diluted primary antibody. After that sections were washed 3x5 minutes with PBS to remove excess antibody and then were incubated at room temperature with

secondary antibody (Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV). Target protein on tissue sections were detected by using Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+ solution and then stopped reaction by running tap water. The tissue section was counterstained with Mayer's hematoxylin and then dehydrated by submerging in increasing concentration of ethanol, subsequently in xylene and mounting the slide. For determination of LIN28B expression, the semiquantitative method was applied. The relative immunointensity of cytoplasmic LIN28B staining was independently scored by two-histopathologists blinded to the patient information. The immunointensity of LIN28B staining in adjacent normal bile duct (within a portal triad area) and bile duct tumor area was scored according to the following criteria: 0= negative staining, 1= weak staining (light yellow), 2= moderate staining (yellow brown) and 3= strong staining (brown), which was reported in previous study [109, 110]. According to previous study, the median value was used as a cut-off value to categorize high and low expression groups [111]. We applied by using intensity score (IS) 2 as a cut-off value. The IS <2 was considered as low LIN28B expression and IS ≥2 was considered as high LIN28B expression.

2.5 Cell viability assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay were applied to determined cell viability. For cells growth analysis, cells were seeded a density of $2x10^3$ cells per well in 96-well culture plate and viable cells were analyzed after day 0, 3 and 5 of experiments. For day 0, cells viability was assayed after seeded cells and allowed cells adhere for at least 6 h. After indicated time, 0.5 mg/ml of MTT diluted in PBS were added to each well and incubated for 3 h at 37 ºC. Subsequently, each well was replaced with 200 μl of DMSO then absorbance at 570 nm by using ELISA reader. For chemotherapeutic drug treatment, cells were seeded at a density of $5x10^3$ cells per well in 96-well plate and allowed cells adhere for overnight. The media was removed and diluted of chemo-drugs in media were added into each well and then cells viability was determined after 72 h of treatment. Absorbance value (OD) was sub-stacked with blank (DMSO) and percentage of cells viability was calculated using the equation, (100 x OD sample)/mean OD untreated cells.

2.6 Soft agar colony formation assay

In brief, base agar solution; 0.8% agarose in culture media was prepared and added 1 ml of base agar solution into 6-well plate then incubated at room temperature until gel completely solidified. The 0.4% top agar was prepared by which diluting 1:1 ratio of base agar with culture media. Cells density $3x10^4$ cells per well were resuspended in 1 ml of 0.4% top agar and seeded on top of base agar, and added 500 μl of media after gel forming. Cells were maintained for 4 weeks and fed cell 2 times per week with 500 μl/well of completed media. Randomized files of each well were captured under ZEISS Apotome microscope with z stack and mosaic programs and all colonies were measured size and counted a number.

2.7 *In vivo* study

For xenograft transplantation assay, 4-6 weeks old male NSG mice were subcutaneously inoculated with $1x10^6$ cells of control- and LIN28B overexpressing MMNK-1 cell lines suspended in matrigel into left and right lower flanks of mice. All mice were euthanized on day 28 of experiment. The tumors size was measured and calculated the tumor volume using the formula, $V= 0.5 \times L \times W^2$ (L= long, and W= short tumor diameter) [112, 113]. All tumor was fixed in 4% paraformaldehyde. After that fixed tissues were processed, embedded and stained with hematoxylin and eosin. Histology of tumor tissues and were observed under light microscope.

2.8 Scratch assay

The cells suspended in serum free media were seeded into each well of the 12-well plate and incubated overnight. Prior to scratching, the medium was removed, wound was created using sterile 200 μl pipette tip, and cells were washed twice with PBS before adding 10% FBS-supplemented medium. The initial wound area and wound area at indicated times of experiments were captured under the microscope and wound gap was measured using ImageJ program**.**

2.9 Boyden chamber assay

For determination of cell invasion capability, firstly, trans-well coated matrigel were prepared by adding 100 μl of 250 μg/ml of matrigel in serum free media into trans-well (8 μm PET membrane) inserted in a well of 24-well plate then incubated at

incubator 37°C for at least 2 h. Subsequently, re-suspended $1x10^4$ cells in serum free media were seeded into a matrigel-coated trans-well insert and lower chamber of 24 well-plate was added with 600 μl of medium containing 10% FBS. After the indicated incubation time, the invaded cells were fixed with 4% paraformaldehyde, permeabilized with absolute methanol, and then stained with 0.05% crystal violet in PBS. Non-invaded cells were discarded by cotton swabs. For the cell migration assay, the procedure of migration assay was performed as abovementioned, differing, cells were seeded into an uncoated matrigel trans-well insert. The migrated and invaded cells were captured and counted.

2.10 Immunofluorescence staining

A glass cover-slips was putted into 24-well plate, washed with 85% ethanol for 15 minutes and washed with 3x5 minutes sterile PBS. Cells were seeded on a glass cover-slips and allowed cell adhere overnight. Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature, aspirated fixative solution and rinsed 3x5 minutes PBS. Cells were permeabilized by adding 0.3% Triton X-100 in PBS then incubated for 30 minutes and incubated for 1 h with blocking buffer (5% BSA in 0.3% Triton X-100 PBS). Cells were incubated with diluted primary antibody in 1% BSA in 0.3% Triton X-100 PBS at 4ºC overnight. Cells were rinsed with 3x5 minutes PBS, 0.05% tween 20 (PBST), then incubated with fluorescent-dye conjugated secondary antibody for 1 h in the dark chamber and subsequently incubated with DAPI (1:1000) in PBST for 5 minutes. Cover-slips were rinsed 3x5 minutes PBST and picked out of plate then mounting the cover-slips on glass slide with ProLong™ Gold Antifade Mountant.

2.11 Sphere formation assay

An assay protocol was conducted as previously reported [114, 115]. Briefly, a total of $1.5x10^3$ cells/well were suspended in mammosphere media consisted of DMEM/F12 supplemented with 1X Gluta max, 1X Antibiotic-antimycotic, 1X B27 and 20 ng/ml of human recombinant epidermal growth factor (hEGF) and human basic fibroblast growth factor (hbFGF) in each, then seeded into 24-well clear flat bottom ultra-low attachment surface culture plate. Cells were cultured for a week in an

incubator setting at 37ºC with 5% CO_2. Mammospheres were counted and measured the size.

2.12 Proteomic analysis

2.12.1 Protein extraction and In-solution digestion. After washing cells with cold PBS, the cells were lysed with mixture of 5% sodium deoxycholate (SDC) and 1X protease inhibitor. Cells were sonicated and centrifuge, the supernatant was collected to further measure the protein concentrations by using BCA protein assay. The sample containing 250 µg of protein was treated with dithiothreitol (DTT) for 30 minutes at 37 °C and iodoacetamide (IA) for 30 minutes at 25 °C in the dark to reduce disulfide bonds and alkylations. The protein was digested with Trypsin (1:50) and incubated at 37 °C overnight. Then, trifluoroacetic acid (0.5%) was added and the samples were incubated for 30 minutes to solubilize the polyglutamine. After drying the samples by vacuum centrifugation, the samples were reconstituted by adding 100 µl of 100 mM tetraethylammonium bromide (TEAB). The amounts of peptide were measured using Pierce Quantitative Fluorometric Peptide Assay.

2.12.2 Peptide labeling and LC-MS/MS analysis. Peptides in the MMNK-1_control sample were labeled with the light isotope reagent (formaldehyde; CH2O and cyanoborohydride; NaBH3CN) and peptides in the MMNK-1_LIN28B sample were labeled with the heavy isotope reagent (13C-labeled formaldehyde; 13CD2O and cyanoborodeuteride; NaBD3CN). The samples were incubated for an hour and the reactions were quenched by adding 25% ammonia solution and formic acid. Then, the two samples were pooled and dried by vacuum centrifugation. To reduce sample complexity, the pooled peptide sample was fractionated into 10 fractions using Pierce High pH Reversed-Phase Peptide Fractionation Kit. All eluted fractions were dried by vacuum centrifugation and subject to LC-MS/MS analyses on a Q Exactive Plus mass spectrometer. The MS procedure was followed as previously described [116].

2.12.3 Data analysis. Raw mass spectra files were analyzed with the MaxQuant software (version 1.6.2.10) and searched against the Human Swiss-Prot Database (20,408 proteins, downloaded December 2018) as well as a list of common protein contaminants (http://www.thegpm.org). Trypsin/P was set as the digestion enzyme with a maximum of two missed cleavages allowed. Carbamidomethylation of

Cysteine and Acetylation at N-terminus were set as fixed modifications while Oxidation of Methionine was set as variable modification. Dimethyl labeling was selected as the quantification mode with dimethyl Lys 0 and N-term 0 = the light labels, dimethyl Lys 4 and N-term 4 = the intermediate labels and Lys 8 and N-term 8 = the heavy labels. The maximum false discovery rate (FDR) was set at 1% for both peptide and protein levels. Heavy-to-light protein expression ratios were extracted from the protein group output of MaxQuant, log-transformed, and subjected to one-sample student's t-test analyses. The list of 234 significant differentially expressed protein groups were selected at FDR of 5% using the Benjamini-Hochberg procedure. Only 2,539 protein groups with at least 2 razor and unique peptides were considered here. Gene set enrichment analysis was performed using web site, https:// academic.oup.com/ nar/ article/ 47/ W1/ W199/ 5494758 and https://academic.oup.com/ nar/ article/ 47/ D1/ D419/ 5165346 for pathway annotation.

2.13 TGF-β cytokine array

After culturing cells for 24 h, the media was collected and centrifuged at 1,000 xg, 15 minutes at 4 ºC then the supernatant was collected. The concentration of cytokines was evaluated using Bio-Plex Pro™ TGF-β Assay according to the manufacturer's instructions. In brief, 1X beads were added into each well of black 96-well plate. The standards, blank, samples were added into pre-coated beads wells and incubated at room temperature with gently shake at 850 rpm for 2 h. Then, the assay plate was rinsed with wash buffer and added 1X detection antibody, then incubated for 1 h at room temperature with shaking at 850 rpm. 1X streptavidin-PE diluted in assay buffer was added and shaking with shaker at 850 rpm for 30 minutes. After indicated time, assay plate was rinsed with wash buffer and beads were suspended by assay buffer. The assay plate was analyzed by using Bio-Plex Pro™ system and acquire data with Bio-Plex manager software v 6.0. The relative concentration of cytokines to standards were shown as pg/ml unit.

2.14 Statistical analysis

The statistical significance of continuous variables was assessed by student's t-test and conducted with GraphPad Prism 8.4.3 Demo software (GraphPad Software, Inc., CA, USA). The *P* value ≤0.05 was considered as statistically significant. The

association between clinicopathological variables and LIN28B expression was analyzed by using chi-square analysis, Fisher's exact test. A two-sided $P \leq 0.05$ was considered statistically significant. The association analysis was performed using SPSS statistics 22.0 for windows.

CHAPTER IV
RESULTS

1. LIN28B is highly expressed in human CCA tissues.

To determine the frequency expression of LIN28 in CCA, the expression level of LIN28A and LIN28B were determined by immunohistochemistry staining. Semi-quantitative analysis of LIN28 expression was determined by two-independent histopathologists. The results show that LIN28A is not expressed in CCA samples (n=20) whereas LIN28B is strongly expressed (Figure 2A). Next, we further evaluated LIN28B expression in 32 cases, those have combining of tumor area and adjacent normal bile duct on the same tissue section. We found that LIN28B is positively expressed in vary CCA-histopathological types, but weakly expressed in normal adjacent bile duct (Figure 2B). The immunofluorescence staining showed LIN28B is expressed in sub-population of tumor cells as indicated by bile duct marker, cytokeratin 7 (CK7) (Figure 2C). The IHC staining showed LIN28B is predominantly localized in the cytoplasmic region with different immunointensity gradient between adjacent normal bile duct and tumor area, then we next determined the expression level of LIN28B according to the semiquantitative method (as describe in method section). To determine the potential association between the expression of LIN28B and clinicopathological variables, we then analyzed by using chi-square analysis, Fisher's exact test. The result showed that LIN28B expression was not significantly associated with any clinical variables (Table 3). However, in the tumor biology, LIN28B might be a tumor promoting factor in CCA by it was frequently found with high expression in the tumor area, whereas low LIN28B expression was found in the adjacent normal bile duct. Together, these data indicate that only LIN28B but not LIN28A was found in CCA tissue and an increasing of LIN28B protein in tumor may assume that LIN28B protein is potentially implicate in the tumorigenesis of CCA.

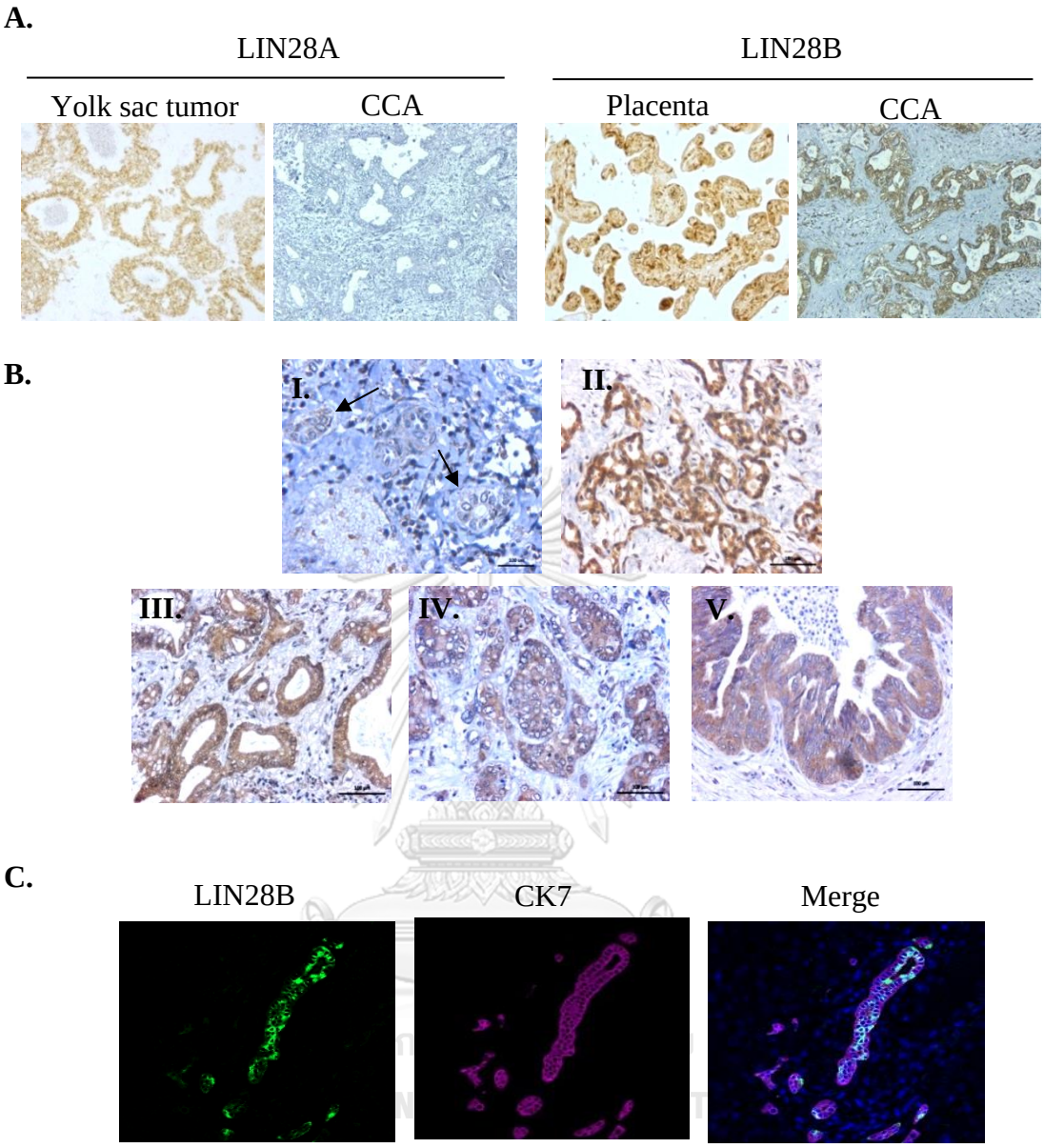


Figure 2. LIN28B is overexpressed in human CCA tissues.

LIN28A and LIN28B expression in CCA patient's tissues were evaluated by immunohistochemistry staining. LIN28A and LIN28B were stained in CCA tissues. The yolk sac tumor and placenta tissues were used as a positive expression of LIN28A and LIN28B, respectively **(A)**. **B**, representative picture of LIN28B staining in CCA tissues, the arrows indicate the adjacent normal bile duct (I), well differentiated tumor type (II), moderate differentiated tumor type (III), poorly differentiated tumor type (IV) and intraductal papillary neoplasm (V). **C**, Representative immunofluorescence images of LIN28B and CK7 expression in CCA tissues. All pictures were scaled as 100 μm.

Table 3. The potential association between LIN28B protein expression and clinicopathological parameters of patients with CCA.

	Adjacent Normal			Tumor		
	Low	High	*P* -value	Low	High	*P* -value
n	24	8		1	31	
Age (years)						
≤ 50	4	0	0.550	0	4	1.000
> 50	20	8		1	27	
Gender						
Male	11	3	1.00	0	14	1.000
Female	13	5		1	17	
Tumor stage						
I-II	6	0	0.296	1	5	0.188
III-IV	18	8		0	26	
Histology						
Well differentiated	12	4	0.541	0	16	0.125
Moderately differentiated	8	4		0	12	
Poorly differentiated	4	0		1	3	
Lymphatic invasion						
No	7	0	0.150	1	6	0.219
Yes	17	8		0	25	

2. Establishment of stable LIN28B-overexpressing MMNK-1 cells.

From the results of LIN28B expression in human CCA patient's tissues and together with the role the role of LIN28B in cancer development by promoting cancer stem cell properties. We hypothesize that LIN28Bmight implicate in malignant transformation of cholangiocytes. Therefore, stable LIN28B-overexpressing normal cholangiocytes, MMNK-1 cells were established to study the tumor-promoting role of LIN28B in cholangiocytes. The efficiency of LIN28B transfection was confirmed by showingLIN28B expression at both mRNA and protein levels (Figure. 3A-B). LIN28B is mainly localized in nucleolus and cytoplasm of LIN28B-overexpressing MMNK-1 cells (Figure 3C).

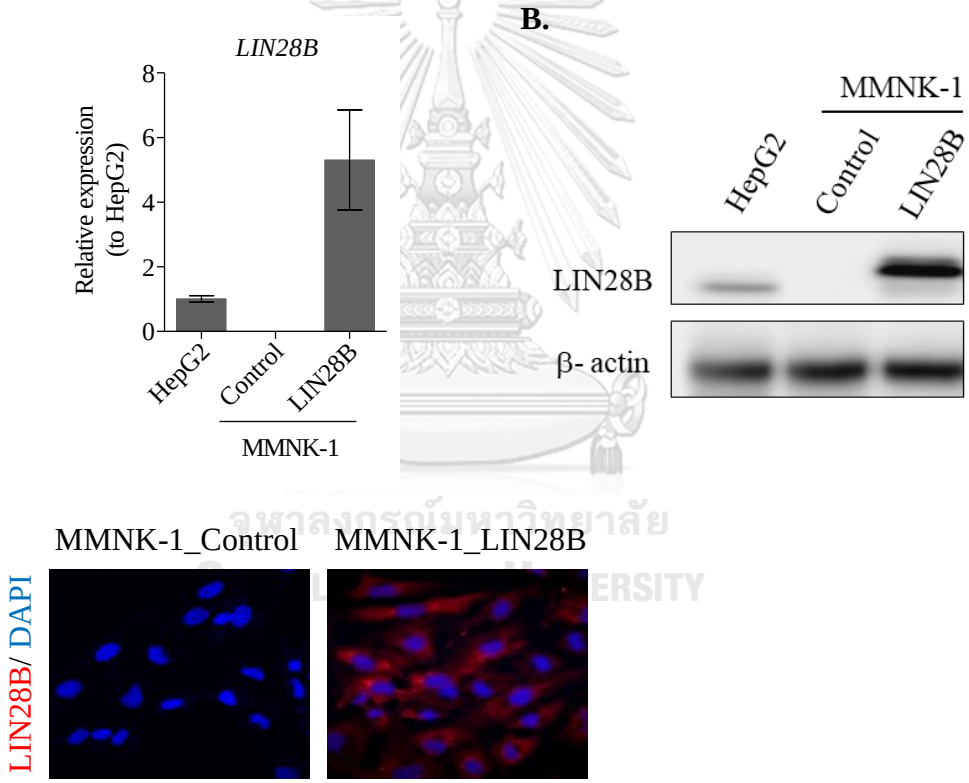


Figure 3. Establishment of stable LIN28B-overexpressing MMNK-1 cells.

LIN28B-overexpressing cells and empty vector-control cells were determined the expression levels of LIN28B mRNA and protein by qRT-PCR and western blot, respectively and HCC cells; HepG2 were used as positive LIN28B expression **(A-B)**. The localization of LIN28B in LIN28B-expressing cells and control cells were demonstrated by immunofluorescence staining **(C)**.

According LIN28B function by which it regulates let-7 miRNAs biogenesis. Hence, to confirm the regulation of LIN28B/let-7 in MMNK-1 cells stably overexpressed LIN28B, mature let-7 miRNAs were determined. The results demonstrate that mature let-7a, b and i expression were significantly decreased in LIN28B-expressing cells compared to control cells (Figure. 4). Collectively, the results indicate that the LIN28B expressing in the context of MMNK-1 cells could functionally suppress let-7 miRNAs.

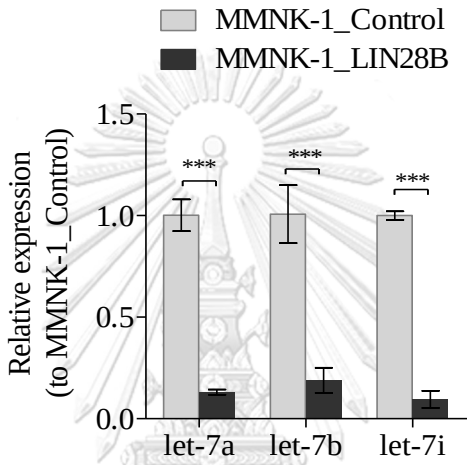


Figure 4. The relative expression of let-7 miRNAs in LIN28B-overexpressing cells.

The expression of let-7 miRNAs, let-7a, let-7b and let-7i were evaluated using miScript Primer Assays and analyzed by qRT-PCR. The value is presented as fold change when normalized to MMNK-1_Control cells. A bar graph is represented as mean±SD and statistical significantly different indicated by *** *P*-value < 0.001.

3. LIN28B enhances cell proliferation and induces a tumorigenic potential of cholangiocytes.

As previous reports demonstrated that LIN28B/let-7 axis mediate cancer cell growth in multiple cancer types. Our study was also performed that LIN28B enhances growth of cholangiocytes by which LIN28B-overexpressing MMNK-1 cells was significantly higher viable cells than control cells at day 3 and 5 of experiment (Figure. 5A). In addition, the expression of several let-7 target genes that important functions in cell cycle progression and gene-mediated cell proliferation was determined. *CCND1*, *CDC25A* and *CDK6* gene was significantly upregulated in LIN28B-overexpressing cells compared to control cells (Figure. 5B). These data show that LIN28B enhances cell proliferation by inducing cell cycle progression in cholangiocytes.

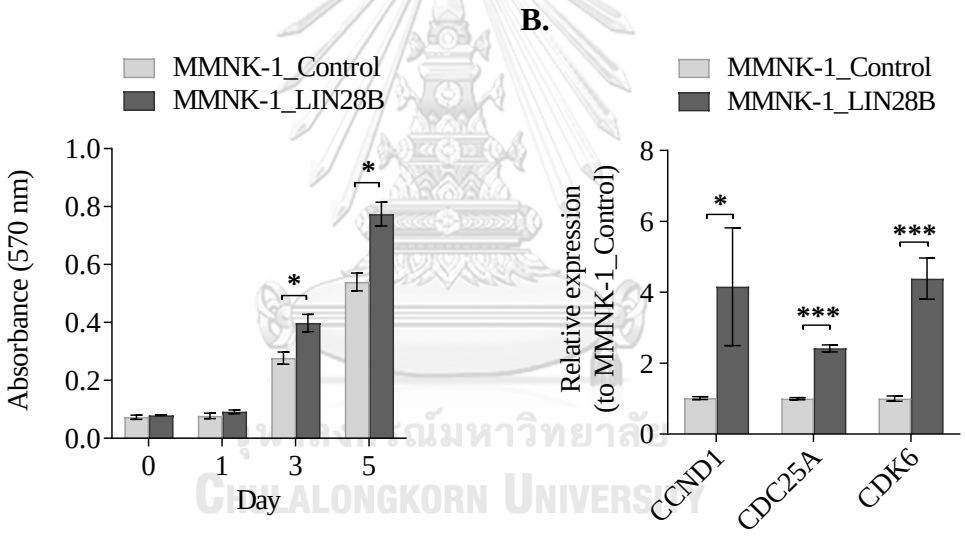


Figure 5. LIN28B induces cell proliferation of cholangiocytes.

MTT assay was used to determine cell viability of LIN28B-overexpressing MMNK-1 cells and control cells at day 0, 1, 3 and 5 of experiment and the absorbance value is relative to the number of viable cells **(A)**. The relative expression level of cell cycle-related genes, *CCND1*, *CDC25A* and *CDK6* were determined by qRT- PCR **(B)**.*P-value<0.05, ***P-value<0.001.

We then evaluated the growth ability of LIN28B-overexpressing MMNK-1 cells in mice xenograft model. MMNK-1 control and overexpressed LIN28B cells was subcutaneously injected at the lower flank of NSG mice. We found that the size of tissue was not obviously different (Figure 6A), but the histology showed accumulating of small bile duct around the border of tissue from LIN28B-overexpressing MMNK-1 cells as demonstrated by positive staining of bile duct marker, cytokeratin 7 (CK7) with normality features of bile ductules (Appendices section; supplementary figure 1). Moreover, when we determined the tissue volume, found that the tissue volume of mice injected LIN28B-overexpressing cells was significantly higher than the control group (Figure. 6B). The results indicate that LIN28B could enhance proliferative capacity of cholangiocytes.

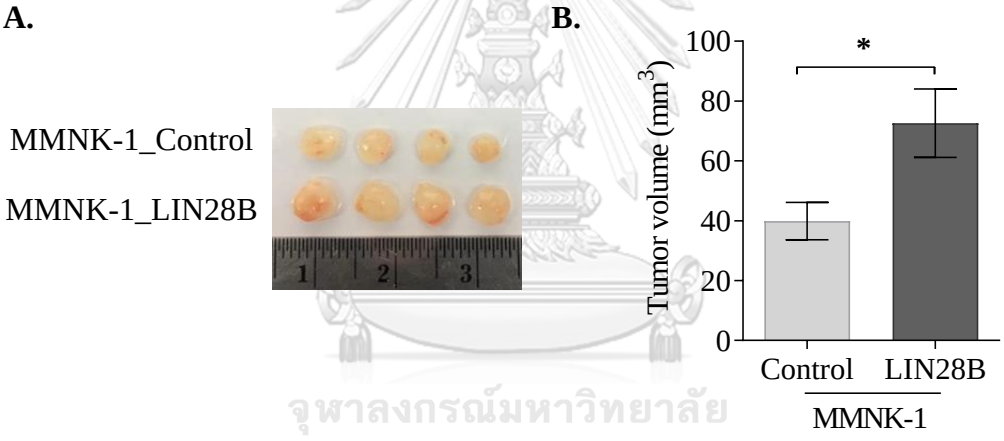


Figure 6. LIN28B promotes growth ability of cholangiocytes in vivo.

The mice were subcutaneously injected with LIN28B-overexpressing cells and control cells (n=4/group) and tissue-bearing mice were collected after day 28 of injected cells **(A)**.The tissue was measured the size and determined the tissue volume (as describe in the method section) **(B)**. **P*-value <0.05.

Furthermore, since LIN28B could promote cellular transformation by which the force LIN28B-overexpression in normal cells could be formed a colony in soft agar. Therefore, we further determined the transforming ability of LIN28B-overexpressing MMNK-1 cells by performing the soft agar colony formation assay. The results show that LIN28B-overexpressing cells had a higher number of colonies than control cells. Moreover, the colony larger than 50 microns was frequently found in LIN28B-overexpressing cells (Figure 7A-B). Therefore, this finding suggests that LIN28B might induce malignant transformation ability in cholangiocytes *in vitro*. Taken together, we may imply that LIN28B was significantly induced proliferation and enhanced the tumor-initiation capacity of cholangiocytes.

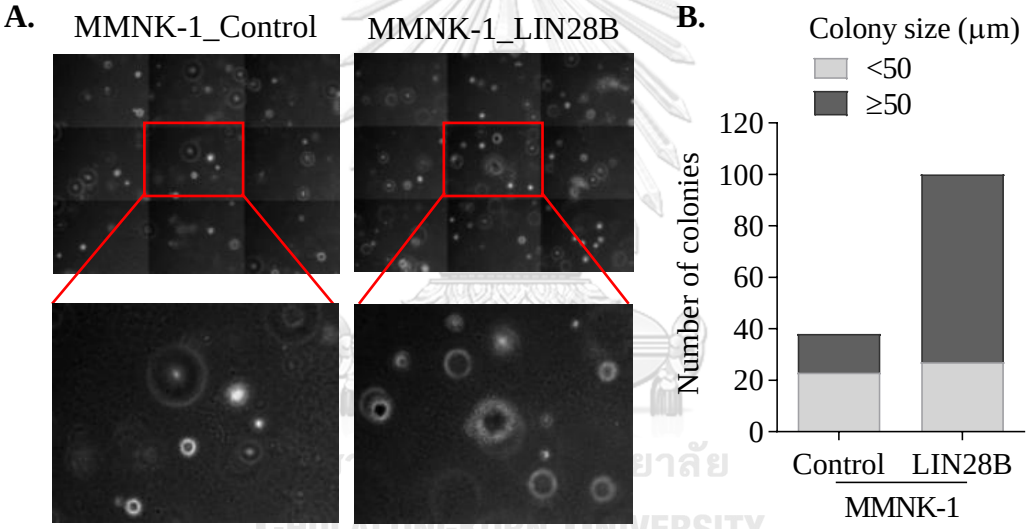


Figure 7. LIN28B promotes colony forming capability in cholangiocytes.

The soft agar colony formation was conducted to examine the capability of LIN28B induces cellular transformation of cholangiocytes. The representative picture of captured 9-focal planes with Z-stack **(A)** and all colonies were counted and measured the size, diameter exceeding 50 µm is considered to be a large colony **(B)**.

4. LIN28B enhances sphere-forming capability in cholangiocytes.

Our results show that LIN28B could induce cell growth and tumorigenicity in cholangiocytes. As known function of LIN28B, it could induce and maintain the cancer stem cell properties. It is likely that LIN28B might induce cancer-initiation capacity in MMNK-1 cells by promoting cancer stem-like property. Hence, to prove this hypothesis, we determined capability of LIN28B-overexpressing MMNK-1 cells to form the sphere, which is a method that used to determine the cell which is having the cancer stem cells property [117]. We found that the number of spheroids in control and LIN28B-overexpressing cells were not different, as shown by counted 26 spheroids of control cells and 27 spheroids of LIN28B-overexpressing cells. However, the size of spheroids in LIN28B-overexpressing cells were obviously larger than control cells (Figure 8A). An average size of spheroids in MMNK-1 stably overexpressed LIN28B cells is 42.63±24.16 μm whereas control cells is 27.17±8.28 μm (Figure 8B). At least 6 spheroids with the size bigger than 50 microns were found in LIN28B-overexpressing cells, but not found in control cells. Collectively, our finding indicates that LIN28B could promote cancer stem-like cell property in cholangiocytes as shown by performing sphere forming capability.

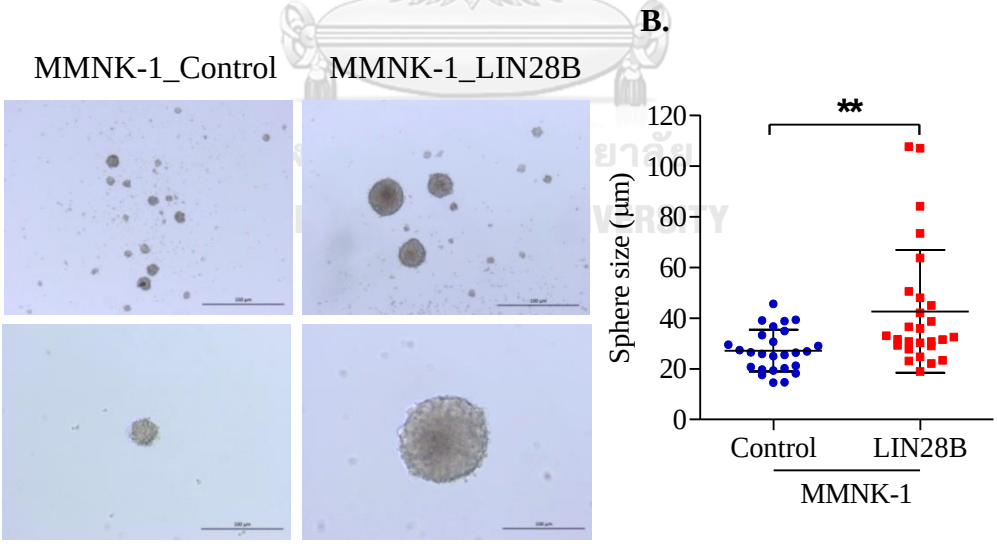


Figure 8. LIN28B induces sphere-forming capability in cholangiocytes.

The representative of spheroids in MMNK-1 control and LIN28B-overexpressing MMNK-1 cells **(A)**. Total number and average size of counting spheroids shown as mean±SD of triplicate wells **(B)**. ***P*-value <0.01.

5. LIN28B promotes migration and invasion of cholangiocytes.

Numerous evidences suggest that cancer stem cells have high metastatic capability and this is a reason why cancer stem cells could be resisted to chemo-/radio therapies. Therefore, the metastasis phenotype is recommended as characteristic of cancer stem cells. Together with previous studies have indicated that LIN28B aberrantly expressed in highly metastatic cells and it was required for metastasis process [118, 119]. Thus, we aim to investigate the metastatic capacity of LIN28B-overexpressing MMNK-1 cells. Cell migration ability of LIN28B-overexpressing MMNK-1 cells and control cells were assayed by cell scratch assay. The results show that the relative wound gap area of LIN28B-overexpressing cells had significantly minimized than control in a time-dependent manner (Figure. 9A-B).

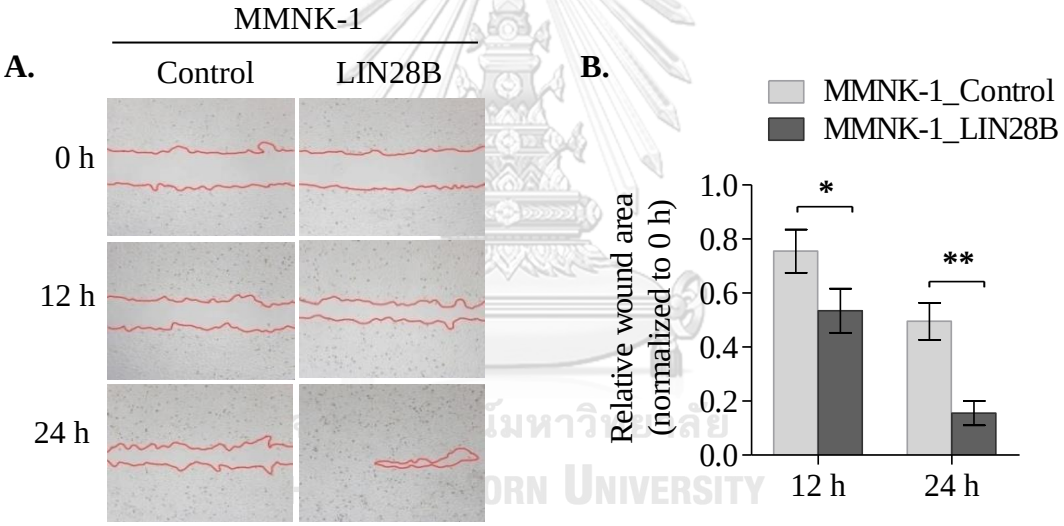


Figure 9. LIN28B promotes migratory ability of cholangiocytes.

Cell scratching assay was performed the ability of LIN28B induce migratory activity of MMNK-1 cells **(A)** and quantification of wound area at 12 h and 24 h was normalized to initial wound area (0 h), n=3 **(B)**. **P*-value <0.05, ** *P*-value <0.01.

Furthermore, to confirm LIN28B induced cell metastatic ability of MMNK-1 cells, the transwell assay was also performed. We found that the number of migrated cells and invaded cells in the stable LIN28B-overexpressing MMNK-1 cells were significantly higher than control cells (Figure. 10A-B).

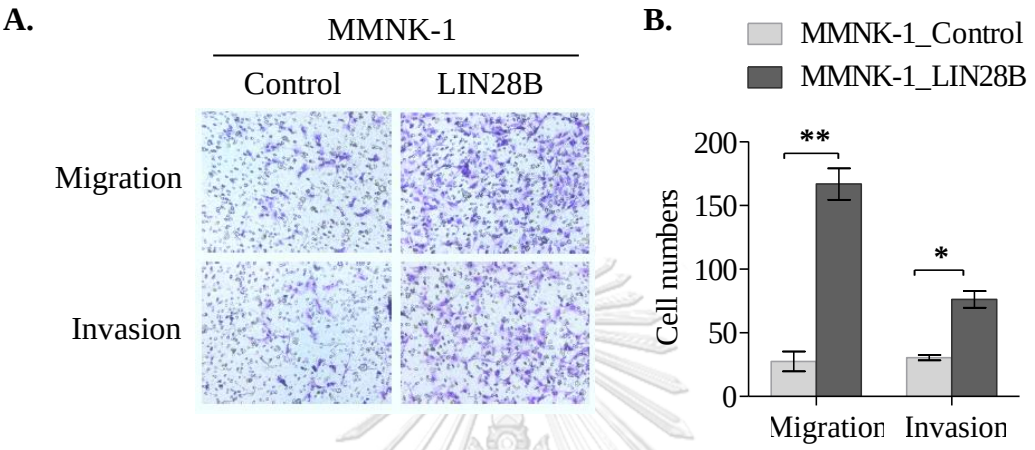


Figure 10. LIN28B enhances migration and invasion ability of cholangiocytes by penetrating the transwell.

Boyden chamber assay was conducted to determine cells migration and invasion **(A)**. The number of migrated and invaded cells was counted after seeding cells for 15 h and 20 h for invasion assay, n=3 **(B)**.*P-value <0.05, ** P-value <0.01.

As these results demonstrate that LIN28B could induce cholangiocytes motility. The EMT is a critical processing to initiate the cells metastasis. Therefore, we ask whether LIN28B promotes the cholangiocytes migration by inducing the EMT-changed. To answer this question, we next performed the mRNA expression of EMT-related genes in LIN28B-overexpressing MMNK-1- and control cells by qRT-PCR. The results show that epithelial marker, E-cadherin was not obviously changed, whereas mesenchymal markers; Vimentin, Fibronectin, Twist1, Snail and Slug genes were significantly increased in LIN28B-overexpressing MMNK-1cells compared to MMNK-1 control cells (Figure. 11). Taken together, these data reveal that LIN28B promotes metastatic capability in cholangiocytes by inducing EMT phenotype.

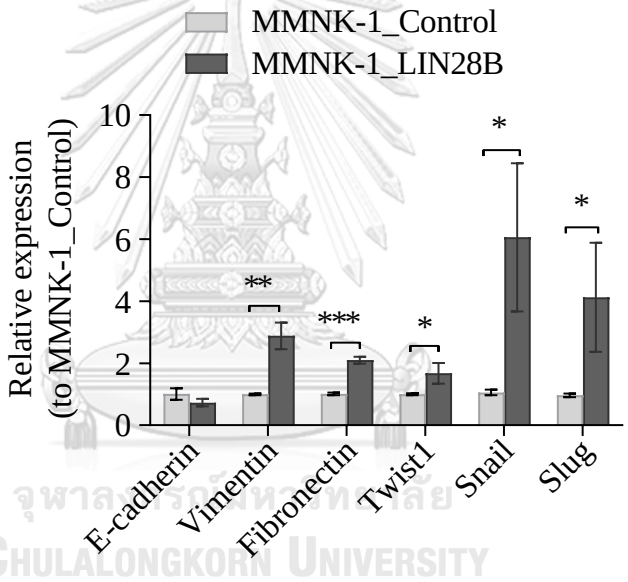


Figure 11. Increasing of mesenchymal related genes expression in LIN28B-overexpressing cholangiocytes.

Quantification of EMT-related genes expression levels in LIN28B-overexpressing cells were analyzed by qRT-PCR and data shown as fold change when normalized to control cells. Bar graphs presented as mean±SD. **P*-value<0.05, ** *P*-value<0.01, ****P*-value<0.001.

6. Protein expression profiling of LIN28B-overexpressing MMNK-1 cells.

In the mechanism, LIN28B could regulate gene expression at post-transcription level, hence, the protein expression should be investigated. The proteomic analysis was conducted to identify proteins expressed in LIN28B-overexpressing MMNK-1 cells. The peptides were annotated and found 234 proteins were differentially expressed with 5% of FDR (Figure. 12).

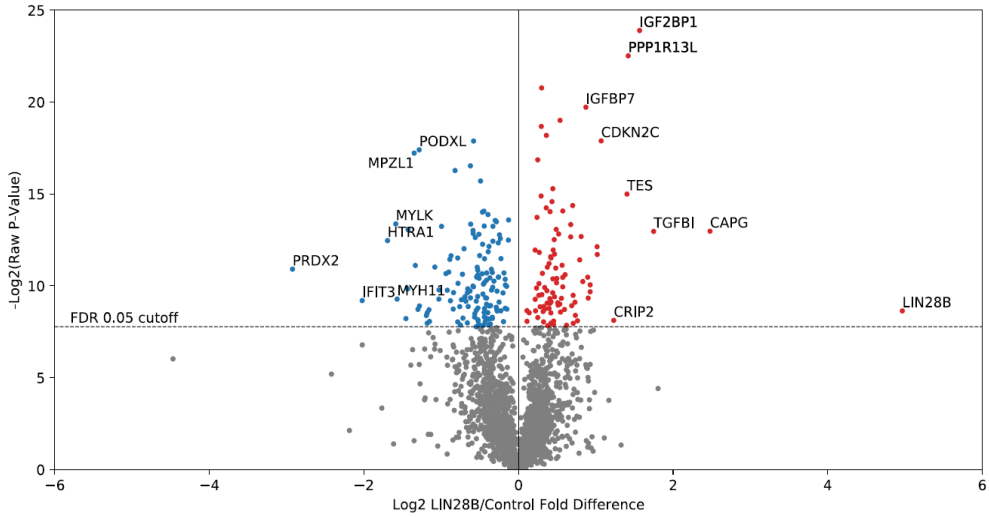


Figure 12. Protein expression profiling in LIN28B-overexpressing MMNK-1 cells.

The volcano plot represents the identified proteins, and categorized into up-regulated and down-regulated expression according to fold change when normalized to control cells. FDR at 0.05 was used as a cutoff value to consider the significant differentially expressed protein.

The transforming growth factor beta induced (TGFβI) was significantly up-regulated, which is a extracellular matrix (ECM) protein and plays important role in many biology processes including cell adhesion, migration, angiogenesis, and inflammation [120]. According to the significant role of TGFβI, we further study the function of TGFβI on the context of LIN28B-induced cancer stem-like properties, EMT in cholangiocytes. We confirmed that TGFβI was highly expressed in LIN28B-overexpressing MMNK-1 cells (Figure. 13).

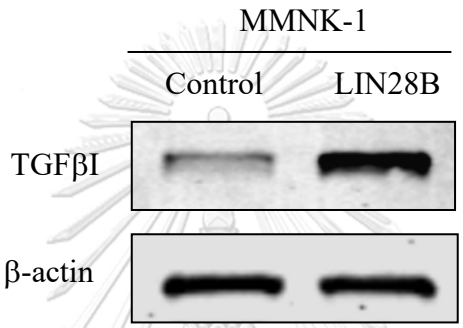


Figure 13. Upregulation of TGFβI in LIN28B-overexpressing MMNK-1 cells

The expression level of TGFβI protein in control and LIN28B-overexpressing MMNK-1 cells was performed by western blot.

7. The migration ability of LIN28B-overexpressing cholangiocytes could be suppressed by inhibiting the TGF-β signaling.

As we know that TGFβI was up-regulated in LIN28B-overexpressing MMNK-1 cells and it known as down-stream target of TGF-β signaling. We hypothesize that TGF-β/ TGFβI signaling might be activated in LIN28B-overexpressing MMNK-1 cells and possibly implicated in promotion of cancer stem-like feature in cholangiocytes. Therefore, we determined the amount of secreted TGF-β complex composed of TGF-β1, 2 and 3 in the culture medium of control cell and LIN28B-overexpressing MMNK-1 cells. We found that the concentration of TGF-β in LIN28B-overexpressing cells was significantly higher than control cells (Figure. 14A).Not only the TGF-β ligands, but its receptor TGF-β receptor 1 (TGFβR1) protein also highly expressed in LIN28B-overexpressing MMNK-1 cells (Figure. 14B). These data indicate that LIN28B expressing cells could more produce TGF-β ligands and enhance TGF-β signaling. Thus, we assume that activation of TGF-β/TGFβI cascade might be involved in LIN28B induced cancer stem-like properties in cholangiocytes.

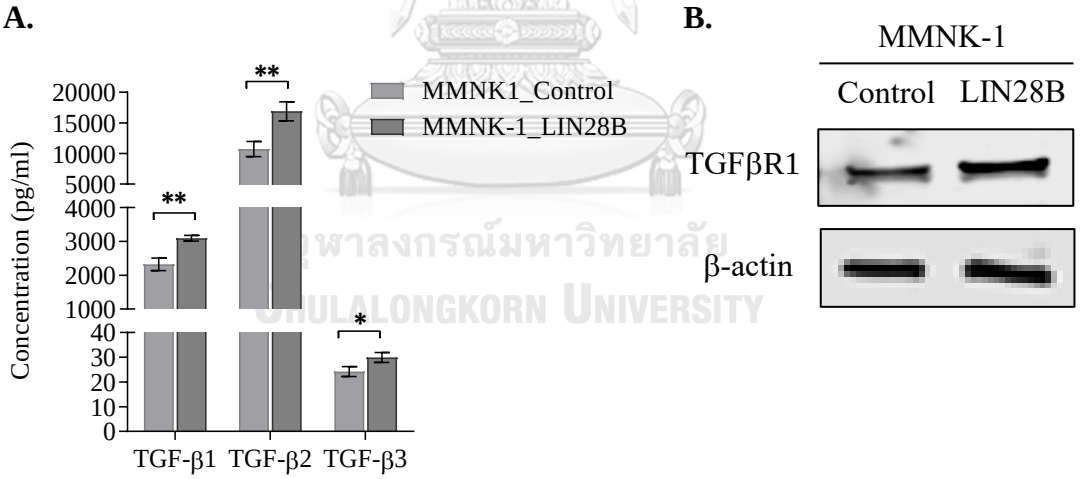


Figure 14. Increasing of TGF-β in LIN28B-overexpressing MMNK-1 cells

The amount of secreted TGF-β complex in culture medium was measured using Bio-Plex Pro™ TGF-β assays **(A)**. The expression of TGFβR1 was detected by western blot **(B)**. Data presented as mean±SD from triplicate wells. **P*-value<0.05, ***P*-value <0.01, *** *P*-value <0.001.

We next focus on the mechanism by which if inhibit the TGF-β/ TGFβI signaling, may have an effect on the oncogenic potential of LIN28B in cholangiocytes. LIN28B expressing cells were treated with each of TGFβR1 inhibitors, SB431542 and Repsox, we found that the expression of TGFβI was decreased compared to control (Figure. 15). It confirm that TGFBI is regulated by TGF-β signaling in our context.

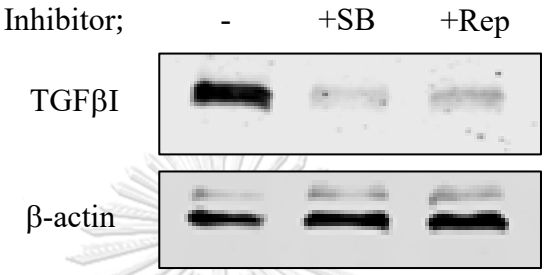


Figure 15. Suppression of TGFβI expression by TGF-β selective inhibitors.

TGFβI expression level in LIN28B-overexpressing MMNK-1 treated with TGFβR1 inhibitors, SB431542 and Repsox for 24 h was determined by western blot.

Then, we evaluated the inhibitory effects of TGF-β/TGFβI signaling on cell migration ability of LIN28B-overexpressing MMNK-1 cells. The treated cells showed decreasing of the migratory activities when compared to untreated cells as shown by having more wound gap area (Figure. 16A). Moreover, significantly down-regulated EMT genes, Vimentin, Twist1, Snail and Fibronectin and increased of epithelial mark, E-cadherin was observed (Figure. 16B).The results indicate that LIN28B induced cancer stem-like property, EMT in cholangiocytes through modulating of TGF-β/TGFβI cascade.

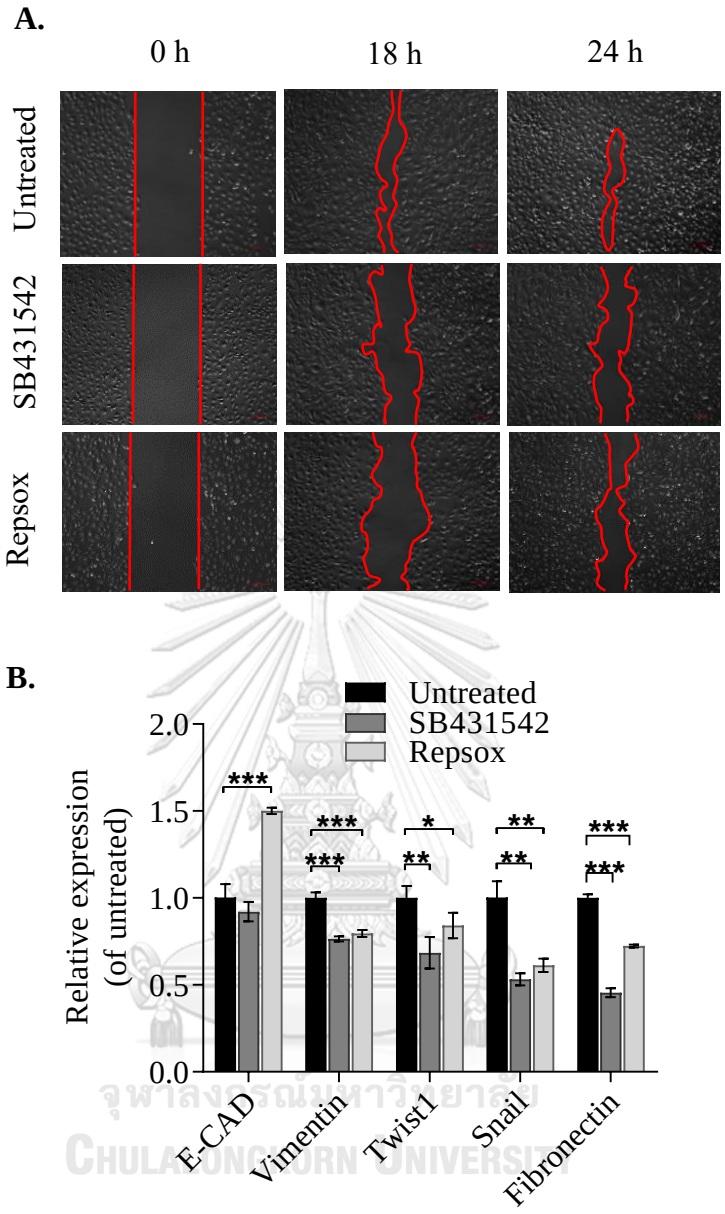


Figure 16. Inhibition of TGFβI by TGF-β selective inhibitors reduce the migration ability of LIN28B-overexpressing MMNK-1 cells.

Cell migration ability of LIN28B-overexpressing MMNK-1 cells treated with SB431542 (10 µM) and Repsox (5 µM) was determined by wound healing assay **(A)**. The genes expression levels were determined using qRT-PCR. E-CAD; E-cadherin **(B)**.*P-value<0.05, **P-value <0.01, *** P-value <0.001.

CHAPTER V
DISCUSSION

The evidence of LIN28B have been demonstrated in multiple types of cancer and associated with poor prognosis. However, the distribution of LIN28B in CCA have not yet been reported. In this study, we present the evidence that LIN28B but not LIN28A is highly expressed in CCA. Therefore, we expect that LIN28B is sufficient to promote cancer stem cell-like properties in bile duct epithelial cells. We demonstrate various changes of the intrinsic programs through paracrine effects of LIN28B overexpressing cells which could induce the tumor initiating capacity in cholangiocytes.

Consistent with mice models have been previously reported that overexpressing LIN28B could induce tumor formation in several cancers such as neuroblastoma [121], Wilms tumor [122], intestinal cancer [123] and liver cancer [7]. In CCA mice model, LIN28B was highly expressed in during tumor development of CCA [9]. It recommends that LIN28B acts as a tumor-initiating mediator. However, it is still unclear whether LIN28B alone is adequate to promote cancer transformation of normal adult cholangiocytes. This study demonstrated that LIN28B overexpression in immortalizedcholangiocyte-MMNK-1 enhanced cell proliferation, migratory capacity and anchorage-independent growth. LIN28Bsuppressed let-7 miRNA and induced genes involved in cell proliferation and EMT which those genes have been documented to be related with tumor progression and poor prognosis CCA [124, 125]. Our results are similar to other forced LIN28B expressing cells. LIN28B was suggested as an oncoprotein and acquired in the processing of normal breast cells transformation by inducing cancer stem-like properties [8]. Thus, although we observed LIN28B IHC staining in both well-differentiated and poorly-differentiated CCA. LIN28B expression is likely marked cancer stem-like sub-population of CCA cells with relatively more aggressive behavior and higher tumor initiating potentials.

Nevertheless, MMNK-1 cell lines are not exactly naive cholangiocytes because p53 signaling was already defective by SV40 and hTERT system that used to establish the immortalization. However, MMNK-1 cells are still maintained the normal cholangiocytes phenotypes and it was widely used in researching for representing as a

normal bile duct epithelial cell [9]. Importantly, we found LIN28B could induce stemness in cholangiocytes by exhibiting the colony-forming capacity. The result suggests that the increase in size may due to cell adhesion and paracrine effects of LIN28B expressing cell. Itis possible that LIN28B could enhance cancer transformation in premalignant cholangiocytes. However, data regarding to LIN28B involvement in precancerous state of CCA is largely unknown and require further studies.

Another cancer stem-like feature is EMT, which is critical step of metastasis process. The responsible factor that mediate EMT in cancer stem cell has been suggested such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and TGF-β [126]. Meanwhile this study demonstrated that TGFβI was increased in LIN28B overexpressing cells. TGFβI is known as extracellular protein and promote tumor aggressiveness by inducing the EMT in several cancer cells types such as pancreatic cancer [127], nasopharyngeal carcinoma [128] and glioma [129]. In addition, TGFβI is also reported as an EMT inducer and promote metastasis of CCA cells [130]. The mechanism by which TGFβI expression is regulated by TGF-β signaling and together with the evidence that TGF-β signaling is responsible for EMT induction by regulating EMT-related genes expression [131]. We demonstrated that TGF-β signaling might be activated in LIN28B overexpressing cholangiocytes by showing increased expression of downstream target TGF-β cytokines and its receptor (TGFβR1). Furthermore, the migration capability of LIN28B overexpressing cells was decreased when inhibit the activity of TGFβR1 by its selective inhibitors. Therefore, we imply that LIN28B induces EMT and migration capacity in cholangiocytes via enhancing of TGF-β signaling.

It seems that our findings support the evidence that LIN28B and TGF-β signaling are linking to promote cancer progression by which TGFβR1 was reported to be a let-7 target and it increased expression in the present of LIN28B [132, 133]. Moreover, recent findings have connected the function between LIN28B and TGF-β in other cell types. For example, LIN28B/ let-7 pathway is involved in TGF-β induced collagen accumulation in mouse mesangial cells by TGF-β regulates LIN28B expression [134]. In the study of immune cell development demonstrated that LIN28B could induce naive fetal T cell differentiation into regulatory T cells (Tregs) through

modulation of TGF-β pathway [135]. Taken together, these previous data and our results provide the relationship between LIN28B/let-7 and TGF-β pathways. Nevertheless, the evidence of LIN28B crosstalk TGF-β signaling in cancer is still limited. Therefore, we propose the possible cross-linking mechanism between LIN28B and TGF-β signaling to promote EMT in cholangiocytes (Figure. 17). In addition, the excessive of EMT-inducing soluble factors like a TGF-β cytokines and secreted TGFβI can turn to bind TGFβR1, resulting in activation of TGF-β signaling. Thus, LIN28B expressing cells might be apparently use an autocrine positive feedback loop via TGF-β pathway to regulate EMT in cholangiocytes. This possible mechanism could explain more understanding that why TGF-β signaling was extremely activated in during malignant transformation of CCA [136].

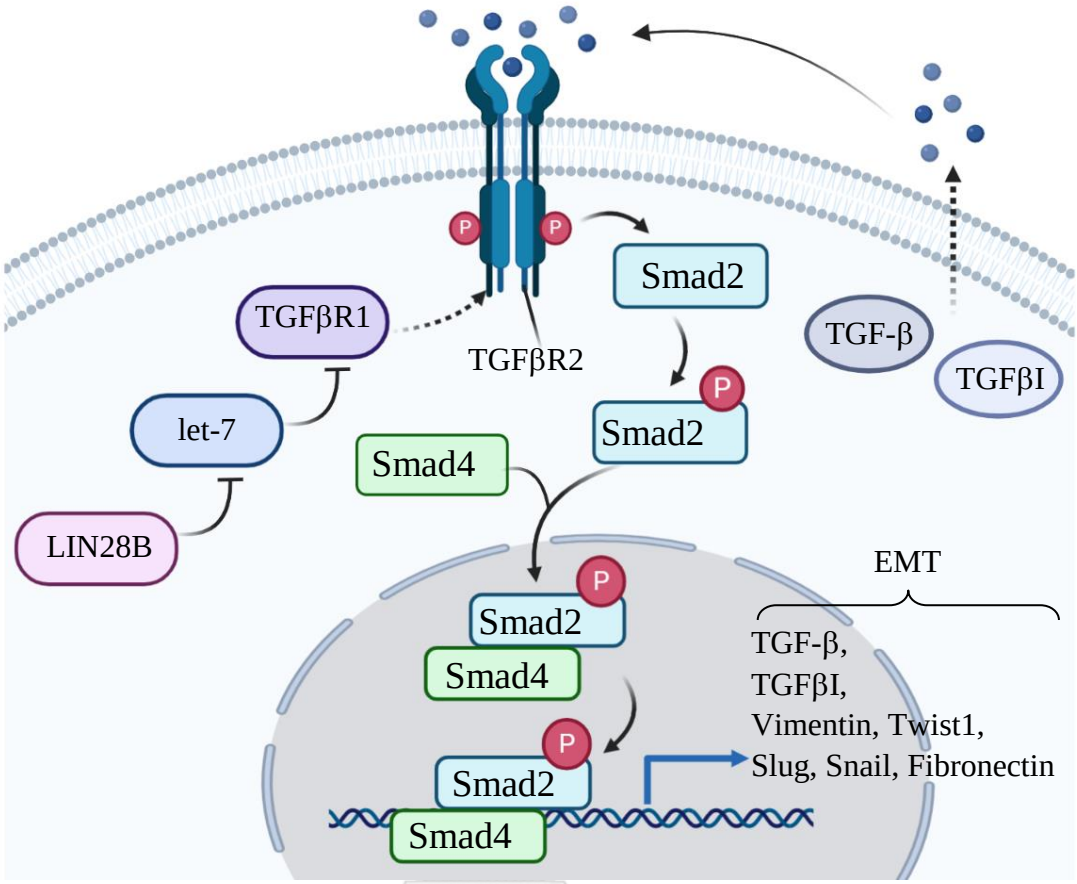


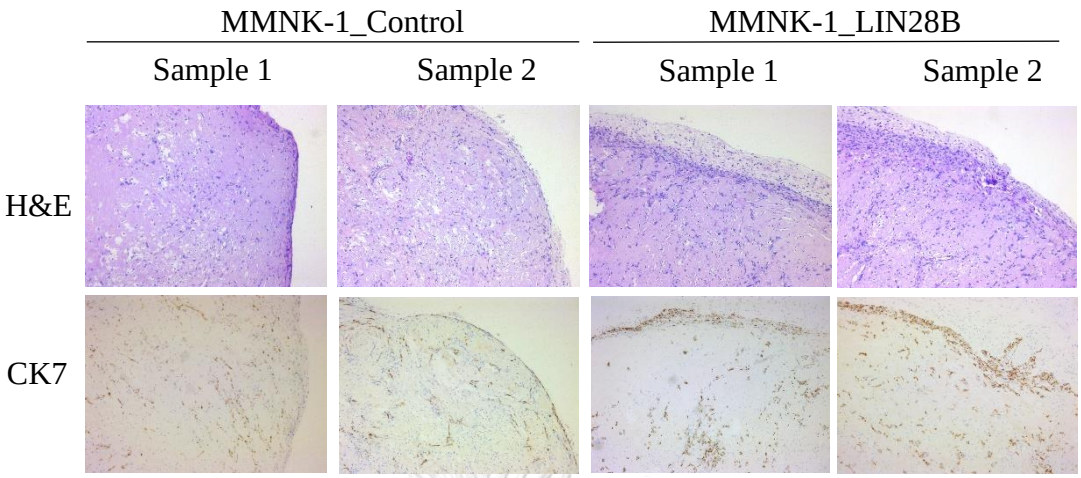
Figure 17. Diagram of possible linking of LIN28B and TGF-βsignaling induce EMT.

Based on the data of previous studies and our results, the mechanism of LIN28B induces EMT in cholangiocytes could be proposed that inhibition of let-7 miRNA by LIN28B could induce the expression of let-7 target, TGFβR1. Enhancement of TGFβR1 expression is potentially further activated the TGF-β signal, leading to stimulate the transcription of several genes such as TGF-β, TGFβI and EMT-related genes (Snail, Twist1, Vimentin, Slug and Fibronectin). These mediators are further promoting the EMT. The positive feedback loop of TGF-β signaling could be created by which the secreted ligands, TGF-β and TGFβI turn to bind its receptor, TGFβR1.

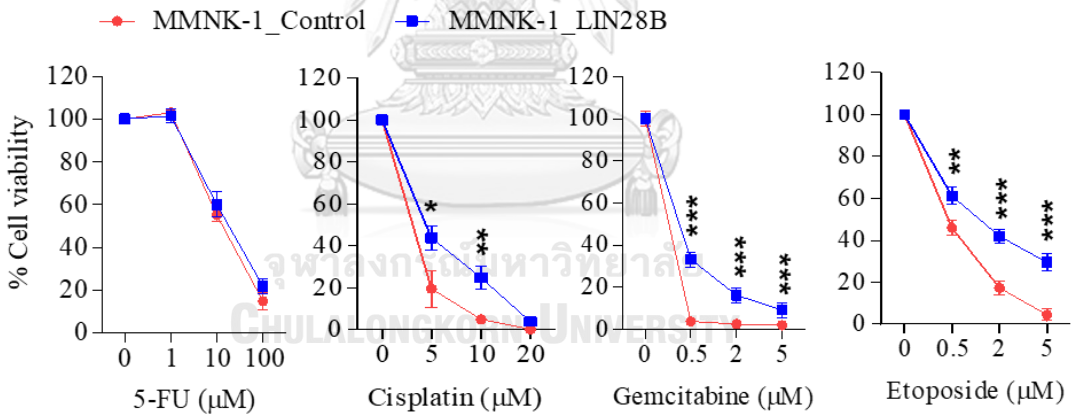
Accumulating evidence clearly suggests that EMT is not only caused cell metastasis, but also contributes to chemoresistance [137, 138]. This study also demonstrated that LIN28B-overexpressing cholangiocytes had a low response to conventional chemotherapeutic drugs, which are commonly used as first- line therapy in CCA (Appendices section; supplementary figure 2). Similar to previous studies showed that the cancer cells undergoing EMT such as colon carcinoma cells [139] and ovarian cancer cells [140] are resisted to conventional chemotherapy. In addition, some EMT-related genes show to be an important factor in promoting anti-cancer drug resistance. For example, Twist1 was required for EMT induction and promoted resistance to chemotherapeutic drugs in prostate cancer cells [141], breast cancer cells [142] and nasopharyngeal carcinoma cells [143]. In contrast, increased chemo-sensitivity was observed in cancer cells depleted Twist1 or Snail genes [144, 145]. Collectively, we suggest that LIN28B overexpressing cholangiocytes resisted chemotherapy might partly due to LIN28B induced EMT. However, the mechanisms by which LIN28B expression leads to enhanced chemoresistance are likely to be multi-factorials. LIN28B could protect cancer cells apoptosis from DNA damage by activating several survival signaling pathways [146, 147] Thus, the other mechanisms of chemoresistance should be further validated in this context.

In conclusion, we present the evidence that LIN28B is potentially marked as one of a cancer stem cell subpopulation of CCA. Various cancer stem-like features resulted from LIN28B pathway activation contribute to enhanced tumor-initiating capacity in cholangiocytes. Mechanistically, LIN28B is possibly enhanced TGF-β signaling to promote EMT in cholangiocytes. Although, this study did not show LIN28B alone is sufficient to drive cholangiocytes transformation. However, we provide the tumorigenic potential of LIN28B in CCA and added valuable information to consider that inhibition of LIN28B pathway is an attractive therapeutic target for LIN28B expressing cancer cells.

APPENDICES



Supplementary figure 1. The Hematoxylin and Eosin (H&E) and IHC staining of Cytokeratin 7 (CK7) in the tissue of MMNK-1 control and LIN28B-overexpressing MMNK-1 cells xenograft mice. Two-tissue samples of each group were taken under the microscope magnification 100X.



Supplementary figure 2. LIN28B-overexpressing cells had a low response to anti-cancer drugs. MTT assay was used to determine the viability of LIN28B-overexpressing MMNK-1 cells and control cells treated chemo-drugs; 5-FU, Cisplatin, Gemcitabine, and Etoposide for 72 h. Data presented as mean±SD, n=3. **P*-value<0.05, ***P*-value <0.01, *** *P*-value <0.001.

Table S1. Chemicals and reagents

Chemicals and reagents	Company
Cell culture	
- Dulbecco's Modified Eagle's Medium	HyClone, GE Healthcare, USA
- RPMI 1640 medium	HyClone, GE Healthcare, USA
- Fetal bovine serum (FBS)	Gibco, Life technologies, USA
- 0.25% Trypsin-EDTA	Gibco, Life technologies, USA
- 100X Antibiotic-Antimycotic	Gibco, Life technologies, USA
- 100X Gluta Max	Gibco, Life technologies, USA
Viral packaging and infection	
- X-tremeGENE HP DNA transfection reagent	Roche, Germany
- Opti-MEM I medium	Gibco, Life technologies, USA
- Polybrene	Sigma-Aldrich, USA
- Puromycin	Sigma-Aldrich, USA
RNA extraction and RT-qPCR	
- TRIzol reagent	Ambion, Life technologies, USA
- miScript II RT kit	Qaigen, Germany
- miScript Primer Assays	Qaigen, Germany
- SYBR green /ROX qPCR master mix	Thermo Scientific, USA
SDS-PAGE and western blotting	
- RIPA lysis buffer	Thermo Scientific, USA
- Halt Protease and Phosphatase Inhibitor Cocktail (100X)	Thermo Scientific, USA
- Pierce™ BCA Protein Assay	Thermo Scientific, USA
- 4X Laemmli sample buffer	Bio-Rad, USA
- 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels	Bio-Rad, USA
- 10X transfer buffer	Bio-Rad, USA
- Odyssey Blocking Buffer (TBS)	LI-COR Biosciences, USA
- IRDye® 800CW Goat anti-Rabbit IgG	LI-COR Biosciences, USA

Chemicals and reagents	Company
MTT assay	
- 5 mg/ml MTT	Sigma-Aldrich, USA
- Dimethysulfoxide (DMSO)	Vivantis, Malaysia
Immunohistochemistry staining	
-Dako REAL™ EnVision™/HRP, Rabbit/Mouse	Dako, Denmark
- Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+ solution	Dako, Denmark
- Mayer's hematoxylin	Merck millipore, Germany
- Xylene, absolute ethanol, ethanol, 30% hydrogen peroxide	Merck millipore, Germany
Antibodies	
- LIN28A (D84C11)XP Rabbit mAb	Cell Signaling Tech, USA
- LIN28B (D4H1 Rabbit mAb	Cell Signaling Tech, USA
- β-actin (13E5) Rabbit mAb	Cell Signaling Tech, USA
- Cytokeratin 7 (EPR1619Y)	Abcam, USA
-TGFBI (EPR12078(B))	Abcam, USA
- TGF-βR1(D-1) sc-518018	Santa Cruz, USA
Boyden chamber assay	
- Trans-well insert	Corning, USA
- Paraformaldehyde	Merck millipore, Germany
- Methanol	Merck millipore, Germany
- Crystal violet powder	Amresco, USA
TGF-β assays	
- Bio-Plex Pro™ TGF-β Assays	Bio-Rad, USA
Immunofluorescence staining	
- ProLong™ Gold Antifade Mountant	Invitrogen, USA
- Bovine serum albumin (BSA)	GE healthcare, USA
- Triton X-100	Vivantis, Malaysia
- Tween20	Vivantis, Malaysia
- DAPI	Thermo Scientific, USA

Chemicals and reagents	Company
Inhibitors and chemo drugs	
- SB431542	Tocris Bioscience, UK
- Repsox	Tocris Bioscience, UK
- 5-Fluorouracil	King Chulalongkorn Hospital
- Cisplatin	King Chulalongkorn Hospital
- Gemcitabine	King Chulalongkorn Hospital
- Etoposide	King Chulalongkorn Hospital
Soft agar colony formation assay	
- Agarose powder	Amresco, USA
***In vivo* assay**	
- Matrigel	Corning, USA
Sphere formation assay	
-DMEM/F12 medium	HyClone, GE Healthcare, USA
- Human rh EGF	Tocris Bioscience, UK
- B-27 Supplement (50X), serum free	Gibco, Life technologies, USA
- Human rhbFGF	Tocris Bioscience, UK
Proteomic assay	
- sodium deoxycholate	Thermo Scientific, USA
- dithiothreitol (DTT)	Thermo Scientific, USA
- iodoacetamide (IA)	Thermo Scientific, USA
- Trypsin	Thermo Scientific, USA
- trifluoroacetic acid	Thermo Scientific, USA
- tetraethylammonium bromide (TEAB)	Thermo Scientific, USA
- Pierce Quantitative Fluorometric Peptide Assay	Thermo Scientific, USA
Proteomic assay	
- isotope reagent	Thermo Scientific, USA
- ammonia solution	Thermo Scientific, USA
- formic acid	Thermo Scientific, USA
- Pierce High pH Reversed-Phase Peptide Fractionation Kit.	Thermo Scientific, USA

Table S2. List of primer for qRT-PCR analysis

Gene	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
LIN28B	CATCTCCATGATAAACCGAGAGG	GTTACCCGTATTGACTCAAGGC
CCND1	AACTACCTGGACCGCTTCCT	CCACTTGAGCTTGTTCACCA
CDC25A	GAGATCGCCTGGGTAATGAA	TGCGGAACTTCTTCAGGTCT
CDK6	AGAGACAGGAGTGGCCTTGA	TGAAAGCAAGCAAACAGGTG
CDH1	TGCCCAGAAAATGAAAAAGG	GTGTATGTGGCAATGCGTTC
VIM	GCAAAGATTCCACTTTGCGT	GAAATTGCAGGAGGAGATGC
FN1	CTGTGACAACTGCCGTAG	CAGCTTCTCCAAGCATCG
TWIST1	GTCCGCAGTCTTACGAGGAG	CCAGCTTGAGGGTCTGAATC
SNAI1	CACTATGCCGCGCTCTTTC	GGTCGTAGGGCTGCTGGAA
SNAI2	CTTTTTCTTGCCCTCACTGC	GCTTCGGAGTGAAGAAATGC
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

Table S3. Immunointensity scoring of LIN28B staining in CCA tissues.

Sample No.	Age (year)	Tumor stage	Tumor histo-types	Assessor 1		Assessor 2		Average score	
				Normal adjacent-tumor	Tumor	Normal adjacent-tumor	Tumor	Normal adjacent-tumor	Tumor
1	45	IV	well differentiated	1	3	1	3	1	3
2	71	IV	well differentiated	2	3	3	3	2.5	3
3	84	II	well differentiated	1	2	1	3	1	2.5
4	77	IV	well differentiated	2	3	2	3	2	3
5	62	IV	well differentiated/papillary	1	3	1	3	1	3
6	69	IV	moderately differentiated/ papillary	1	3	1	3	1	3
7	54	IV	well differentiated	1	3	1	3	1	3
8	64	III	well differentiated	1	2	2	3	1.5	2.5
9	77	III	poorly differentiated	1	2	1	2	1	2
10	60	IV	well differentiated	1	3	2	3	1.5	3
11	65	III	moderately differentiated	2	3	2	3	2	3
12	51	IV	well differentiated	2	3	2	3	2	3
13	70	IV	moderate differentiated	3	3	2	3	2.5	3
14	77	IV	moderately differentiated	1	3	1	3	1	3
15	52	IV	moderately differentiated	1	3	1	3	1	3
16	47	IV	moderately differentiated	1	2	1	3	1	2.5
17	81	IV	moderately differentiated	1	3	1	3	1	3
18	59	IV	moderately differentiated	1	3	1	3	1	3
19	68	IV	moderately differentiated	1	3	1	3	1	3
20	61	II	well differentiated	1	3	1	3	1	3
21	55	II	poorly differentiated	1	1	2	2	1.5	1.5
22	62	IV	well differentiated	1	3	1	3	1	3
23	46	IV	moderately differentiated	1	3	2	3	1.5	3
24	78	II	poorly differentiated	1	2	1	3	1	2.5
25	88	IV	well differentiated	1	3	1	3	1	3
26	76	IV	moderately differentiated	2	3	2	3	2	3
27	89	IV	well differentiated	2	3	2	3	2	3
28	60	IV	well differentiated	1	3	1	3	1	3

Sample No.	Age (year)	Tumor stage	Tumor histo-types	Assessor 1		Assessor 2		Average score	
				Normal adjacent-tumor	Tumor	Normal adjacent-tumor	Tumor	Normal adjacent-tumor	Tumor
29	63	IV	moderately differentiated	2	3	2	3	2	3
30	75	IV	well differentiated	1	2	2	3	1.5	2.5
31	43	II	poorly differentiated	1	2	1	3	1	2.5
32	57	II	well differentiated	1	3	2	3	1.5	3

REFERENCES

1. Khan, S.A., et al., *Guidelines for the diagnosis and treatment of cholangiocarcinoma: an update.* Gut, 2012. **61**(12): p. 1657-69.
2. Morise, Z., et al., *Surgery and chemotherapy for intrahepatic cholangiocarcinoma.* World J Hepatol, 2010. **2**(2): p. 58-64.
3. Shaib, Y. and H.B. El-Serag, *The epidemiology of cholangiocarcinoma.* Semin Liver Dis, 2004. **24**(2): p. 115-25.
4. Maemura, K., S. Natsugoe, and S. Takao, *Molecular mechanism of cholangiocarcinoma carcinogenesis.* J Hepatobiliary Pancreat Sci, 2014. **21**(10): p. 754-60.
5. Bussing, I., F.J. Slack, and H. Grosshans, *let-7 microRNAs in development, stem cells and cancer.* Trends Mol Med, 2008. **14**(9): p. 400-9.
6. Balzeau, J., et al., *The LIN28/let-7 Pathway in Cancer.* Front Genet, 2017. **8**: p. 31.
7. Nguyen, L.H., et al., *Lin28b is sufficient to drive liver cancer and necessary for its maintenance in murine models.* Cancer Cell, 2014. **26**(2): p. 248-61.
8. Gao, Y., et al., *IMP3 expression is associated with poor outcome and epigenetic deregulation in intrahepatic cholangiocarcinoma.* Hum Pathol, 2014. **45**(6): p. 1184-91.
9. Yang, H., et al., *A mouse model of cholestasis-associated cholangiocarcinoma and transcription factors involved in progression.* Gastroenterology, 2011. **141**(1): p. 378-88, 388 e1-4.
10. Uttaravichien, T., et al., *Intrahepatic cholangiocarcinoma in Thailand.* J Hepatobiliary Pancreat Surg, 1999. **6**(2): p. 128-35.
11. Gatto, M., et al., *Cholangiocarcinoma: update and future perspectives.* Dig Liver Dis, 2010. **42**(4): p. 253-60.
12. Khan, S.A., et al., *Cholangiocarcinoma.* Lancet, 2005. **366**(9493): p. 1303-14.
13. Weigt, J. and P. Malfertheiner, *Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer.* Expert Rev Gastroenterol Hepatol, 2010. **4**(4): p. 395-7.
14. Vatanasapt, V., S. Sriamporn, and P. Vatanasapt, *Cancer control in Thailand.* Jpn J Clin Oncol, 2002. **32 Suppl**: p. S82-91.
15. Fava, G., *Molecular mechanisms of cholangiocarcinoma.* World J Gastrointest Pathophysiol, 2010. **1**(1): p. 12-22.
16. Yongvanit, P., S. Pinlaor, and H. Bartsch, *Oxidative and nitrative DNA damage: Key events in opisthorchiasis-induced carcinogenesis.* Parasitol Int, 2011.
17. Sripa, B., et al., *Liver fluke induces cholangiocarcinoma.* PLoS Med, 2007. **4**(7): p. e201.
18. Pinlaor, S., et al., *Oxidative and nitrative stress in Opisthorchis viverrini-infected hamsters: an indirect effect after praziquantel treatment.* Am J Trop Med Hyg, 2008. **78**(4): p. 564-73.
19. Okuda, K., Y. Nakanuma, and M. Miyazaki, *Cholangiocarcinoma: recent progress. Part 1: epidemiology and etiology.* J Gastroenterol Hepatol, 2002. **17**(10): p. 1049-55.
20. Shin, H.R., et al., *Epidemiology of cholangiocarcinoma: an update focusing on risk factors.* Cancer Sci, 2010. **101**(3): p. 579-85.

21. Kamangar, F., G.M. Dores, and W.F. Anderson, *Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world.* J Clin Oncol, 2006. **24**(14): p. 2137-50.
22. Sriamporn, S., et al., *Prevalence of Opisthorchis viverrini infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand.* Trop Med Int Health, 2004. **9**(5): p. 588-94.
23. Sripa, B. and C. Pairojkul, *Cholangiocarcinoma: lessons from Thailand.* Curr Opin Gastroenterol, 2008. **24**(3): p. 349-56.
24. Andersen, J.B., *Molecular pathogenesis of intrahepatic cholangiocarcinoma.* J Hepatobiliary Pancreat Sci, 2015. **22**(2): p. 101-13.
25. Ong, C.K., et al., *Exome sequencing of liver fluke-associated cholangiocarcinoma.* Nat Genet, 2012. **44**(6): p. 690-3.
26. Robertson, S., et al., *The frequency of KRAS and BRAF mutations in intrahepatic cholangiocarcinomas and their correlation with clinical outcome.* Hum Pathol, 2013. **44**(12): p. 2768-73.
27. Mott, J.L. and G.J. Gores, *Targeting IL-6 in cholangiocarcinoma therapy.* Am J Gastroenterol, 2007. **102**(10): p. 2171-2.
28. Isomoto, H., et al., *Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells.* Hepatology, 2005. **42**(6): p. 1329-38.
29. Johnson, C., et al., *Interleukin-6 and its receptor, key players in hepatobiliary inflammation and cancer.* Transl Gastrointest Cancer, 2012. **1**(1): p. 58-70.
30. Isomoto, H., et al., *Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing.* Gastroenterology, 2007. **132**(1): p. 384-96.
31. Kobayashi, S., et al., *Interleukin-6 contributes to Mcl-1 up-regulation and TRAIL resistance via an Akt-signaling pathway in cholangiocarcinoma cells.* Gastroenterology, 2005. **128**(7): p. 2054-65.
32. Park, J., et al., *Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line.* Hepatology, 1999. **30**(5): p. 1128-33.
33. Loilome, W., et al., *Activated macrophages promote Wnt/beta-catenin signaling in cholangiocarcinoma cells.* Tumour Biol, 2014. **35**(6): p. 5357-67.
34. Shen, D.Y., et al., *Inhibition of Wnt/beta-catenin signaling downregulates P-glycoprotein and reverses multi-drug resistance of cholangiocarcinoma.* Cancer Sci, 2013. **104**(10): p. 1303-8.
35. Rizvi, S., et al., *Cholangiocarcinoma: molecular pathways and therapeutic opportunities.* Semin Liver Dis, 2014. **34**(4): p. 456-64.
36. Chen, L., et al., *The role of microRNA expression pattern in human intrahepatic cholangiocarcinoma.* Journal of Hepatology, 2009. **50**(2): p. 358-69.
37. Lin, K.Y., et al., *Genome-wide screen identified let-7c/miR-99a/miR-125b regulating tumor progression and stem-like properties in cholangiocarcinoma.* Oncogene, 2016. **35**(26): p. 3376-86.
38. Papaconstantinou, I., et al., *The role of microRNAs in liver cancer.* Eur J Gastroenterol Hepatol, 2012. **24**(3): p. 223-8.

39. Moss, E.G., R.C. Lee, and V. Ambros, *The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA.* Cell, 1997. **88**(5): p. 637-46.
40. Guo, Y., et al., *Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma.* Gene, 2006. **384**: p. 51-61.
41. Piskounova, E., et al., *Lin28A and Lin28B inhibit let-7 microRNA biogenesis by distinct mechanisms.* Cell, 2011. **147**(5): p. 1066-79.
42. Molenaar, J.J., et al., *LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression.* Nature Genetics, 2012. **44**(11): p. 1199-206.
43. Takashima, Y., et al., *Suppression of lethal-7b and miR-125a/b Maturation by Lin28b Enables Maintenance of Stem Cell Properties in Hepatoblasts.* Hepatology, 2016. **64**(1): p. 245-60.
44. Wan, Y., et al., *miR-125b promotes cell proliferation by directly targeting Lin28 in glioblastoma stem cells with low expression levels of miR-125b.* Neuroreport, 2014. **25**(5): p. 289-96.
45. Wang, J., et al., *MicroRNA-125b/Lin28 pathway contributes to the mesendodermal fate decision of embryonic stem cells.* Stem Cells and Development, 2012. **21**(9): p. 1524-37.
46. Borrego-Diaz, E., et al., *A potential regulatory loop between Lin28B:miR212 in androgen-independent prostate cancer.* Int J Oncol, 2014. **45**(6): p. 2421-9.
47. Jiang, X., et al., *Blockade of miR-150 maturation by MLL-fusion/MYC/LIN-28 is required for MLL-associated leukemia.* Cancer Cell, 2012. **22**(4): p. 524-35.
48. Polesskaya, A., et al., *Lin-28 binds IGF-2 mRNA and participates in skeletal myogenesis by increasing translation efficiency.* Genes Dev, 2007. **21**(9): p. 1125-38.
49. Qiu, C., et al., *Lin28-mediated post-transcriptional regulation of Oct4 expression in human embryonic stem cells.* Nucleic Acids Research, 2010. **38**(4): p. 1240-8.
50. Xu, B., K. Zhang, and Y. Huang, *Lin28 modulates cell growth and associates with a subset of cell cycle regulator mRNAs in mouse embryonic stem cells.* Rna-a Publication of the Rna Society, 2009. **15**(3): p. 357-61.
51. Xu, B. and Y. Huang, *Histone H2a mRNA interacts with Lin28 and contains a Lin28-dependent posttranscriptional regulatory element.* Nucleic Acids Research, 2009. **37**(13): p. 4256-63.
52. Hafner, M., et al., *Identification of mRNAs bound and regulated by human LIN28 proteins and molecular requirements for RNA recognition.* Rna-a Publication of the Rna Society, 2013. **19**(5): p. 613-26.
53. Graf, R., et al., *Identification of LIN28B-bound mRNAs reveals features of target recognition and regulation.* Rna Biology, 2013. **10**(7): p. 1146-59.
54. Wilbert, M.L., et al., *LIN28 binds messenger RNAs at GGAGA motifs and regulates splicing factor abundance.* Molecular Cell, 2012. **48**(2): p. 195-206.
55. Vadla, B., et al., *lin-28 controls the succession of cell fate choices via two distinct activities.* Plos Genetics, 2012. **8**(3): p. e1002588.
56. Moss, E.G. and L. Tang, *Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites.* Developmental Biology, 2003. **258**(2): p. 432-42.

57. Zhu, H., et al., *Lin28a transgenic mice manifest size and puberty phenotypes identified in human genetic association studies.* Nature Genetics, 2010. **42**(7): p. 626-30.
58. West, J.A., et al., *A role for Lin28 in primordial germ-cell development and germ-cell malignancy.* Nature, 2009. **460**(7257): p. 909-13.
59. Yu, J., et al., *Induced pluripotent stem cell lines derived from human somatic cells.* Science, 2007. **318**(5858): p. 1917-20.
60. Faas, L., et al., *Lin28 proteins are required for germ layer specification in Xenopus.* Development, 2013. **140**(5): p. 976-86.
61. Thornton, J.E. and R.I. Gregory, *How does Lin28 let-7 control development and disease?* Trends in Cell Biology, 2012. **22**(9): p. 474-482.
62. Zhu, H., et al., *The Lin28/let-7 axis regulates glucose metabolism.* Cell, 2011. **147**(1): p. 81-94.
63. Peng, S., et al., *Genome-wide studies reveal that Lin28 enhances the translation of genes important for growth and survival of human embryonic stem cells.* Stem Cells, 2011. **29**(3): p. 496-504.
64. Wang, J., et al., *Dependence of mouse embryonic stem cells on threonine catabolism.* Science, 2009. **325**(5939): p. 435-9.
65. Zhang, W.C., et al., *Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis.* Cell, 2012. **148**(1-2): p. 259-72.
66. Ma, X., et al., *Lin28/let-7 axis regulates aerobic glycolysis and cancer progression via PDK1.* Nature Communications, 2014. **5**: p. 5212.
67. Shyh-Chang, N., G.Q. Daley, and L.C. Cantley, *Stem cell metabolism in tissue development and aging.* Development, 2013. **140**(12): p. 2535-47.
68. Murray, M.J., et al., *LIN28 Expression in malignant germ cell tumors downregulates let-7 and increases oncogene levels.* Cancer Res, 2013. **73**(15): p. 4872-84.
69. Cao, D., et al., *RNA-binding protein LIN28 is a marker for testicular germ cell tumors.* Hum Pathol, 2011. **42**(5): p. 710-8.
70. Chang, T.C., et al., *Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation.* Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3384-9.
71. Tummala, R., et al., *Lin28 promotes growth of prostate cancer cells and activates the androgen receptor.* American Journal of Pathology, 2013. **183**(1): p. 288-95.
72. Iliopoulos, D., H.A. Hirsch, and K. Struhl, *An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation.* Cell, 2009. **139**(4): p. 693-706.
73. King, C.E., et al., *LIN28B promotes colon cancer progression and metastasis.* Cancer Research, 2011. **71**(12): p. 4260-8.
74. Xue, D., et al., *RNA-binding protein LIN28 is a sensitive marker of ovarian primitive germ cell tumours.* Histopathology, 2011. **59**(3): p. 452-9.
75. Hsu, K.F., et al., *Overexpression of the RNA-binding proteins Lin28B and IGF2BP3 (IMP3) is associated with chemoresistance and poor disease outcome in ovarian cancer.* Br J Cancer, 2015. **113**(3): p. 414-24.
76. Tu, H.C., et al., *LIN28 cooperates with WNT signaling to drive invasive intestinal and colorectal adenocarcinoma in mice and humans.* Genes Dev, 2015. **29**(10): p. 1074-86.

77. Hamano, R., et al., *High expression of Lin28 is associated with tumour aggressiveness and poor prognosis of patients in oesophagus cancer.* Br J Cancer, 2012. **106**(8): p. 1415-23.
78. Wang, Y.C., et al., *Lin-28B expression promotes transformation and invasion in human hepatocellular carcinoma.* Carcinogenesis, 2010. **31**(9): p. 1516-22.
79. Viswanathan, S.R., et al., *Lin28 promotes transformation and is associated with advanced human malignancies.* Nature Genetics, 2009. **41**(7): p. 843-U109.
80. Cairo, S., et al., *Stem cell-like micro-RNA signature driven by Myc in aggressive liver cancer.* Proc Natl Acad Sci U S A, 2010. **107**(47): p. 20471-6.
81. Pan, L., et al., *Lin-28 reactivation is required for let-7 repression and proliferation in human small cell lung cancer cells.* Mol Cell Biochem, 2011. **355**(1-2): p. 257-63.
82. Meder, L., et al., *LIN28B enhanced tumorigenesis in an autochthonous KRAS(G12V)-driven lung carcinoma mouse model.* Oncogene, 2018. **37**(20): p. 2746-2756.
83. Lozier, A.M., et al., *Targeting ornithine decarboxylase reverses the LIN28/Let-7 axis and inhibits glycolytic metabolism in neuroblastoma.* Oncotarget, 2015. **6**(1): p. 196-206.
84. Qin, R., et al., *LIN28 is involved in glioma carcinogenesis and predicts outcomes of glioblastoma multiforme patients.* Plos One, 2014. **9**(1): p. e86446.
85. Cleynen, I. and W.J. Van de Ven, *The HMGA proteins: a myriad of functions (Review).* Int J Oncol, 2008. **32**(2): p. 289-305.
86. Wang, X., et al., *Regulation of let-7 and its target oncogenes (Review).* Oncol Lett, 2012. **3**(5): p. 955-960.
87. Deng, K., et al., *The lncRNA-MYC regulatory network in cancer.* Tumour Biol, 2014. **35**(10): p. 9497-503.
88. Li, Y., et al., *The Lin28/let-7a/c-Myc pathway plays a role in non-muscle invasive bladder cancer.* Cell and Tissue Research, 2013. **354**(2): p. 533-41.
89. Santarpia, L., S.M. Lippman, and A.K. El-Naggar, *Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy.* Expert Opin Ther Targets, 2012. **16**(1): p. 103-19.
90. Oh, J.S., et al., *Lin28-let7 modulates radiosensitivity of human cancer cells with activation of K-Ras.* Int J Radiat Oncol Biol Phys, 2010. **76**(1): p. 5-8.
91. Pallante, P., et al., *High mobility group a proteins as tumor markers.* Front Med (Lausanne), 2015. **2**: p. 15.
92. Sterenczak, K.A., et al., *HMGA1 and HMGA2 expression and comparative analyses of HMGA2, Lin28 and let-7 miRNAs in oral squamous cell carcinoma.* Bmc Cancer, 2014. **14**: p. 694.
93. Wu, T., et al., *Increased expression of Lin28B associates with poor prognosis in patients with oral squamous cell carcinoma.* Plos One, 2013. **8**(12): p. e83869.
94. Lok, T., et al., *Immunohistochemical distinction between intrahepatic cholangiocarcinoma and pancreatic ductal adenocarcinoma.* Hum Pathol, 2014. **45**(2): p. 394-400.
95. Yu, Z., et al., *Cancer stem cells.* Int J Biochem Cell Biol, 2012. **44**(12): p. 2144-51.
96. Zhang, J., et al., *LIN28 Regulates Stem Cell Metabolism and Conversion to Primed Pluripotency.* Cell Stem Cell, 2016. **19**(1): p. 66-80.

97. Moss, E.G. and L. Tang, *Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites.* Dev Biol, 2003. **258**(2): p. 432-42.
98. Thornton, J.E. and R.I. Gregory, *How does Lin28 let-7 control development and disease?* Trends Cell Biol, 2012. **22**(9): p. 474-82.
99. Kong, D., et al., *Epithelial to mesenchymal transition is mechanistically linked with stem cell signatures in prostate cancer cells.* Plos One, 2010. **5**(8): p. e12445.
100. Albino, D., et al., *Activation of the Lin28/let-7 Axis by Loss of ESE3/EHF Promotes a Tumorigenic and Stem-like Phenotype in Prostate Cancer.* Cancer Res, 2016. **76**(12): p. 3629-43.
101. Zhou, J., et al., *LIN28B Activation by PRL-3 Promotes Leukemogenesis and a Stem Cell-like Transcriptional Program in AML.* Mol Cancer Res, 2017. **15**(3): p. 294-303.
102. Yang, X., et al., *Double-negative feedback loop between reprogramming factor LIN28 and microRNA let-7 regulates aldehyde dehydrogenase 1-positive cancer stem cells.* Cancer Res, 2010. **70**(22): p. 9463-72.
103. Meirelles, K., et al., *Human ovarian cancer stem/progenitor cells are stimulated by doxorubicin but inhibited by Mullerian inhibiting substance.* Proc Natl Acad Sci U S A, 2012. **109**(7): p. 2358-63.
104. Hayashi, S., et al., *Lin28a is a putative factor in regulating cancer stem cell-like properties in side population cells of oral squamous cell carcinoma.* Exp Cell Res, 2013. **319**(8): p. 1220-8.
105. Chien, C.S., et al., *Lin28B/Let-7 Regulates Expression of Oct4 and Sox2 and Reprograms Oral Squamous Cell Carcinoma Cells to a Stem-like State.* Cancer Res, 2015. **75**(12): p. 2553-65.
106. Peng, F., et al., *H19/let-7/LIN28 reciprocal negative regulatory circuit promotes breast cancer stem cell maintenance.* Cell Death Dis, 2017. **8**(1): p. e2569.
107. Cai, W.Y., et al., *The Wnt-beta-catenin pathway represses let-7 microRNA expression through transactivation of Lin28 to augment breast cancer stem cell expansion.* J Cell Sci, 2013. **126**(Pt 13): p. 2877-89.
108. Cheng, S.W., et al., *Lin28B is an oncofetal circulating cancer stem cell-like marker associated with recurrence of hepatocellular carcinoma.* Plos One, 2013. **8**(11): p. e80053.
109. Alajez, N.M., et al., *Lin28b promotes head and neck cancer progression via modulation of the insulin-like growth factor survival pathway.* Oncotarget, 2012. **3**(12): p. 1641-52.
110. Kugel, S., et al., *SIRT6 Suppresses Pancreatic Cancer through Control of Lin28b.* Cell, 2016. **165**(6): p. 1401-1415.
111. Hu, Q., et al., *Lin28B is a novel prognostic marker in gastric adenocarcinoma.* Int J Clin Exp Pathol, 2014. **7**(8): p. 5083-92.
112. Zhong, Y., et al., *The interaction of Lin28A/Rho associated coiled-coil containing protein kinase2 accelerates the malignancy of ovarian cancer.* Oncogene, 2019. **38**(9): p. 1381-1397.
113. Teng, R., et al., *Overexpression of Lin28 Decreases the Chemosensitivity of Gastric Cancer Cells to Oxaliplatin, Paclitaxel, Doxorubicin, and Fluorouracil in Part via microRNA-107.* PLoS One, 2015. **10**(12): p. e0143716.

114. Shaw, F.L., et al., *A detailed mammosphere assay protocol for the quantification of breast stem cell activity.* J Mammary Gland Biol Neoplasia, 2012. **17**(2): p. 111-7.
115. Manuel Iglesias, J., et al., *Mammosphere formation in breast carcinoma cell lines depends upon expression of E-cadherin.* Plos One, 2013. **8**(10): p. e77281.
116. Makjaroen, J., et al., *Comprehensive Proteomics Identification of IFN-lambda3-regulated Antiviral Proteins in HBV-transfected Cells.* Mol Cell Proteomics, 2018. **17**(11): p. 2197-2215.
117. Bahmad, H.F., et al., *Sphere-Formation Assay: Three-Dimensional in vitro Culturing of Prostate Cancer Stem/Progenitor Sphere-Forming Cells.* Front Oncol, 2018. **8**: p. 347.
118. King, C.E., et al., *LIN28B promotes colon cancer progression and metastasis.* Cancer Res, 2011. **71**(12): p. 4260-8.
119. Wang, Y., et al., *Lin28B facilitates the progression and metastasis of pancreatic ductal adenocarcinoma.* Oncotarget, 2017. **8**(36): p. 60414-60428.
120. Thapa, N., B.H. Lee, and I.S. Kim, *TGFBIp/betaig-h3 protein: a versatile matrix molecule induced by TGF-beta.* Int J Biochem Cell Biol, 2007. **39**(12): p. 2183-94.
121. Molenaar, J.J., et al., *LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression.* Nat Genet, 2012. **44**(11): p. 1199-206.
122. Urbach, A., et al., *Lin28 sustains early renal progenitors and induces Wilms tumor.* Genes Dev, 2014. **28**(9): p. 971-82.
123. Madison, B.B., et al., *LIN28B promotes growth and tumorigenesis of the intestinal epithelium via Let-7.* Genes Dev, 2013. **27**(20): p. 2233-45.
124. Wang, M., et al., *Isolation and characterization of tumorigenic extrahepatic cholangiocarcinoma cells with stem cell-like properties.* Int J Cancer, 2011. **128**(1): p. 72-81.
125. Vaquero, J., et al., *Epithelial-mesenchymal transition in cholangiocarcinoma: From clinical evidence to regulatory networks.* J Hepatol, 2017. **66**(2): p. 424-441.
126. Moustakas, A. and C.H. Heldin, *Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression.* Cancer Sci, 2007. **98**(10): p. 1512-20.
127. Sato, T., et al., *Identification and characterization of transforming growth factor beta-induced in circulating tumor cell subline from pancreatic cancer cell line.* Cancer Sci, 2018. **109**(11): p. 3623-3633.
128. Bissey, P.A., et al., *Dysregulation of the MiR-449b target TGFBI alters the TGFbeta pathway to induce cisplatin resistance in nasopharyngeal carcinoma.* Oncogenesis, 2018. **7**(5): p. 40.
129. Guo, S.K., et al., *Enhanced Expression of TGFBI Promotes the Proliferation and Migration of Glioma Cells.* Cell Physiol Biochem, 2018. **49**(3): p. 1097-1109.
130. Yoo, H.J., et al., *Genetic and expression alterations in association with the sarcomatous change of cholangiocarcinoma cells.* Exp Mol Med, 2009. **41**(2): p. 102-15.
131. Yokobori, T. and M. Nishiyama, *TGF-beta Signaling in Gastrointestinal Cancers: Progress in Basic and Clinical Research.* J Clin Med, 2017. **6**(1).

132. Ottaviani, S., et al., *TGF-beta induces miR-100 and miR-125b but blocks let-7a through LIN28B controlling PDAC progression.* Nat Commun, 2018. **9**(1): p. 1845.
133. Chen, P.Y., et al., *FGF regulates TGF-beta signaling and endothelial-to-mesenchymal transition via control of let-7 miRNA expression.* Cell Rep, 2012. **2**(6): p. 1684-96.
134. Park, J.T., et al., *Repression of let-7 by transforming growth factor-beta1-induced Lin28 upregulates collagen expression in glomerular mesangial cells under diabetic conditions.* Am J Physiol Renal Physiol, 2014. **307**(12): p. F1390-403.
135. Bronevetsky, Y., T.D. Burt, and J.M. McCune, *Lin28b Regulates Fetal Regulatory T Cell Differentiation through Modulation of TGF-beta Signaling.* J Immunol, 2016. **197**(11): p. 4344-4350.
136. Papoutsoglou, P., C. Louis, and C. Coulouarn, *Transforming Growth Factor-Beta (TGFbeta) Signaling Pathway in Cholangiocarcinoma.* Cells, 2019. **8**(9).
137. Thiery, J.P., et al., *Epithelial-mesenchymal transitions in development and disease.* Cell, 2009. **139**(5): p. 871-90.
138. Singh, A. and J. Settleman, *EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer.* Oncogene, 2010. **29**(34): p. 4741-51.
139. Yang, A.D., et al., *Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines.* Clin Cancer Res, 2006. **12**(14 Pt 1): p. 4147-53.
140. Kajiyama, H., et al., *Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells.* Int J Oncol, 2007. **31**(2): p. 277-83.
141. Kwok, W.K., et al., *Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target.* Cancer Res, 2005. **65**(12): p. 5153-62.
142. Cheng, G.Z., et al., *Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel.* Cancer Res, 2007. **67**(5): p. 1979-87.
143. Wang, X., et al., *Identification of a novel function of TWIST, a bHLH protein, in the development of acquired taxol resistance in human cancer cells.* Oncogene, 2004. **23**(2): p. 474-82.
144. Zhuo, W.L., et al., *Short interfering RNA directed against TWIST, a novel zinc finger transcription factor, increases A549 cell sensitivity to cisplatin via MAPK/mitochondrial pathway.* Biochem Biophys Res Commun, 2008. **369**(4): p. 1098-102.
145. Zhuo, W., et al., *Knockdown of Snail, a novel zinc finger transcription factor, via RNA interference increases A549 cell sensitivity to cisplatin via JNK/mitochondrial pathway.* Lung Cancer, 2008. **62**(1): p. 8-14.
146. Lv, K., et al., *Lin28 mediates paclitaxel resistance by modulating p21, Rb and Let-7a miRNA in breast cancer cells.* PLoS One, 2012. **7**(7): p. e40008.
147. Tian, N., et al., *Lin28/let-7/Bcl-xL pathway: the underlying mechanism of drug resistance in Hep3B cells.* Oncol Rep, 2014. **32**(3): p. 1050-6.

VITA

NAME	Nattapong Puthdee
DATE OF BIRTH	17 June 1987
PLACE OF BIRTH	Sakon Nakhon
INSTITUTIONS ATTENDED	Department of Biochemistry, Faculty of Medicine, Chulalongkorn University
HOME ADDRESS	61/ 3 Kudruakham sub-district, Wanonniwat District, Sakon Nakhon, 47120
PUBLICATION	"Berberine Induces Cell Cycle Arrest in Cholangiocarcinoma Cell Lines via Inhibition of NF-κB and STAT3 Pathways", Puthdee N, Seubwai W, Vaeteewoottacharn K, Boonmars T, Cha'on U, Phoomak C, Wongkham S. Biol Pharm Bull. 2017;40(6):751-757. doi: 10.1248/bpb.b16-00428. "Establishment of an allo-transplantable hamster cholangiocarcinoma cell line and its application for in vivo screening of anti-cancer drugs", Puthdee N, Vaeteewoottacharn K, Seubwai W, Wonkchalee O, Kaewkong W, Juasook A, Pinlaor S, Pairojkul C, Wongkham C, Okada S, Boonmars T, Wongkham S. Korean J Parasitol. 2013 Dec;51(6):711-7. doi: 10.3347/kjp.2013.51.6.711. Epub 2013 Dec 31.