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**Conjugated Bile Acid and Sphingosine 1-phosphate prompt Cholangiocarcinoma Cell Growth via Releasing Exosomes**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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## List of Abbreviation

CA : Cholic acid.....	20
CAF : Cancer-associated myofibroblasts.....	38
CCA : Cholangiocarcinoma.....	5
CDCA : Chenodeoxycholic acid.....	20
DCA : Deoxycholic acid.....	20
dCCA : Distal Cholangiocarcinoma .....	5
ECM : Extracellular matrix.....	18
HCC : Hepatocellular carcinoma .....	5
HPCs : Hepatic progenitor cells.....	15
ICCA : Intrahepatic Cholangiocarcinoma .....	5
LCA : Lithocholic acid .....	20
pCCA : Perihilar Cholangiocarcinoma .....	5
PDGF : Platelet-derived growth factor .....	38
S1P : Sphingosine 1 phosphate .....	31
SCT : Secretin.....	42
SCTR : Secretin receptor .....	42
SDF-1 : stromal cell-derived factor 1-alpha .....	38
SDF-1 : Stromal cell-derived factor, 1-alpha.....	38
TCA : Taurochenodeoxycholic acid .....	31
TGF- $\beta$ : Transforming growth factor beta 1 .....	38
$\alpha$ -SMA : $\alpha$ -Smooth muscle actin .....	38

## Abstract

### **Conjugated Bile Acid and Sphingosine 1-phosphate Prompt Cholangiocarcinoma Cell Growth via Releasing Exosomes**

By Waad Alruwaili, MSc.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University  
Virginia Commonwealth University, 2019

Major Director: Huiping Zhou, Professor, Department of Microbiology and Immunology

Cholangiocarcinoma (CCA) is a fatal primary malignancy that is formed in the bile ducts. Cancer-associated myofibroblasts play a crucial role in CCA proliferation and invasion. Furthermore, there is a growing interest in the role of the exosome in the interaction between the cancer-associated myofibroblasts and cholangiocarcinoma which lead to CCA growth. However how cholangiocarcinoma-derived exosome affect the cancer-associated myofibroblasts in the tumor microenvironment remain unknown. In this study, we examined whether exosome produced by cholangiocarcinoma could involve in the prompt of CCA cells growth by regulation of myofibroblast. We found that cholangiocarcinoma-derived exosome could prompt elevated  $\alpha$ -smooth muscle actin and stromal cell-derived factor one expression that induces myofibroblast proliferation. We then demonstrated that cholangiocarcinoma-derived exosome upregulated periostin expression that plays an important role in cancer metastasis. In 3D organotypic rat CCA coculture model, TCA and S1P considerably increase the growth of CCA cell. Conclusion: cholangiocarcinoma-derived exosome trigger cancer-associated myofibroblasts proliferation in the tumor microenvironment that leads to prompt CCA growth.

## **Chapter: 1 Introduction**

### **1.1 Cancer Biology**

Cancer is characterized by uncontrolled cell growth and proliferation. Cancer cells grow and divide out control and spread and destroy normal body tissue. In addition, cancer cells acquire new features, including alterations in cell structure, size, cell adhesion properties, replication time, and the production of new enzymes. Cancers are generally classified according to the organ in which they originate, and there are more than 100 types of cancer. Although cancer is an extremely complicated and highly diverse disease with multiple types, a group of features are common among nearly all malignancies. Those characteristics are called cancer hallmarks. These hallmarks represent molecular and biochemical changes in cells that are common in most cancer types and are acquired to change a normal cell into a malignant cell <sup>1</sup> (Fig. 1).

A tumor can be defined as non-cancerous (benign) or malignant based on its growth and behavior. Benign tumors grow slowly, do not invade nearby tissues and are not life-threatening unless they interrupt the function of an organ or tissue. However, in malignant, cells can spread from where they first formed (primary cancer) to a new location in the body through blood circulation or lymph vessels to form new tumors in other areas of the body.

**Figure 1. Diagrammatic Representation of the Hallmarks of Cancer.** This diagram illustrates the six hallmarks of cancer through different mechanisms <sup>2</sup>. This image was adapted from Hanahan et al. 2011.

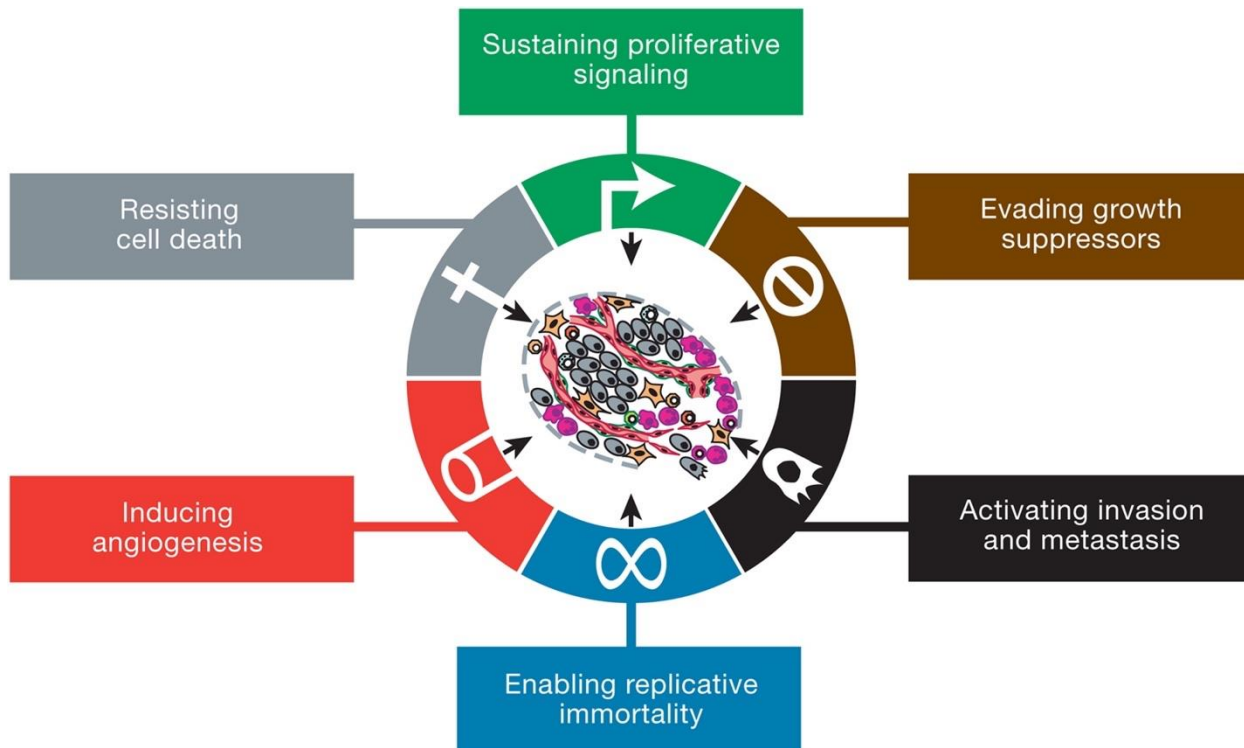


Figure 1 Diagrammatic Representation of The Hallmarks of Cancer.

### **1.1.1 Causes and Risk Factors of Cancer**

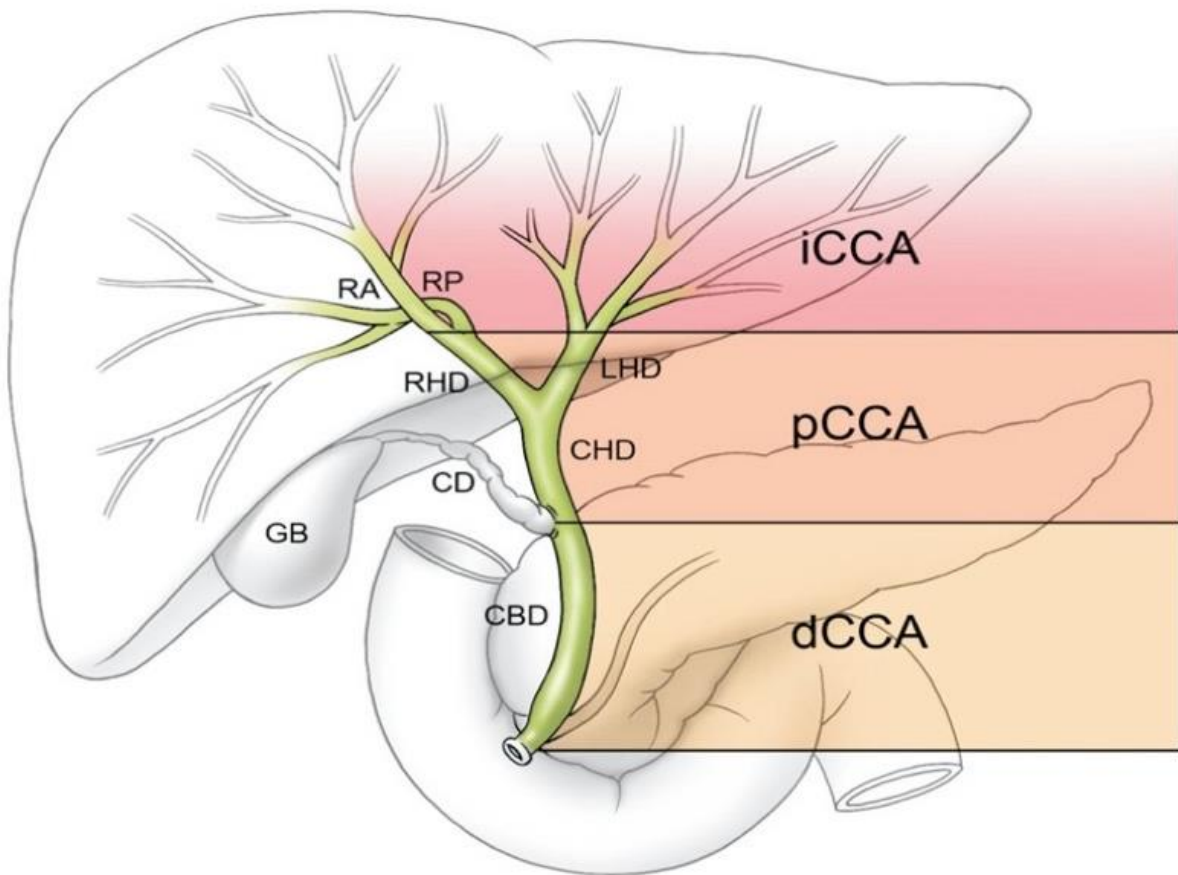
Cancer is a complicated group of diseases that can be associated with several causes. At its core, cancer is caused by changes (mutations) to the DNA within cells. This mutation directs the cell to grow and divide more rapidly. Behaviors such as smoking, excess alcohol consumption, overexposure to ultraviolet (UV) light from the sun, unhealthy diet choices, obesity, and physical inactivity can lead to cancer. Furthermore, a handful of viruses may play a role in cancer development. For instance, hepatitis B infection can lead to hepatocellular carcinoma <sup>3</sup>, Epstein-Barr infection is linked to various types of cancer <sup>4</sup> and human papillomavirus is the primary cause of cervical cancer <sup>5</sup>. However, it is not yet completely understood how many mutations must accumulate for cancer to occur.

## 1.2 Cholangiocarcinoma

Cholangiocarcinoma (CCA), or bile duct carcinoma arising from the cholangiocytes, which form a network of ducts responsible for the transport of bile acid and the production of bicarbonate lining the biliary tree. CCA is the second most common primary liver malignancy after hepatocellular carcinoma (HCC) and the most common biliary tract malignancy. Based on the anatomical localization, CCA tumors are classified into intrahepatic (ICCA), which are defined as a CCA located in bile ducts inside the liver; distal (dCCA), which are limited to the region between the origin of the cystic duct and ampulla of Vater and perihilar; and perihilar (pCCA), which are found in the area between the right and left bile ducts leaving the liver and connect to form the common hepatic duct, as shown in (Figure 2) However, this classification does not show the distinctive biological properties of CCA <sup>6</sup>. Most CCA tumors are in the perihilar and distal region, while ICC represents roughly 8–10% of all CCAs <sup>7</sup>.

**Figure 2. CCA classification.** CCA is classified based on its anatomical location inside the biliary tree into intrahepatic(iCCA), perihilar (pCCA), and distal (dCCA) Cholangiocarcinoma<sup>8</sup> This image was adapted from Blechacz. 2017.





Visual Art: © 2014 The University of Texas MD Anderson Cancer Center

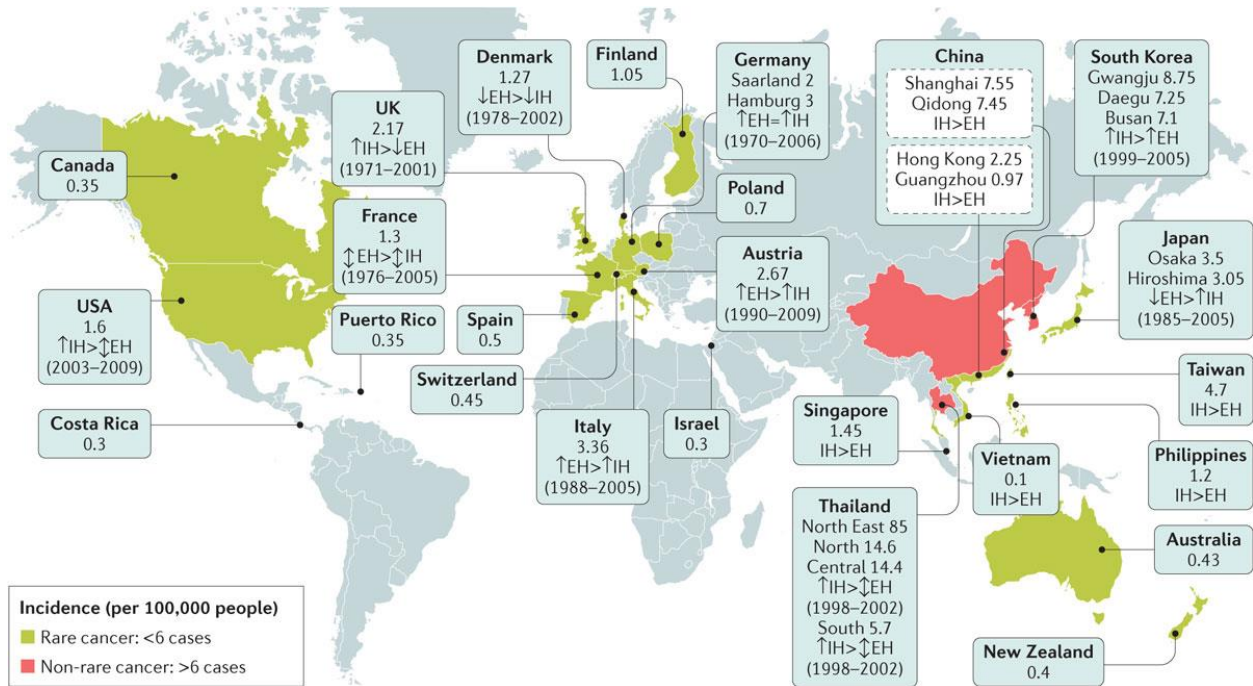
Figure 2 CCA classification

### 1.2.1 Epidemiology

An increasing number of studies have reported that the incidence and mortality of CCA have increased worldwide. For instance, in the United States, the CCA incidence rate increased 165% in 30 years to 0.95 cases per 100,000<sup>9,10</sup>. In addition, in the last decade, the CCA incidence has increased and the mortality rates were 9% greater in Europe<sup>11</sup>. The highest reported CCA prevalence was in northeast Thailand (85/100,000) and it is around 85% of the total primitive liver cancers<sup>12</sup>, demonstrating a distinct variation in its geographical distribution (Figure 3).

CCA symptoms do not develop until cancer has reached a more serious stage, and the clinical diagnosis is challenging because it is clinically silent in the patient and largely shows non-specific symptoms<sup>13</sup>. Furthermore, a substantial problem encountered with CCA is that it is usually diagnosed when intrahepatic or lymph node metastasis has already occurred and the only potentially curative treatment existing is surgical resection. However, the 5-year survival rate after surgery is less than 45%, and recurrence rates are very high (49%–64%)<sup>14</sup>.

**Figure 3. Worldwide incidence of CCA.** This map illustrates the incidence of CCA from 1971-2009. Pink color shows countries with higher incidence, while green color show countries with a low incidence <sup>15</sup>. This image was adapted from Banales et al., 2016.



Nature Reviews | Gastroenterology & Hepatology

Figure 3 Worldwide incidence of CCA

## 1.2.2 Risk factors

CCA development is usually not associated with a known risk factor, and CCA can grow in a noncirrhotic liver<sup>16</sup>. However, numerous established risk factors predispose to CCA, and the geographical distribution of CCA reflects the prevalence of its risk factors. Risk factors can include infection with hepatobiliary flukes, primary sclerosing cholangitis, and biliary duct cysts. Additionally, other risks factors are currently being studied such as smoking, obesity and diabetes mellitus.

### 1.2.2.1 Liver flukes

The hepatobiliary flukes *Opisthorchis viverrini* (*O. viverrini*) and *Clonorchis sinensis* (*C. sinensis*) are well-established CCA risk factors. Humans are infected with these flukes through the consumption of raw or undercooked foods (salted, pickled, or smoked). Both species have similar life cycles and are small flatworms that mature in the biliary duct system. Eggs produced by adult worms pass down the bile duct and exit in the feces. If the eggs reach a freshwater body, they can be swallowed by snails, which are the primary intermediate host, where they hatch and then develop into cercariae and then enter the flesh of freshwater fish, the secondary intermediate host, where they mature into metacercariae.

Chronic infection with *O. viverrini* and *C. sinensis* and persistent inflammation are crucial factors in human cancer development<sup>17</sup>. This scenario leads to biliary duct ulceration and chronic inflammation, which induce oxidative and nitrative tissue and DNA damage; these could be driving forces that lead to cholangiocarcinogenesis<sup>18</sup>.

The development of CCA is geographically associated with the liver fluke, particularly in the East Asia. *Opisthorchis viverrini* is endemic in several regions such as northeast Thailand, Laos,

and Cambodia, whereas *Clonorchis sinensis* is common in Korea, China, Taiwan and Vietnam<sup>19</sup>.

### **1.2.2.2 Primary sclerosing cholangitis**

Primary sclerosing cholangitis (PSC) is an autoimmune disease that is characterized by chronic inflammation resulting from strictures and stenosis (narrowing) of extrahepatic and/or intrahepatic bile ducts. The majority of patients do not show any symptoms. Fatigue and upper abdominal discomfort are common symptoms. In advance stages, patients can develop pruritus and jaundice due to cholestasis.

PSC is associated with the development of CCA. Chronic inflammation and the proliferation of progenitor cells are postulated mechanisms of CCA formation<sup>7</sup>. Patients diagnosed with PSC have been reported to have a lifetime prevalence of CCA ranging between 5% and 10%, and approximately half of the patients with CCA are diagnosed within 26 months following a diagnosis of PSC<sup>20</sup>.

### **1.2.2.3 Biliary stones**

Biliary stones have been associated with an increased risk of CCA. Most biliary stones are found in the gallbladder. However, they can occasionally cross the cystic duct to the intrahepatic bile ducts proximal to the right and left hepatic duct and lead to hepatolithiasis. They can also proceed to the extrahepatic bile ducts and cause a condition called choledocholithiasis. Furthermore, an estimated 7% of patients who have hepatolithiasis progress to intrahepatic CCA<sup>7</sup>.

The incidence of hepatolithiasis is low in Western countries but is a more common risk factor in numerous Asian countries where the incidence ranges from 2% to 25%<sup>21</sup>. Hepatolithiasis is a well-known risk factor for ICC.

#### **1.2.2.4 Biliary Tract Disorders**

Choledochal cysts are rare congenital dilatations of the extrahepatic and intrahepatic bile ducts and can be single or multiple. Ductal dilation occurs when pancreatic enzymes reflux into the biliary system following increased intraductal pressure and inflammation. Numerous hepatobiliary complications are associated with bile duct cysts such as cirrhosis, pancreatitis, cholelithiasis, and pancreatic duct obstruction. Bile duct cysts are also an established risk factor for CCA. Pancreatic enzymes reflux, cholestasis, and elevated bile acid concentrations have been postulated to further prompt malignant formation in patients with choledochal cysts <sup>22</sup>.

The prevalence of CCA in Asians with bile duct cysts is approximately 18%, which is higher than that in the United States where the incidence is approximately 6% <sup>23</sup>. Complete choledochal cyst removal can lower the risk of cancer, but choledochal cysts patients continue to have a greater chance of developing CCA than people who do not have choledochal cysts <sup>23</sup>.

#### **1.2.2.5 Chemical carcinogens**

Exposure to certain chemicals has been strongly associated with an increased risk of developing CCA. These include dioxin, asbestos, radon, and thorium dioxide, commercially labeled as Thorotrast <sup>9</sup>. Thorotrast is a radiographic contrast agent that was used worldwide from 1930 to 1950 as an intravascular contrast agent but was banned in 1960. Thorotrast exposure is strongly associated with a 300-fold increased risk of developing CCA <sup>9</sup>, and the average latency period associated with Thorotrast is 16-45 years after exposure. Thorotrast can deposit in the reticuloendothelial system involving the spleen, liver, bone marrow and, lymph nodes <sup>24</sup>. Nevertheless, the association between radiation exposure and hepatobiliary malignancy has not been fully established.

### **1.2.2.6 Possible Risk Factors**

A number of additional factors have been suggested to influence the risk of developing cholangiocarcinoma. More specifically, chronic viral hepatitis and cirrhosis are associated with CCA. An increasing number of studies have found that hepatitis C can be a contributing factor to iCCA<sup>25</sup>. Additionally, diabetes, obesity, tobacco smoking, and alcoholic liver disease have been implicated in CCA<sup>23</sup>. Despite the well-established association between the risk factors and CCA, the majority of patients develop CCA in the absence of identifiable risk factors.

### **1.2.3 Tumor phenotypes**

CCA presents very heterogeneous malignancies from morphological, histological, and biological standpoints. Classically, CCA has been divided into intrahepatic, perihilar and distal CCA. However, these are also classified based on their macroscopic growth pattern into mass-forming, periductal-infiltrating or intraductal-papillary as illustrated in (Figure 4). In addition, CCAs can exist as a single or mixed growth form. The masses located in the liver parenchyma enter the hepatic parenchyma through the portal venous system and invade lymphatic vessels at advanced stages. This represents the most common growth type<sup>13</sup>. The periductal-infiltrating type primarily extends along and within the bile duct. Furthermore, Histologically, 90%-95% of CCA are adenocarcinomas. The tumors show grades of differentiation (poor to moderate) with greatly desmoplastic stroma and distinct mucin expression<sup>8</sup>.

Although CCA originates from transformed biliary epithelial cells (cholangiocytes), hepatic progenitor cells (HPCs) have been suggested to also represent the cell of origin<sup>26</sup>. Tumorigenesis in CCA is a complex multistep process involving conversion from hyperplasia to dysplasia and finally to carcinoma. Chronic inflammation and partial blocking of bile flow cause chronic cholestasis and prolonged biliary cell exposure to the carcinogenetic elements in bile that

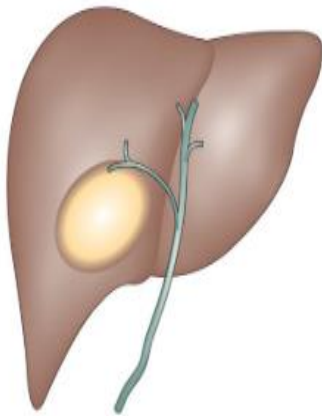


are associated with bile duct obstruction (BDO). Nevertheless, the specific mechanisms of how bile acid signals may promote CCA and how they are involved in invasive biliary tumor growth are not yet completely understood.

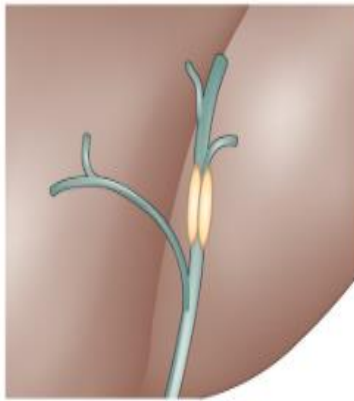
**Figure 4. Classification of CCA Based on Its Gross Morphologic.** CCA can be classified into three forms of growth: mass-forming, periductal-infiltrating, and intraductal-growth<sup>13</sup>.

Illustrations adapted from Blechacz et al., 2011.

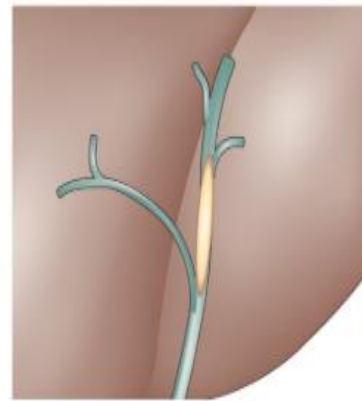
Mass-forming type



Periductal-infiltrating type



Intraductal-growth type



*Figure 4 Classification of CCA Based on Its Gross Morphologic.*

### **1.3 Metastasis and the tumor microenvironment**

Metastasis is the primary cause of mortality in patients with cancer. Cancer metastasis is the spread of tumor cells through the bloodstream or the lymphatic system and eventually forming a secondary tumor in other organs or tissues. Importantly, The majority of CCA-related deaths result from the spread of CCA cancer cells to other organs, and it has a poor-outcome due to the propensity of cancer cells to metastasize<sup>27</sup>.

Although genetic characteristics of the cancer are considered as the leading causes driving metastasis, it is also important to understanding how tumor microenvironment (TME) influence tumor progression. The TME involves of extracellular matrix (ECM), immune cells and fibroblasts.

Each component of the TME has a specific function in inducing cancer initiation, progression, invasion, and metastasis. Many TME components have been well-known to play a role in metastasis, but continuous activation of myofibroblasts plays a significant role in metastasis. In addition, myofibroblasts is the most prevalent component in CCA tumor microenvironment<sup>28</sup>.

Furthermore, the interaction between the cancer cells and myofibroblasts has a critical role in tumor development and metastasis. Yet, the mechanisms responsible for this inter-cellular communication are not widely understood so a component that is vital to our understanding is how the inter-cellular communication occurs between tumor cells and myofibroblasts that lead to CCA progression and metastasis. Cell–microenvironment interaction mediated by secreted vesicles termed exosomes is widely considered<sup>29</sup>. However, given that the role of exosomes during CCA metastasis is not elucidated, and the mechanism by which exosomes affect fibroblast remains vague. Thus, it is important to find if the exosomes mediated the

communication between tumor cells and myofibroblasts to promote cancer development and metastasis in CCA.

## 1.4 Bile Acid

### 1.4.1 Synthesis and Conjugation:

Bile acids are detergent molecules synthesized from cholesterol in a multistep process. Bile acids are amphipathic steroid molecules that can serve as powerful detergents for the absorption of lipids and fat-soluble vitamins in the intestine. Bile acids are synthesized from cholesterol in the liver through two pathways, the classical or neutral pathway and the alternative or acidic pathway. Both pathways produce the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA). In humans, the classical pathway is the primary source of bile acid biosynthesis and accounts for approximately 90% of total bile acids. The classic pathway involves the hydroxylation of cholesterol at the 7 position via ER enzyme cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) and produces 7-hydroxycholesterol in the rate-limiting step in the reaction to yield two primary bile acids, CA and CDCA, in the liver. A 12 $\alpha$ -hydroxylase is necessary to catalyze CA synthesis, and without CYP8B1, CDCA synthesis will occur. There is also an “acidic pathway” or alternative pathway that initiates the hydroxylation of cholesterol at the 27 position by the mitochondrial enzyme sterol 27-hydroxylase to yield chenodeoxycholic acid.

Primary bile acids that are formed in the liver from cholesterol are further converted by gut bacteria to secondary bile acid deoxycholic acid (DCA) and lithocholic acid (LCA) from CA and CDCA, respectively. Before being released into the biliary tree, these bile acids are conjugated with the amino acid taurine or glycine. Cholic acid and chenodeoxycholic acid are conjugated with glycine to synthesis glycocholic acid and glycochenodeoxycholic acid or with taurine to synthesis taurocholic acid and taurochenodeoxycholic acid. This conjugation increases hydrophilicity, which enhances solubility in bile and assists in bile flow.

## **1.5 Exosome**

Exosomes are the smallest group in the extracellular vesicles. Exosomes are small vesicles that are characterized by a size range from 40 nm to 100 nm in diameter and are having identical lipid bi-layer membrane from the original cell<sup>30</sup>. The size of exosomes limits their analysis, and they require the use of electron microscopy instead of light microscopy. Exosomes contain protein and RNA and have the ability to carry their cargo between cells. Trams et al. first described the exosome via electron microscopy (EM) in 1981 as exfoliated vesicles with ectoenzyme activity<sup>31</sup>. Two years later, Harding and Stahl Show that rat reticulocyte can produce small vesicles and tubules<sup>32</sup>.

### **1.5.1 Exosome origin, biogenesis, and secretion**

Exosomes are a group of heterogeneous vesicles called extracellular vesicles (EVs), which are produced and secreted by a multitude of cell types<sup>33</sup>. EVs are distinct in terms of size and biogenesis, and they include the exosome as well as apoptotic bodies and microvesicles. Apoptotic bodies are characteristic membrane blebs measuring 1000-5000 nm in diameter those are produced and released by cells undergoing apoptosis. In contrast, microvesicles are 150-1000 nm and are produced by “pinching off” from the plasma membrane. Exosomes are formed in the endocytic tract within the multivesicular body (MVB) compartments. This formation occurs intracellularly inside the endosomal/lysosomal system, which performs functions that are important for intracellular protein transferring between diverse organelles and the cell membrane. Exosomes are then released from the cell via exocytosis. There are four steps included in exosome biogenesis: initiation, endocytosis, MVBs formation, and exosome secretion.

The endosomal system also includes the exosome biogenesis processing of endocytic

vesicles, early endosomes, late endosomes, and lysosomes. The biogenesis of exosomes begins in reverse budding, causing the vesicles to carry cytosol and reveal the extracellular space of surface receptors<sup>34</sup>. The exosomes then intrinsically fuse with the plasma membrane by Ca<sup>++</sup>-dependent activation or the activation of Rab-GTPases<sup>35</sup>. Next, Rab25 controls exosome binding to and tethering with the plasma membrane and Rab27b exosome release<sup>36,37</sup>.

### **1.5.2 The molecular composition of exosomes**

Exosomes are known to have a variety of unique constituent-like lipids, proteins and nucleic acids derived from the original cells. A general overview of molecules typically found in exosomes is shown in (Figure 5)

Numerous proteins repeatedly detected in exosomal preparations are considered to be exosome markers<sup>38</sup>. Such as, tetraspanins (CD81, CD82, CD9, CD63), heat shock proteins, membrane transport and fusion proteins (Alix, TSG101).

Microvesicles contain a wide array of functional RNA involving mRNA and small noncoding RNA (miRNA)<sup>39</sup>. Functional mRNAs can convey to target cells where they are translated into proteins that alter cellular signaling. An increasing number of studies have found that different types of cells, for example, dendrite cells, B- and T-lymphocytes, and stomach cancer cells, release exosomes that contain RNA<sup>40,41</sup>.



**Figure 5. Typical content of exosome.** A schematic diagram of the overall constituents of exosome <sup>42</sup>. This image was adapted from Hu et al. 2012.

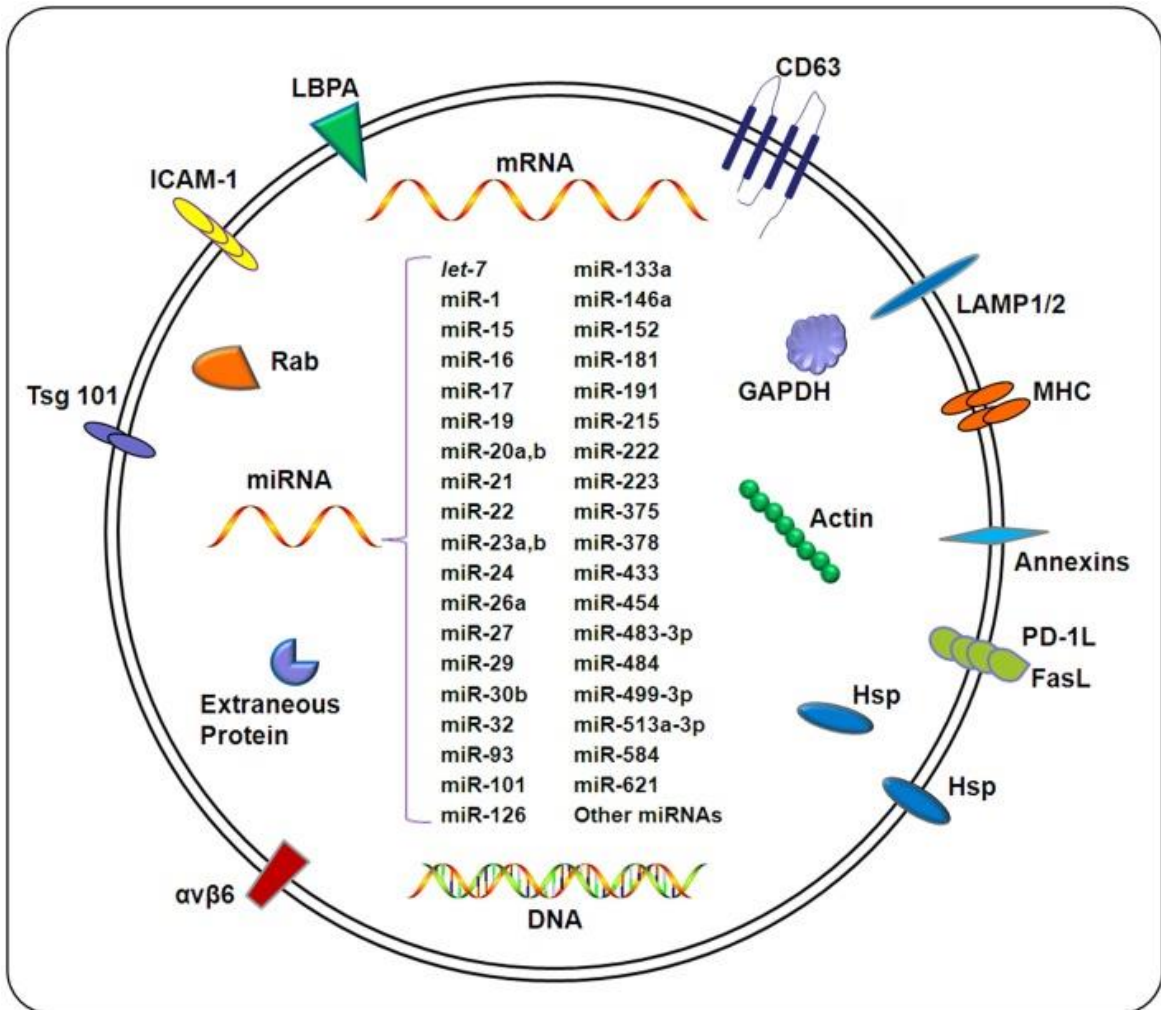


Figure 5 Typical content of exosome

### **1.5.3 Importance in normal physiology**

Exosomes are shed by most cell types, circulate in different bodily fluids (e.g., urine, blood, and saliva) and move their load to recipient cells. The presence of exosomes in normal biological fluids indicates that they are involved in physiological processes. However, the role of exosomes in normal physiology depends on their cell/tissue of origin.

A role for exosomes during pregnancy has been proposed <sup>43</sup>. Mitchell et al. showed that the human placenta produces exosomes in the maternal circulation at approximately 6–7 weeks into gestation. Hence, exosomes could play a vital role in signaling by facilitating communication between maternal and fetal circulation during pregnancy. One example of these roles is the maternal-fetal tolerance that is crucial during gestation. The placenta-derived exosomes impair maternal T-cell signaling and support maternal immune tolerance to the fetus <sup>44</sup>. In addition, the exosome complex has a significant impact on both innate and acquired immune system components. For instance, NK cells can produce exosomes that are cytotoxic against cancer cells and that stimulate immune cells <sup>45</sup>.

#### **1.5.4 Systemic effects of tumor-derived exosomes**

Metastasis begins with the dissemination of cells from the primary neoplasm to remote organs followed by their uncontrolled growth. In certain metastatic sites, the cross talk between primary tumors, stromal constituents, and the immune system is essential for the creation of a “premetastatic niche”<sup>46</sup>. Exosomes that facilitate cellular communication can be used by tumor cells in pre-metastatic niche formation. However, continuing research indicates that exosomes play a role in cancer development and progression. Recent studies proposed that cancer cells and host-stromal cells are augmented by tumor-derived exosomes to define organ-specific metastasis and acquire metastatic phenotypes<sup>47-50</sup>. Bone marrow MSC-derived exosomes (MSC-exosomes) can upregulate the vascular endothelial growth factor (VEGF) expression in cancer cells that promote tumor proliferation<sup>51</sup>. Peinado et al. stated that melanoma-derived exosomes support metastasis by augmenting bone marrow-derived progenitor cells (BMDCs)<sup>49</sup>. More recent evidence shows that exosomes that are secreted from myofibroblasts promote metastasis in breast cancer cells<sup>52</sup>. Therefore, understanding the role of the exosome in pathogenesis and metastasis on the systemic, cellular and molecular levels are vital goals of cancer research.

## **1.6 Aim of the study**

The previous study carried out by our lab shows that conjugated bile acid can induce CCA growth and invasion via S1PR2<sup>53</sup>. In addition, knowing that exosome plays a role in cell-cell communication and also, the significant influence of the exosome to modify the tumor microenvironment, I hypothesized that Sphingosine 1-phosphate prompt CCA cells growth by releasing exosome. To examine this hypothesis, I studied the following aims:

### **Aim 1: CCA-derived exosome can influence tumor microenvironment.**

1. Isolation and purification of CCA-derived exosome.
2. Demonstrating that CCA cells produce exosome.
3. Treatment of CCA associated myofibroblast (TDEsp-TDF<sub>E4</sub>) with exosome from CCA cells (BDEsp-TDE<sub>H10</sub>) pretreated with Sphingosine 1-phosphate.
4. Showing that CCA-derived exosome increases the production of growth factors from the myofibroblast.

### **Aim 2: CCA-derived exosome mediates the growth of CCA.**

- 1- Isolation of exosome following treatment of CCA cells (BDEsp-TDE<sub>H10</sub>) with Sphingosine 1-phosphate.
- 2- Establishment of CCA associated myofibroblast (TDEsp-TDF<sub>E4</sub>) and CCA cells (BDEsp-TDE<sub>H10</sub>) in 3D organotypic CA coculture model.
- 3- Evaluate CCA spheroid numbers.

### **Aim 3: Sphingosine 1-phosphate influence the content of the CCA-derived exosome.**

1. Treatment of CCA cells with Sphingosine 1-phosphate.
2. Isolation and purification of CCA-derived exosome.
3. Evaluation of the exosome content using RT-PCR.

## 2 Chapter 2: Materials and Methods

### 2.1 Cell lines used in this study

Two rat CCA cell lines were used in the present study. BDEsp tumor-derived BDEsp-TDE<sub>H10</sub> CCA cell strain (clone H10), and BDEsp tumor-derived BDEsp-TDF<sub>E4</sub> cancer-associated myofibroblastic cell strain.

#### 2.1.1 Thawing

Cryovial containing the frozen Cells were removed from liquid nitrogen tank and directly thawed in a 37°C water bath for 1 min. The cells were then transferred to a centrifuge tube containing a pre-warmed medium suitable for the cells and spun gently at 1350 g for 5 min. The cells were then resuspended in medium and transferred into the suitable culture dish. After a given time to enable attachment to the surface of the petri dish (overnight), the culture media was changed to remove the remaining DMSO.

#### 2.1.2 Freezing

Cells were gently detached from the culture dish following the same technique in the the subculture and resuspended in a complete growth medium. After counting with a cell counter and trypan blue exclusion, the cells were resuspended in culture media with DMSO and separated into tubes with one million cells each. To evade fracturing of the cell membrane due to rapid freezing, the tubes were placed in a -80°C freezer. The frozen tubes were moved to liquid nitrogen tanks for long-term storage.

### **2.1.3 Cell culturing**

Cells were maintained as adherent monolayers in a tissue culture dish in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution to prevent bacterial growth, insulin (1  $\mu\text{mol/L}$ ) and transferrin (5  $\mu\text{g/mL}$ ). For exosome isolation, the DMEM medium was supplemented with fetal bovine serum pre-depleted with exosomes by ultracentrifugation. myofibroblasts were cultured with the same supplements in the DMEM medium. All cells were cultured under sterile conditions at 37°C with 5% CO<sub>2</sub> in humidified cell culture incubators.

### **2.1.4 Subculturing**

The cells were consistently propagated as soon as they reached 80-90% confluence. Briefly, the cell culture media from the culture dish was removed. Trypsin was then added to detach the cells, and the dish was incubated for 3-5 min at 37°C. The cell was then moved to a tube with equivalent volumes of pre-warmed complete growth medium. This tube was centrifuged at 1350  $\times$  g for 5 min. The pellets were resuspended in growth medium. The suitable amount was pipetted into new cell culture dishes.

## **2.2 Exosome purification by differential centrifugation**

Exosomes were purified from the supernatant of BDEsp-TDE<sub>H10</sub> cell culture media following a protocol consisting of three successive series of centrifugation.  $7 \times 10^6$  cells were plated in a 150 mm Petri dish in DMEM supplemented with .1% FBS. The following day, the media were changed to exosome-free DMEM and treated with Taurochenodeoxycholic acid (TCA) (100  $\mu$ M), Sphingosine 1 phosphate (S1P) (100 nM), and deoxycholic acid (DCA) (50  $\mu$ M). The cells were grown to 80-85% confluence during the 48-hour culturing period.

The culture media was centrifuged at low speed at 2000 x g for 15 min at 4°C to clear from cell debris. The supernatant was transferred to polypropylene tubes (Beckman Coulter) and centrifuged at 16,000 x g for 20 min at 4°C using a WX Ultra 100 from Thermo Fisher Scientific (Waltham, MA). The supernatant was then filtered through a 0.22- $\mu$ m cellulose acetate membrane 50 ml filter system (Corning Incorporated), and the media was further centrifuged at 100000 x g for 90 min to generate the exosome pellet. The exosome pellet was dissolved in sterile phosphate-buffered saline (PBS) and stored at -80°C for further use.

## **2.3 Western blotting and immunodetection**

### **2.3.1 Cell lysates**

Confluent cells were washed with cold PBS and scraped from the culture dish. The cell was transferred in a tube and centrifuged at 10000 RPM for 5 min at 4°C. The PBS was discarded, and RIPA buffer was added to the cell pellet. The sample was vortexed at 4°C for 2 min and centrifuged at 10000 RPM for 5 min at 4°C. The supernatant then was transferred to a new tube. Finally, samples were stored at -80°C.



### **2.3.2 Gel electrophoresis**

Based on the Bradford estimate of protein concentrations, 70 µg of protein was loaded on a gel. An equal amount of the denatured samples and Precision Plus Protein™ All Blue Plus 2 Standard (Bio-Rad) were loaded into the wells of a 10% gel. The gel was inserted into an electrophoresis chamber that was loaded with running buffer. The proteins were separated by molecular weight at 80 V through the stacking gel and then run at 120 V until the dye front reaches the bottom of the gel (approximately 90 min). After separation, the gel was transmitted to the blotting device.

### **2.3.3 Blotting**

A wet blotting technique was used to move the proteins from the gel to 0.45 µm nitrocellulose membranes (Thermo, USA). The pre-run gel that was transmitted on the nitrocellulose membrane then inserted between two cassettes. During the transfer, any trapped air bubbles were removed by a blotting roller. The sandwich was then immersed in the transfer buffer. The device performed the transfer in a closed circuit according to the programmed parameters.

### **2.3.4 Blocking the membrane**

The membrane was blocked to avoid non-specific binding of the primary and/or secondary antibodies to the membrane. Immunoblots were blocked for 1 hour at RT under agitation with 5% non-fat milk in TBS buffer to prevent unspecific antibody binding to the membrane. Last, the membranes were rinsed for 5 seconds in TBST after incubation.

### **2.3.5 Antibody incubation**

Antibodies were used to detect the proteins of interest. The membrane was incubated with primary antibody (1:500) diluted in TBST buffer containing 0.1% bovine serum albumin

(BSA) overnight at 4°C under agitation. The membrane was washed three times using TBST for 15 min each to remove excess antibody solution. The membrane was then incubated with a secondary antibody (1:2000) for 1 h at room temperature and subsequently washed three times for 15 min. The antibodies of western blot are listed in Table 1.

### 2.3.6 Detection

Detection was aided by horseradish peroxidase–conjugated secondary antibodies and using HRP-conjugated secondary antibodies and ECL reagents (Thermo, USA). Images were captured and analyzed by the Bio-Rad Gel Doc XR+ Imaging System (CA, USA).

Some of the membranes were stripped and re-incubated with diverse antibodies. Stripping helped remove bound primary and secondary antibodies from a membrane.

<b>Antibody</b>	<b>Species</b>	<b>Source</b>	<b>Catalog #</b>	<b>Application/dilution</b>
CD63	Mouse	DSHB University of Iowa	H5C6	(1:500)
GRP78	Rabbit	Santa Cruz	Sc-1368	(1:500)
β -actin(JLA20)	Mouse	DSHB University of Iowa	JLA20	(1:500)

1. The antibodies of western blot.

## **2.4 RT-PCR Analysis**

### **2.4.1 Total RNA Extraction**

Total RNA was isolated From CCA exosome using TRIzol reagent (QIAGEN, Inc, Valencia, CA) and first stander of complementary DNA (cDNA) was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). In brief, 700  $\mu$ L of TRIzol™ Reagent was added directly to the culture dish to lyse the cells. Samples were incubated for 5 min at RT to allow complete separation of the nucleoproteins complex. Chloroform was then added, and the mixture was incubated for 15 min, then centrifuged for 15 min at  $12,000 \times g$  at  $4^{\circ}\text{C}$ . Next, the aqueous phase, which contains the RNA, was transferred to a new tube. Isopropanol was added, incubated for 10 minutes and centrifuged for 10 minutes at  $12,000 \times g$  at  $4^{\circ}\text{C}$ . The pellet was resuspended in 75% ethanol, then centrifuged for 5 minutes at  $7500 \times g$  at  $4^{\circ}\text{C}$ . Finally, the pellet was allowed to air dry for 5 min then resuspended with RNase-free water. The extracted total RNA was stored at  $-80^{\circ}\text{C}$  for further use.

### **2.4.2 RT-PCR Analysis**

RNA extraction was quantified using Nano Drop 2000 (Thermo Scientific, Willington, DE) and reverse transcribed into first-strand cDNA and was run on a thermo cycler (Bio-Rad CFX Connect™, Hercules, CA). The amplified reaction products were visualized by running them on DNA Gels for 15 minutes.

The mRNA levels of the targeted genes were detected by real-time PCR using iQTM SYBR Green Supermix reagents (Bio-Rad, Hercules, CA) and normalized using HPRT1 or  $\beta$ -Actin as an internal control. The primer sequences of real-time PCR are listed in Table 2. L  
SEP

Gene	Species	Accession#	Forward primer (5' to 3')	Reverse primer (5' to 3')
S1PR1	Rat	NM_017301	CTCCGACTATGGCAACTATG	GCTAACAGGTCCGAGAGG
S1PR2	Rat	NM_017192	GGAACACTACAATTACACCAAG	GCGATTAGCACCCAGAAGG
S1PR3	Rat	XM_225216	GGAGGGCAGTATGTTCGTAG	GGGCAAGATGGTAGAGCAG
SCT	Rat	NM_022670	AGCGAGGAGGACACAGAA	ACCACAGCAAGCAGAGTT
SCTR	Rat	NM_031115	TCCGAAGTTCCTCCTGAT	TCTGACCATCCATCTTGTG
SDF-1	Rat	NM_022177	ATGTCGCCAGAGCCAACG	CCACTTTAATTTTCGGGTCAATGC
POSTN	Rat	NM_001108550	AGAATGGAAGGGATGAAAG	GCGAAGTATGTGTAGGAC
TGF- $\beta$ 1	Rat	X52498	GAGAAGAACTGCTGTGTA	GGTTGTGTTGGTTGTAGA
PDGFRb	Rat	NM_031525	CTAATGAGACTGACAATGACT	AGGAGATGGTGGGAAGAAG
$\alpha$ -SMA	Rat	NM_031004	GAGTGATGGTTGGAATGG	TCGTTATAGAAGGAGTGGT
HPRT1	Rat	NM_012583	CCAGCGTCGTGATTAGTG	GCCTCCCATCTCCTTCAT
$\beta$ -Actin	Rat	NM_031144	TATCGGCAATGAGCGGTTCC	AGCACTGTGTTGGCATAGAGG

2. The primer sequences of real-time PCR

### 2.5 3-D Organic culturing.

BDEsp-TDF<sub>E4</sub> at an Initial plating density of  $4 \times 10^5$  cells and BDEsp-TDE<sub>H10</sub> at an Initial plating density  $8 \times 10^5$  were mixed together with an appropriate volume of rat tail type I collagen gel matrix (BD Biosciences) and plate in six-well culture plates. The mixture was incubated at 37C for 1 hour. Gel matrix was removed to 60-mm plate and culture overnight. After 24 hours fresh medium contains either exo-TCA, exo-DCA or exo-S1P were add to the culture. The culture medium was replaced at every 48 hours intervals. Finally, the gels were fixed immediately in 10% buffered formalin, embedded, sectioned and stained with hematoxylin and eosin.

### 2.6 Statistical Analysis.

All the results were got from at least three experiments. The results are reported as mean standard deviation. The one-way analysis of variance (ANOVA) was used to analyze the data. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Soft Inc., San Diego, CA). The Value of Significance was set at  $P < 0.05$ .

### 3 Chapter 3: Result

#### 3.1 Purification and Characterization of Exosomes Derived from Different CCA Cell Lines.

Based on exosomes' distinctive size and density, exosomes were extracted and purified from the CCA cell line (BDEsp-TDE<sub>H10</sub>). Next, transmission electron microscopy (TEM) and western blot analysis were used to characterize exosome isolation and purification. TEM analysis shows that the BDE-TDE exosomes had a distinctive saucer-shape structure that is surrounded by a lipid bilayer, and the TDE-released exosomes ranged from 50 to 150 nm in diameter, as shown in Fig. 6A

The Western blotting analysis further proved that the purified exosomes express CD63, the common marker of exosomes (Fig. 6B). Further, to confirm the purification of the exosome isolate, the GRP78 (ER marker) was examined. The data show (in Fig. 6B) the absence of GRP78 in the exosome samples in all cell lines' media.

Furthermore, Dynamic Light Scattering (DLS) analysis shows that the size of the BDEsp-TDE<sub>H10</sub>-released exosomes is around 100 nm in diameter. Interestingly, TCA (but not the other bile acid) slightly increased the number of exosomes (Fig. 6C).

Overall, DLS data and the Western blot data indicated that the vesicles released by CCA cells line are exosomes. Indeed, our extraction method, which involved successive centrifugation steps, yields purified exosomes free from cell debris.

### **3.2 BDEsp-TDEH<sub>10</sub> CCA Exosomes Affect BDEsp-TDF<sub>E4</sub> CAFs and Promote Their Secretory Factor Production.**

Exosomes are cell-derived vesicles that can act as essential intercellular communicators in the local tumor microenvironment<sup>54</sup>. Given that CAFs participate in tumor proliferation and invasion, we asked whether BDEsp-TDEH<sub>10</sub>-derived exosomes might have an effect on the CAF. To analyze whether the proliferation of CAFs was affected by CCA cancer-derived exosomes, a Cancer-associated myofibroblasts (BDEsp-TDF<sub>E4</sub>) was cultured in DMEM containing BDEsp-TDEH<sub>10</sub> cell-derived exosomes in the presence or absence of S1P (1 mg/mL) for 24 hours.

Using quantitative reverse-transcriptase PCR (qRT-PCR) analysis we confirmed the elevated expression of  $\alpha$ -Smooth muscle actin ( $\alpha$ -SMA) mRNA in BDEsp-TDF<sub>E4</sub> that was treated with BDEsp-TDEH<sub>10</sub> cancer-cell-derived exosomes treated with S1P (Fig. 7A). Moreover, a stromal cell-derived factor 1-alpha (SDF-1), a chemokine that plays an important role in the invasion and migration of CCA cells<sup>55</sup>. A qRT-PCR was used to determine the mRNA expression levels of SDF-1 in the absence or presence of S1P BDEsp-TDEH<sub>10</sub> cancer- cell-derived exosomes. Interestingly, the purified exosomes from BDEsp-TDEH<sub>10</sub> treated with S1P slightly increased the SDF-1 expression in BDEsp-TDF<sub>E4</sub> (Fig. 7B). Data showed that, compared with the untreated group, treatment with S1P cancer exosomes resulted in slight increases in SDF-1. These results indicate that CCA-derived exosomes play a role in regulating genes involved in invasion and migration.

Numerous factors produced by activated Cancer-associated myofibroblasts can considerably influence the CCA growth<sup>55</sup>. We studied whether the CCA exosome affects the production of these factors. A real-time RT-PCR was used to determine the mRNA expression levels of Transforming growth factor beta 1 (TGF-  $\beta$ ) and Platelet-deriving growth factor

(PDGF) in the absence or presence of S1P BDEsp-TDEH<sub>10</sub> cancer- cell-derived exosomes. Data showed that there is no significant increase in the expression in the TGF-  $\beta$  and PDGF in the BDEsp-TDF<sub>E4</sub> that treated with S1P cancer exosomes (Fig. 7C, 7D).

### 3.3 Effect of CCA-Derived Exosomes on Periostin Expression

Periostin is a matricellular protein. The overexpression of the periostin gene was found in different types of human cancer<sup>56</sup> which could play a crucial role in the progression and metastasization of cancer. Furthermore, it has been reported that in human and rat cholangiocarcinoma, the CCA-derived fibroblasts positive for  $\alpha$ -SMA are the exclusive origin of POSTN that is released in the desmoplastic stroma<sup>57</sup>

To determine the role of exosome on the POSTN expression, exosomes were first purified from the conditioned media of BDEsp-TDEH<sub>10</sub> treated with S1P (100 nM) for 48 hours. Then, a cancer-associated myofibroblasts (BDEsp-TDF<sub>E4</sub>) was cultured in a medium containing PBS or a BDEsp-TDEH<sub>10</sub> derived exosome (1mg/ml) for 24 hours. The results revealed that the periostin mRNA level in the cancer-associated myofibroblasts (BDEsp-TDF<sub>E4</sub>) treated with BDEsp-TDEH<sub>10</sub>-derived exosomes that were treated with S1P were higher in comparison with those in cells treated with BDEsp-TDEH<sub>10</sub>-derived exosomes that were treated with PBS, according to the RT-qPCR data (Fig. 8B). These results suggest a potential link between periostin expression and cholangiocarcinoma-derived exosomes treated with S1P.



### **3.4 CCA-Released Exosomes and BDEsp-TDFE4 CAFs Induce BDEsp-TDEH<sub>10</sub> CCA Cell Growth in Co-Culture**

After we showed that BDEsp-TDEH<sub>10</sub>-cell-derived exosomes affect the BDEsp-TDFE<sub>4</sub> CAFs, we set out to examine whether cancer-derived exosomes can stimulate the BDEsp-TDEH<sub>10</sub> growth in the co-culture. We used a 3-D co-culture model of organotypic CCA growth to examine this postulation. BDEsp-TDEH<sub>10</sub> CCA cells were cultured with BDEsp-TDFE<sub>4</sub> in a rat type 1 collagen-gel matrix that forms into 3-D duct-like structures, and a fresh medium with 0.10% FBS containing either BDEsp-TDEH<sub>10</sub>-released exosome treated with TCA (100  $\mu$ M), DCA (50  $\mu$ M) or S1P (100 nM) were added to the cultures. Subsequently, the medium was changed every 48 hours for an eight-day treatment. These results show that BDEsp-TDEH<sub>10</sub>-derived exosomes that were treated with TCA and S1P significantly increased the number of CCA cell spheroid/duct-like structures when rat BDEsp-TDEH<sub>10</sub> CCA cells were co-cultured with BDEsp-TDFE<sub>4</sub> in the collagen-gel matrix (Figs. 9A, B). The result of the 3-D co-culture model of organotypic CCA growth revealed that CCA-derived exosomes treated with TCA or S1P significantly increased CCA growth.

### 3.5 Sphingosine 1-Phosphate Increase the Release of Exosomal S1PR2.

Sphingosine 1-phosphate (S1P) is a bioactive lipid that is formed by the sphingosine kinase phosphorylation and binds to a family of five S1P-specific G protein coupled receptors (S1P<sub>1-5</sub>) that can be expressed in wide variety of tissues <sup>58</sup>. Our previous study found that CBA prompted progression and invasion of CCA by the activation of both ERK1/2 and Akt signaling <sup>59</sup>. In addition, it has been reported that breast cancer cells exosomes contain S1PR2 <sup>60</sup>. Therefore, we considered the possibility that S1P receptors could be released in the exosome from CCA cells.

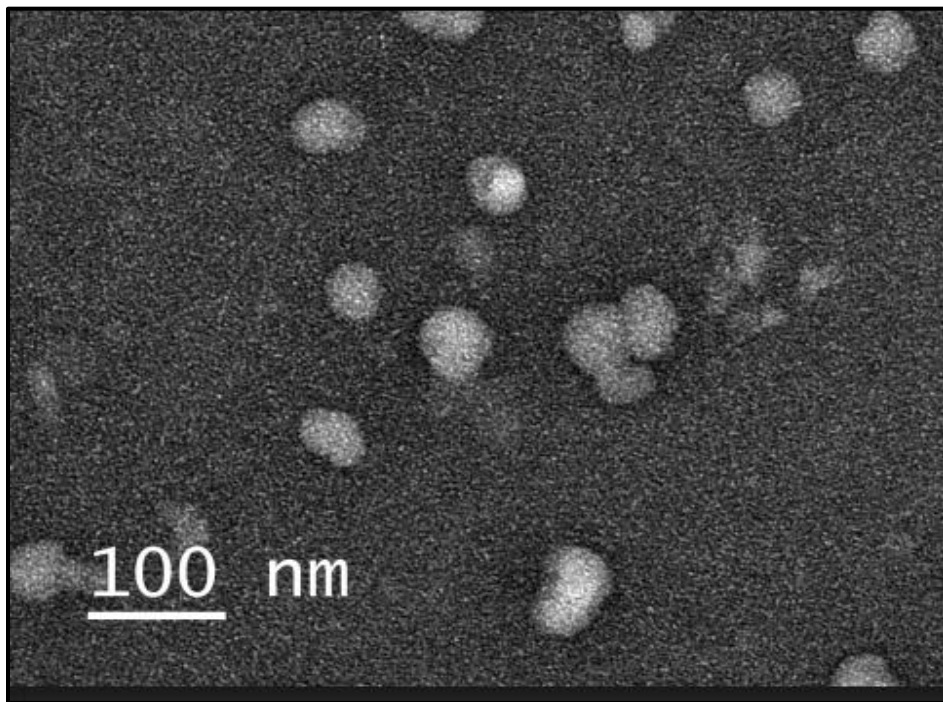
To determine the effect of conjugated bile acid and S1P on exosomes produced by CCA cells, BDEsp-TDE<sub>H10</sub> treated with TCA, DCA and S1P for 48 hours. Then, qRT-PCR was used to determine the mRNA expression of the receptors in the CCA cell line. As shown in Fig. 10, S1P significantly increased S1PR2 levels in exosomes but not S1PR1 and S1PR3 (Fig.10). This was of interest because our lab has previously demonstrated that S1PR2 is the most prevalent sphingosine 1-phosphate receptors in cholangiocarcinoma cells <sup>59</sup>.

Moreover, the secretin receptor (SCTR) is another receptor that might play a role in the CCA growth. SCTR is a G protein-coupled receptor <sup>61</sup>. SCTR has been found to be highly expressed in cholangiocarcinoma <sup>62,63</sup>. We therefore considered the possibility that SCTR could shed in the exosome from CCA cell line.

BDEsp-TDE<sub>H10</sub> cells were treated with TCA, DCA and S1P for 48 hours, and culture media were harvested for exosome isolation. The mRNA expression levels of SCT and SCTR were detected by real-time RT-PCR. From the results, it is clear that S1P had no significant effect on the SCT and SCTR levels in the cholangiocarcinoma-released exosome (Fig.11).

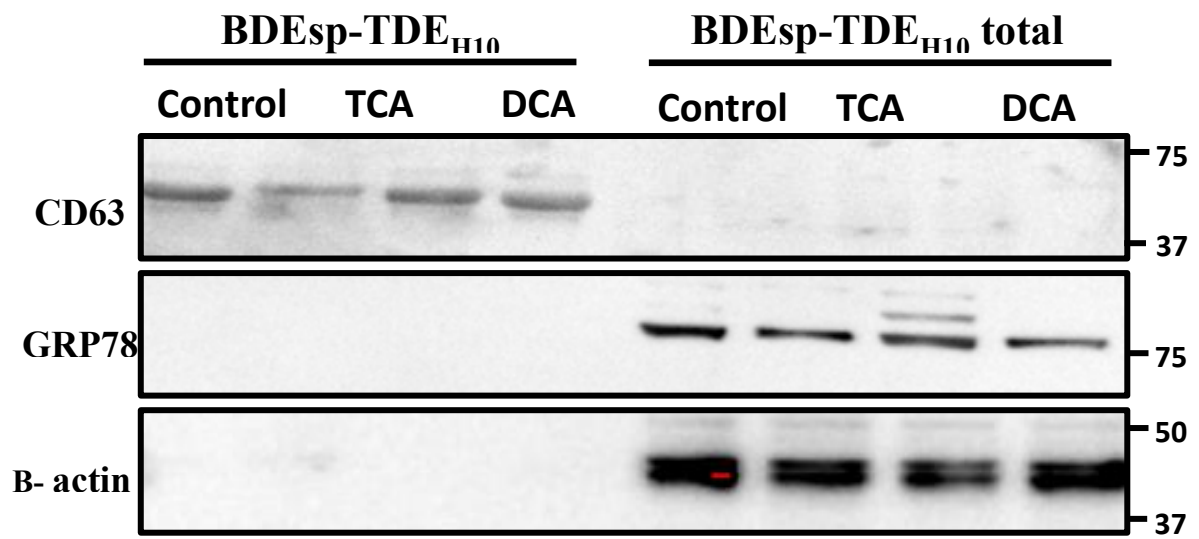
**Figure 6. Purification and Characterization of Exosomes.** BDEsp-TDEH10 cells were plated with exosome-free DMEM medium and then treated with control, TCA (100  $\mu$ M), DCA (50  $\mu$ M), or S1P (100 nM), for 48 hours. (A) Exosome isolated from BDE-TDE medium imaged by TEM. (B) Western blotting analysis of protein composition of exosomes. Protein expression levels of exosomal surface markers CD63 and GRP78 were determined by immunoblotting analysis in isolated exosomes from BDEsp-TDE<sub>H10</sub> cell-culture medium and total cell lysates. (C) DLS measurements of exosomes' size distribution and number released from BDEsp-TDE<sub>H10</sub> cells.

**6A**

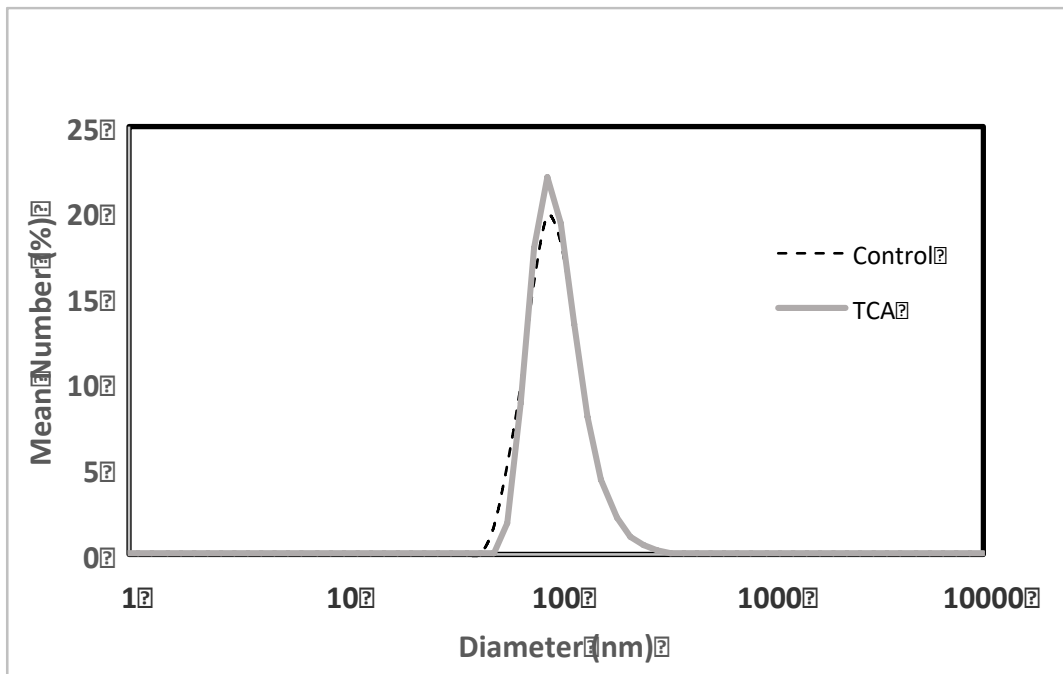


*Figure 6 Purification and Characterization of Exosomes*

6B



6C



**Figure 7. Cancer-derived exosomes induce CAF markers' expression.** (A) Relative  $\alpha$ -SMA mRNA expression levels in BDEsp-TDF<sub>E4</sub> treated with S1P exosomes isolated from BDEsp-TDE<sub>H10</sub>. (B) Relative SDF-1 mRNA expression levels in BDEsp-TDF<sub>E4</sub> derived exosomes were determined by real-time RT-PCR and normalized using HPRT1 as an internal control. (C) Relative TGF- $\beta$  mRNA expression levels in BDEsp-TDF<sub>E4</sub> in the presence or absence of tumor exosome. (D) mRNA levels of PDGF were determined by real-time RT-PCR and normalized to HPRT1 as an internal control.

7A

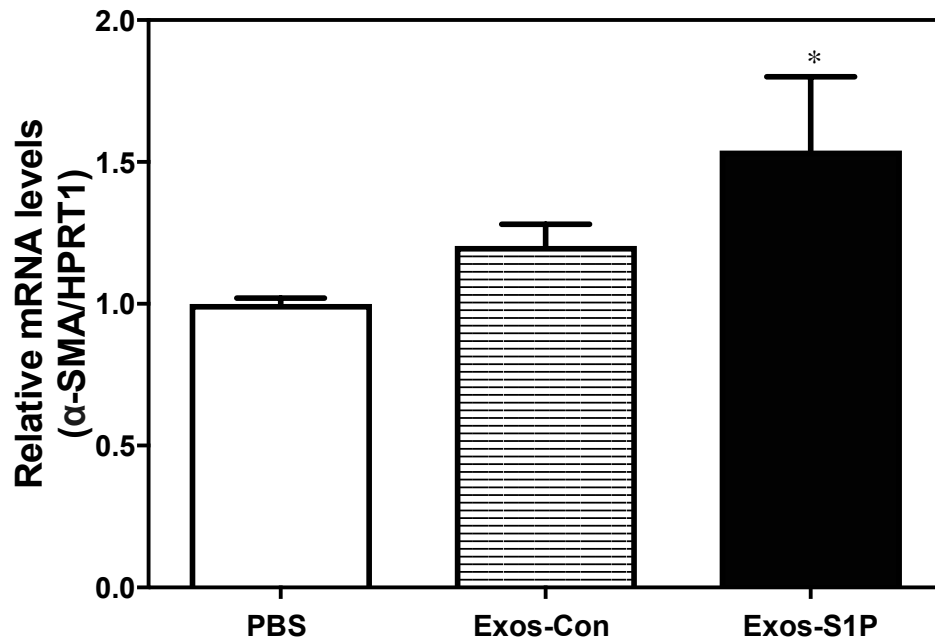
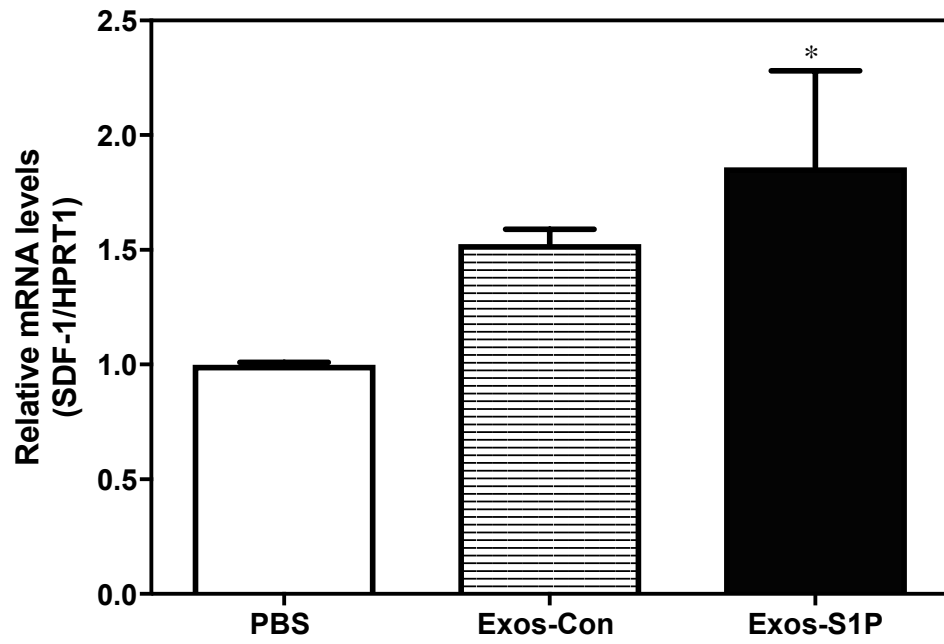


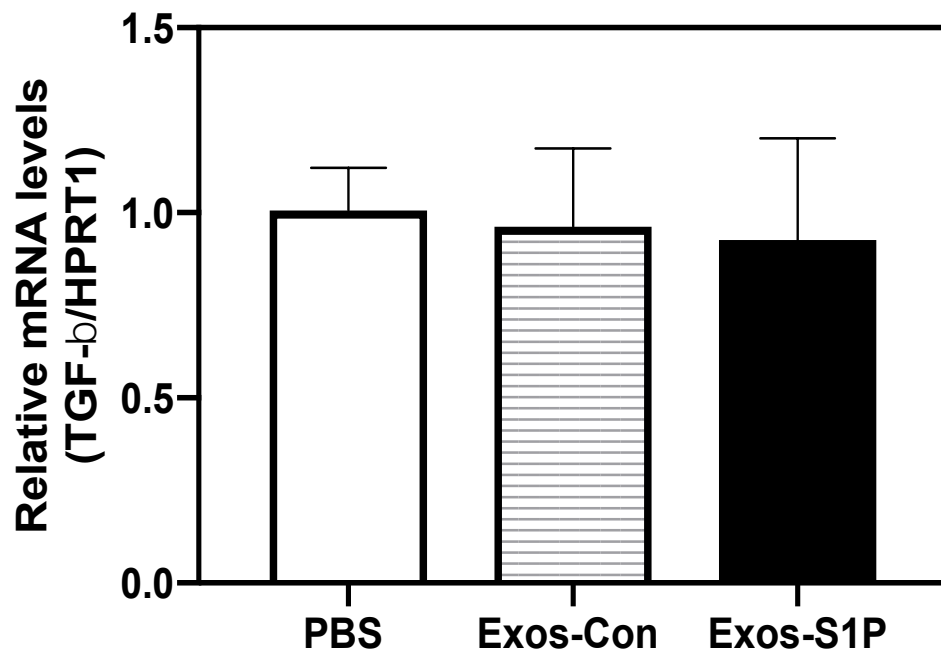
Figure 7 Cancer-derived exosomes induce CAF markers' expression.



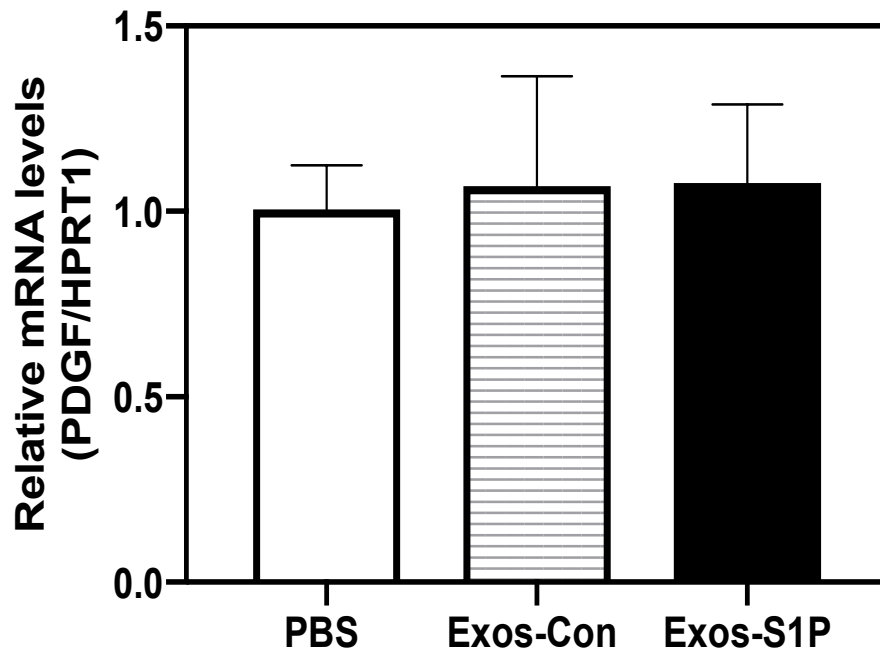
7B



7C



7D



**Figure 8. Effect of exosome on POSTN expression in cholangiocarcinoma.** BDEsp-TDF<sub>E4</sub> cells were treated with CCA exosome for 24 hours. mRNA levels of POSTN were determined by real-time RT-PCR and normalized to HPRT1 as an internal control.

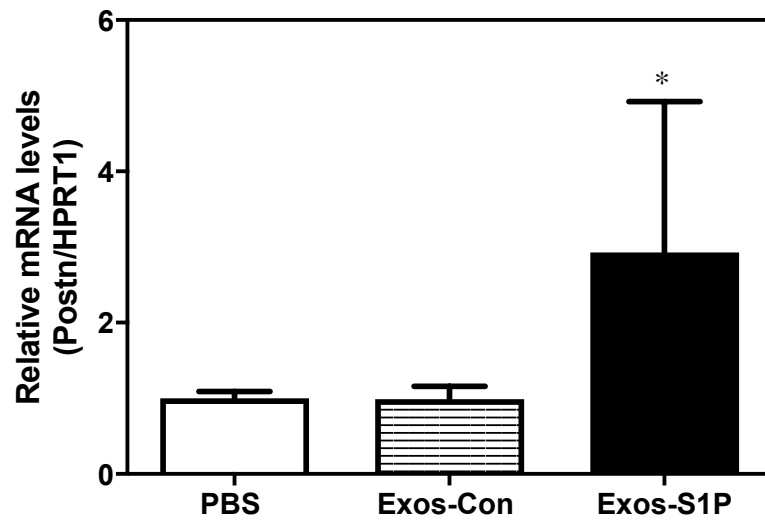
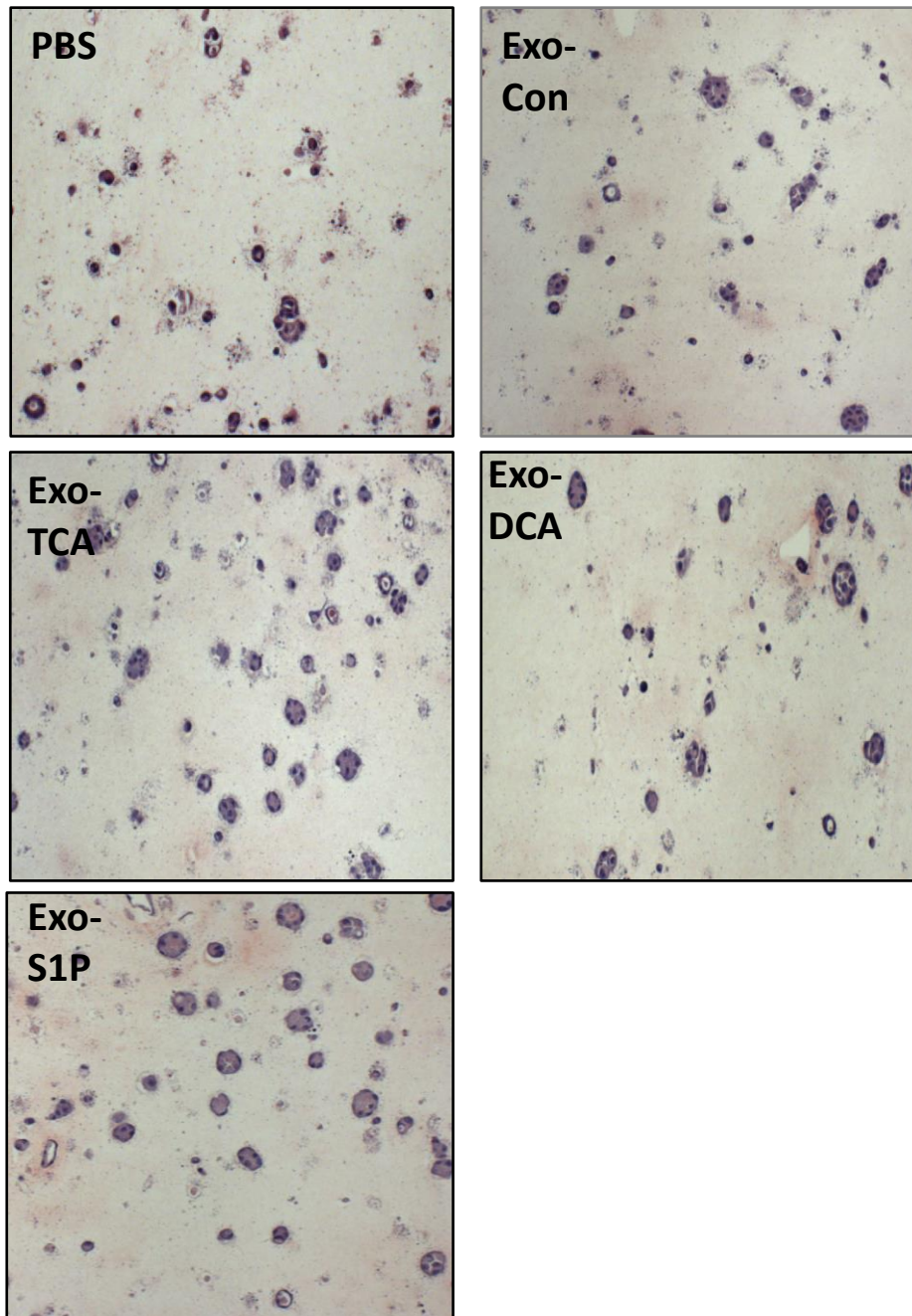


Figure 8. Effect of exosome on POSTN expression in cholangiocarcinoma

**Figure 9. Effect of BDEsp-TDE<sub>H10</sub> exosome on the growth of spheroid structures formed in three-dimensional organotypic culture of BDEsp-TDEH10 and BDEsp-TDFE4 cells.** Cells were treated with BDEsp-TDE<sub>H10</sub> exosome pretreated with or without TCA (100  $\mu$ M), DCA (50  $\mu$ M), or S1P (100 nM) for eight days. (A) Representative images of H&E staining of duct-like structures formed in vehicle control versus exosome treatment groups with TCA, DCA or S1P (B) The mean number of spheroid/duct-like structures/cm<sup>2</sup>. \*\*P < 0.01; \*\*\*\*P < 0.001, compared to control group.

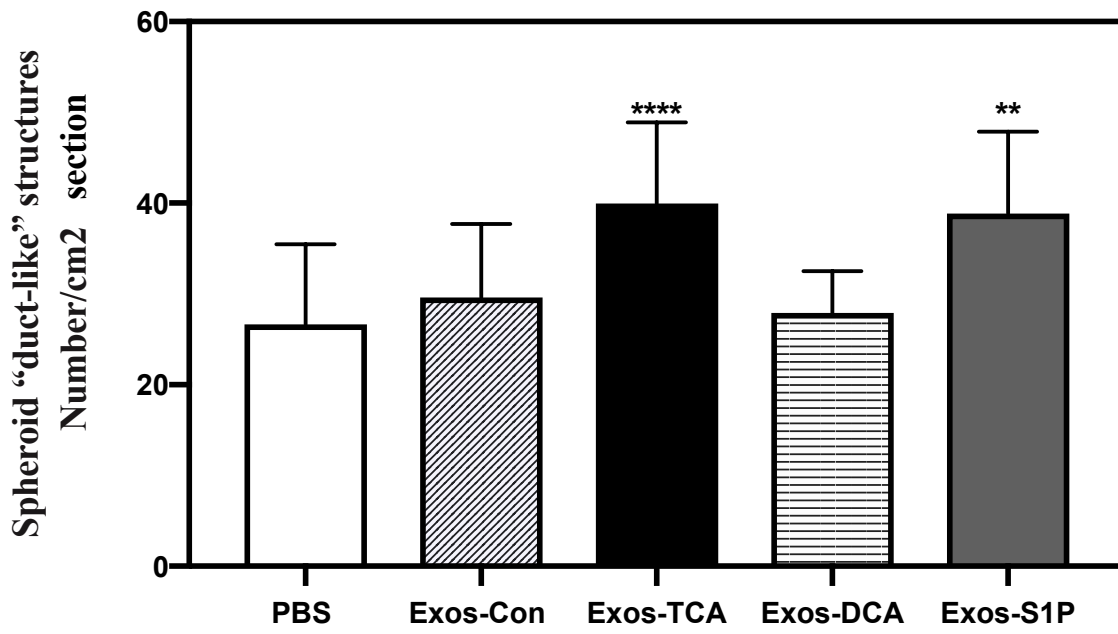
9A

**BDEsp-TDF<sub>E4</sub> + BDEsp-TDE<sub>H10</sub>**



*Figure 9* Effect of BDEsp-TDEH10 exosome on the growth of spheroid structures formed in three-dimensional organotypic culture of BDEsp-TDEH10 and BDEsp-TDFE4 cells.

# 9B





**Figure 10. Differential expression of S1PRs in CCA exosome.** Total RNA exosome was isolated from a rat BDEsp-TDEH10 cell, that was pretreated with TCA (100  $\mu$ M), DCA (50  $\mu$ M), or S1P (100 nM), for 48 hours. mRNA levels of S1PRs were determined by real-time RT-PCR (A) S1PR1. (B) S1PR2. (C) S1PR3 and normalized using  $\beta$ -Actin as an internal control. \*\*P < 0.01 compared to control group.

10A

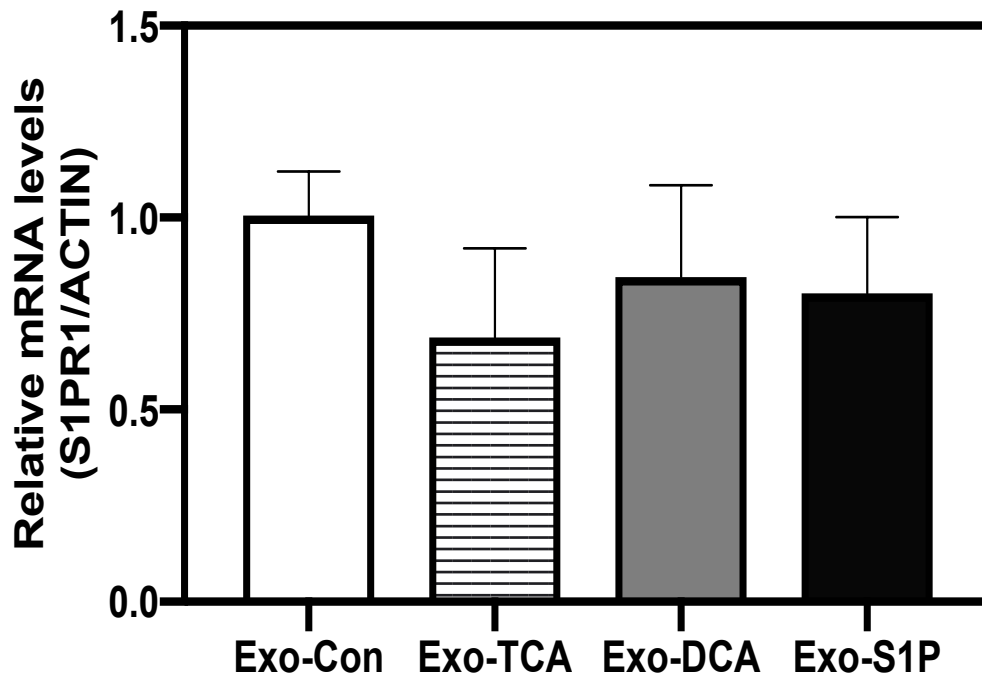
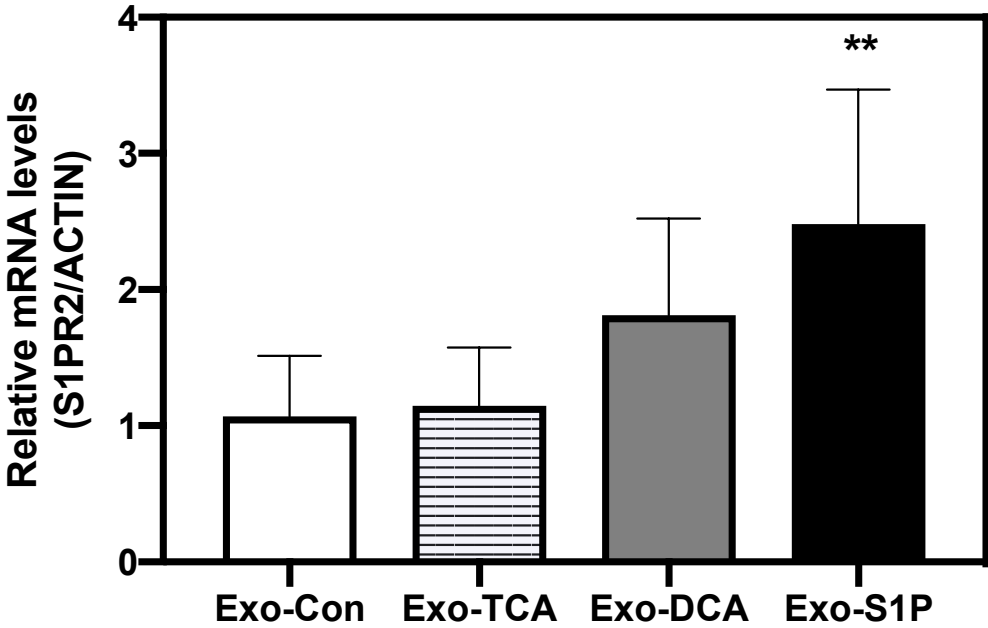
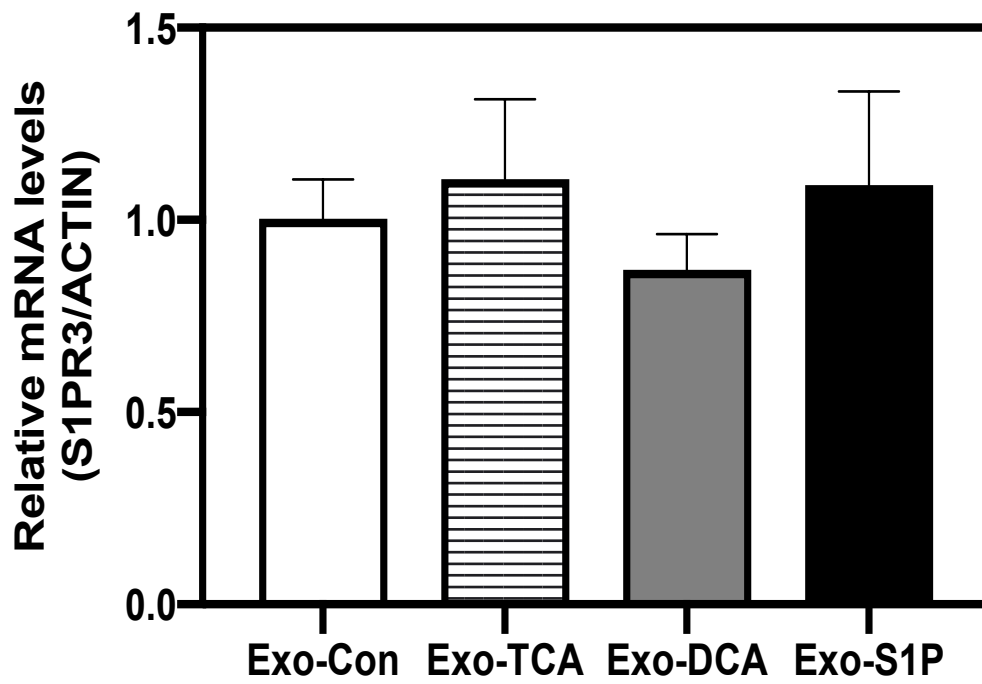


Figure 10 Differential expression of S1PRs in CCA exosome

10B



10C



**Figure11. Expression of SCT and SCTR in CCA exosome.** Relative (A) SCT and (B) SCTR mRNA levels in isolated exosomes were determined by real-time RT-PCR and normalized using  $\beta$ -Actin as an internal control.

11A

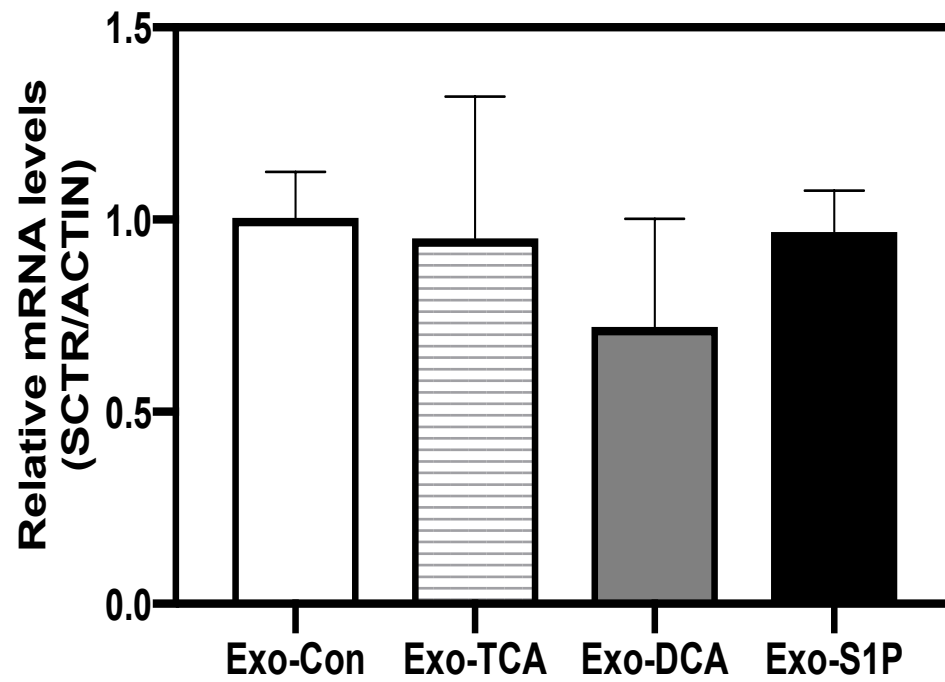
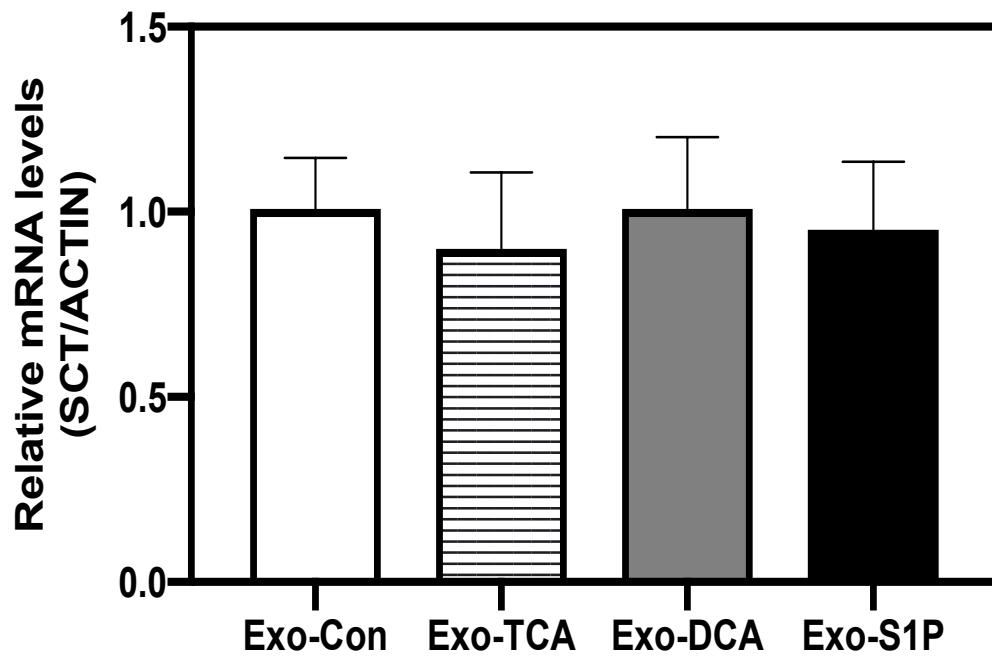


Figure 11 Expression of SCT and SCTR in CCA exosome

11B



## 4 Discussion

In recent years, there has been growing interest in the role of the exosome in the tumor microenvironment. A growing body of literature has shown that tumor-derived exosomes considerably influence the cancer-associated myofibroblasts<sup>64-67</sup>. Therefore, tumor-derived exosomes can alter the microenvironment to be more hospitable for the tumor. This study examined whether exosomes released by CCA cancer cells were involved in modulating cancer-associated myofibroblasts and increase CCA growth.

An exosome can be extracted by different methods. However, the major challenge for studying the cancer-derived exosome among these methods is to prevent intracellular microvesicles contaminants. In this study, we used differential ultracentrifugation as an isolation method. To validate the purity of isolated CCA-derived exosomes, we examined the presence of the exosome marker, the CD63 protein, and the ER marker (GRP78); which should not be present in exosomes. Our data show that CD63 was detected in the CCA-derived exosome extracts, while GRP78 was not present in the in the exosome samples. Furthermore, the result of the DLS analysis proves that the exosome size is approximately 100 nm in diameter, which agreed with the sizes previously reported<sup>68</sup>. The presence of CD63 and the lack of GRP78 in the samples, and the appropriate size of the exosome strongly suggest that our method is effective at separating CCA-derived exosome from another cellular component. This method can be used in future studies on CCA- derived exosome influence on the microenvironment.



Cancer-associated myofibroblasts (CAF) behavior is altered to significantly modify the tumor microenvironment, leading to the promotion of tumor growth, progression, and metastasis<sup>69</sup>. Moreover, tumors can use exosomes to influence myofibroblasts behavior, making exosomes an important player in the intracellular communication in the tumor microenvironment. However, there are few studies that show exosomes potentially influence on the CAF. To determine whether CCA-derived exosomes can alter CAF cells' production of pro-malignant factors, we used BDEsp-TDF<sub>E4</sub> as a model. CCA cells were treated with S1P and their exosomes isolated, these exosomes were used to treat BDEsp-TDF<sub>E4</sub> leading to an increased expression of  $\alpha$ -SMA mRNA; suggesting CCA-derived exosomes cause an increase in myofibroblasts cell's proliferation and growth advancing the progression of the tumor microenvironment. Moreover, these BDEsp-TDF<sub>E4</sub> cells also had an increase in SDF-1 mRNA levels. This is significant because SDF-1 is a soluble growth factor that is secreted by CAFs and it plays an important role in CCA invasion<sup>70</sup>. Most importantly we determined that CCA-derived exosome treatment of BDEsp-TDF<sub>E4</sub> significantly increase the expression of periostin (Fig. 7). It has been reported that periostin is involved in cholangiocarcinoma invasion<sup>71</sup>. Taken together, this data suggests that CCA-derived exosomes induce pro-malignant traits in myofibroblasts, progressing the tumor microenvironment by increasing the myofibroblasts production of soluble growth factors that promote tumor growth and increase metastasis. However, underlying mechanisms still need to be examined in future studies.

The majority of the data was based on an in vitro culture system. In order to further study the interaction between CCA-derived exosome and CAF, a 3D organotypic CCA co-culture model was employed to mimics the vivo microenvironment. CCA cells were treated with TCA

or S1P, the resulting exosomes produced were used to treat the 3D organotypic model, significantly increased the number of spheroid/duct-like in the 3D structures. This data suggests that exposure to tumor exosome increase CCA cells growth.

A number of studies have found that S1P could influence the tumor microenvironment by regulating CAF. For instance, Previous studies demonstrated that SPHK1/S1P can control communication between melanoma cancer cells and CAF <sup>72</sup>. Recently, Ashref et al. (2018) have reported that breast-released exosome contains S1PR2 that are internalized by myofibroblasts, leading to the increased activation of the S1PR2 pathway associated with growth in the myofibroblasts. <sup>60</sup>. In the present study, we further examined the presence of S1PR in the CCA-released exosome. RT-qPCR was used to determine the mRNA expression of S1PR1, S1PR2, and S1PR3 in the exosomes. It was found that treating CCA with S1P significantly increased the S1PR2 mRNA levels in the released exosomes (Fig. 9). Nonetheless, our limited finding suggests that CCA-derived exosomal-S1PR2 might increase the growth factors level in the myofibroblasts leading the progression of the tumor microenvironment. The question remains how CAF internalized the CCA-released exosomal S1PR2.

In conclusion, we have found that CCA-derived exosomes can induce pro-malignant traits of CAF cells. This is primarily due to the increase of growth factor production in CAF cells that have been exposed to CCA-derived exosomes, leading to an increase in tumor growth and metastasis. Equally importantly, the exposing CCA with bile acids and S1P, known promoters of CCA development, leads to the production of CCA-derived exosomes that are more capable of inducing pro-malignant traits in CAF. CCA-derived exosomes could be the missing link between how bile acid build-up in the bile duct results in the alters behavior of myofibroblasts while in the presence of CCA.

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