CHAPTER V
DISCUSSIONS AND CONCLUSION

5.1 Discussions

Cancer-associated fibroblasts have been recognized for their impact in the genesis, promotion and progression of many carcinomas and highlighted in several reviews (De Wever et al., 2008; Micke and Ostman, 2005). CCA is a cancer originated from biliary epithelial cells and notoriously associated with dense desmoplastic stroma with activated fibroblasts (Chuaysri et al., 2009; Okamura et al., 2005). Relatively little, however, is known about the contribution of the stromal fibroblasts in CCA. Though Chuaysri and colleagues have reported, for the first time, the effect of CCA stromal fibroblasts, with and without direct interaction with cancer cells to induce cancer cell proliferation (Chuaysri et al., 2009), the molecular mechanism of this evidence is still unexplored.

Herein, the gene expression profile of CCA-derived fibroblasts was focused in order to investigate the molecular mechanism of how fibroblasts induce a favorable microenvironment to promote cancer especially in term of fibroblast-derived substances. In addition, lists of genes having altered expression level in CCA-derived fibroblasts were obtained and the proposed function of their protein products in cancer progression were proposed. Lastly, fibroblast-derived PN was confirmed its impact as diagnostic and prognostic factors in CCA. Role and mechanism of PN to promote cell proliferation and invasion in CCA tissues were investigated.

Even though the current study is limited to a single cancer fibroblast line isolated from a single CCA patient, the validity of array results was strengthened by comparing gene expression levels in cancer fibroblasts to the two lines of normal fibroblasts; one isolated from the same CCA patient and the other from a second patient. Only genes in cancer fibroblasts altered from both normal fibroblast lines were investigated as the common up- or down-regulated genes. This is to provide evidence that the fibroblasts used in our study are valid representatives of fibroblasts found in CCA.

5.1.1 Comparison of gene expression profile of CCA-associated fibroblast and other cancers-derived fibroblasts

By comparing gene profiles in fibroblasts from CCA with those of other tumor types, it is suggested that CCA fibroblasts display not only common genotypes for activated cells but also unique characteristics. Genes involved in metabolism of cells needed to be up-regulated in order to support the active function of CCA stromal fibroblasts to produce many supporting proteins in the cancer environment. Neuropeptide Y receptor Y1 has been indicated to receive the activation signal to induce neuroproliferation (Howell et al., 2003) and doublecortin-like kinase 1, a microtubule associated active protein kinase expressed in growth cones of postmitotic neurons (Burgess and Reiner, 2000) may help facilitate fibroblast proliferation.

In similar to human basal cell carcinoma fibroblasts (Micke et al., 2007), *SPARC* or osteonectin, was also over-expressed in CCA-associated fibroblasts. SPARC-null mice were recently demonstrated to resist UV induced squamous cell carcinoma, suggesting a tumor-promoting role of SPARC (Aycock et al., 2004). In contrast to the cancer-associated fibroblasts in metastatic colon cancer to the liver which showed down-regulation of stromal cell-derive factor-1 (*SDF-1*) (Nakagawa et al., 2004), CCA-derived fibroblasts had up-regulated *SDF-1* (data not shown). SDF-1 is mentioned to promote cancer progression including growth (Liu et al., 2010) and metastasis (Zhao and Guo, 2010). Its up-regulation in CCA fibroblast may contribute to cancer progression.

5.1.2 Gene expression profile of CCA-fibroblast reveals genes related to cancer progression

5.1.2.1 Up-regulated gene profile

Focus on the up-regulated genes with high intensities (Table 4-1), CCA-associated fibroblasts showed several interesting functions involved in cancer progression. Serpin peptidase inhibitor, clade B member 2 (*SERPINE2*) or plasminogen activator inhibitor type 2 (*PAI2*) is involved in cancer invasion and metastasis by controlling serine protease urokinase plasminogen activator (Croucher et al., 2008). In a recent review, several studies led to the suggestion that the significance of *PAI2* expression on prognosis of cancers is organ context-dependent.

In breast cancer, *PAI2* was expressed in both stromal and tumor cells and associated with prolonged disease-free survival (Croucher et al., 2008). In contrast, high levels of PAI2 in endometrial cancer were reported to correlate with the invasion potential of the cancer (Osmak et al., 2001). S100 calcium binding protein A4 (*S100A4*) has been revealed as the metastasis-inducing protein (Schmidt-Hansen et al., 2004). Genes such as procollagen C endopeptidase enhancer 2 (*PCPE2*) were also detected which may involve in collagen synthesis (Steiglitz, Keene, and Greenspan, 2002) which supports the function of fibroblasts in CCA to promote desmoplatic reaction.

However, arylacetamide deacetylase (*DAC*) which ranked at the first for up-regulated gene may have no effect on cancer promotion. DAC shares homology with hormone-sensitive lipase and can potentially participate in hepatic lipid metabolism (Probst et al., 1994). In addition it can activate arylamine substrates to ultimate carcinogens (Probst et al., 1994). Though there have been no reports about the expression *DAC* in liver fibroblast, the up-regulation of this gene in CCA-derived fibroblast may imply the possible effect of fibroblasts to help toxify or detoxify carcinogenic substances hepatocytes.

Regarding up-regulated genes classified in signal transduction, formyl peptide receptor-like 2 (*FPRL2*) showed the highest up-regulated level in CCA-associated fibroblasts. FPRL2 has been mentioned to induce the recruitment of eosinophils and macrophage differentiation following trigger by formyl 2 ligand in innate immune system (Devosse et al., 2009). FPRL2 may be an important receptor for fibroblast differentiation and recruitment activity. Up-regulation of cyclin D2 and cyclin A1 which drive through G1 and S phase of the cell cycle, respectively (Wolgemuth, 2008), ensured the hyper-proliferative capability of fibroblast embedded in CCA tissues.

Several mitogen-encoded genes were up-regulated in CCA-derived fibroblasts including jagged 1 (*JAGL1*) which has been shown to promote prostate cancer cell proliferation (Wang et al., 2010b). Epiregulin (*ER*) is revealed the proliferation of liver cancer cells (Regales et al., 2009) and laminin alpha 5 (*LAMA5*) can facilitate tumor growth *in vivo* (Mizushima et al., 2002). These mitogens secreted from cancer fibroblasts and influent cancer cell proliferation via paracrine effect.

In conclusion, the up-regulated gene expression profile in CCA-associated fibroblasts imply two main functions; firstly the capability of fibroblast to be recruited, proliferate, and survive in the cancer; secondly the function of fibroblast-derived substances to facilitate a favorable microenvironment for cancer progression.

5.1.2.2 Down-regulated gene profile

Bone morphogenetic protein 2 (*BMP2A*), a multi-functional growth factor belonging to the TGF-β superfamily was decreased in CCA fibroblasts as previously reported in breast cancer-derived fibroblasts (Singer et al., 2008). BMP2A has been elucidated to induce hypophosphorylation of retinoblastoma causing cell cycle arrest (Tomari et al., 2005). Hence, decreased *BMP2A* in the CCA microenvironment may promote cancer cells to enter the cell cycle.

Moreover, a decreased level of interleukin 24 (*IL-24*), an apoptotic inducible cytokine in cancer tissues, attenuates cancer cells from undergoing apoptosis (Zheng et al., 2008). In other words, decreasing level of IL-24 in CCA-derived fibroblast may confer tumor survival.

Being intracellular protein and membrane receptor, respectively, response gene to complement 32 (*RGC32*), a novel p53-inducible gene, and bradykinin receptor B1 (*BRADYB1*) have been shown fibroblast cell proliferation inhibition (Saigusa et al., 2007; Zou et al., 2008). In addition, decreased *retinoblastoma 1* which encoded a crucial negative regulator of cell proliferation (Sherr and McCormick, 2002) together with the depletion of *glypican 4* expression which its soluble form has been shown to inhibit cell proliferation by blocking other mitogens to bind receptors (Hagihara et al., 2000) implied that CCA-associated fibroblasts have lost many proteins acting as cell proliferation break.

In conclusion, down-regulated genes encoded proteins, if acting inside fibroblasts, can inhibit the proliferation of fibroblasts themselves which confirm the hyper-proliferation of activated fibroblasts in CCA. But if they exist in the extracellular region, they may involve in inhibition of cancer cell proliferation which strengthens the roles of fibroblast-derived proteins released into a tumor environment to induce a high proliferative capability of cancer cells.

5.1.3 Gene expression profile of CCA fibroblasts reveals genes related to cancer inhibition

Fibroblasts have been proposed the bipolar effects in cancers (Mueller and Fusenig, 2004). As well as promotion of tumor aggressiveness, fibroblasts have been indicated roles in tumor suppression. In our microarray results, ADAMTS-like 1 (*ADAMTSR1*) was over-expressed in CCA-derived fibroblasts. The ADAMTS-like proteins have been discussed as the enhancers of ADAMTS proteases (Hirohata et al., 2002). Since some ADAMTS have been proven to be anti-angiogenic factors (Vazquez et al., 1999) partly via the trapping of VEGF by thrombospondin motifs of ADAMTS (Luque, Carpizo, and Iruela-Arispe, 2003). So up-regulation of *ADAMTSR1* in fibroblasts may inhibit angiogenesis.

Moreover, *stromelysin-1* or *MMP-3* which can degrade ECM and induce cancer invasion and metastasis, showed the decreased expression in fibroblasts. Epidermal growth factor (*EGF*) has been indicated a strong effect to induce cancer cell proliferation (Chu et al., 2010), we found that it was decreased in CCA fibroblasts and may attenuate mitogenic effect of fibroblast on cancer cells. However, proliferation of CCA cells may go on by compensation effect of EGF which is mainly produced from tumor cells themselves.

Taken together, the increased expression of ADAMTSR1 and the decreased expression of MMP-3 and EGF may highlight fibroblasts in term of suppressing CCA progression.

In conclusion, the study herein has firstly identified genes which may explain roles of fibroblasts embedded in CCA tissues. To our data, though most of the differential genes in CCA-derived fibroblasts support their pro-tumorigenic effect, some genes may encode proteins acting in the anti-tumorigenic manner (Fig 5-1).

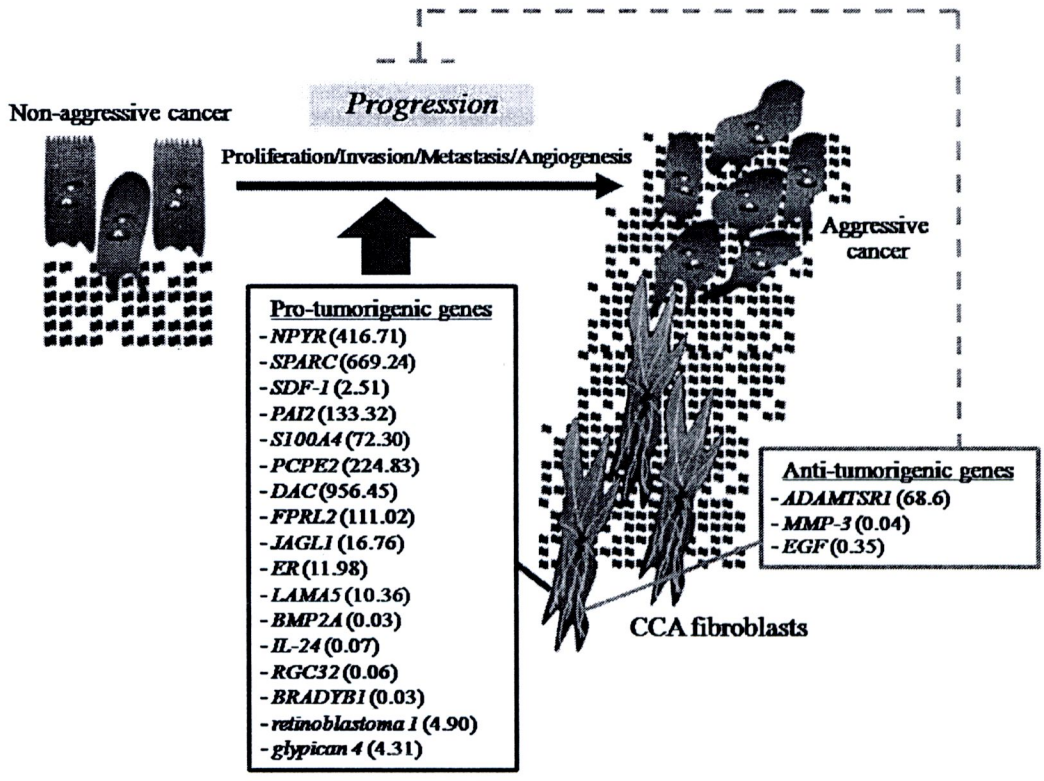

Figure 5-1 Bipolar effects of CCA-derived fibroblasts. The fibroblasts do have gene expression profile which can promote and inhibit cancer progression. The pro- and anti-tumorigenic genes expressed in CCA fibroblasts were indicated for example. The number in parentheses represent fold of gene expression level in CCA fibroblast over those in non-tumorigenic liver fibroblasts (Modified from De Wever et al., 2008).

5.1.4 Gene in CCA-associated fibroblasts encoded tumorigenic secreted

Theoretically, proteins secreted from fibroblasts having interplay with cancer cells could be detected in the extracellular region and be involved in ECM organization and biosynthesis. Firstly, we focused our interest on genes encoded secreted proteins in which these substances can act as the paracrine on the surrounding cancer cells. In addition, we selected the genes which their products have been previously reported about their tumorigenic effects. Using these criteria, *ADAM12*, *AREG*, *AGN2*, *ER*, *JAGL1*, *LAMA5*, *NOV*, *PDGF-A*, *PN*, *RL*, and *SCG2* were selected to explore. *AREG*, *ER*, *JAGL1*, and *LAMA5* are predominantly reported

for proliferation induction in cancer cells (Castillo et al., 2006; Gonzales et al., 1999; Morita et al., 2007; Purow et al., 2005). *PDGF-A*, *NOV*, *AGN2*, and *SCG2* are involved in angiogenesis (Kirchmair et al., 2004; Lin et al., 2003; Lobov, Brooks, and Lang, 2002; Shikada et al., 2005), whereas *ADAM12* and *RL* play an important role in cell motility, invasion and metastasis (Hashimoto-Torii et al., 2008; Le Pabic et al., 2003). For *PN*, many carcinogenic functions including cell proliferation, invasion, metastasis and angiogenesis have been demonstrated (Gillan et al., 2002; Kudo et al., 2006; Shao et al., 2004; Siriwardena et al., 2006; Tai, Dai, and Chen, 2005). However, this study employed real time PCR to verify the up-regulation of these genes, and found that only *ADAM12*, *AREG*, *ER*, *JAGL1*, *PDGF-A*, *PN*, and *SCG2* were significantly increased in their expression levels in CCA-drived fibroblasts and may promote CCA progression through activation of cancer growth, invasion and angiogenesis.

5.1.4.1 Genes involved in promotion of cancer cell proliferation

(1) Amphiregulin (*AREG*), a heparin binding EGFR ligand, has been proposed as a mitogenic factor in some cancers. In hepatocellular carcinoma, *AREG* was up-regulated and behaved as a mitogenic and anti-apoptotic factor (Castillo et al., 2006). Moreover, the mechanism of AREG in regulation of cell proliferation through EGFR via ERK and AKT pathways was proven in pancreatic cancer which thereby proposed AREG as a therapeutic target (Yotsumoto et al., 2010).

(2) Epiregulin (*ER*), a member of the EGF family, has been demonstrated to play a role in the proliferation of epithelial cells in both normal (Shirakata et al., 2000; Varley et al., 2005) and tumorigenic condition (McIntyre et al., 2010). In hepatocellular carcinoma, ER plays a part with N-ras to promote cancer cell growth by driving cell cycle progression via AKT and ERK1/2 pathway (Regales et al., 2009). Additionally, ER was over-expressed in hTERT-transformed fibroblasts and supported fibroblast cell proliferation as autocrine mode (Lindvall et al., 2003). These information suggest that ER may play important roles in initiation and progression of cancer.

(3) Jagged-1 (*JAGL1*), a ligand of Notch receptor family, has been shown a high expression level in cancer tissues and correlation to head and neck

cancer (Lin et al., 2010) and glioma (Purow et al., 2005) patient survival. The update report suggested that JAGL1 influences cell proliferation by activation of AKT, mTOR, and NF-κB signaling pathways in prostate cancer (Wang et al., 2010b).

5.1.4.2 Genes involved in promotion of cell motility and invasion

(1) A disintegrin and metalloprotease 12 (*ADAM12*) is a member of the ADAMs family having more than 33 members with diverse expression profiles and cellular functions (Seals DF et al., 2003). In hepatocellular carcinoma, *ADAM12* was up-regulated and correlated with increased MMP2 expression and activity (Le Pabic et al., 2003). Hence, it has been proposed that ADAM12 expression may be associated with tumor aggressiveness by co-function with MMP2 to promote cancer invasion.

5.1.4.3 Genes involved in promotion of angiogenesis

(1) Platelet-derived growth factors-alpha (*PDGF-A*), a polypeptide growth factors, is commonly expressed in epithelial muscle and neuron cells (Trojanowska, 2008). One of PDGF-A members, PDGF-AA is an important regulator of the frequency and level of VEGF expression during the transition from a precancerous lesion to advanced cancer (Shihada et al., 2005). This data suggest that the PDGF-AA/VEGF axis may be a ubiquitous autocrine system for enhancing angiogenic signals.

(2) Secretogranin 2 (*SCG2*) is an abundant protein in neuroendocrine storage vesicles and a member of the chromogranin/secretogranin family (Kirchmair R, 1993). *In vitro* study showed that SCG2-induced capillary tube formation was dose dependent manner (Kirchmair et al., 2004). Moreover, SCG2 could also stimulate proliferation and exert anti-apoptotic effect by activating of PI3K/AKT and MAPK pathways in endothelial cells.

In addition to genes mentioned above and the proposed activities of their protein products, we also performed secretome analysis of CCA fibroblast compared to those of non-tumorigenic liver fibroblast by antibody array approach (Appendix F). Among 507 proteins containing in the array chip, we found that 123 proteins were up-regulated for 2-fold or more in CCA fibroblast and were categorized into cytokines, receptors, growth factors, MMPs and phosphorylated proteins. Some proteins have been revealed their tumorigenic properties.

Interestingly, endoglin (CD105) is found to be the highest up-regulated proteins released from CCA-associated fibroblast. CD105 has been proposed as an neo-angiogenic marker in some cancers (Michael Tachezy et al., 2010). A variety of secreted proteins in particular cytokines, chemokines, and MMPs have been shown in the secretome profile of CCA fibroblasts which is an information storehouse for further exploration.

Taken all together, the profiles of genes and secreted proteins explored in this study highlight the carcinogenic roles of fibroblast-derived substances in CCA progression. These mentioned genes and proteins are of great interest to be explored their effects in CCA in the future. The better understanding on fibroblast-derived substances, the more possibility to propose fibroblasts and their induced-tumorigenic effects as the targets for inhibition of CCA progression.

5.1.5 Fibroblast-derived PN and roles in CCA

PN, a multifunctional extracellular matrix protein, has been detected in several cancers in which is secreted from either cancer cells or stromal cells. With many carcinogenic functions including cell proliferation, invasion, metastasis and angiogenesis (Gillan et al., 2002; Kudo et al., 2006; Shao et al., 2004; Siriwardena et al., 2006; Tai, Dai, and Chen, 2005) and the fact that PN has never been mentioned in CCA, we determined that PN should be the first target to explore.

5.1.5.1 PN expression in CCA

Using different biological preparations of CCA-associated fibroblasts confirmed the increased levels of PN at both mRNA and protein. Most of CCA tissues of all differentiated types had high levels of PN and expressed exclusively in α-SMA positive fibroblasts whereas, absence of PN was found in cancer cells. It can be concluded that PN detected in CCA tissues is only of fibroblast origin as reported in some cancers (Fukushima et al., 2008; Kikuchi et al., 2008).

In cancers of head and neck, ovary and colon, PN was found in cancer cells and has been proposed to induce tumorigenic properties of cancer cells via an autocrine mechanism (Bao et al., 2004; Gillan et al., 2002; Kudo et al., 2006). Hence results from the present study allow the speculation to propose a phenomenon that fibroblast-derived PN in CCA may affect cancer cells by a paracrine mode and has a promising role in cancer promotion.

5.1.5.2 High level of PN correlates with poor prognosis

A high level of PN in fibroblasts was an independent risk factor in CCA patients and those having high PN had significantly low cumulative survival time after surgery. Supported evidence from bile duct carcinomas investigated by another group shown that PN expression had significantly associated with patient survival even though, it was observed in both epithelial and stromal cells (Riener et al., 2009). PN might therefore be applied as a poor prognostic marker in patients suffering from CCA. Unlike masson's trichome staining that indicates abundant of collagen in fibrosis tissue, PN is specific expressed in CCA fibroblasts hence, it may be better used to predict severity of CCA than those of non-specific fibroblast staining. Additionally, high PN expression in CCA tissues compared to no-to-low signal in benign and hepatocellular carcinoma indicate that either tissue or serum PN may help to distinguish CCA from benign conditions and closely-related liver cancer and may use as the prognostic or predictive marker as previously reported in other cancers (Hong et al., 2009; Sasaki et al., 2001a; Sasaki et al., 2003).

5.1.5.3 PN induces CCA proliferation and invasion via ITGα5β1

To exhibit the tumorigenic impacts of PN on CCA cells, recombinant PN was employed as extracellular PN to mimic the paracrine effect of PN produced from cancer stromal fibroblasts to induce CCA cell proliferation and invasion. Though receptors ITGαvβ3 and ITGαvβ5 have been shown to be the receptors for PN in several cancer cells (Bao et al., 2004; Gillan et al., 2002; Shao et al., 2004), PN promoted invasiveness of pancreatic cancer cells via the β4 integrin (Baril et al., 2007). This suggests the cell type dependent on a specific ITG responded to PN. Herein we revealed that PN-induced cell proliferation and invasion could be mediated through ITGα5β1. As the well known receptor for fibronectin, the apparent reason for ITGα5 presented in CCA cells may be to support the abundance of fibronectin found in CCA as proven previously (Chen et al., 2003).

Regarding the fact that ITGα5 can only be dimerized with β1 subunit and fibronectin-ITGα5β1 ligation has been revealed to support cell survival (Zhang et al., 1995) and induce invasion and angiogenesis (Tuomi et al., 2009; Zeng et al., 2009). This supports our findings that using either RNAi against ITGα5 or anti-ITGα5β1, these ITGα5β1-deficient cells have less capability to response to PN-

induced invasion. In conclusion, the results suggest that CCA cells need ITGα5β1 to mediated PN-activated cell invasion. However, the slight increased of PN-induced invasion in cells with ITGα5β1 blockage may imply the other ITGs play role in CCA cell response to PN as such studied in our lab is ITGα6β4 (data not shown).

However, the anti-TGα5β1 antibody-treated cells had less intrinsic capability to invade comparing to parental cells. The possible explanations are (a) interaction of ITGα5β1 and fibronectin in the Matrigel of the invasion chamber may influence the invasion of cells and (b) inappropriate antibody concentration used to block membrane ITGα5β1. Though ITGα5β1 is suggested for PN activated CCA cell invasion, the further experiments are needed to confirm this finding and ITGα6β4-mediated pathway may of great challenge to explore.

5.1.5.4 PN-mediated ITGα5β1 via AKT but not ERK in CCA invasion

PN has been shown strong effect to stimulate activated form of AKT and ERK1/2 in pancreatic cancer cells (Erkan et al., 2007). To reveal the involvement of AKT and ERK pathways in PN-induced invasion through ITGα5β1 receptor, cells with transient knockdown ITGα5β1 were used. In KKU-M213 CCA cells, PN could not induce pAKT if cells were previously treated with si*ITGα5* in concomitant to the decreased invasive capability of cells induced by PN. This result suggests the possible of PN to activate cell invasion through ITGα5β1-AKT dependent pathway.

As for the ERK pathway, a slightly decreased level of pERK in PN-activated *ITGα5*-knockdown cells indicated the less involvement of this pathway in PN-induced invasion. In support with our findings, PN was proposed to drive tumor survival and angiogenesis through PI3K/AKT pathway in colon cancer via ITGαvβ3 (Bao et al., 2004) and promote the invasiveness of tumor cells through phosphorylation of FAK and PI3K/AKT kinase pathway but not ERK1/2 in pancreatic cancer via ITGα6β4 (Baril et al., 2007). These evidences suggest that though PN can enhance tumorigenesis through different ITGs in many cancers, AKT may be a key signaling transduction molecule to achieve cancer progression.

In conclusion, though using specific PI3K/AKT and ERK/MEK inhibitors are needed to identify the downstream signal pathway of PN-ITGα5β1

induced CCA invasion, our results suggest the ITGα5β1-AKT dependent pathway in fibroblast-derived PN induced CCA invasion. Regarding the unique ITGα5β1-PN in CCA invasion, using anti-ITGα5β1 antibody may help for inhibit tumor progression as anti-ITGαvβ5 reported in neuroblastoma (Bonfoco et al., 2000).

In addition, other ITGs including ITGα6β4 expressed on bile duct cancer cells were detected in our lab (unpublished data). In some cancers, activation of ITGα6β4 resulted in induction of cell motility and helped cancer cells to invade (Baril et al., 2007). In addition, ITGαvβ6 showed strongly expressed in bile duct cancer cells and has been demonstrated as the differential diagnosis marker with high specificity for CCA (Patsenker et al., 2010). This is of great challenging to explore the molecular mechanism underlying PN-induced CCA progression through different heterodimer of ITGs to better understand the specific profile of PN-induced signaling pathway in CCA cells.

5.1.5.5 Proposed effect of fibroblast-derived PN in *O. viverrini*-driven CCA

TGF-β has been proposed to induce the expression of PN (Horiuchi et al., 1999). *O. viverrini* excretory/secretory product (ESP) has recently been shown to be the stimulator of fibroblast proliferation via the TGF-β-mediated signal transduction pathway (Thuwajit et al., 2006). To reveal the relation of parasitic ESP and PN production, normal liver fibroblasts were treated with ESP and PN was then measured. Interestingly, we found the increased PN expression in fibroblasts exposed to ESP (Appendix G). It is interesting to propose that in the early stage of CCA formation, parasitic substances, in addition to contain the mitogen (Smout et al., 2009; Thuwajit et al., 2004), they may activate stromal fibroblasts (Fig 5-2). Later, these activated fibroblasts may help promote carcinogenesis. In addition, in late stage of cancer, fibroblast-derived PN could be more induced by TGF-β produced from CCA cells (Ohira et al., 2006) and in part facilitate cancer progression.

Though *in vivo* experiments are needed to confirm, fibroblast-derived PN may influent *O. viverrini*-associated CCA at the early stage of cancer as well as to promote cancer progression in the later time. With this information, targeting the stroma in CCA may not only be effective in treatment of primary,

invasive and metastatic tumors, but may also play role in prevention of tumor development.

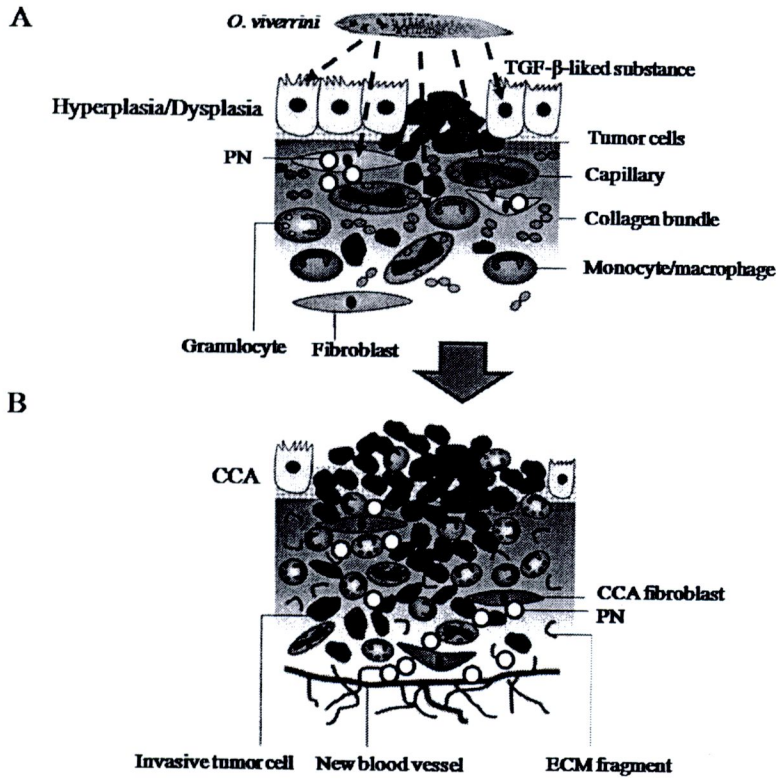

Figure 5-2 Proposed mechanism of fibroblast-derived PN in *O. viverrini*-associated CCA. Parasite infection mechanically and chemically induces hyperplasia/dysplasia of bile duct epithelial cells and fibroblasts (A). TGF-β-liked substances stimulate PN expression in stromal fibroblasts and may help CCA formation. After CCA development, PN may secrete from CCA-derived fibroblasts and facilitate CCA progression through the induction of proliferation, invasion and angiogenesis (B) (Modified from Mbeunkui et al., 2009).

5.2 Conclusion

This study is the first to describe gene expression profile of CCA-derived fibroblasts. Understanding fibroblasts in CCA at the molecular level by the functions of certain up- and down-regulated genes has been revealed and has suggested certain groups of genes in controlling cancer cell proliferation, invasion, metastasis and

angiogenesis (Fig 5-3). These findings provide evidence that fibroblasts are important sources of tumorigenic substances, particularly PN, when produced into the microenvironment of CCA. High levels of PN are found in most CCA patients and can be used as a poor prognostic marker. In addition, the level of PN can be used to distinguish CCA from other benign liver conditions and hepatocellular carcinoma. The interaction of fibroblast-derived PN and CCA cells helps to promote cell proliferation and invasion via ITGα5β1 and AKT pathway. Targeting fibroblasts or fibroblast-derived-PN-stimulated pathways in cancer cells to attenuate the tumorigenic induction of PN is a further challenge to inhibit CCA progression.

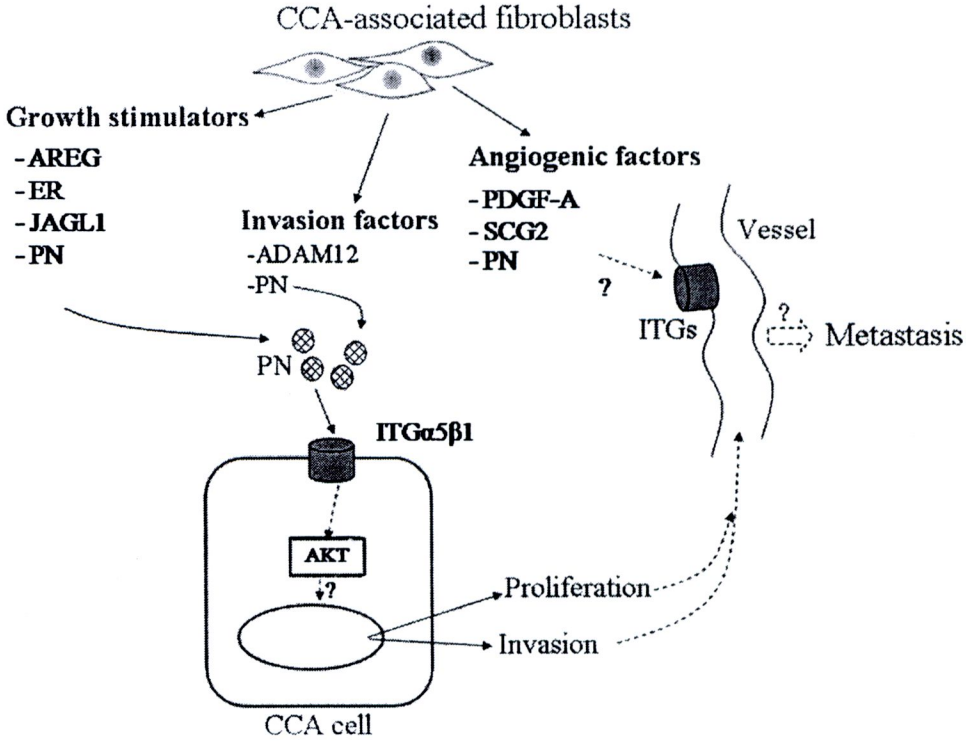

Figure 5-3 Proposed impacts of CCA-associated fibroblasts revealed in this study. A schematic representation of the main alterations in CCA-associated fibroblasts revealed in this study. The biological functions of protein products from the up-regulated genes in fibroblasts are represented. Tumorigenic effects of PN on CCA cancer cells are also proposed.

5.3 Limitation and suggestions for further studies

To complete understanding in roles of fibroblasts in CCA progression, these further explorations are challenged to perform.

5.3.1 The present study is limited to a single cancer fibroblast line isolated from one CCA patient. Expanding of sample sizes isolated from different feature of patients may facilitate more informative data of CCA-derived fibroblasts. In addition, using laser capture microdissected-fibroblasts may ensure the real characters of fibroblasts embedded in CCA tissues.

5.3.2 The effect of AREG, ER, JAGL1, ADAM12, PDGF-A and SCG2 are of great interest to explore in CCA which will give more complete roles of fibroblast-derived substances in various aspects of cancer progression and provide the proper therapeutic targets for CCA patients.

5.3.3 To ensure that PN can be an effective prognostic marker for CCA, measurement of PN level in CCA serum is promising to perform in comparison to both of normal persons and patients suffering from other related cancers.

5.3.4 The application of specific inhibitors to confirm whether PN activates ITGα5β1 through PI3K/AKT pathway is needed to perform. Moreover, ITGα6β4-mediated cell invasion is challenged to prove in cell exposed to PN. These will complete profile of PN-induced CCA invasion through ITGs and propose PN and signal pathways as therapeutic targets for CCA patients. PN-induced angiogenesis and MMP expressions as published in other cancers are in the waiting list for investigation.

5.3.5 Several proteins found in the conditioned-media of CCA-associated fibroblasts are listed for further investigations. Fibroblast-derived growth factors, cytokines, chemokines, and MMPs should be emphasized in the near future for their exact functions on CCA cells.