**A DISSERTATION**

**FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

# STUDY THE ROLE OF SPALT-LIKE GENE IN CANCER PROGRESSION

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**August, 2018**

**STUDY THE ROLE OF SPALT-LIKE GENE IN CANCER PROGRESSION**

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A thesis submitted in partial fulfillment

of the requirement for the degree of Doctor of Philosophy

August, 2018

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**To lab members and all of my friends and family, many thanks for all of your love and support.**

**ACKNOWLEDGEMENTS**

I owe a great amount of thanks to my primary mentor, professor Dong-Kee Jeong. Your enthusiasm for science inspires me and guides me tackling any problem in my future scientific career. I also owe a lot of thanks to my co-mentor Dr. Taeho Kwon. Your advices in research direct me to goals and I have learned many things from your skills. I would like to thank to all members of the GENG lab who have engaged me in great discussions and are constantly challenging me to take my research to the next level. I would like to thank my friends from my country, who stay together with me at the moment i need helps. Last, but certainly not least, I want to say thanks to my family. Thanks for supporting me in good times and bad, I could not have done this without you.

## TABLE OF CONTENTS

## SUMMARY

*Spalt*-like (Sall) gene family were originally identified in *Drosophila,* which has two main gene groups, including *spalt major (salm)* and *spalt related (salr).* These genes also play important roles in vertebrate development and any abnormal might create genetic disorders. In human, *Spalt* genes have four gene members, including SALL1, SALL2, SALL3 and SALL4, which are tightly involved in some of human diseases such as Townes-Brocks syndrome (TBS) Okihiro syndrome, Holt-Oram syndrome or cancers.

SALL4 is located at chromosome 20 q13.2 and recently defined by three isoforms including: SALL4A, SALL4B, SALL4C. SALL4 is nuclear protein and mediated by Nuclear Localization Signal (NLS) located at ZF1 zone (acid amine 64-67). SALL4 is expressed at early stage and important to embryo development. In adult, SALL4 is localized at germ cells. However, SALL4 is correlated with variety of cancers such as leukemia, gastric cancer, colorectal cancer, lung cancer, breast cancer, glioma, endometrial cancer, liver cancer and esophageal squamous carcinoma. SALL4 is also a reliable marker in germ cell tumor.

Evidences showed that SALL4 gets involved in many signaling pathways. SALL4 co-operates with Nucleosome Remodeling Deacetylase (NuRD) complex to suppress PTEN expression or drives the expressions of leukemia oncogenes (HOXA9, MEIS1). SALL4 is believed to activate and co-operate with stemness genes including Oct-4, Nanog, Sox-2. Furthermore, some of evidences showed SALL4 involvement to beta-catenin, hedgehog signaling or drug resistance.

In chapter 1, we demonstrated that SALL4 was tightly involved in proliferation of pancreatic malignant cells *in vitro* and *in vivo*. Results also indicated that SALL4 regulated strongly the metastatic phenotype in PDAC cell lines including migration, invasion or EMT protein markers.

Therefore, SALL4 might be a potential factor to master EMT and proliferation in PDAC cells. Furthermore, SALL4 was associated with reactive oxygen species (ROS) regulation in pancreatic ductal adenocarcinoma (PDAC) phenotype. The expression of SALL4 was linear to Prx III. Downregulation and upregulation of SALL4 result in responses of Prx III. Upstreaming the pathway, we found that SALL4 enhanced FoxM1 activity which in turns upregulated the expression of Prx III. Therefore, the expression of SALL4 tends to reduce ROS level in PDAC cells and favors metastasis.

In chapter 2, we have found the role of SALL4 to the drug resistance of pancreatic cancer stem cells (PCSCs). In the double positive $CD44^+$ $CD24^+$ subpopulation from BxPC-3 and PANC-1, we observed the upregulation of SALL4. Downregulation of SALL4 resulted in the decreases of stemness genes, clonogenicity capacity and tumor sphere forming ability. Moreover, SALL4 downregulation sensitizes PCSCs to gemcitabine treatment. Some of drug resistant genes were attenuated by SALL4 silencing. Upstreaming pathway, by somehow SALL4 governs Notch1 signaling which in turns regulates drug resistance via Nrf2 axis.

The application of herbal plants in anti-cancer has been developed and achieved some first-fruits. Herbal plants are used to inhibit tumorigenesis alone or in combination with conventional drugs. In this chapter 3, we examined the effects of seven herbal plants mixture extract – named as BRM270 to suppress tumorigenesis induced by pancreas body-derived cancer stem cells (bPCSCs). Results showed that BRM270 prevented the proliferation of $CD133^+$ $CD44^+$ bPCSCs in *vitro* and in *vivo*. Some of stem cell factor genes (SALL4, Oct-4, Sox-2, Nanog, CD133, CD44) were downregulated by BRM270, resulting in suppression of clonogenicity, tumor sphere forming ability. Furthermore, BRM270 repressed the expressions of metastatic markers and restrained cell mobility of bPCSCs. Further analysis indicated that BRM720 targeted Shh

signaling, leading to downregulations of epithelial-mesenchymal transition (EMT) genes. These evidences consolidate BRM270 potential in anti-tumorigenesis, especially pancreatic cancer stem cells.

In overall conclusion, this study first time indicated that SALL4 promotes metastasis in pancreatic cancer cells via enhance EMT genes expression and tends to lower ROS via FoxM1/ Prx III. SALL4 maintains the expression of stemness genes both in cancer cells and cancer stem cells. Furthermore, SALL4 gets involved in drug resistance in PCSCs via Notch1 signaling. This study strongly recommended the potential role of SALL4 and it can be the promising target in pancreatic ductal adenocarcinoma (PDAC) treatment. Furthermore, BRM270 can be an ideal adjuvant to inhibit SALL4 contribution and to prevent pancreatic tumorigenesis.

# 초 록

Spalt 유사(Sall) 유전자 군은 초파리에서 확인 되었고, spalt (salm), spalt 과 관련(salr)된 두 가지 주요 유전자 그룹을 가지고 있다. 이 유전자들은 척추 동물 발달에 중요한 역할을 하지만 비정상적인 것은 유전 질환을 일으킬 수 있다. 인간의 Spalt 유전자에는 타운즈 브록스 증후군, 오키히로 증후군, 홀트-오람 증후군 또는 암과 같이 인간 질병의 일부에 밀접하게 연관된 SALL1, SALL2, SALL3 및 SALL4 를 비롯한 4 개의 유전자 구성원이 있다.

SALL4 는 염색체 20q13.2 에 위치하고 있으며 최근 SALL4A, SALL4B, SALL4C 등 3 가지 동형 단백질로 정의한다. SALL4 는 핵 단백질이며 ZF1 구역 (산성 아민 64-67)에 위치한 핵 이행 신호(Nuclear Localization Signal)에 의해 조절된다. SALL4 는 초기 단계에서 발현되며 태아 발달에 중요하다. 성인에서 SALL4 는 생식 세포에 국한되지만 백혈병, 위암, 대장암, 폐암, 유방암, 신경아교종, 자궁내막 암, 간암 및 식도 편평 상피암과 같은 다양한 암과 관련이 있다. 또한 SALL4 는 생식 세포 종양에서 신뢰할 수 있는 마커이다.

기존의 연구에는 SALL4 가 많은 신호 전달 경로에 관여한다고 보고되었다. SALL4 는 Nucleus Remodeling Deacetylase (NuRD) 복합체와 상호 작용하여 PTEN 발현을 억제하거나 백혈병 발암 유전자 (HOXA9, MEIS1)의 발현을 유도한다. SALL4 는 Oct-4, Nanog, Sox2 를 포함한 줄기세포능 유전자로 활성화되고 상호 작용한다고 알려져 있다. 또한, 일부 연구에서는 SALL4 가 베타 - 카테닌, 헤치호그 시그널링 또는 약물 내성에 관여 함을 보고하였다.

제 1 장에서는 SALL4 가 *in vitro* 및 *in vivo* 에서 췌장 악성 세포 증식과 밀접하게 연관되어 있음을 보여 주었다. 또한 SALL4 가 상피간엽이행(EMT) 단백질 마커를 포함하는 췌장관세포암(PDAC) 세포주에서 전이성 표현형을 강력하게 조절하는 것을 관찰하였다. 따라서 SALL4 는 PDAC 에서 EMT 와 세포 증식을 지배 할 수 있는 잠재적인 요인이며, PDAC 표현형에서 활성산소 종 (ROS) 조절과도 관련이 있다. SALL4 의 하향 조절 및 상향 조절은 Prx Ⅲ 의 반응을 유도한다. 우리는 SALL4 를 상향조절 했을 때 FoxM1 활동을 강화 시켜서 Prx Ⅲ 의 발현을 증가시키는 것을 발견했다. 따라서, SALL4 의 발현은 PDAC 세포에서 ROS 수준을 감소시키는 경향이 있으며 암의 전이를 일으킨다.

제 2 장에서 우리는 췌장암 줄기 세포 (PCSC)의 약물 내성에 대한 SALL4 의 역할을 발견했다. BxPC-3 및 Panc-1 의 이중 양성 $CD44^+$ $CD24^+$ 하위 집단에서 SALL4 의 상향 조절을 관찰했다. SALL4 의 하향 조절은 줄기세포능 유전자, 집락 형성 및 종양 형성 능력의 감소를 보였고, 젬시타빈 치료에 PCSC 를 반응하게 하였다. 약물 내성 유전자의 일부는 SALL4 발현 억제에 의해 약화되었다. SALL4 상향 조절은 Nrf2 축을 통해 약물 내성을 차례로 조절하는 Notch1 신호를 제어한다.

암 치료를 위해 약용식물을 이용한 기술이 개발되었고, 단독으로 또는 통상적으로 함께 종양 형성을 억제하는데 사용되었다. 제 3 장에서는 췌장 체내 암 줄기 세포 (bPCSCs)에 의해 유발 된 종양 발생을 억제하기 위해 7 가지 약초 혼합 추출물의 효과를 조사했다. 그 결과 천연물복합물질가 *in vitro* 와 *in vivo* 상에서 CD133 + CD44 + bPCSC 의 증식을 방지한다는 것을 보여 주었다. 줄기 세포 인자 유전자 (SALL4, Oct-4, Sox2, nanog, CD133, CD44)의 일부는 BRM270 에 의해 발현이 감소되어 종양 형성 능력을 억제했다. 또한, 천연물복합물질은 bPCSC 의 전이성 마커의 발현을 억제하고 암 세포의 이동성을 억제했다. 추가 분석에 따르면 천연물복합물질은 Shh 신호 전달을 표적으로 삼아 EMT 유전자의 하향 조절을 유도했다. 이와 같은 증거들은 항 종양 형성, 특히 췌장암 줄기 세포에서 천연물복합물질 잠재력을 강화한다. 결론적으로 SALL4 는

EMT 유전자 발현을 통해 췌장암 세포 전이를 촉진하고 FoxM1 / Prx Ⅲ를 통해 ROS 를 낮추는 것으로 확인되었다. SALL4 는 암세포와 암 줄기 세포에서 줄기세포능 유전자의 발현을 유지하고 Notch1 신호 전달을 통해 PCSC 의 약물 내성에 관여한다. 따라서 본 연구결과를 통해 SALL4 의 잠재적인 역할을 강력히 권장하며 췌장관세포암 치료를 위한 표적이 될 수 있을 것으로 사료된다. 또한, 천연물복합물질은 SALL4 의 협력을 억제하고 췌장 종양 형성을 예방하는데 이상적인 보조제가 될 수 있을 것이다.

## LIST OF TABLES

## LIST OF FIGURES

**INTRODUCTION** **Biology review of Spalt-like genes and SALL4**

## Spalt-like (Sall) gene families and their roles in development and cancer

### *Spalt*-like gene family

*Spalt*-like (Sall) gene family were originally identified in *Drosophila,* which has two main gene groups, including *spalt major (salm)* and *spalt related (salr)* (Sweetman and Munsterberg, 2006). These genes also play important roles in vertebrate development and any abnormal might create genetic disorders. During evolution, these proteins are modified into four groups of genes which are distributed in many species (Figure 1.1). The phylogenetic tree indicated that there are four genes in human called *SALL1-4*, four genes in mouse *Sall1-4*, three in chick, five in *Xenopus*, three in zebrafish and 1 in Medaka (Sweetman and Munsterberg, 2006).

### The role of *Spalt* genes in vertebrate development

Many reports suggested *Spalt* genes get involved in limbs, nervous system and several organs (kidney, heart). Indeed, several homologues are expressed during limbs development in many vertebrates such as mouse, chick or *Xenopus* (Barembaum and Bronner-Fraser, 2004; Buck et al., 2001; Farrell and Munsterberg, 2000; Hollemann et al., 1996; Kohlhase, 2000; Neff et al., 2005; Ott et al., 1996).

To heart development, expressions of Spalt-like genes have been found in mouse, zebrafish, or chick (Buck et al., 2001; Camp et al., 2003; Ott et al., 1996; Sweetman et al., 2005). Abnormal of *Spalt*-like genes are reported to be related to heart defects (Kohlhase et al., 2003a; Sweetman et al., 2005). Furthermore, *Spalt*-like genes are required for neural and kidney

development (Barembaum and Bronner-Fraser, 2004; Kiefer et al., 2003; Nishinakamura et al., 2001; Onai et al., 2004; Parrish et al., 2004; Powell and Michaelis, 1999).

Taken together, these evidences confirmed the important role of *Spalt*-like genes during embryonic development and any defects of these genes might create organs failure.

**Structure and function of spalt-genes**

*Structure of Spalt genes*

As shown in Figure 1.1, *Spalt* genes in human have four gene members, including SALL1, SALL2, SALL3 and SALL4. General predicted structures of human *Spalt*-like genes are described in Figure 1.2.

The *Spalt* proteins have a typical structure including N-terminal zinc finger (ZF) domain of C2HC type, glutamine-rich region (Q-rich) and various number of zinc fingers domains, which depends on SALL protein type (Figure 1.2).

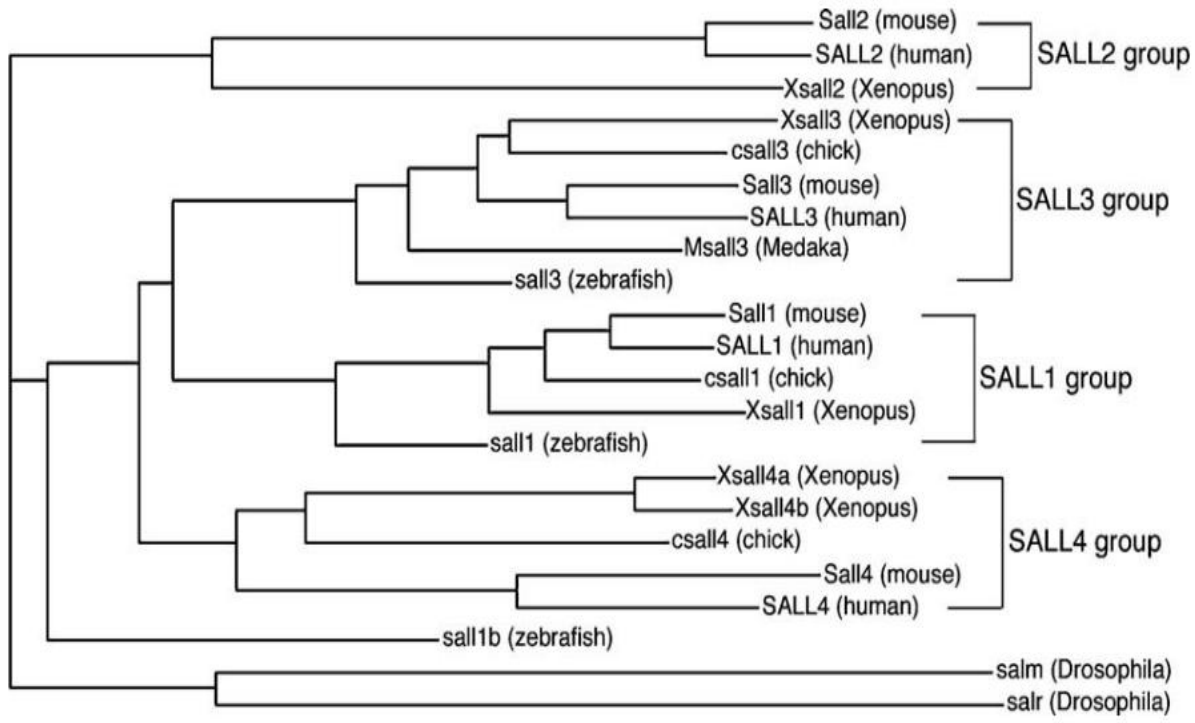



**Figure 1.1 Clustal alignment of vertebrate spalt protein sequences.** *Spalt*-like (Sall) protein families are divided into four groups of genes. In human Spalt genes are called SALL1-4, corresponding in mouse Sall1-4, three in chick are csall1-4. In *Xenopus*, these proteins are named as Xsall1-4, sall1a-b and sall3 in zebrafish and one candidate in Medaka, Msall3. (Adopted from Sweetman et al. Developmental Biology 293 (2006) 285–293)

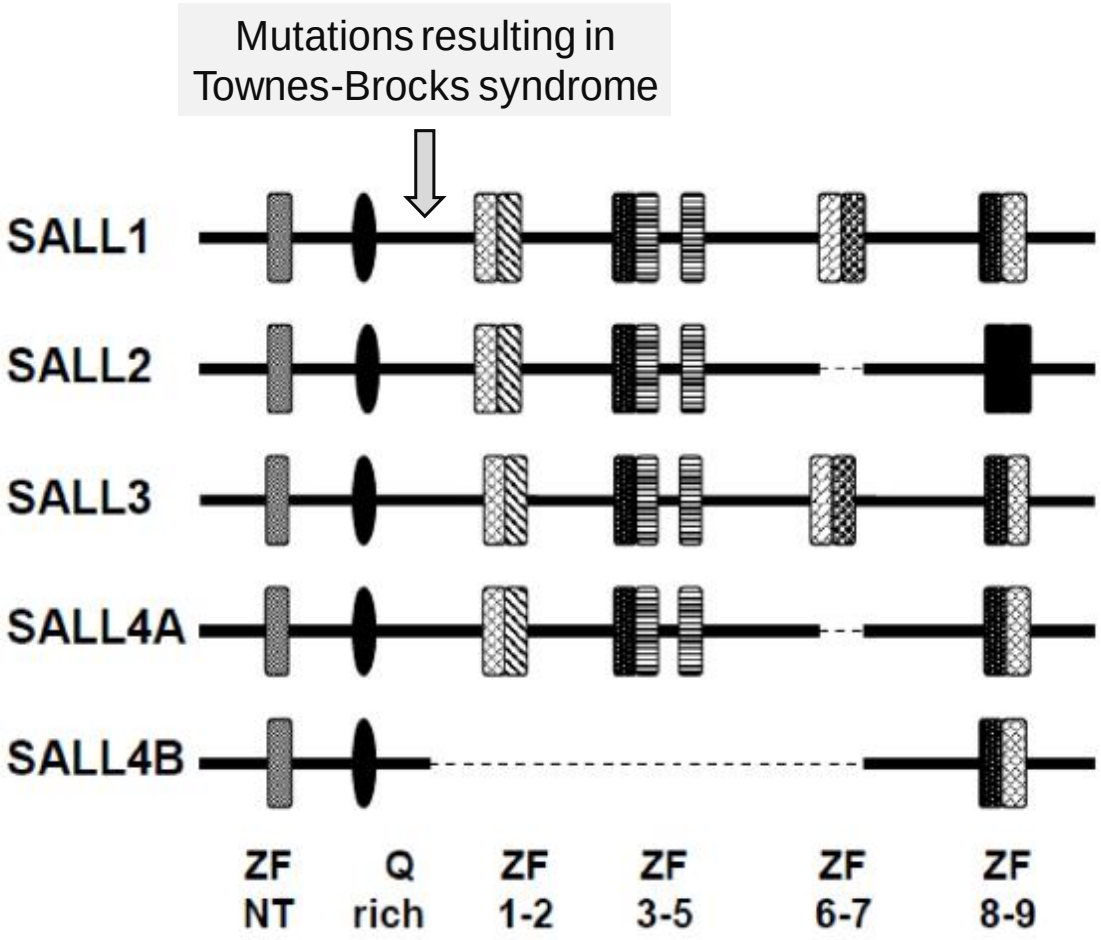



**Figure 1.2 Predicted structures of SALL proteins in human.** The common structure of *Spalt* proteins is including N-terminal zinc finger (ZF) domain of C2HC type, glutamine-rich region (Q-rich) and various number of zinc fingers domains.

*Regulating target genes*

*Spalt*-genes proteins are reported as transcriptional factors. GAL4 promoter is found to be bound with domains in SALL1, Sall1, csall (Kiefer et al., 2002; Netzer et al., 2001; Sweetman et al., 2003). SALL2 is also reported as transcriptional factor of p21 promoter (Li et al., 2004) or SALL1 regulates beta-catenin/Wnt signaling (Sato et al., 2004). Furthermore, SALL4 are thought to enhance expressions of Nanog, Oct4, Sox-2 and other proteins (BMI-1, HOXA9) via its DNA binding domain (Gao et al., 2013b; Tanimura et al., 2013; Tatetsu et al., 2016; Yang et al., 2007; Zhang, X. et al., 2015). There are still many promoters regulated by *Spalt*-genes are being assayed to gain better understanding about the activities of *Spalt* genes.

**The participation of *Spalt* genes to human diseases**

Townes-Brocks syndrome (TBS) is an autosomal dominant disorder with symptoms such as preaxial polydactyly and triphalangeal thumbs, external ear defects and sensorineural hearing loss, imperforate anus, kidney and heart defects (Powell and Michaelis, 1999). Kohlhase *et al.* indicated that mutations in SALL1 leads to TBS in human (Kohlhase et al., 1998). These mutations induce premature stop codons in 5' region, leading to the termination of translation and the incomplete protein function at Glutamine rich region or first set zinc finger (Figure 2). Recently, evidence showed some TBS patients possessing entire deletion of SALL1 allele (Borozdin et al., 2006), suggesting that truncated protein has a stronger effect and cause severe phenotype which is believed to induce TBS. Other evidences also supposed that truncated SALL1 interacts with other SALL proteins and by that way induces TBS phenotype during embryonic development (Dong et al., 2003; Kiefer et al., 2003; Sweetman et al., 2003).

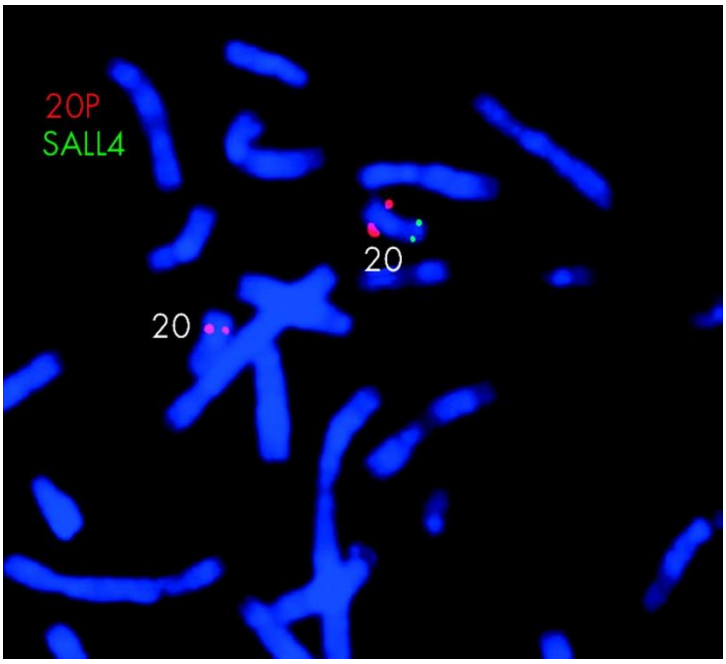



**Figure 1.3 Fish analysis of chromosome sample.** Red is representative for probe, which is specific for chromosome 20p11.2 and Green is representative for SALL4. In hybridization in situ, one allele is deleted in one chromosome while two chromosomes can be detected (Borozdin et al., 2004).

Moreover, SALL4 mutants are believed to be a cause for Okihiro syndrome, Holt-Oram syndrome (Borozdin et al., 2004; Kohlhase et al., 2002b; Kohlhase et al., 2003b). These syndromes defect to limb and heart or eye and kidney developments. Mutations in SALL4 are not clustered in some certain regions and haploinsuffiency showed to be the cause of these disorders (Kohlhase et al., 2005). Furthermore, SALL4 is also believed as one of candidate genes which cause premature ovarian failures in human (Wang, B. et al., 2009).

### ***Spalt*-like genes and human cancer**

Many isoforms of Spalt genes are associated to cancer development. For instance, SALL1 is highly expressed while SALL2 was down-regulated in Wilms tumor (Ma et al., 2001a; Ma et al., 2001b). Furthermore, SALL1 might get involved in tumorigenesis via Wnt signaling pathway (Sato et al., 2004). Many reports indicated that SALL4 have been related to cancer development in many types of cancer such as hepatocarcinoma, lung cancer, glioma, germ cell tumor, gastric cancer, breast cancer or leukemia (Tatetsu et al., 2016; Zhang, X. et al., 2015).

**SALL4, the view from development, stem cells to cancer in human**

## SALL4 isoforms and structures

SALL4 is located at 20q13.2 and recently defined by three isoforms including: SALL4A, SALL4B, SALL4C. SALL4A is composed of four exons, which contains four zinc finger (ZF) domains and Glutamine (Q) rich domain as other *Spalt* genes (described in Figure 1.2). This is existed as full length. During splicing process, full length is modified 2 isoforms. SALL4B is composed of Exon 1, a part of Exon 2 and Exon 3 and Exon 4 while Exon 2 is sliced out in SALL4C (Figure 2.1).

SALL4 is nuclear protein mediated by Nuclear Localization Signal (NLS) on its sequence. NLS is located at ZF1 zone (acid amine 64-67). Substitution between Lysine into Arginine at acid amine 64 interfere SALL4 localization in *vitro* and in *vivo* (Wu et al., 2014). Evidence showed that the role of Q rich domain is to be essential to the interactions between *Spalt* proteins and this sequence is highly conserved in all invertebrate and vertebrate (Sweetman et al., 2003). Other evidence indicated that ZF4 domain is very important to SALL4 attachment on heterochromatin (Sakaki-Yumoto et al., 2006). SALL4A and B might form homodimers or heterodimers and regulates the expressions of downstream genes in nucleus (Rao et al., 2010).

Modifications at post-translation of SALL4 including phosphorylation, ubiquitination or sumoylation have been reported (Wilson et al., 2012; Yang, F. et al., 2012; Zoumaro-Djayoon et al., 2011). However, there are many dark corners still illusive.

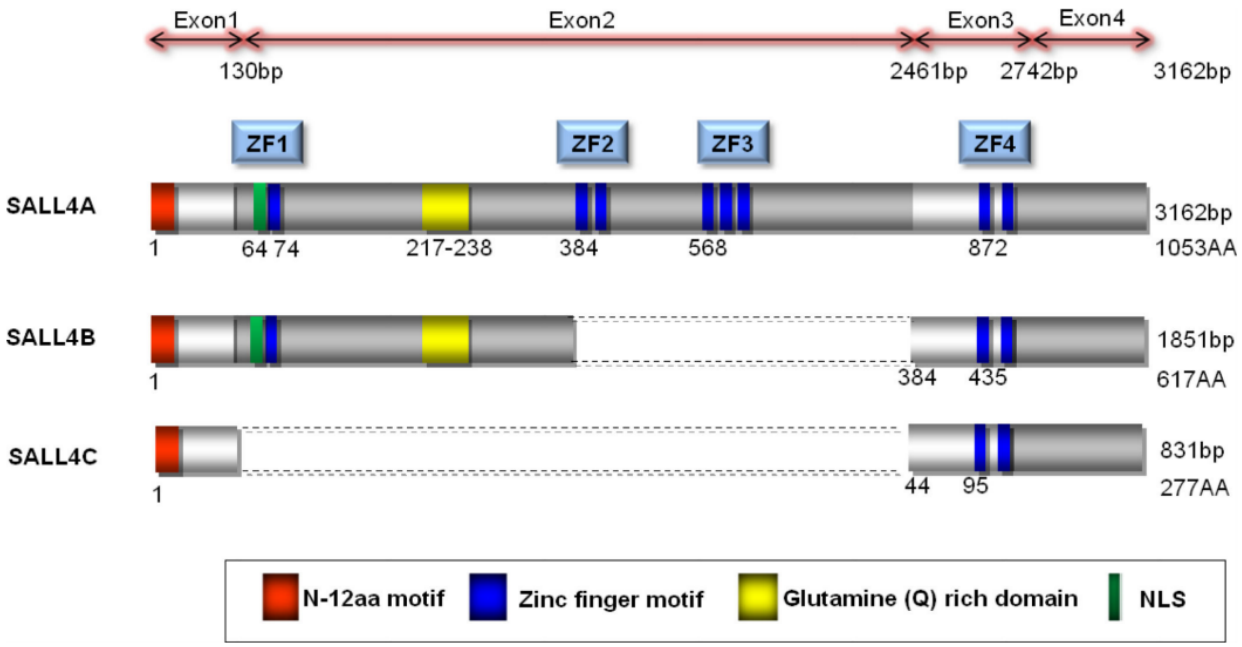



**Figure 2.1 Structures of human SALL4 isoforms.** SALL4 gene contains four exons (Exon 1, Exon 2, Exon 3, Exon 4). Isoform SALL4A (full length of SALL4) contains N-12aa motif, four zinc finger (ZF) domains and Glutamine (Q) rich domain. During splicing in transcription, two new isoforms SALLB and SALLC are created. SALLB is constituted from Q rich domain, ZF1 and ZF4 but lack of ZF2 and ZF3. In SALL4C structure, Exon 2 is sliced out. **(**Adopted from Tatesu et al. 2016 (Tatetsu et al., 2016))

**SALL4 expression in development**

There is limitation in human embryos research due to ethic issues. Therefore, murine embryos have been used as an alternative model for SALL4 function research in human. In mice, Sall4 expression is found at early stage of embryos, for instance, at two-cell and 8-16 cell stage of embryos (Elling et al., 2006). At late of blastocyst, Sall4 is found at the inner cell mass (ICM) and trophectoderm. The expression of Sall4 becomes lower and finally focus expressing only in some places such as midbrain, limb buds, tail bud or genital tubercle at stage of E11.5 (Kohlhase et al., 2002a; Koshiba-Takeuchi et al., 2006). At postnatal stage, Sall4 expression is bunched at germ cells (Cao et al., 2009b; Eildermann et al., 2012; Miettinen et al., 2014) (Figure 2.2). Similarly, SALL4 is also found in adult human at testis or ovary (Kohlhase et al., 2002b). However, human hematopoietic stem cells are also believed to express SALL4 (Gao et al., 2013b).

Isoforms of SALL4 are also specifically expressed and patterning in various tissues. Indeed, SALL4A but not SALL4B, is found at fetal livers whereas both isoforms can be found in hepatocellular carcinoma (HCC) cell lines (Oikawa et al., 2009; Oikawa et al., 2013).

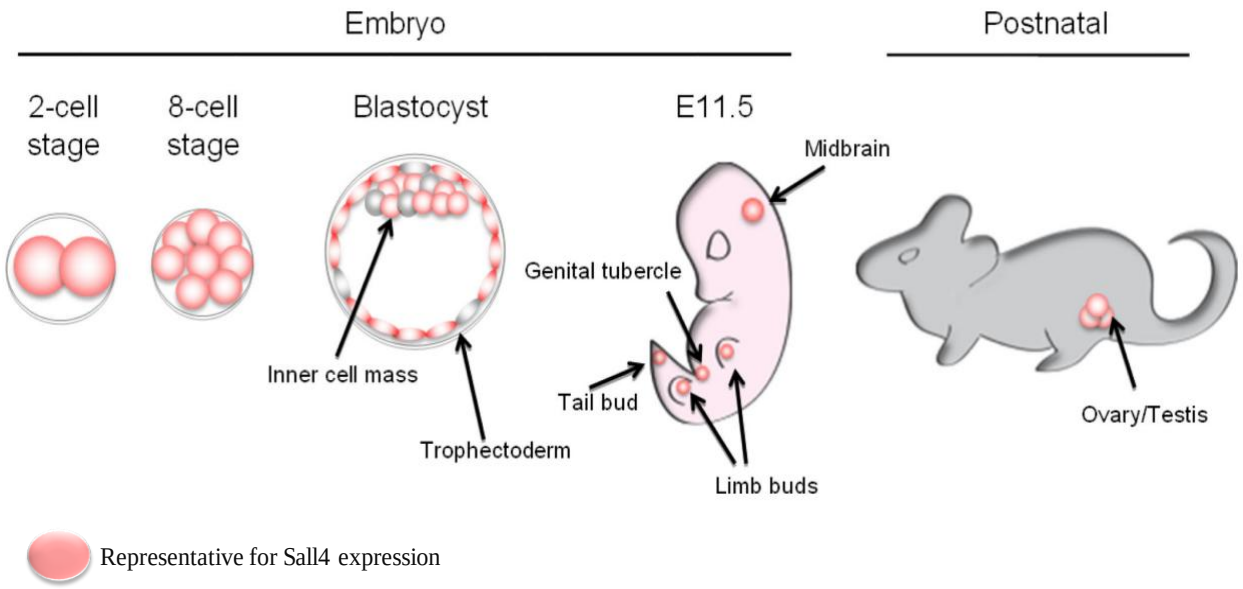



**Figure 2.2 Expression of Sall4 in mice development.** Sall4 is expressed at early stage of embryos (two-eight cells embryo) and its expression starts degenerated at late of blastocyst. At the E11.5 day, Sall4 is only expressed at midbrain, genital tubercle, tail bud and in some limb buds. In adult mice, Sall4 expression is only localized at ovary or testis. In human, SALL4 expression is also found at germ cells.

**SALL4 in stem cells**

In murine model, Sall4 is believed to participate in reprograming mouse embryonic fibroblast (MEF) into induced pluripotent stem (iPS) cells (Tsubooka et al., 2009). Sall4 enhances iPS reprograming efficiency consistently and stably (Wong et al., 2008). Conventional stemness factors (Oct4, Sox-2, Klf4, c-Myc) can be replaced by the combination of Sall4, Nanog, Esrrb and LIN28 or Sall4, Sall1, Utf1, Nanog and c-Myc to transform adult cells into iPS cells (Buganim et al., 2014; Mansour et al., 2012).

Furthermore, hypomethylation at CpG island of SALL4 is also observed in its promoter at Exon1/ Intron 1 in leukemia-derived iPS cells, suggesting that SALL4 is essential to the generation of iPS cells. Other studies indicated that Sall4 existence is important to embryonic stem (ES) cells in MEF free medium (Zhang et al., 2006) and their fates (Lim et al., 2008; Tanimura et al., 2013; Wu et al., 2006; Yang et al., 2010; Zhou et al., 2007).

**SALL4 is in correlation with cancer**

In hematological cancer patients with poor diagnose, high-risk myelodysplastic syndrome (MDS) is correlated with high expression of SALL4 (Wang et al., 2013). Epigenetic changes at SALL4 gene for example abnormal hypomethylation is also observed in patient samples (Lin et al., 2013; Ma et al., 2013). In leukemia cell lines, SALL4 is abundantly present in primary acute myeloid leukemia (AML) and myeloid leukemia cell lines (Ma et al., 2006).

For solid tumor, many evidences showed that SALL4 is reactivated in various types of cancer and associated with drug resistance, metastasis and proliferation or survival such as: gastric cancer (Osada et al., 2014; Ushiku et al., 2010; Zhang et al., 2014), colorectal cancer

(Forghanifard et al., 2013), lung cancer (Kobayashi et al., 2011a), breast cancer (Kobayashi et al., 2011b; Yue et al., 2015), glioma (Zhang, L. et al., 2015), endometrial cancer (Li et al., 2015; Liu, L. et al., 2015), esophageal squamous carcinoma (Forghanifard et al., 2014). Furthermore, SALL4 expression presents at various types of germ cell tumors and becomes reliable marker for diagnosis (Camparo and Comperat, 2013; Cao et al., 2011; Cao et al., 2009a; Cao et al., 2009b; Mei et al., 2009).

SALL4 is also believed as a cause of aggression in HCC patients. HCC patients with SALL4 positive display higher metastasis capacity and stemness gene markers (Shibahara et al., 2014). SALL4 is also found in serum and served as diagnostic marker for tumor relapse and overall survival rate (Han et al., 2014).

**Interactions and signaling pathways**

*Interactions*

SALL4 is reported to epigenetically co-operate with some protein partners. In nucleus, SALL4 interacts with Nucleosome Remodeling Deacetylase (NuRD) complex and recruits this complex to deacetylate Phosphatase and tensin homolog (PTEN) promoter region (Lu et al., 2009). As a result, expression of PTEN becomes lower due to SALL4 regulation. The NuRD is the complex and composed by different proteins which have defined such as histone deacetylase HDAC1/2, ATP-dependent remodeling enzymes CHD3/4…(Lai and Wade, 2011). Treatment of interactive competitor to SALL4/ NuRD is able to rescue PTEN expression (Gao et al., 2013a; Yong et al., 2013). These evidences provide new strategy to restore PTEN in cancer treatment.

Furthermore, SALL4 is also thought to be engaged with DNA methyltransferases (DNMTs) which are transferred methyl residue to target DNA and controls some downstream genes like PTEN (Yang, J. et al., 2012) (Figure 2.3 A).

Moreover, SALL4-NuRD premises to recruit lysine specific demethylase 1 (LSD1), a histone demethylase that specifically targets histone H3K4 to nip methyl residue at target site and facilitate gene expressions. In leukemia model, by recruiting Mixed lineage leukemia (MLL) protein complex, SALL4 is considered as co-activator of leukemia oncogenes such as HOXA9, MEIS1 and results in more severe leukemogenesis (Liu et al., 2013; Shi et al., 2004) (Figure 2.3B).

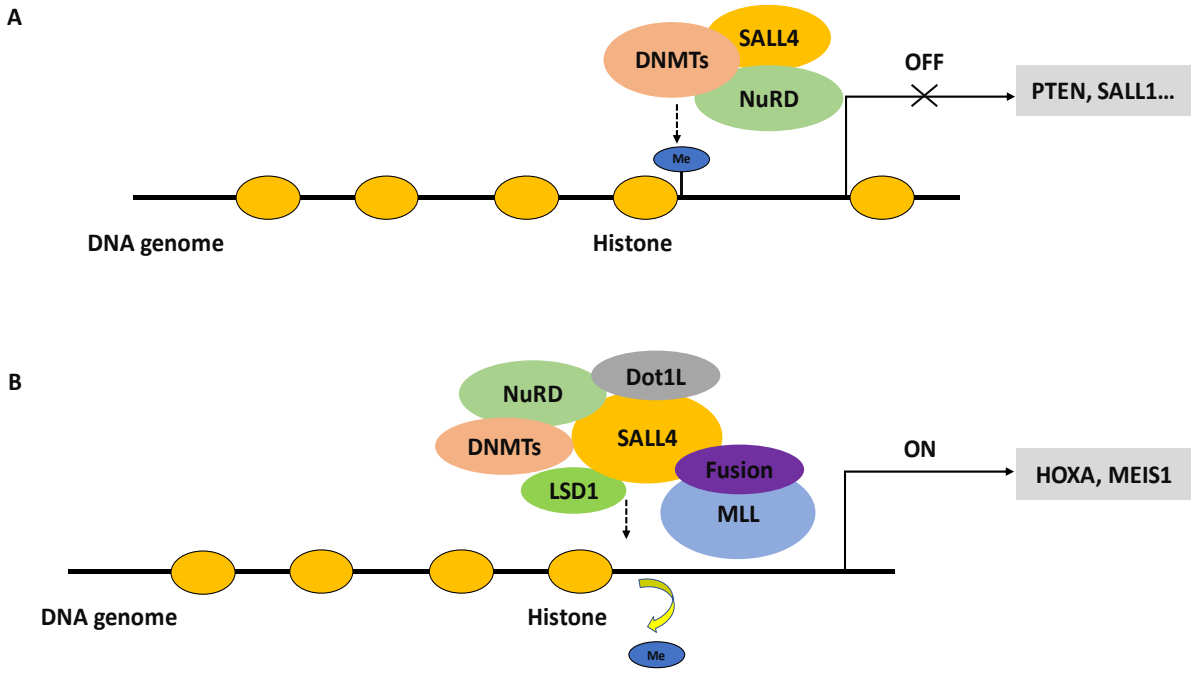



**Figure 2.3 SALL4 and epigenetic factor interactions.** (A) SALL4 epigenetically suppresses the expressions of PTEN, SALL1 via interacting with DNA methyltransferases (DNMT) and Nucleosome Remodeling Deacetylase (NuRD) complex. This results in methylation and inhibition of transcription at target site. (B) SALL4/NuRD/DNMT complex recruits Lysine specific demethylase 1 (LSD1) and Mixed lineage Leukemia (MLL). Therefore, demethylation occurs and drives the expressions of HOXA and MEIS1. This chain of activity induces severe leukemia.

SALL4 seems to be a major pluripotential regulator in murin embryonic stem cells by interacting with many stemness factors including Oct-4, Nanog, or Sox-2 and co-regulates pluripotential in stem cells (Yang et al., 2008). Indeed, SALL4 binds direclty to Sox-2/Oct4 complex and this complex occupies Oct-Sox element in mouse ESCs (Tanimura et al., 2013; van den Berg et al., 2010). Besides, one report indicated that SALL4 interacts with Nanog and co-governs at Nanog binding sites in embrynonic stem cell genome (Wu et al., 2006). Other report suggested that Oct4/SALL4/Nanog triad might govern the development of pre-implanting mammalian embryos (Tan et al., 2013). Moreover, SALL4 enables recruiting polycomb complex protein such as BMI-1 and drives chromatin structure to mediate cell fate in embryonic cells (Abboud et al., 2015). Therefore, there is no doubt to confirm the role of SALL4 to maintain the pluripotency in embryonic stem cells.

*Regulation of signaling pathways*

One study reported that SALL4 enables binding to its promoter and auto-regulates feed-back itself expression (Yang et al., 2010). The study also figured out that SALL4 works as an antagonist at other SALL gene family members (SALL1 and SALL3) and results in the downregulations of these SALL genes (Lu et al., 2009; Yang et al., 2010) (Figure 2.4). Not only interacting with stemness proteins, SALL4 is also able to up-regulate Nanog, Oct4 or Sox-2 (Yang et al., 2008). Furthermore, SALL4 enables BMI-1 expression (Shen et al., 2012; Wang et al., 2013; Yang et al., 2007) or HOXA9 (Gao et al., 2013b; Li, A. et al., 2013) in normal hematopoietic and leukemia cells (Figure 2.3 and Figure 2.4). These evidences indicated the indispensable function of SALL4 to stemness maintenance by signaling pathways.

SALL4 is able to enhance drug resistance in cancer cells by up-regulating ATP-binding cassette multidrug transporters (ABCA3 and ABCB1) in leukemia cancer cells (Jeong et al., 2011). Knocking down SALL4 expression leads to the decrease of side population (SP) cells. Further assays detailed that SALL4 binds to ABCA3 promoter region and directly activates this gene whereas indirectly upregulates ABCG2 (Figure 2.4). Moreover, SALL4 is also considered as an agent to increase some surface markers like EpCAM (Oikawa et al., 2013; Zeng et al., 2014) or CD44 (Yuan et al., 2016) or it acts as a regulator of some genes such as Snail (Zeng et al., 2014), CXCR4 (Oikawa et al., 2013), Twist (Oikawa et al., 2013), E-cadherin (Deng et al., 2015; Liu, L. et al., 2015; Zhang et al., 2014), Vimentin (Itou et al., 2013), or ZEB1 (Itou et al., 2013) which are related to epithelial - mesenchymal transition (EMT) (Figure 2.4).

Many evidences showed that SALL4 has a strong link to Beta-catenin pathway. Indeed, there is association between SALL4 and Beta-catenin in colorectal cancer (Hao et al., 2016), or in acute myeloid leukemia (AML) (Ma et al., 2006), in myelodyplastic syndromes (Shuai et al., 2009) or in choriocarcinoma (Zhao et al., 2018) and esophageal squamous cell carcinoma (He et al., 2016). Further assay indicated that SALL4 and Beta-catenin co-localizes and interacts with each other (Hao et al., 2016; Ma et al., 2006) and consequently upregulates the expressions of c-Myc or cyclin D1 (Shuai et al., 2009; Zhao et al., 2018) or EMT genes (E-cadherin or Vimentin) (He et al., 2016) (Figure 2.4).

SALL4 is also believed to regulate Sonic Hedgehog (Shh) pathway (Akiyama et al., 2015). The study showed that SALL4-Gli3 interaction is very important to the development of limb skeletal elements. This study intensifies the role of SALL4 to development via Shh pathway. Furthermore, SALL4 is asscociated with Gli1 expression in colorectal cancer and may interact with Shh pathway (Cheng et al., 2015). Interactions between SALL4 and Shh signaling have not

yet defined. However, therapies both target SALL4 and Shh signaling pathway may be a promising approach in perspective.

**Conclusion**

Cell fate is controlled by genetics and possibly changed by epigenetic factor. SALL4 has been involved in many interactions and pathways which determine pathway cells go through. Much understanding about SALL4 function in development and carcinogenesis would bring the chance to apply its benefits for tissue regeneration and to inhibit tumor growth. SALL4 is a bridge between embryonic stem cells, development and cancer. Acting as a trans-activator epigenetically and as a regulator for oncogenes, inhibition of SALL4 would be a promising approach in various types of cancer.

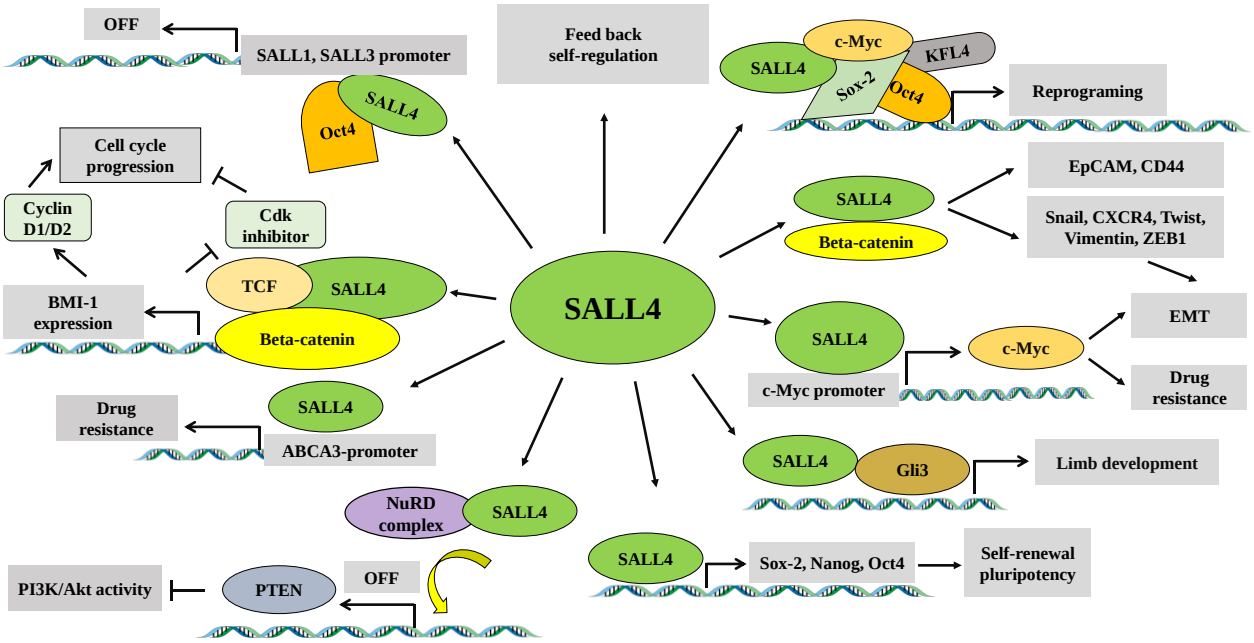



**Figure 2.4 Interactions and signaling pathways involved by SALL4.** SALL4 gets involved in many signaling pathways such as facilitating Sox-2, Nanog, Oct4 expressions to maintain self-renewal pluripotency. SALL4 co-operates with Sox-2, Oct4, KLF4, and c-Myc for cell reprogramming. SALL4 promotes ABCA3 or c-Myc expressions via enhancing promoter activity, results in drug resistance in cancer. Some cell surface or EMT markers such as Snail, CXCR4, Twist, Vimentin, ZEB1, c-Myc or EpCAM, CD44 are driven via SALL4 itself or interaction with beta-catenin, results in high metastasis capacity in cancer cell. SALL4 also governs BMI-1 expression and cell cycle through interacting with beta-catenin and SALL4 also switches off some of target genes (SALL1, SALL3, PTEN). SALL4 also regulates itself expression and targets Shh signaling pathway via interaction with Gli3 to promote limb development in embryo.

## CHAPTER 1

### SALL4/FoxM1/PRX III axis regulates Reactive Oxygen Species in pancreatic ductal adenocarcinoma phenotype


### Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a major malignant phenotype in pancreatic cancer, which is one of the most death causes by cancer in the world. PDAC developed from pancreatic intra-epithelial neoplasms (PanINs) is poorly diagnosed at early stages. Beside of high drug resistance, metastasis is the great concern during pancreatic cancer treatment. In this study, we found the role of Spalt like protein 4 (SALL4) to PDAC progression, mobility and its regulation to reactive oxygen species (ROS) via FoxM1/ Prx III axis. It is possible that SALL4 mainly induces endothelial-mesenchymal transition (EMT) phenotype and decreases oxidative stress to facilitate metastasis efficiency in PDAC cells. Therefore, SALL4 might be a promising marker for PDAC treatment and targeting SALL4 would benefit anti-proliferative and anti-metastasis therapies.



**Keywords:**

SALL4, pancreatic ductal adenocarcinoma, metastasis, FoxM1, Peroxiredoxin III.

**Introduction**

Pancreatic cancer is one of the most death causes in United States due to its high mortality and inefficacy treatments. Pancreas is composed of endocrine and exocrine ductal and both of them exhibit cancer. However, tumor in endocrine pancreas is not popular. Most of cases are pancreatic ductal adenocarcinoma (PDAC) and at advanced stages when surgery is impossible (Ercan et al., 2017). With current treatment by chemotherapies (Gemcitabine, 5-Flurocil plus leucovorin), cancer patients are still suffered from low survival rate. The main obstacle is poor diagnostic tests at early stage of PDAC and as a result, most of patients are found at late stage with local or advanced metastasis. Therefore, inhibition of pancreatic cancer metastasis is one of urgent target in treatment.

Pancreatic intra-epithelial neoplasm (PanINs) are the precursor stage of PDAC and are resulted from chronic pancreatic inflammation (Hernandez-Munoz et al., 2008). PanINs are classified in different stages including: PanIN1A, PanIN-1B, PanIN-2, PanIN-3 and finally transformed into PDAC phenotype (Figure 3.1). Approximately 90% of PDAC belongs to K-RAS mutation (Hingorani et al., 2005). Coordinating with mutations in tumor repressor genes such as cyclin-dependent kinase inhibitor 2A (CDKN2A) or mothers against decapentaplegic homolog 4 (SMAD4) or TP53 facilitates for the transformation of malignance (Bardeesy et al., 2006; Feldmann et al., 2007). Furthermore, other factors such as mutations in genome-maintenance genes or abnormalities in telomere length or epigenetics, alterations in expression of apomucin (MUC1, MUC2, MUC5) and Cox-2 might create PanINs (Koorstra et al., 2008).

In term of morphology, PDAC phenotype is consisted of epithelial cells and dense-fibrotic stroma. The high dense stroma is believed to constitute 90% tumor volume and acts as a barrier for drug delivery or other therapies (Ercan et al., 2017).

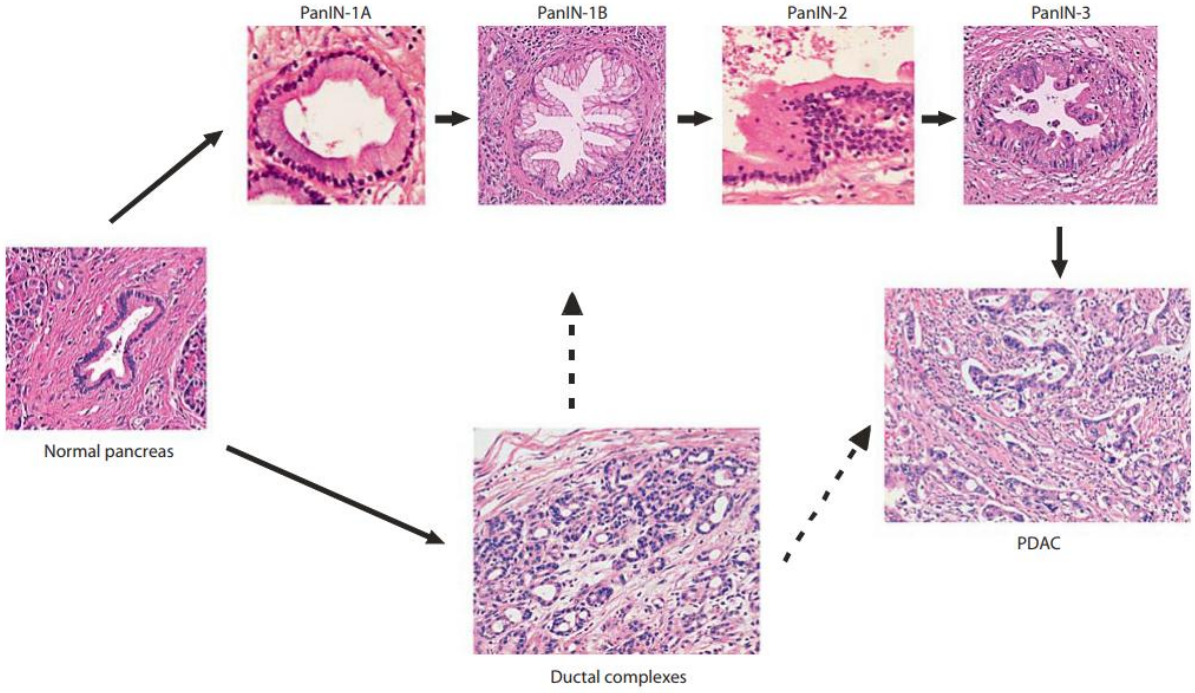



**Figure 3.1 Intermediate stages of before pancreatic ductal adenocarcinoma**. Normal pancreas transforms into two states, including pancreatic intra-epithelial neoplasm (PanIN)-1A or ductal complexes. Ductal complexes can transform directly into PDAC phenotype or indirectly PanIN-1B/ PanIN-2 and PanIN-3 before PDAC (Hernandez-Munoz et al., 2008).

*Peroxiredoxin protein family*

Peroxiredoxin (PRDX) proteins are thiol-specific antioxidant enzymes and catalyze peroxide reduction of by their cysteine-containing core sites (Park et al., 2016) (Figure 3.2). In mammalian system PRDX protein family is consisted of six proteins, which are named from PRDX1-6 (Nicolussi et al., 2017). PRDXs are divided into 3 subclasses: PRDX1-4 contain 1-Cys and typical 2-Cys redox-active cysteine residue; PRDX5 possesses 1-Cys and atypical 2-Cys while PRDX6 possesses only 1-Cys group (Nicolussi et al., 2017) (Figure 3.3). PRDXs protein family is important to cell signaling and metabolism and served as redox signaling regulator. Therefore, PRDX proteins are tightly involved in cell proliferation, apoptosis, lipid metabolism, immune responses or embryonic development (Park et al., 2016). Recently, several studies indicated that PRDXs are associated with various types of cancer such as lung, glioblastoma, colorectal cancer, prostate cancer or ovarian cancer and along with that NF-kB, STAT3, Wnt/Beta-catenin or MAPK pathways have been reported (Nicolussi et al., 2017; Park et al., 2016). Thus, better understanding about PRDXs would benefit for anti-cancer therapies.

*Peroxiredoxin III protein*

Peroxiredoxin III (Prx III) or PRDX3 acts as a 1-Cys and typical 2-Cys redox-active cysteine residue enzyme and is responsible for mitochondrial homeostasis and neoplastic transformation (Nicolussi et al., 2017; Park et al., 2016). Located in mitochondria and uses Thioredoxin 2 (Trx2) as electron donor for their activities (Li and Yu, 2015), Prx III is believed as a main redox regulator of mitochondrial $H_2O_2$ (Chang et al., 2004; Cunniff et al., 2014). Prx III coordinates with MAP3K13 to regulate cytosol NF-kB activities and prevents cells from oxidative damages (Park et al., 2016) (Figure 3.4).

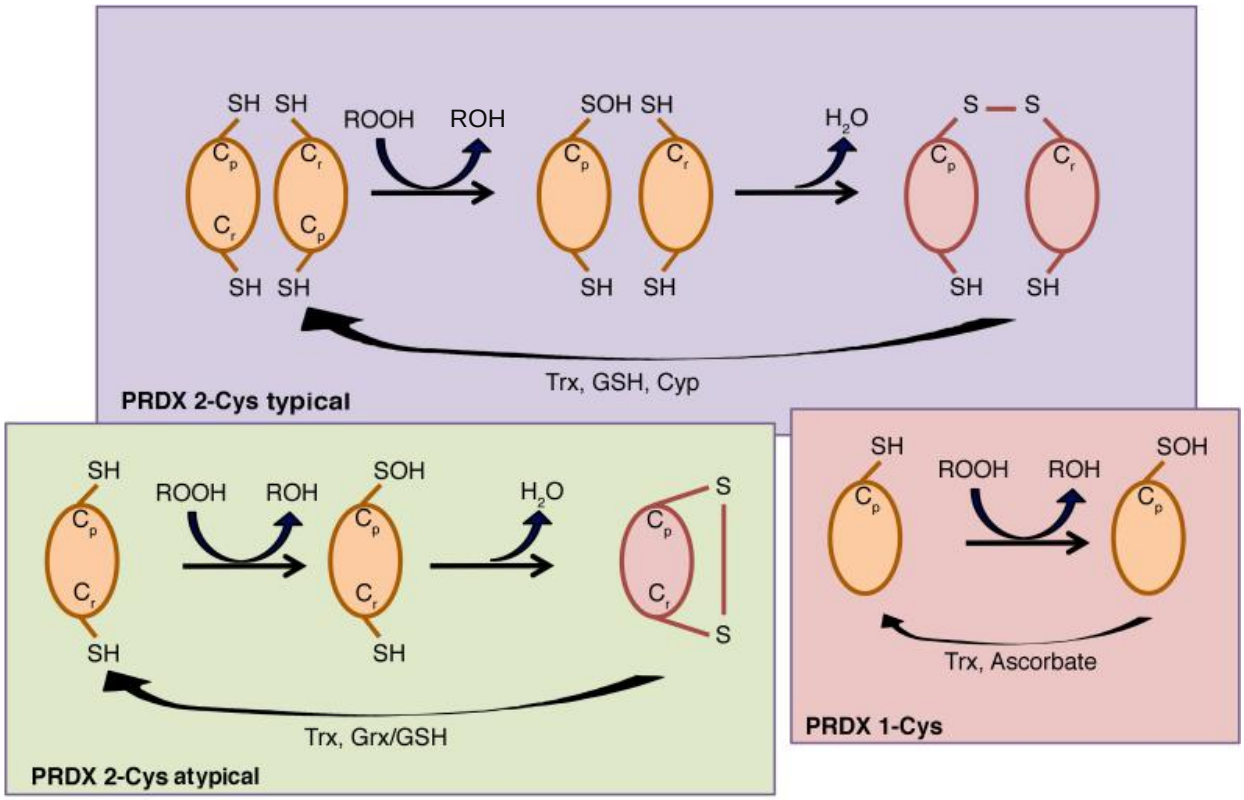



**Figure 3.3 PRDX subclasses and their mechanisms.** PRDX proteins are composed of three subclasses via their structures. Proteins PRDX1-4 are 2-Cys typical and dimer, included two subunits are reverse to each other. The 1st subunit $C_p$ residue in oxidation state reacts with the 2nd subunit $C_r$ residue and forms disulfide bond. By the involvements of Trx, GSH or Cyp, PRXDs restructure into innate forms. In monomer 2-Cys atypical PRDX5, $C_p$ reacts with $C_r$ in the same subunit after oxidation. In PRDX6, there is only $C_p$ interacts with peroxide species and received electron from donor (Trx or Ascorbate) and returns to initial state. Cyp, cyclophilin; Grx, glutaredoxin; GSH, reduced glutathione; ROOH, peroxide; $C_p$, peroxidatic Cys; $C_r$, resolving cysteine; Trx, thioredoxin (Nicolussi et al., 2017).

In many types of cancer, the promotions of Prx III to carcinogenesis have reported. Prx III is believed to affect cell proliferation and cell cycle in breast cancer cells and involved in prostate cancer resistance (Whitaker et al., 2013). Prx III also protects cervical cancer from apoptosis (Li, L. et al., 2013) and responsible for drug resistance in ovarian cancer (Li and Yu, 2015). Prx III is thought to maintain the survival of endometrial CSCs (Song et al., 2017). Furthermore, Prx III is upregulated in laryngeal squamous cell carcinoma (Zhang et al., 2017). All these studies indicated that Prx III is associated with aggressive phenotype. In the context of fast growing and drug treatment, level of reactive oxygene species (ROS) becomes upregulated, the expression of PRDXs family (Prx III) is very important to favor malignant cell growth.

Many activators of Prx III have been reported. Sirtuin 1 (SIRT1), a class III histone deacetylase is able to drive expression of Prx III via the formation of PGC-1α/FoxO3a transcriptional complex in bovine aortic epithelial cells (Olmos et al., 2013). Prx III is also regulated by forkhead box protein 1 (FoxM1) in colon CSCs (Song et al., 2015). Moreover, microRNAs are also involved in Prx III maintenance in cancer cells (Jiang et al., 2015; Li and Yu, 2015).

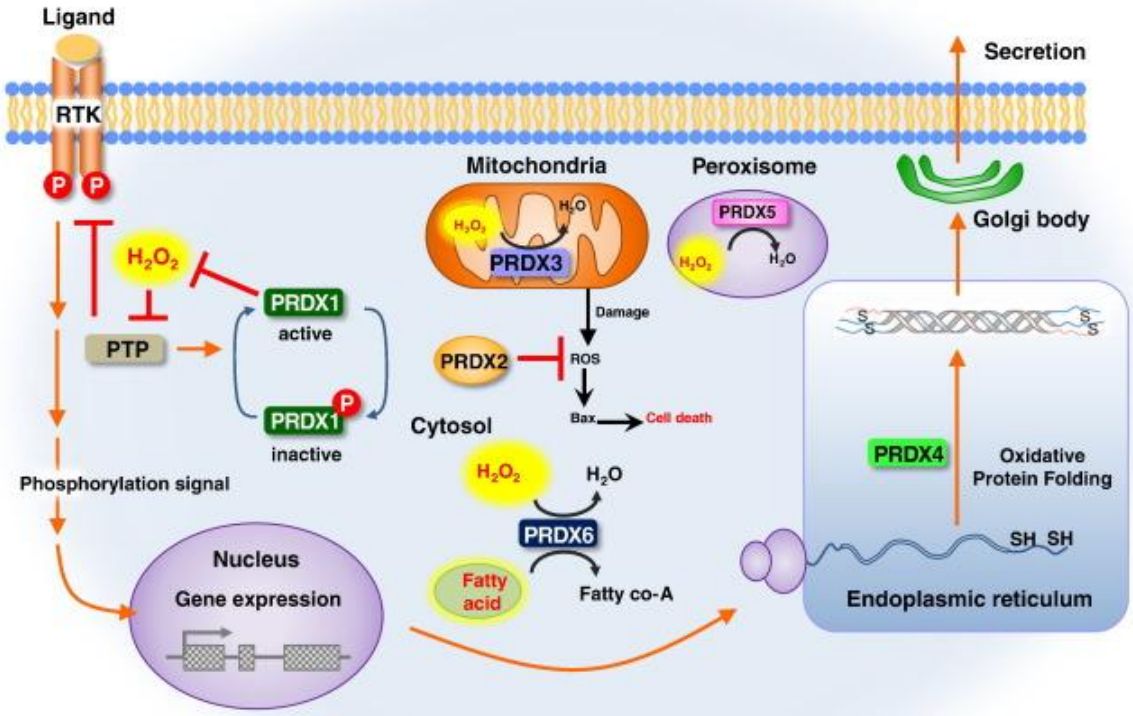



**Figure 3.4 PRDXs distribution and their functions in cell metabolism**. Receptor Tyrosine Kinase (RTK) activated by its ligand can drive signaling pathway related to cell growth. Phosphotyrosine phosphatase (PTP) inhibits RTK activation and helps transform inactive PRDX1 to active, which suppresses $H_2O_2$ in cells. Therefore, in this case $H_2O_2$ maintains RTK signaling pathway and promotes cell growth. PRDX2 represses the apoptosis-induced Bax activation via its function in ROS scavenging. PRDX3 localizes at mitochondria to eliminate ROS. PRDX4 is engaged to endoplasmic reticulum, modified and secreted into serum to circulate in whole body. PRDX5 represents at peroxisome and works as an antioxidant of $H_2O_2$ and alkyl hydroperoxides. PRDX6 acts with $H_2O_2$, short fatty acid or its derivatives (Park et al., 2016).

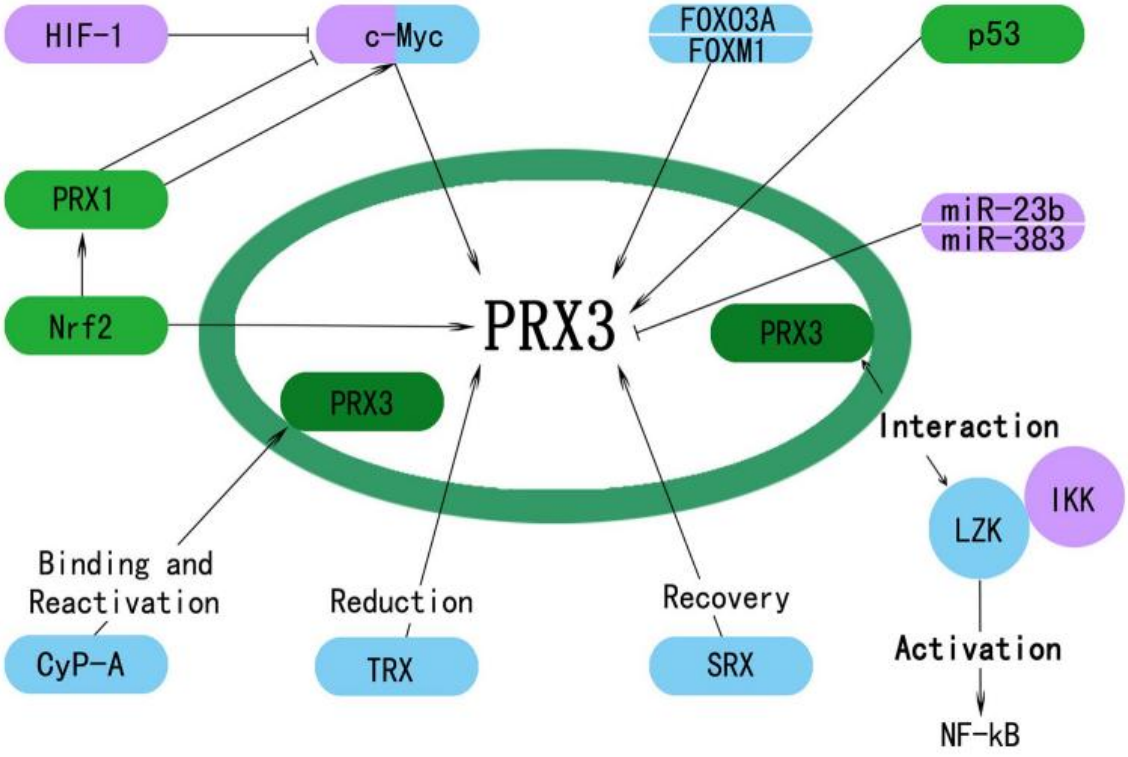



**Figure 3.5 PRX III and its interactions.** Oxidative stress promotes the activations of p53, Nrf2, FoxM1 and these enhance Prx III expression. Prx III is also upregulated by c-Myc, which is inactivated by HIF-1 and activated by Nrf2/PRX1. MicroRNA miR-23b and miR-383 are potential inhibitor of PRX III. The interaction between PRX III and IkB kinase (IKK) induces activation of NF-kB and leading to anti-apoptosis. PRX III also interacts with SRX or TRX to transform its oxidative state or is promoted by cytosolic cyclophilin A (CyP-A) (Li and Yu, 2015).

*Forkhead box protein 1*

Forkhead box protein 1 (FoxM1) is a member in Forkhead family which has conserved DNA binding domain named as Forkhead (Laoukili et al., 2007). These proteins are classified by their identity similarity in DNA binding domain and to FoxM1, this ratio is 45%. FoxM1 binds to DNA at consensus region TAAACA and shares this recognizing region to other forkhead protein family in *in vitro* (Korver et al., 1997; Pierrou et al., 1994). Due to splicing at transcription level, FoxM1 is varied into 3 subtypes, including Class A (exons and alternative region A1 and A2), Class B (exons and none of A1 or A2) and Class C (exons and only A1) (Figure 3.6).

FoxM1 is believed to enhance proliferation, cell cycle regulator (Koo et al., 2012; Laoukili et al., 2007). Moreover, FoxM1 plays role in DNA damage and sensecence (Alvarez-Fernandez and Medema, 2013; Zona et al., 2014) and by these characteristics, FoxM1 becomes one of the factors crucial for tumorigenesis and drug resistance (Zona et al., 2014). Other evidences also indicated that FoxM1 maintains Oct4, Nanog, Sox-2 expression which faciliate for self renewal in cancer cells (Koo et al., 2012). By driving vascular endothelial growth factor (VEGF) or EMT genes, FoxM1 also triggers tumor angiogenesis and cell mobility and these are critical steps for tumor metastasis (Koo et al., 2012). Furthermore, FoxM1 is also involved in carcinogensis of cervical squamous carcinoma, breast cancer, or basal cell carcinoma (Luscher-Firzlaff et al., 1999; Teh et al., 2002; Wonsey and Follettie, 2005). Evidences also indicated FoxM1 promotes prostate cancer and lung adenocarcinoma or hepatocellular carcinoma growth (Kalin et al., 2006; Kalinichenko et al., 2004; Kalinina et al., 2003; Kim et al., 2006). These evidences suggest FoxM1 becomes reliable marker for prodiagnosis (Nandi et al., 2017) and provide a new approach in anti-tumorigenesis via FoxM1 inhibition.

In pancreatic cancer, evidences showed that FoxM1 participates at tumor initiation and at whole stages of PDAC (Quan et al., 2013). Indeed, during the transformation from PanINs to PDAC, there are many factors govern such as tumor suppressor 53 (TP53), cyclin-dependent kinase inhibitor 2a (CDKN2A), SMAD4 or KRAS. Some mutations of these genes are believed as the major causes induce PDAC phenotype. Evidences showed that FoxM1 is the target of Ras/Raf/MAPK which induces its nuclear translocation and trans-activating activity of FOXM1. Futhermore, FoxM1 also hyperacitvate PI3K/AKT pathway and loss of function of TP53. In epigenetics, FoxM1 suppresses CDKN2A expression by its hypermethylation activity on promoter. These evidences confirmed that during PanIN-PDAC transition, FoxM1 is tightly involved.

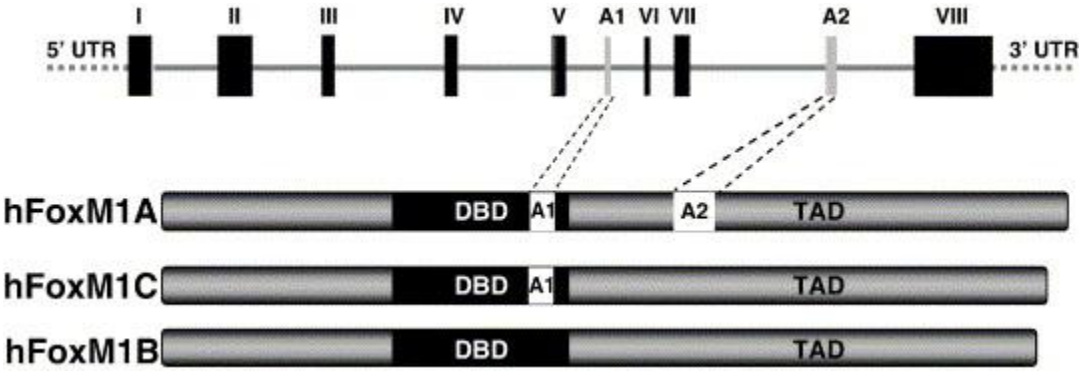



**Figure 3.6 Variants of human FoxM1 protein**. A1 and A2 Exons are variants among FoxM1 proteins. Exon A1 lengths 15 amino acids and locates at C-terminal of DNA binding domain (DBD) while Exon A2 lengths 38 amino acids and inserts at C-terminal transactivation domain (TAD) of protein. Insertions of Exons A1 and A2 create FoxM1 isoforms A, B and C. (Laoukili et al., 2007).

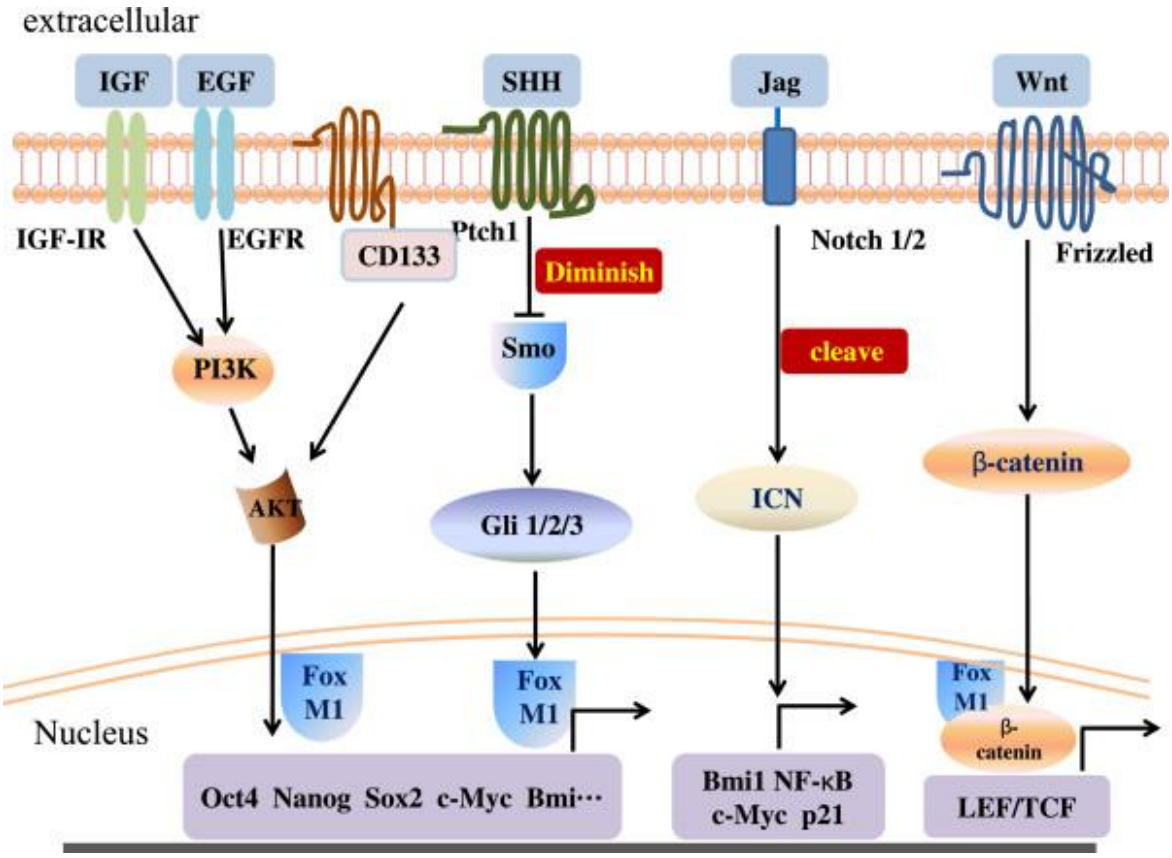



**Figure 3.7 The involvement of FoxM1 to different signaling pathways.** FoxM1 gets involved in activation of Shh/ Gli pathway and maintains stemness traits via driving stem cell factors such as Oct4, Nanog, Sox-2, c-Myc or Bmi1. At present, there is no evidence showing the correlation between Notch signaling pathway and FoxM1. However, FoxM1 and Notch signaling pathway share some target genes might induce cancer aggression. FoxM1acts as a partner of Beta-catenin and determines target genes in Wnt/beta catenin at factor T-cell factor/lymphocyte enhancer factor (TCF/LEF) site. CD133 and phosphorylation of Akt might cause FoxM1 activation, resulting in expressions of target genes. IGF, insulin-like growth factor; EGF, epidermal growth factor; Jag, Jagged; IGF-1R, insulin-like growth factor-1 receptor; EGFR, epidermal growth factor receptor; ICN, intracellular domain of Notch (Quan et al., 2013).

## Materials and methods

### Cell culture

AsPC-1, BxPC-3 and PANC-1 cells purchased from American Type Culture Collection (ATCC) were cultured in DMEM or RPMI (Gibco, CA, US), supplemented with 10% FBS (Welgene, Korea) and 1% anti-mycotic agent. Cells were maintained in a humidified atmosphere of 5% $CO_2$ in the incubator at 37 ℃ and passaged twice per week.

### siRNA transfection

BxPC-3 cells in log phase reached 80% confluence before proceeding siRNA transfection by using RNAimax Lipofectamine. In brief, diluted siRNA and diluted RNAimax reagent were mixed with 1:1 ratio, followed by 5 min incubation at room temperature before transfection. siRNA-RNAimax complex was added to cells in serum and antibiotics free medium.

### Clonogenicity assay

Cells ($1 \times 10^3$) were seeded onto 6-well plates at 37 $^0$C/ 5% $CO_2$. After 7 days of incubation in DMEM supplemented with 10% FBS, cells were fixed with 3.7% PFA and stained with 0.05% Crystal violet, followed by washing with 1X PBS before observation.

### *In vitro* cell migration and invasion assays

A cell migration assay was performed using 8-µm pore-sized hanging cell-inserts (Merck Millipore, MA, US). Cells ($1 \times 10^5$) in 0.5% FBS-DMEM were seeded in the upper chamber while the lower chamber was filled with 20% FBS. After 48 h of incubation, the migrating cells were stained with 0.05% crystal violet (w/v). The number of migrated cells in the lower surface of the membrane was counted under a microscope in five random fields at 100 ×. For the cell

invasion assay, all procedures carried out were the same as that in the migration assay, except that in this case, a Matrigel matrix growth factor reduced basement (BD Biosciences, NJ, US) (3.5 mg/ml) was coated on the upper chamber, according to the manufacturer's protocol.

**Immunocytochemical staining**

Cells after seeding 24 h, were fixed using 3.7% paraformaldehyde (PFA). Before overnight staining with the primary antibodies of interest (CD133, CD44, c-Myc, CXCR4, Nanog, Oct4, Sall4, Sox-2), cells were blocked by PBST 1X with 3% BSA. After washing twice with PBST 1X, the secondary antibodies were added, followed by a 2-h incubation. Cells were stained for 15 min with 4', 6-diamidino-2-phenylindole (DAPI) before microscopic observation.

**Western blotting**

The protein content of the cell lysates was determined using the BCA assay. Proteins were separated on by 12% SDS-PAGE, transferred electrophoretically (Bio-Rad, CA, USA) onto a polyvinylidene fluoride (PVDF) membrane, and blocked with 5% non-fat milk powder (w/v) in phosphate-buffered saline 0.1% Tween-20 (PBST) for 1 hour at room temperature; membrane was followed by incubation with primary antibodies or with anti-GADPH mouse monoclonal antibody as an internal control overnight at 4℃ and with appropriate HRP-conjugated secondary antibodies for 4 h at room temperature. The bands were captured using an ImageQuant™ LAS 4000 mini Fujifilm camera.

**Table 1: List of antibodies for Western blotting**

| No. | Antibodies | Company | Catalogue number | Source |
|---|---|---|---|---|
| 1 | CD133 | Proteintech | 18470-1-AP | Rabbit polyclonal |
| 2 | CD44 | Santa cruz | sc-7297 | Mouse monoclonal |
| 3 | CD24 | Bioss | bs-4890R | Rabbit polyclonal |
| 4 | SALL4 | Abfrontier | YF-MA11603 | Mouse monoclonal |
| 5 | c-Myc | Santa cruz | sc-40 | Mouse monoclonal |
| 6 | CXCR4 | Abfrontier | YF-MA16239 | Mouse monoclonal |
| 7 | GADPH | Abfrontier | LF-MA0038 | Rabbit polyclonal |
| 8 | Nanog | Santa cruz | sc-33759 | Mouse monoclonal |
| 9 | N-cadherin | Abfrontier | LF-MA50067 | Mouse monoclonal |
| 10 | Oct-4 | Santa cruz | sc-9081 | Rabbit polyclonal |
| 11 | Sox-2 | Santa cruz | sc-20088 | Mouse monoclonal |
| 12 | Vimentin | Santa-cruz | sc-6260 | Mouse monoclonal |
| 13 | ERK1/2 | Abfrontier | LF-MA0178 | Mouse monoclonal |
| 14 | pERK1/2 | Abfrontier | LF-PA0199 | Rabbit polyclonal |
| 15 | FoxM1 | Abfrontier | LF-MA10387 | Mouse monoclonal |
| 16 | Prx III | Abfrontier | LF-PA0030 | Rabbit polyclonal |

***In vivo* evaluation**

$5 \times 10^6$ cells were injected into the flanks of 6-week-old nude female BALB/c-nu mice. The tumor sizes were measured every 7 days using calipers. The tumor volume ($V = W \times L \times H/2$) was evaluated by length (L), height (H), width (W). Mice were sacrificed after day 35 of cell injection.

**Statistical analysis**

Statistical analysis was performed using the Graphpad Prism software (Version 6.02). Data are expressed as mean ± standard deviation (SD). Experimental differences were examined by ANOVA and Student's *t*-tests, as appropriate. *P* values of <0.05 were considered to indicate statistically significant data.

## Results

### SALL4 expression pertains to mobility in PDAC cells

To validate the role of SALL4 in PDAC phenotype, we examined expression of SALL4 among three PDAC cell lines (BxPC-3, PANC-1, AsPC-1). We found that SALL4 expression was significantly higher in BxPC-3 as compared to PANC-1 and AsPC-1 (Figure 3.8 A-B). Previous reports showed that SALL4 regulates stemness properties and cell metastasis in various types of cancers (Forghanifard et al., 2014; Forghanifard et al., 2013; Fujimoto et al., 2014; Kobayashi et al., 2011a; Kobayashi et al., 2011b; Ma et al., 2006; Miettinen et al., 2014; Yong et al., 2013; Yue et al., 2015; Zhang et al., 2014; Zhang, L. et al., 2015). Therefore, we performed clonogenicity, migration and invasion assays among three PDAC cell lines. Results indicated BxPC-3 forming colonies abundantly as compared to PANC-1 and AsPC-1.

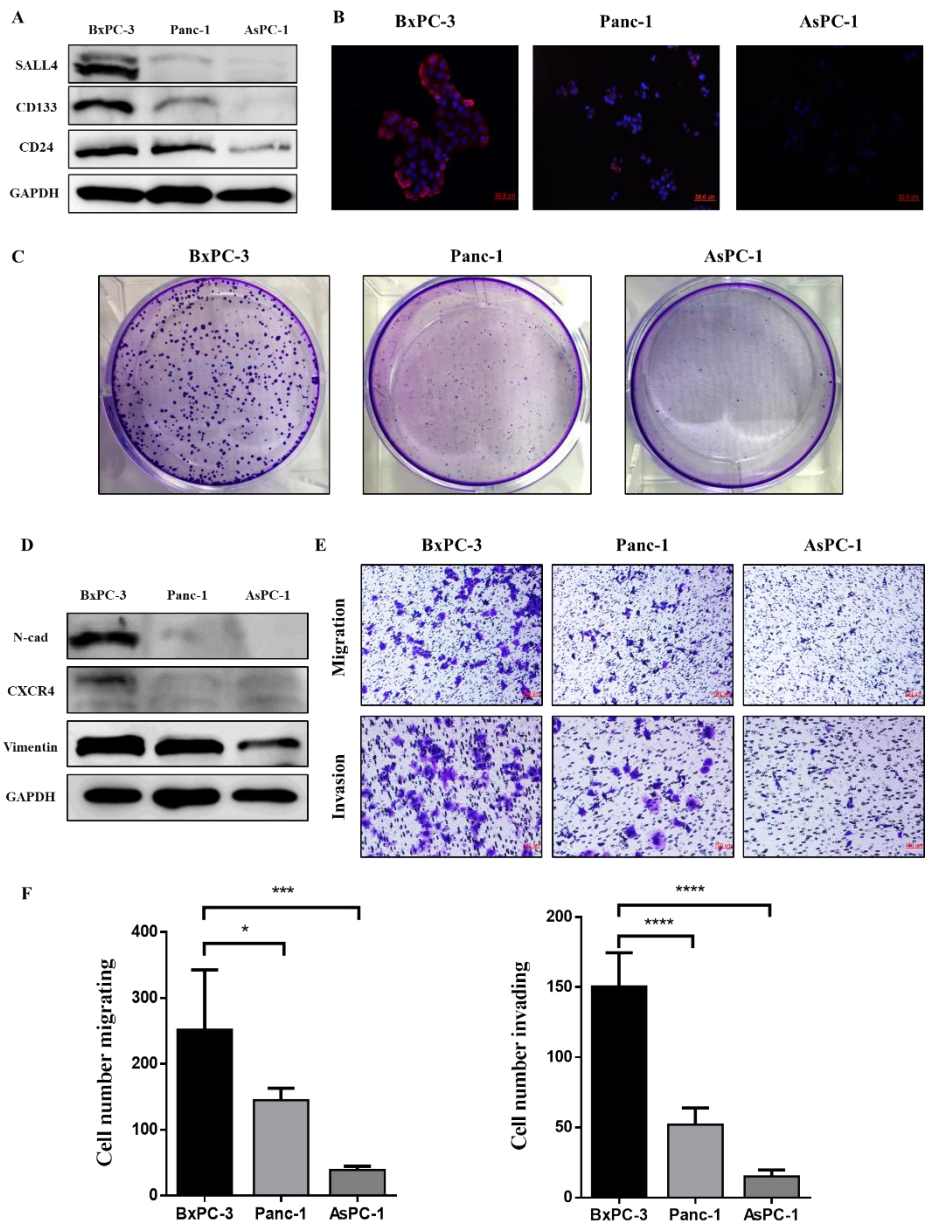



**Figure 3.8 Expression of SALL4 in human PDAC cell lines.** A, Western blotting of SALL4, CD133 and CD24 proteins among PDAC cell lines. B, Immunocytochemistry staining of SALL4 in PDAC cell lines. C, Clonogenicity of PDAC cell lines. D, Western blotting of EMT markers among PDAC cell lines. E, Migration and invasion of PDAC cell lines. F, Number of cells migrating and invading in PDAC cell lines. P value < 0.05 is considered as significance.

(Figure 3.8 C). Furthermore, SALL4 expression was involved in the upregulations of N-cad, CXCR4, Vimentin (Figure 3.8 D) and as a result, numbers of migrating and invading cells in BxPC-3 were highest (Figure 3.8 E-F). These data suggested that SALL4 might pertain to the ability of cell mobility in PDAC cells.

**SALL4 knockdown suppresses stemness and metastatic phenotypes in PDAC cells**

Next, we knocked down the expression of SALL4 in BxPC-3 by using siRNA to examine responsive genes. The downregulation of SALL4 was examined by immunocytochemistry staining and western blotting. Results showed that level of SALL4 was attenuated after knockdown (Figure 3.9 A-B). Furthermore, other cancer stem cells surface markers such as CD133, CD44 and CD24 were downregulated (Figure 3.9 B). To verify the effects of SALL4 knockdown to stemness properties, we performed clonogenicity assay and western blotting. Results showed that colony forming ability was reduced in SALL4 siRNA-transfected cells as compared to control siRNA (Figure 3.9 C). Western blotting showed the downregulations of stemness genes including Sox-2, Nanog, c-Myc (Figure 3.9 D). Next, we further determined SALL4 knockdown effects to metastatic phenotype. Results indicated that SALL4 knocked down by siRNA affected to the migration and invasion of BxPC-3 (Figure 3.9 E) and showed the delay in wound closuring (Figure 3.9 F). Western blotting results further indicated that epithelial-mesenchymal transition (EMT) markers were downregulated after SALL4 siRNA transfection (Figure 3.9 G). These data suggested that SALL4 downregulation suppresses stemness and metastatic phenotypes in PDAC cells.

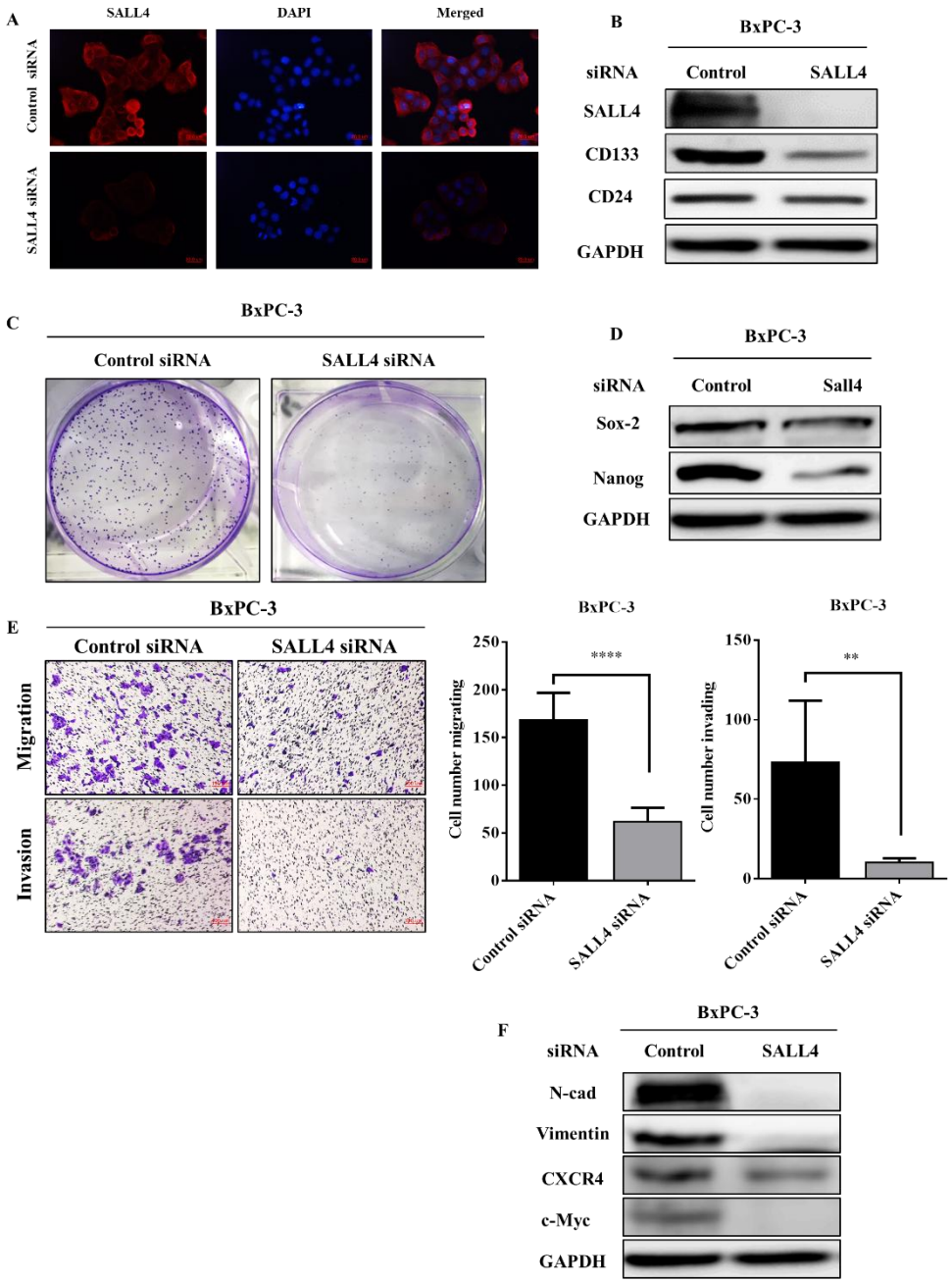



**Figure 3.9 SALL4 downregulation inhibits stemness traits and cell mobility in PDAC.** A, Immunocytochemistry staining of SALL4 in cells transfected with siRNA control vs SALL4 siRNA. B, Western blotting of SALL4, CD133 and CD24 in lysates transfected with control siRNA vs SALL4 siRNA. C, Clonogenicity of BxPC-3 in siRNA control vs SALL4 siRNA. D,

Western blotting of stemness genes in lysates transfected with siRNA control vs SALL4 siRNA. E, Migration and invasion of cells transfected with siRNA control vs SALL4 siRNA. F, Western blotting of EMT markers in lysates transfected with siRNA control vs SALL4 siRNA. *P value* < 0.05 is considered as significance.

**SALL4 overexpression enhances stemness trait and cell mobility in PDAC cells**

Next, we checked whether the overexpression of SALL4 in two PDAC cell lines change the stemness trait and cell mobility. Cells at 48 h post-transfection were used for further analysis. In Figure 3.10 A, cells after transfection with pENTER-SALL4 showed the increase of SALL4 expression as compared to mock vector. Western blot also indicated SALL4 upregulation and along with that CD133 and CD24 were also increased (Figure 3.10 B). Clonongenicity assay showed that colony forming ability was enhanced significantly as compared to mock control (Figure 3.10 C). Furthermore, upregulations of Sox-2 , Nanog, c-Myc were also observed. Figure 3.10 D, E indicated that the number of cell migrating and invading are dramatically increased versus mock control. However, only Vimentin was upregulated by SALL4 overexpression while N-cad, CXCR4 and c-Myc were unchangeable (Figure 3.10 F). Taken together, these data suggested that SALL4 overexpression enhances self-renewal capacity and mobility in PDAC phenotype.

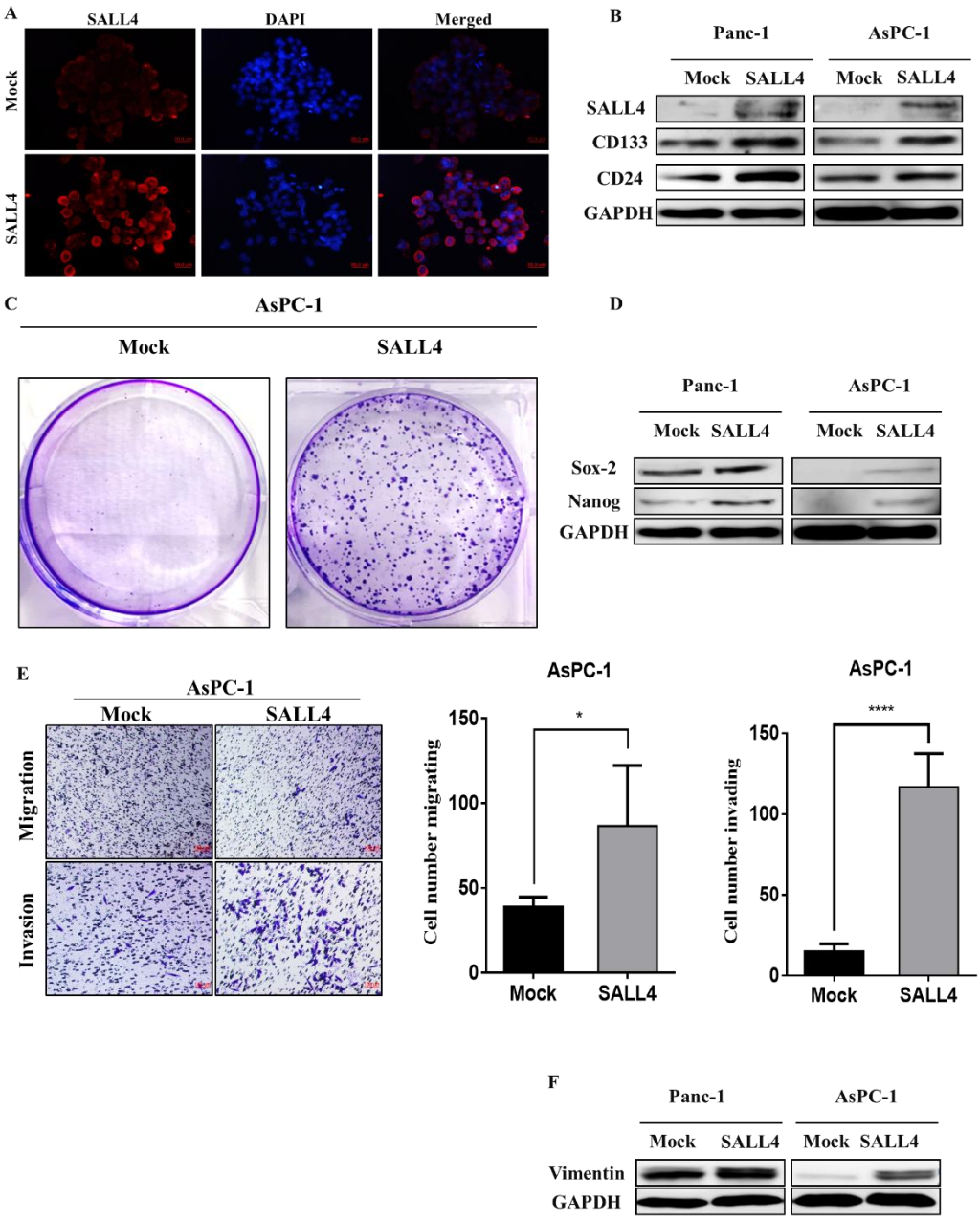



**Figure 3.10 SALL4 overexpression promotes PDAC stemness traits and cell mobility.** A, Immunocytochemistry staining of SALL4 expression in cells transfected with mock vs SALL4 vector. B, Western blotting of SALL4, CD133 and CD24 in lysates transfected with Mock vs SALL4 vector. C, Clonogenicity of AsPC-1 cells transfected with mock vs SALL4 vector. D,

Western blotting of stemness genes in lysates transfected with mock vs SALL4 vector. E, Migration and invasion assay of AsPC-1 cells transfected with mock vs SALL4 vector. F, Western blotting of EMT marker Vimentin in lysates transfected with mock vs SALL4 vector. *P value* < 0.05 is considered as significance.

**SALL4 promotes tumor growth in PDAC phenotype**

Many evidences showed that SALL4 plays important role to tumor growth in many types of cancer (Tatetsu et al., 2016; Zhang, X. et al., 2015). In this study, we have examined the role of SALL4 to PDAC tumor growth. Results showed that downregulation of SALL4 affected to tumor size, weight, volume as compared to control (Figure 3.11 A). In contrast, when introducing SALL4 vector to PDAC cells, sizes, weights or volumes of tumors grew significantly as compare to mock groups (Figure 3.11 B). These data suggested that SALL4 presence notably promotes to PDAC tumorigenesis.

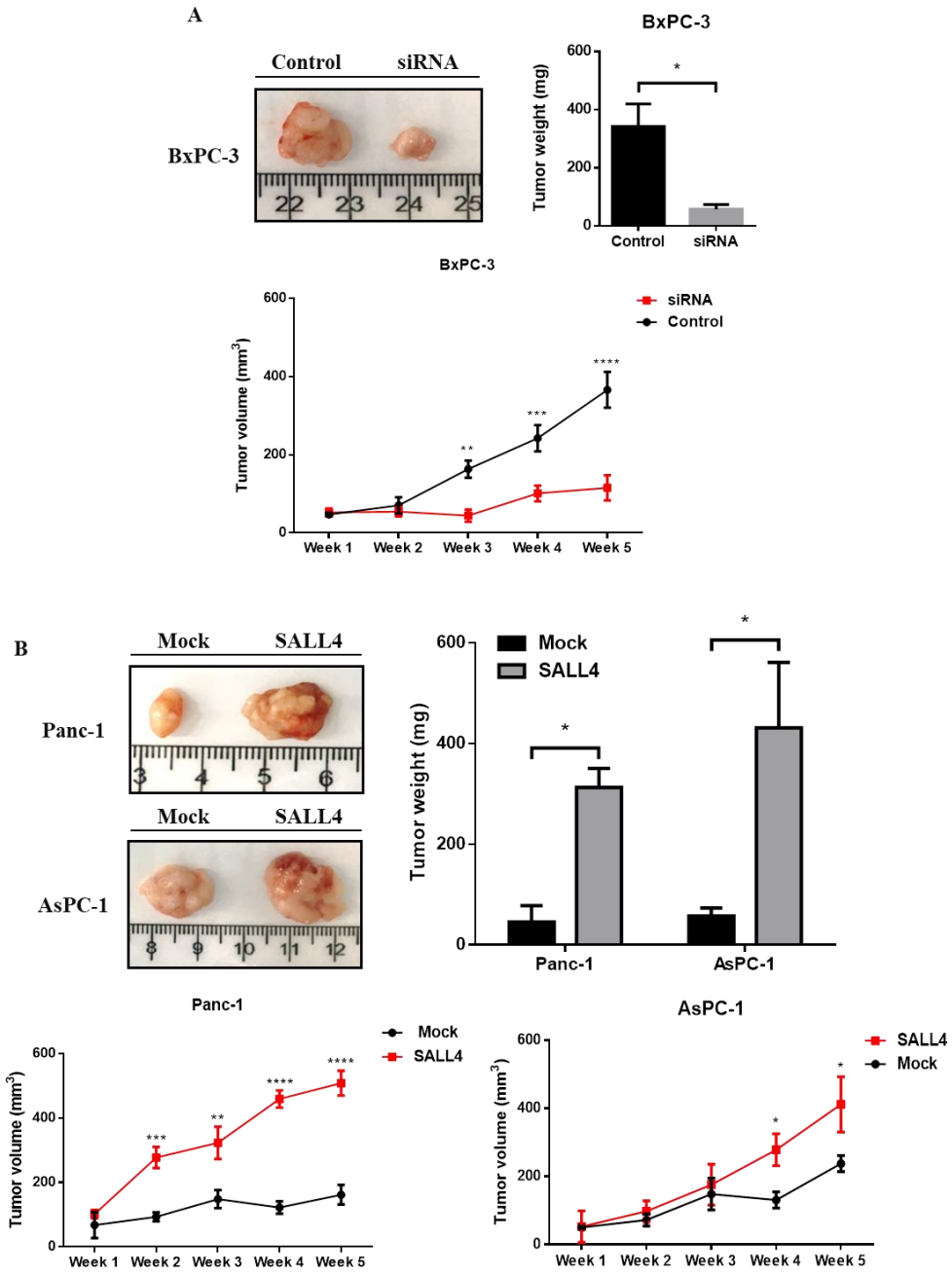



**Figure 3.11 Promotion of SALL4 to PDC tumorigenesis.** A, tumor resections/ weights/ volume in control vs siRNA treatment. B, tumor resections/ weights/ volume in mock vs SALL4 vector. *P value* < 0.05 is considered as significance.

**SALL4 regulates reactive oxygen species in PDAC cells**

ROS homeostasis is very important to cancer cell metabolism (Kim et al., 2016; Panieri and Santoro, 2016). In this study, we have check ROS level among three PDAC cell lines. Results showed that ROS in BxPC-3 was lowest as compared to PANC-1 and AsPC-1 (Figure 3.12 A). The downregulation of SALL4 rescued ROS level in the PDAC cells (Figure 3.12 B). However, SALL4 overexpression was able to reinforce ROS scavenging capacity in PDAC (Figure 3.12 C). These data suggested that SALL4 might regulate ROS homeostasis in PDAC cells in directly or indirectly way.

**SALL4 modulates Prx III activity via FoxM1 activation**

ROS homeostasis in cells is mainly controlled by oxidase enzymes, for instance Peroxiredoxins (Kim et al., 2016; Nicolussi et al., 2017; Ow et al., 2017; Park et al., 2016). Data above showed that SALL4 regulates ROS in PDAC cells. To address the pathway SALL4 involved in, we have screened the expressions of Prx proteins among three PDAC cell lines. Results showed that Prx III was expressed in all cell line and highest at BxPC-3 (Figure 3.13 A). Furthermore, we also found that FoxM1, one of direct activator of Prx III, was also linear to SALL4 and Prx III expressions (Figure 3.13 A and B).

Downregulation of SALL4 by siRNA showed the decrease of Prx III and FoxM1 while in converse way, there were upregulations of FoxM1 and Prx III (Figure 3.13 C). In term of activation signaling, there was dephosphorylation of extracellular-signal-regulated kinase 1/2 (ERK1/2) when SALL4 expression was silenced. Moreover, overexpression of SALL4 enhanced ERK1/2 phosphorylation (Figure 3.13 C). These data suggested that SALL4 might regulates ROS homeostasis by activation of FoxM1/Prx III via ERK1/2 phosphorylation.

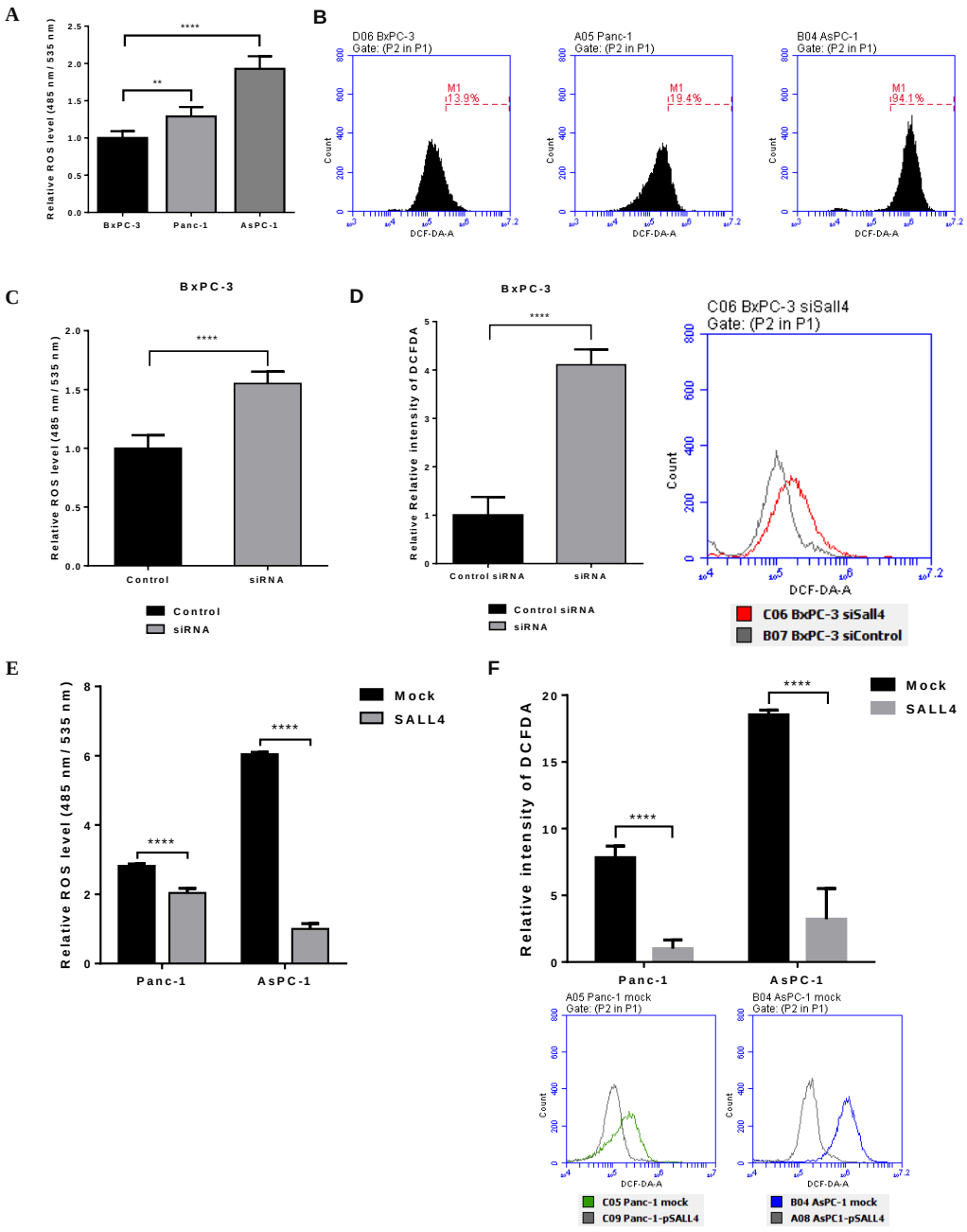



**Figure 3.12 SALL4 regulates ROS in PDAC cells.** A, levels of ROS in PDAC cell lines. B, FACS analysis of ROS among PDAC cell lines by 2',7' –dichlorofluorescin diacetate (DCF-DA). C, ROS level in control vs siRNA. D, FACS analysis of ROS measured by DCF-DA. E, ROS level in cells transfected with mock vs SALL4 vector. F, FACS analysis of ROS in cells transfected with mock vs SALL4 vector, measured by DCF-DA.

**Discussion**

PDAC phenotype is incurable due to two main causes: the more chemoresistant and highly metastatic cancer (Adamska et al., 2017; Das and Batra, 2015). Present therapies including surgery, radiation, chemotherapy and immunotherapy are almost ineffective. Recently, the multi-drugs combined therapies based gemcitabine becomes effective and gained some primary achievements like lowering cancer symptoms or increasing survival rate (Adamska et al., 2017). However, these combinations depend on constitution of each patient and usually create side effects. Therefore, better understanding in PDAC-related genes functions and precisely targeting these determinant oncogenes are the promising goals.

Besides, metastasis issue squeezes to PDAC management and recently circulating tumor cells (CTCs) are detected in mouse model and patient samples (Pimienta et al., 2017). These evidences urge to find new approaches to detect PDAC at early stage of metastasis, or to define the major factors in EMT process and to discover novel inhibitor targeting effectively these factors. In this study, we have examined the role of SALL4 to PDAC phenotype and results showed that SALL4 gets tightly involved in proliferation of pancreatic malignant cells *in vitro* and *in vivo*. These data are conformed to previous reports about SALL4 function in other cancer cell lines (Tatetsu et al., 2016). Results also indicated that SALL4 regulates strongly the metastatic phenotype in PDAC cell lines including migration, invasion or EMT protein markers. Therefore, SALL4 might be a novel factor mastering EMT and proliferation in PDAC cells. The level of ROS is ascendingly from BxPC-3, PANC-1 to AsPC-1. It seems like a trend of some factors regulating ROS homeostasis in PDAC cells. Therefore, we checked the expressions of peroxiredoxin protein family and we found that among Prx I-VI, there is only Prx III corresponding to this trend.

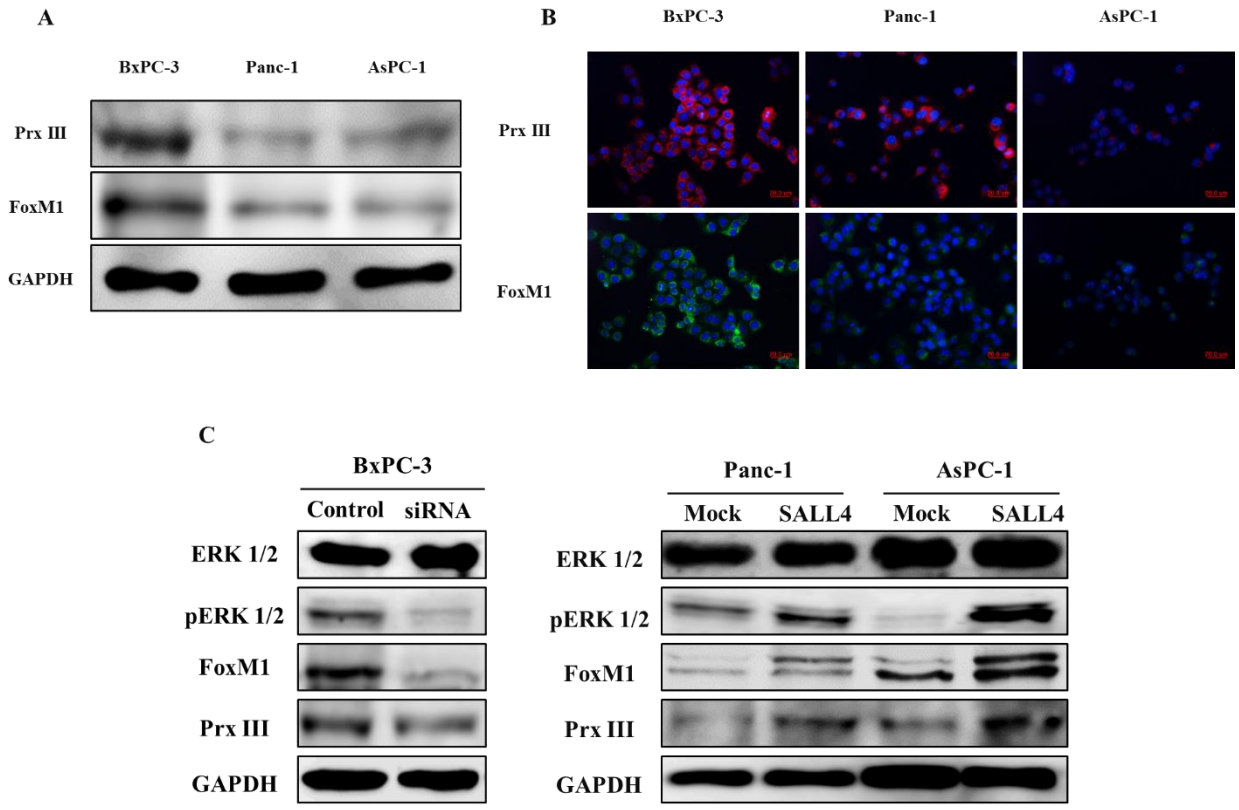



**Figure 3.13 SALL4 regulates ROS via FoxM1/Prx III axis.** A, Western blotting of Prx III and FoxM1 among PDAC cell lines. B., Immunocytochemistry staining of Prx III and FoxM1 among PDAC cell lines. C, Western blotting of ERK1/2, Prx III and FoxM1 in lysates as downregulating and overexpressing SALL4.

Furthermore, silence or overexpression of SALL4 enables modulating ROS status in PDAC cells. Thus, these evidences interpreted that lowest cellular ROS level in BxPC-3 cells is probably due to the activity of Prx III in mitochondria. Upstreaming of Prx III, FoxM1 is one of trans-activator and regulated by SALL4 activity. It is primarily concluded that SALL4 is unfavorable to ROS accumulation via Prx III activity but enhancing metastatic phenotype in PDAC cell lines. This also explains why low expression of SALL4 induced higher ROS level in PANC-1 and AsPC-1 while lowered cell number migrating or invading.

These facts are compromised with the study of Piskounova et al. 2015 (Piskounova et al., 2015). The study showed that high cellular ROS level is not critical for metastasis. Cancer cells from blood and metastatic sites represent higher level of ROS. However, treatment with antioxidant N-acetyl-cysteine (NAC) enhances the presence of CTCs and metastasis phenotype. CTCs with lower oxidative stress tolerance are killed at multiple stages of metastasis and therefore metastasizing efficiency becomes low once oxidative stress is high. Indeed, cells at late stage of EMT start leaving out of extracellular matrix (ECM) and have to face with many barriers including lower glucose supply, ATP depletion or oxidative stress (Gong et al., 2015; Micalizzi et al., 2017; Regmi et al., 2017). Therefore, only few tumor cells can form new tumor (Micalizzi et al., 2017). In this case, some CTCs can self-modulate oxidative stress tolerance to adapt new environment during blood stream circulating and form new tumors at secondary sites.

Many evidences indicated that metastasis efficiency is correlated with peroxidase enzymes and the expressions of these enzymes enhance oxidative stress tolerance in CTCs during metastasis (Kamarajugadda et al., 2013; Nguyen et al., 2016; Qu et al., 2011; Schafer et al., 2009). These evidences leave a question whether ROS scavenging is able to facilitate metastasis. Furthermore, administrations of antioxidant drugs seem to promote tumor metastasis (Le Gal et

al., 2015; Wang et al., 2016). Thus, targeting peroxidase induced-oxidative stress tolerance in PDAC can be an exit way in anti-metastasis vicious circle.

In our study, we demonstrated that SALL4 not only maintains stemness traits in PDAC phenotype but also regulates EMT markers, which are critical for early metastasis. Furthermore, SALL4 suppresses ROS status in cells via mitochondrial peroxidase Prx III activities. This event consequently incorporates with EMT markers, promoting PDAC metastasis. Therefore, inhibition of SALL4 might be a novel target in anti-metastasis and anti-proliferative therapies.

**Conclusion**

To conclude SALL4 activities to PDAC phenotype, these data suggested that SALL4 maintains the expressions of stemness genes in PDAC cell lines. Furthermore, SALL4 expression enhances PDAC mobility and SALL4 regulates ROS homeostasis in cell via FoxM1/Prx III axis. Many corners to explore SALL4 interactions to FoxM1/ Prx III axis are still elusive. Thus, it is necessary to define the mechanism of triangle SALL4/FoxM1 and Prx III, which might provide better understanding of SALL4 function in PDAC and bridge its novel therapeutic application to pancreatic cancer treatment.

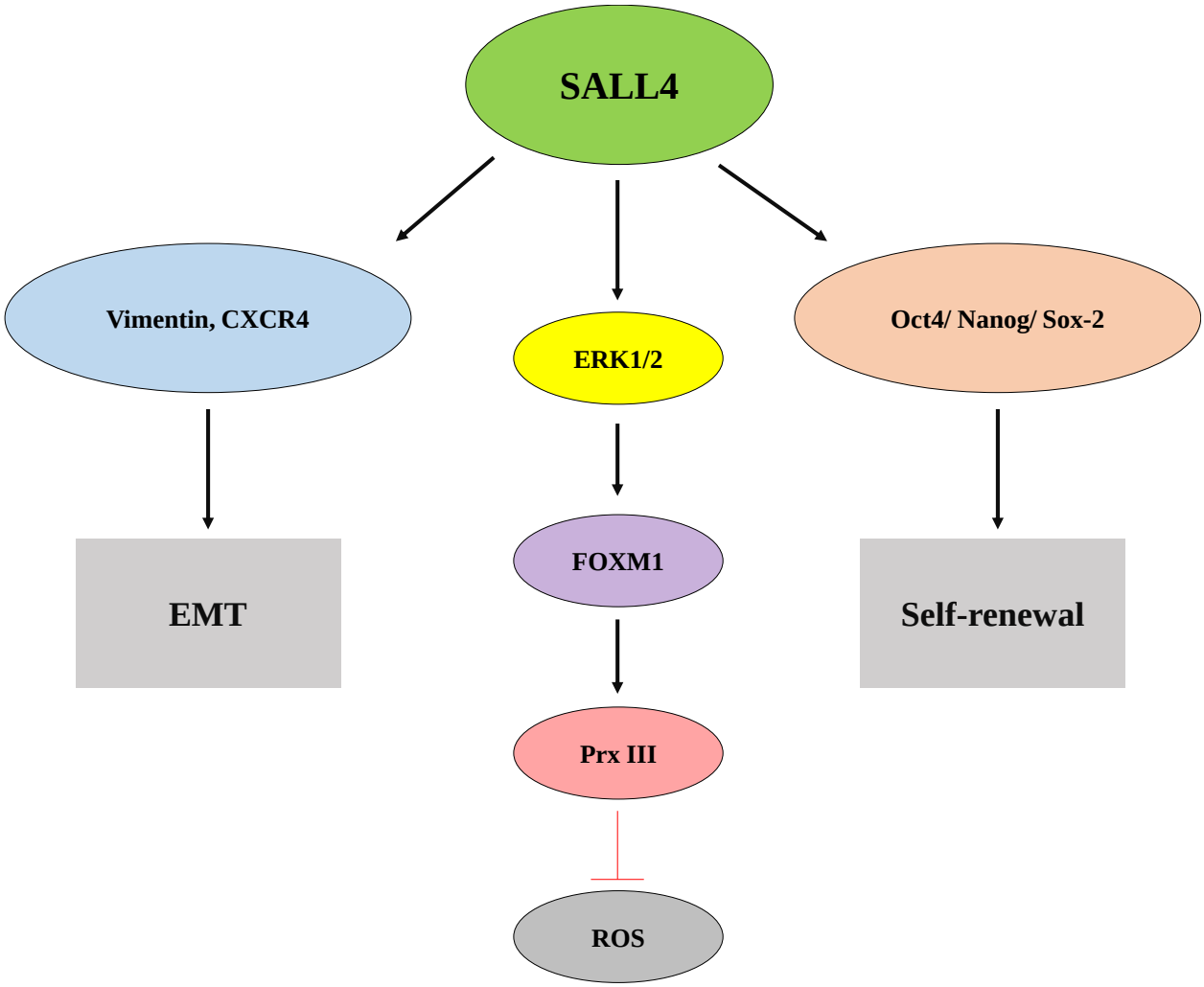



**Figure 3.14 schematic mechanism of SALL4 activity in PDAC phenotype.** SALL4 promotes EMT via expressions of Vimentin, CXCR4. SALL4 maintains self-renewal via Oct-4/Sox-2/ Nanog. SALL4 drives the phosphorylation of ERK1/2, leading to activation of FoxM1/Prx III and inhibits ROS level in PDAC cells.

## CHAPTER 2

### SALL4 role to the drug resistance of pancreatic cancer stem cells


### Abstract

Pancreatic cancer stem cells (PCSCs) play important role to self-renewal, metastasis or drug resistance. Many cancer stem cell markers such as CD133, CD44, CD24, ESA, Nestin, c-Met or ALDH have defined and help enrich PCSC subpopulation for further analysis. SALL4 serves as a fetal oncogene to maintain stemness traits, cell proliferation and tightly involved in the poor diagnose or aggressive phenotypes in variety of cancers. In this study, we have found the upregulation of SALL4 in $CD44^+$ $CD24^+$ PCSCs subpopulation. Results showed the key role of SALL4 in clonogenicity, tumor sphere formation and stemness genes. Downregulation of SALL4 sensitized PCSCs to gemcitabine treatment. Furthermore, drug resistance markers (Nrf2, Keap1, ABCG2) were attenuated when introducing SALL4 siRNA. Furthermore, analysis indicated that SALL4 regulates Notch1 expression and therefore governs the NICD target genes including drug resistant genes. Taken together, this finding first reports the correlation between SALL4 and Notch1 and this interlink might be the promising target in PCSC treatment.



**Keywords: SALL4, Pancreatic cancer stem cells, drug resistance, Notch1.**

## Introduction

### Pancreatic cancer stem cells (PCSCs)

Cancer stem cells (CSCs) account for small number of tumor cells, which have ability to differentiate into cancer cells, refresh themselves via self-renewal process and high tumorigenic cells (Figure 4.1). With surface markers, CSCs can be isolated from non-tumorigenic cells (Bao et al., 2013). CSCs have been found in many types of cancer such as breast, prostate, head and neck, colon, liver, bladder, or lung (Bao et al., 2013; O'Brien et al., 2010).

In pancreatic cancer, there are many *in vitro* assays to define CSCs subpopulation based on their characteristics. They might be featured as sphere forming ability, dye exclusion (Hoechst dye), intracellular enzyme activity (aldehyde dehydrogenase1-ALDH1), clonogenicity or stem cell markers. In term of surface markers, some of main markers have been defined. These are including CD133, CD44, CD24, ESA (epithelial-specific antigen), EpCAM which have been considered as PCSC markers (Ercan et al., 2017; Fitzgerald and McCubrey, 2014; Zhan et al., 2015).

Recent therapies are used for treatment alone or in combination, including surgery, radiation, or chemotherapy. However, these combinations eliminate cancer incompletely and usually accompanying with side effects. Furthermore, therapies are often resisted once tumor recurrences and metastasis (Pimienta et al., 2017). Therefore, it is urgently to figure out the drug resistant mechanisms or key effectors during metastasis in CSCs whereby to investigate novel therapies.

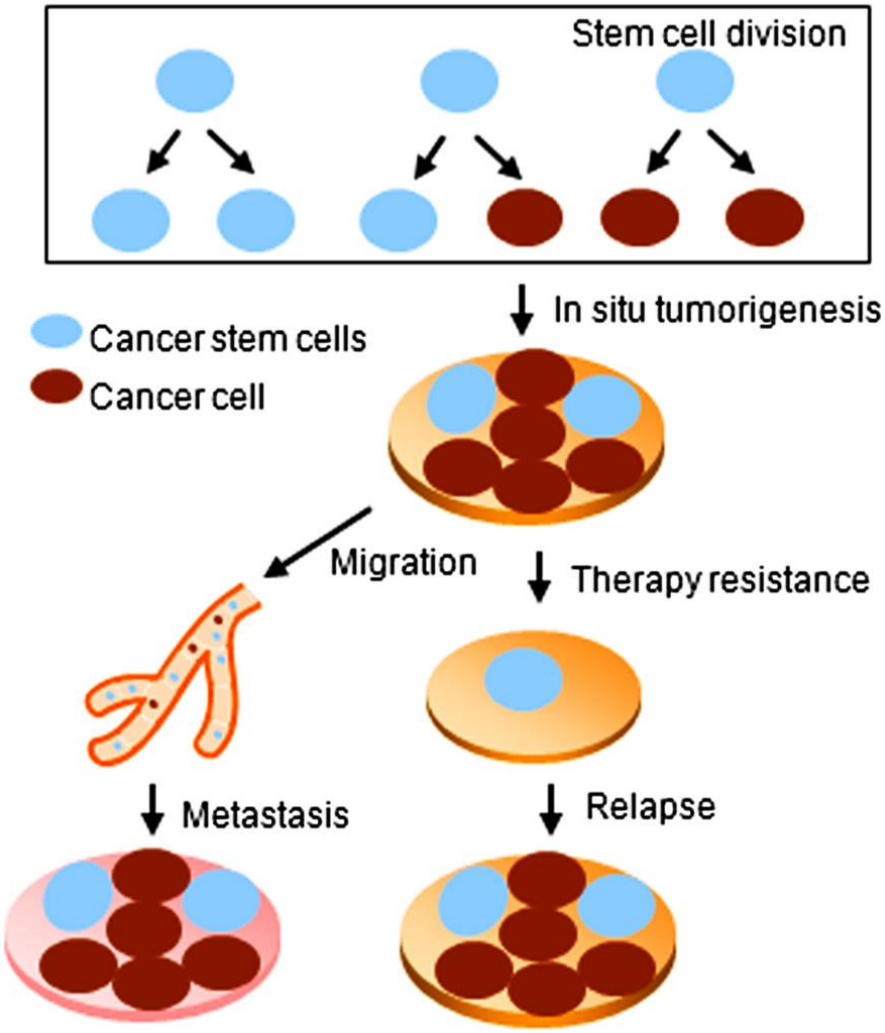



**Figure 4.1 Cancer stem cells regulate tumor generation, drug resistance, relapse and metastasis.** Cancer stem cells (CSCs) have ability to self-renewal that enables duplicating into two CSC or cancer cells and CSC or cancer cells only. These cells form tumor and some of them might migrate via blood stream or lymphatic vessels to localize at new sites of body. This event is named as metastasis. Some of CSCs are resistant to therapies and cause tumor recurrence after treatment. Adopted and modified from Yu et al. 2012 (Yu et al., 2012).

**Notch signaling pathway in pancreatic cancer**

Notch signaling is essential to cell-cell and crucial for cell proliferation, development and homeostasis (Ranganathan et al., 2011). Pre-Notch protein undergoes cleaving process at Golgi apparatus (S1 cleavage enzyme, Furin-like convertase) before representing on plasma membrane. Notch proteins are trans-membranes receptors including Notch-1, Notch-2, Notch-3 and Notch-4, which match with their ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged-1 and Jagged-2) (Masek and Andersson, 2017; Miele, 2006) (Figure 4.2).

After ligands binding, Notch receptors are activated by tumor necrosis factor-alpha-converting enzyme (TACE) and releasing Notch ectodomain (S2 cleavage). This state facilitates cleaving process induced by γ-secretase enzyme, which localizes at plasma membrane and endosome, leading to the formation of active Notch intracellular domain (NICD). This active fragment would drive the expressions of target genes by recruiting complex proteins like coactivator mastermind (MAM) and complex DNA-binding protein CSL (CBF1/Su(H)/Lag2) (Guo et al., 2014; Hori et al., 2013) (Figure 4.3).

In NICD structure, there are functional sub-domains and positions for phosphorylation, ubiquitylation, hydroxylation or acetylation (Andersson et al., 2011), which represent different post-translational modifications (Figure 4.4). These sub-domains are including Rbp-associated molecule domain (RAM), playing important role to mediate interaction between NICD and CSL, Nuclear localization signal (NLS), Ankyrin repeats (ANK), transactivation domain (TAD) and Proline/Glutamic acid/Serine/Threonine (PEST) degradation domain (Andersson et al., 2011).

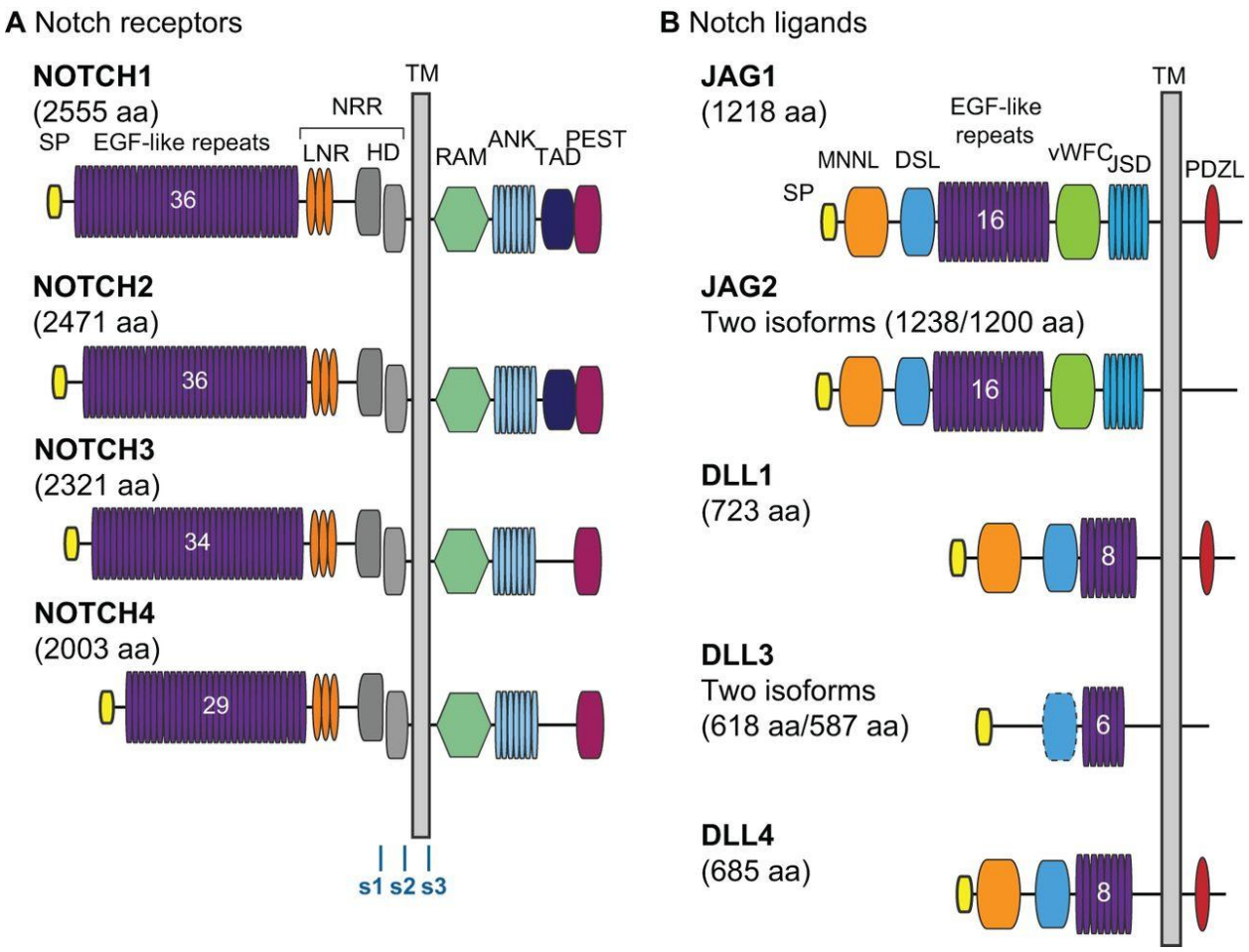



**Figure 4.2 Notch and Notch classical ligand proteins.** Dispositions of protein domains are described in Notch receptor (A) and ligands (B). ANK, ankyrin repeats; DLL, Delta-like protein; DSL, Delta/Serrate/LAG-2 domain; EGF, epidermal growth factor; HD, heterodimerization domain; JAG, jagged; JSD, Jagged Serrate domain; LNR, Lin-Notch repeats; MNNL, Notch ligand N-terminal domain; NRR, negative regulatory region; PDZL, PDZ ligand domain (PDZ, post synaptic density protein (PSD95)); PEST, proline (P), glutamic acid (E), serine (S) and threonine (T) degradation domain; RAM, Rbp-associated molecule domain; s, cleavage site; SP, signal peptide; TAD, transactivation domain; TM, transmembrane domain; vWFC, von Willebrand factor type C domain. Adopted from (Masek and Andersson, 2017).

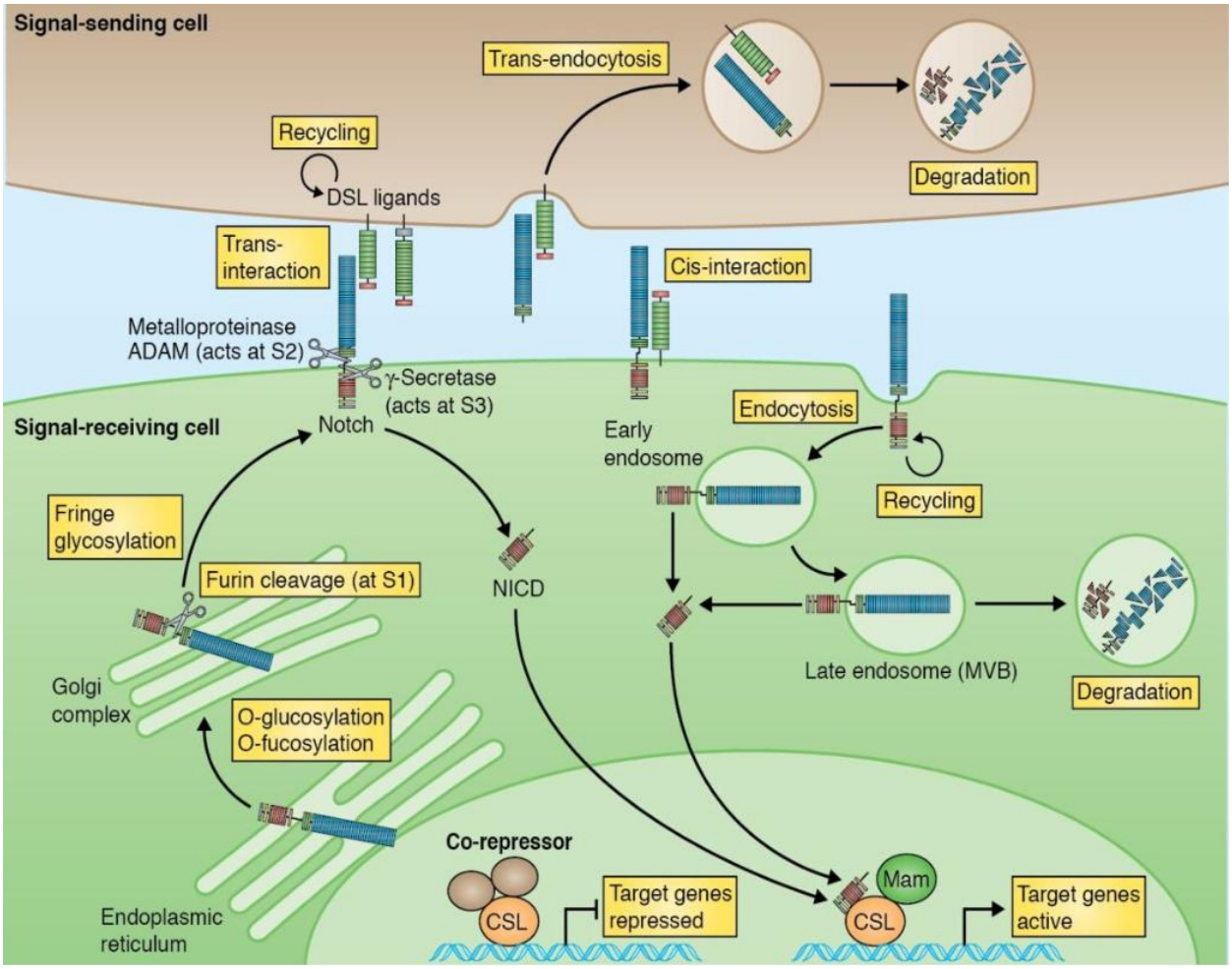



**Figure 4.3 Overview of Notch signaling.** Notch signaling is activated by cell-cell interactions. Notch receptors undergo post-translational modifications at endoplasmic reticulum and Golgi apparatus. Cells with Notch ligands can send signal to receiving cells, subsequently driving Notch intracellular domain (NICD) activation. NICD activation is a cleaving by γ-secretase at plasma membrane (trans-interaction) or endosome (cis-interaction). As a result, NICD translocates into nucleus to "hit" co-repressor of CBF1/Su(H)/Lag2 complex (CSL) and recruit master mind (Mam) protein for target gene expressions. Adopted from (Hori et al., 2013).

Phosphorylation plays important role to NICD activity. Phosphorylated at ANK repeats C-terminal of NICD would inhibit Notch2 activity, leading to downregulation of Hairy and enhancer of split homolog-1 (Hes1) (Espinosa et al., 2003). However, this phosphorylation would stabilize NICD derived from Notch1 (Foltz et al., 2002). Phosphorylations at PEST domain are useful for ubiquitylating initiation (Andersson et al., 2011). Ubiquitylation process at some certain positions in NICD would drive to protein degradation. For instance, ubiquitylation at PEST domain promotes rapid degradation of NICD. Any abnormal in ubiquitylation at this domain would lead to the extent half-life of NICD and therefore facilitate tumorigenesis (Andersson et al., 2011). Furthermore, some evidences found the hydroxylation and acetylation of NICD as shown in Figure 4.4, however their functions are not fully understood (Andersson et al., 2011).

Different ligands activate corresponding Notch receptor and result in different target genes (Rusanescu et al., 2008). Classical ligands including Jagged -1 and -2 (Jag-1 and Jag-2) and Delta-like DLL1 and DLL4 can activate respective Notch1/2/3/4 receptors (Figure 4.5), excepting for DLL-3 which is believed as an inhibitor of Notch receptor (Chapman et al., 2011; Kunnimalaiyaan and Chen, 2007; Saunders et al., 2015). Atypical notch ligands such as Delta-like homologue (Dlk1), F3/contactin 1, DNER or BN3/contacting activate NICD to promote the expressions of neuron-specific genes (Rusanescu et al., 2008). Thus, depending on ligands would determine the expressions of target genes, which might contribute differently to tumorigenesis and subsequently determine target gene therapies.

Many target genes are defined under the trigger of Notch signaling. NICD and its co-activators drive many genes relates to tumor growth such as c-Myc, cyclin D1, Hes, Hey, Cox-2, or crucial genes for invasion/ migration/ metastasis (VEGF, MMP9) (Ranganathan et al., 2011)

(Figure 4.6). Furthermore, Notch signaling is also believed to regulate other signaling such as Akt, protein kinase D1 (PKD1), NF-κB, Src, wnt/beta catenin or hedgehog signaling pathway. These signaling pathways are very crucial for tumorigenesis (Gao et al., 2017).

Increasing number of evidences showed that Notch signaling gets involved in pancreatic cancer. Evidences showed that Notch signaling governs the EMT in pancreatic cancer (Brabletz et al., 2011; Wang, Z. et al., 2009). Downregulation of Jag1 or inactivation of Notch signaling would lead to the inhibition of tumor sphere, tumor growth and PCSC depletion (Brabletz et al., 2011; Mizuma et al., 2012; Yen et al., 2012). Furthermore, Notch1 expression is correlated to the expression of CD44, EpCAM surface markers (Abel et al., 2014; Yabuuchi et al., 2013). PDAC cells positive with Notch2 expresses higher levels of Oct-4, Nanog, Pancreatic and duodenal homeobox 1 (PDX1) (Zhou et al., 2013). Furthermore, activation of Notch signaling results in the expression of other target genes related to tumor growth or metastasis (Gao et al., 2017). Therefore, developments of therapies targeting Notch signaling would be a promising approach for pancreatic cancer patients.

Some of strategies have been employed to inhibit Notch signaling pathway in pancreatic cancer. Activation of NICD requires the involvement of γ-secretase and therefore γ-secretase inhibitors (GSIs) have been explored for treatment (Gao et al., 2017). GSIs inhibit metastasis traits in pancreatic cancer, cell proliferation and tumor growth. However, GSIs also display their toxicities and side effects (Espinoza and Miele, 2013). Other evidences suggest alternative natural compounds to inhibit pancreatic carcinogenesis via suppressing Notch signaling pathway (Gao et al., 2017), for instance, Quinomycin (Ponnurangam et al., 2016), Genistein (Bi et al., 2018), Sulforaphane (Kallifatidis et al., 2011). These evidences premise for Notch inhibiting anti-cancer therapies by natural compounds.

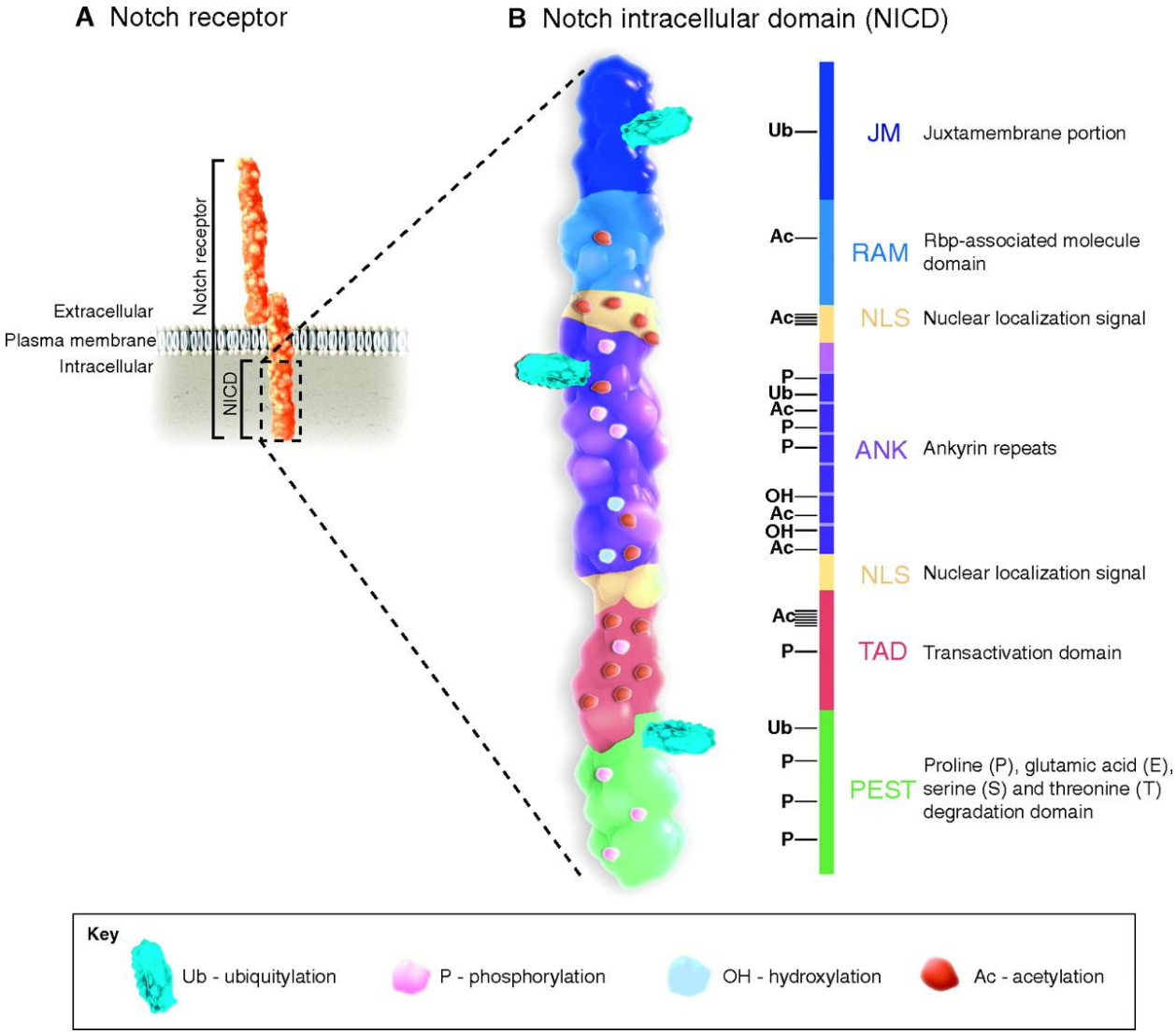



**Figure 4.4 Notch intracellular domain structure and its post-translation modifications.** A, the structure of Notch is heterodimer transmembrane, consisting of extracellular domain and intracellular domain which later can be separated by γ-secretase. B, NICD is included functional domains Juxtamembrane portion (JM), Rbp-associated molecule domain (RAM), Ankyrin repeats (AKN), trans-activation (TAD), PEST and two nuclear localization signal (NLS). Adopted from (Andersson et al., 2011).

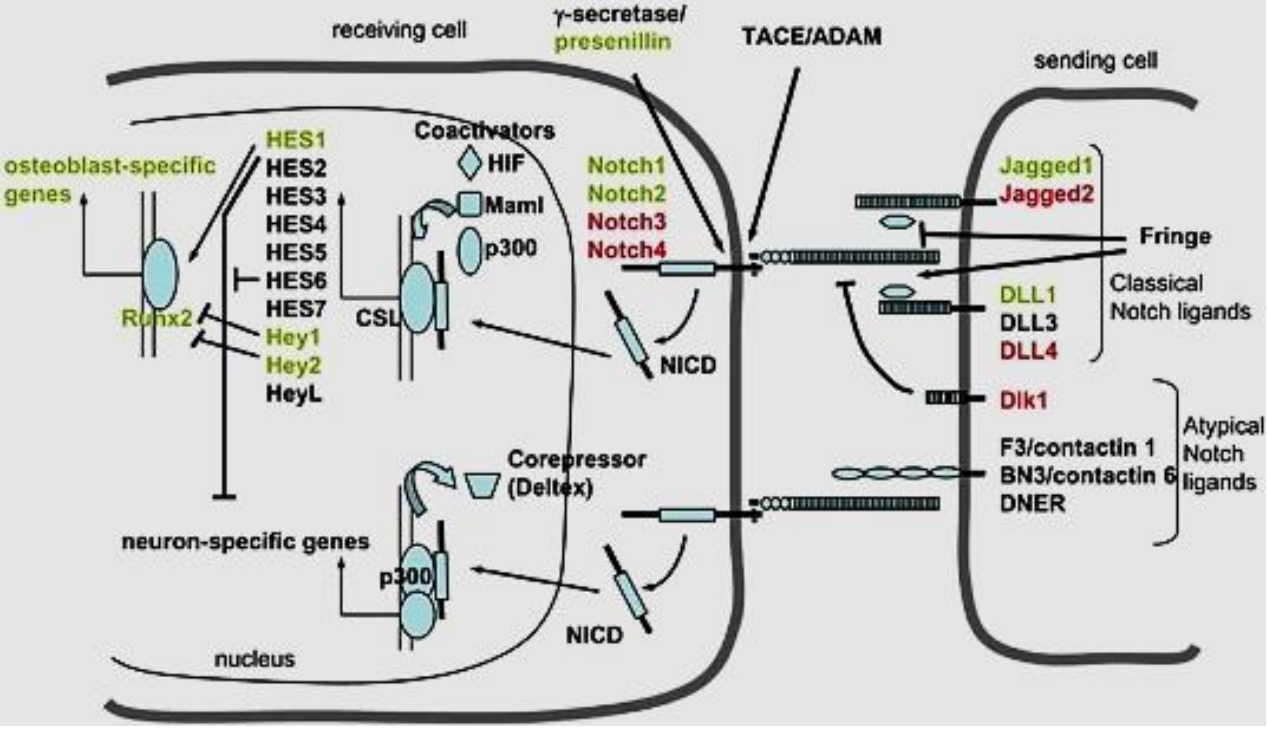



**Figure 4.5 Cell fate and different interactions of Notch ligands.** Different ligands bind to different Notch receptors would activate different corresponding downstream genes and determine cell fate. For instance, Jag-1 and DLL1 are responsible for Notch1-2 activation, leading to the different regulations of Hes1 or Hey1/2 and Runx2, afterwards regulating osteogenesis. Similarly, Hes1 prefers to enhance osteogenesis while inhibiting neurogenesis. In contrast, Hes6 promotes neurogenesis via suppressing Hes1 activity. In classical or canonical ligands, enzyme Fringe will facilitate DLL binding to Notch receptor while inhibiting Jag1-2 and therefore Fringe effect partly determines cell fate. Atypical or non-canonical ligands are related to the expressions of neurogenesis specific genes whereby Deltex are hit out of open reading frame. Canonical Notch signaling inhibits non-canonical Notch signaling, results in cell proliferation by CSL/Hes1 pathway and inhibition of neuronal differentiation. Adopted from (Rusanescu et al., 2008).

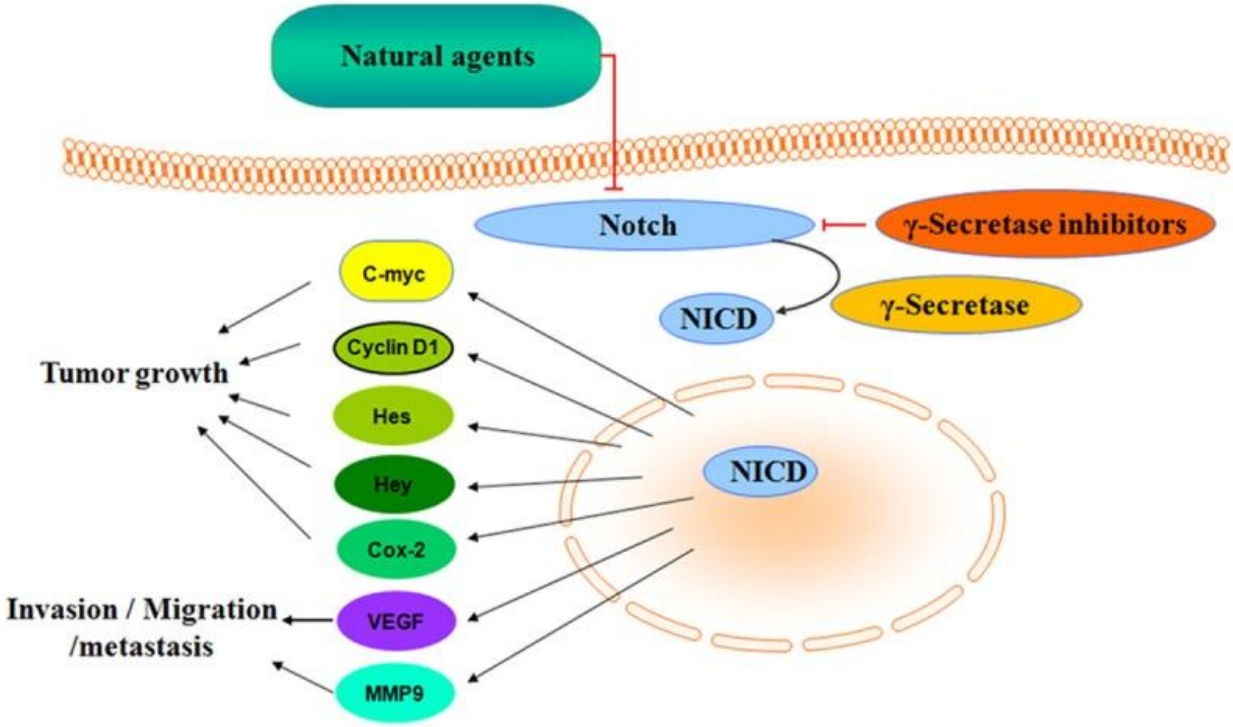



**Figure 4.6 Contributions of Notch-induced downstream genes to tumorigenesis.** Activated by γ-secretase, Notch-ICD translocates into nucleus and drives expressions of target genes, which are involved in tumor growth or metastasis. Enzyme γ- secretase is responsible for NICD activation, thus γ-secretase inhibitors (GSIs) or natural agents (Sulforaphane, genistein, Quinomycin) would be promising target therapies. Adopted from (Gao et al., 2017).

## Materials and methods

### Cell culture

BxPC-3 and PANC-1 cells were cultured in DMEM (Gibco, CA, US) supplemented with 10% FBS (Welgene, Korea) and 1% anti-mycotic agent, and maintained in a humidified atmosphere of 5% $CO_2$ in the incubator at 37 ℃; the cells were passaged twice per week.

### Isolation of pancreatic cancer stem cells

Cells in the log phase were used for Magnetic activated cell sorting (MACS) with CD44 and CD24 microbeads (Miltenyic biotech, Germany), as per the manufacturer's protocol. Afterwards, cells were stained with anti-CD44 and anti-CD24 antibodies, $CD44^+$ $CD24^+$ BxPC-3 and PANC-1 CSCs were maintained in DMEM/F12 supplemented with and B27 1X (Invitrogen, CA, US), 10 ng/ml hEGF, (Sigma-Aldrich, MO, US) and 10 ng/ml bFGF (KOMA biotech, Seoul, Korea).

### Flow cytometry analysis

After isolation by MACS, the cells were subjected to further analysis. For confirmation of CD44 and CD24 presences after MACS, cells were stained with CD44-APC and CD24-FITC antibodies (Miltenyic biotech, Germany) as per the manufacturer's protocol. For the apoptosis assay, after 48 h Gemcitabine treatment, the cells were resuspended in 100 µl of Annexin V-binding buffer containing 5 µl Annexin V-FITC-conjugated antibody and 5 µl propidium iodide for exactly 15 min in the dark at room temperature. Cells were then analyzed using the BD Accuri C6 cytometer (BD Biosciences, NJ, US).

**Cell viability**

Cells ($5 \times 10^3$) were seeded onto a 96-well plate and incubated for 24 h before treatment with the indicated Gemcitabine concentrations. After 48 h, cytotoxic effects were measured using an EZ-cytox kit (Daeil lab, Seoul, Korea) according to the manufacturer's protocol. The cell viability results are presented as the ratios of the optical densities of the treated groups and the vehicle control group at 450 nm ($OD_{450}$); this was calculated using the following formula: (%) cell viability = (OD treatment groups or control groups/ OD vehicle control group) × 100%.

**Immunocytochemical staining**

Single spheres were fixed using 3.7% paraformaldehyde (PFA). Before overnight staining with the primary antibodies of interest (CD44, CD24, SALL4), cells were blocked by PBST 1X with 3% BSA. After washing twice with PBST 1X, the secondary antibodies were added, followed by a 2-h incubation. Cells were stained for 10 min with 4',6-diamidino-2-phenylindole (DAPI) before microscopic observation.

**Clonogenicity assay**

Cells ($1 \times 10^3$) were seeded onto 6-well plates at 37 $^0$C/ 5% $CO_2$. After 7 days of incubation in DMEM supplemented with 10% FBS, cells were fixed with 3.7% PFA and stained with 0.05% Crystal violet, followed by washing with 1X PBS before observation.

**Sphere formation**

Cells ($1 \times 10^3$) in serum-free medium (DMEM/F12 supplemented with 2% B27, 10 ng/ml hEGF and 10 ng/ml bFGF and 1% antimycotic) were seeded onto ultralow attachment 6-well

plates (Corning, NY, US). After 7 days of incubation, the spheres were viewed using a microscope.

**Western blotting**

The protein content of the cell lysates was determined using the BCA assay. Lysates were separated on by 12% SDS-PAGE, transferred electrophoretically (Bio-Rad, CA, USA) onto a polyvinylidene fluoride (PVDF) membrane and blocked with 5% non-fat milk powder (w/v) in phosphate-buffered saline 0.1% Tween-20 (PBST) for 1 hour at room temperature. Membrane was followed by incubating the membrane with primary antibodies or with anti-GADPH mouse monoclonal antibody as an internal control overnight at 4 ℃ and with appropriate HRP-conjugated secondary antibodies for 2 h at room temperature. The bands were captured using an ImageQuant™ LAS 4000 mini Fujifilm camera.

**Statistical analysis**

Statistical analysis was performed using the Graphpad Prism software (Version 6.02). Data are expressed as mean ± standard deviation (SD). Experimental differences were examined by ANOVA and Student's *t*-tests, as appropriate. *P* values of $<0.05$ were considered to indicate statistically significant data.

**Table 2: List of antibodies for Western blotting**

| No. | Antibodies | Company | Catalogue number | Source |
|---|---|---|---|---|
| 1 | CD133 | Proteintech | 18470-1-AP | Rabbit polyclonal |
| 2 | CD44 | Santa cruz | sc-7297 | Mouse monoclonal |
| 3 | CD24 | Bioss | bs-4890R | Rabbit polyclonal |
| 4 | SALL4 | Abfrontier | YF-MA11603 | Mouse monoclonal |
| 5 | c-Myc | Santa cruz | sc-40 | Mouse monoclonal |
| 6 | CXCR4 | Abfrontier | YF-MA16239 | Mouse monoclonal |
| 7 | GADPH | Abfrontier | LF-MA0038 | Rabbit polyclonal |
| 8 | Nanog | Santa cruz | sc-33759 | Mouse monoclonal |
| 9 | N-cadherin | Abfrontier | LF-MA50067 | Mouse monoclonal |
| 10 | Oct-4 | Santa cruz | sc-9081 | Rabbit polyclonal |
| 11 | Sox-2 | Santa cruz | sc-20088 | Mouse monoclonal |
| 12 | Notch1 | Abcam | ab52627 | Rabbit polyclonal |
| 13 | Hes1 | Abfrontier | *YF-MA11051* | Mouse monoclonal |
| 14 | Nrf2 | Bioss | bs-1074R | Rabbit polyclonal |
| 15 | Keap1 | Bioss | bs-3648R | Rabbit polyclonal |
| 16 | ABCG2 | Santa-cruz | Sc-58222 | Mouse monoclonal |

## Results

### SALL4 is strongly expressed in $CD44^+$ $CD24^+$ PCSCs

Pancreatic cancer stem cells (PCSCs) are defined as many types of surface markers, including CD44, CD24, CD133, epithelium specific antigen (ESA), aldehyde dehydrogenase (ALDH) or Hoechst dye exclusion (side population). These markers were demonstrated their effects to drug resistance, tumor relapse, tumor growth or metastasis (Fitzgerald and McCubrey, 2014). In our study, we isolated double positive $CD44^+$ $CD24^+$ to figure out SALL4 function to PCSCs. Results showed that CD44 and CD24 in double positive were higher than double negative (Figure 4.7 A-B). Immunocytochemistry staining confirmed the expressions of CD44 and CD24 in spheres of double positive BxPC-3 and PANC-1 cells (Figure 4.7 C). Western blotting indicated that expression levels of CD44 and CD24 in double positive were significantly higher than double negative (Figure 4.7 D). More interestingly, SALL4 expression was also higher in PCSCs subpopulation. These data suggested that we have isolated successfully double positive $CD44^+$ $CD24^+$ PCSCs and SALL4 is strongly expressed in this subpopulation.

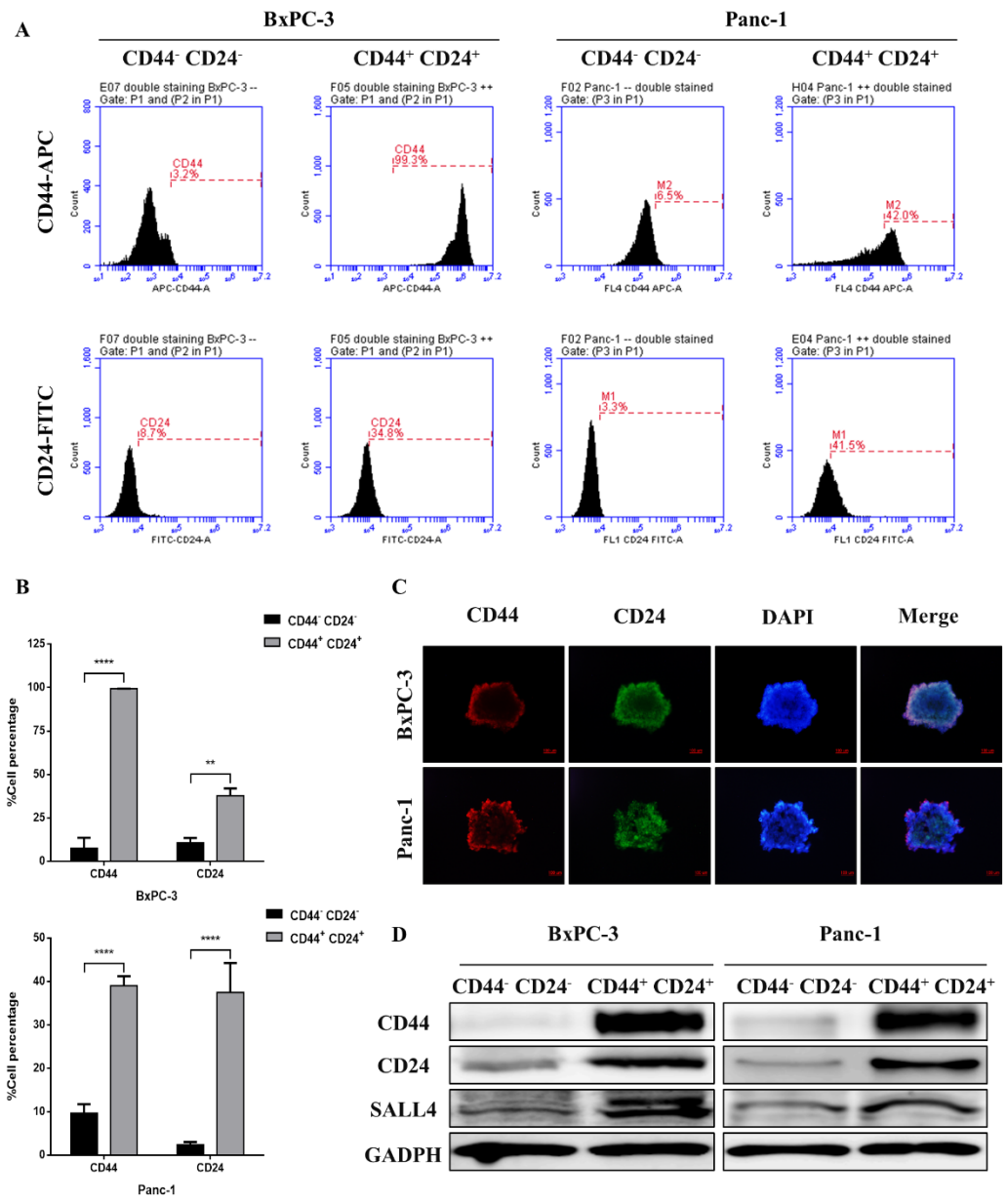



**Figure 4.7 Upregulation of SALL4 in CD44$^+$ CD24$^+$ PCSCs**. A, FACS analysis of double positive cells subpopulation with CD44-APC and CD24-FITC after magnetic activated cell sorting (MACS). B, Levels of CD44 and CD24 expressions analyzed by qPCR. C, Immunocytochemistry staining of CD44, CD24 in tumor spheres. D, Western blotting of CD44, CD24 and SALL4 in protein lysates. *P value* < 0.05 is considered as significance.

**SALL4 governs stemness properties of PCSCs *in vitro***

SALL4 is a well-known regulator of self-renewal capacity. Downregulation of SALL4 would lead to the suppressions of stemness genes such as Sox-2, Nanog or Oct-4 (Tatetsu et al., 2016). Therefore, we downregulated the expression of SALL4 by siRNA in PCSCs. Results showed that colony forming capacity of double positives were inhibited when introducing SALL4 siRNA (Figure 4.8 A). Sphere forming assay also indicated that double positives formed giant sphere in control group as compared to SALL4 siRNA treatment (Figure 4.8 B). Further analysis in western blot showed that stemness genes including CD133, Sox-2 and Oct-4 were downregulated in double positives versus non-treated SALL4 (Figure 4.8 C). Taken together, these data suggested that SALL4 regulates the expressions of stemness genes, resulting in regulation of self-renewal capacity in PCSCs.

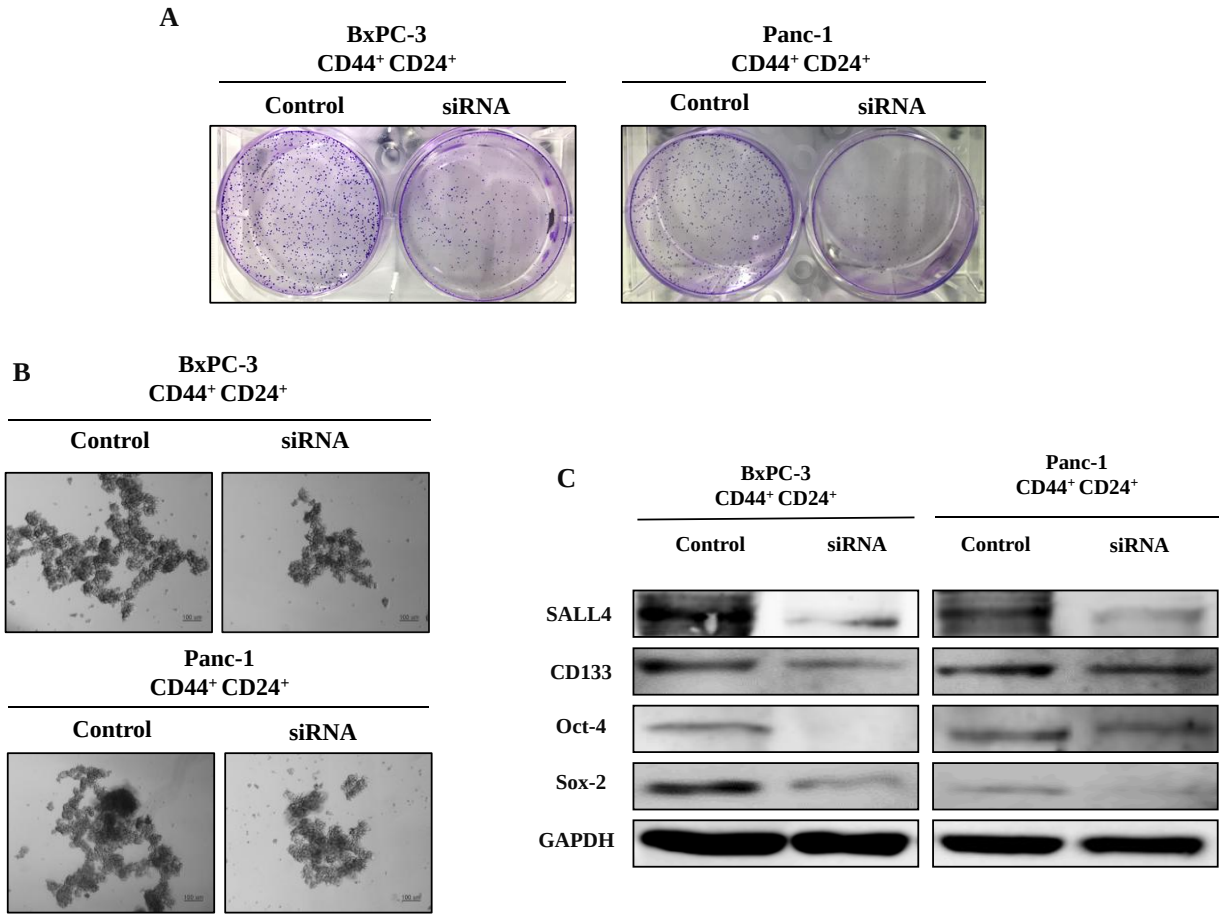



**Figure 4.8 SALL4 gets involved in the malignance of PCSCs.** A, Clonogenicity of PCSCs cells transfected with control siRNA vs SALL4 siRNA. B, Tumor spheres of PCSCs as SALL4 silenced. C, Western blotting of stemness genes in control siRNA vs SALL4 siRNA.

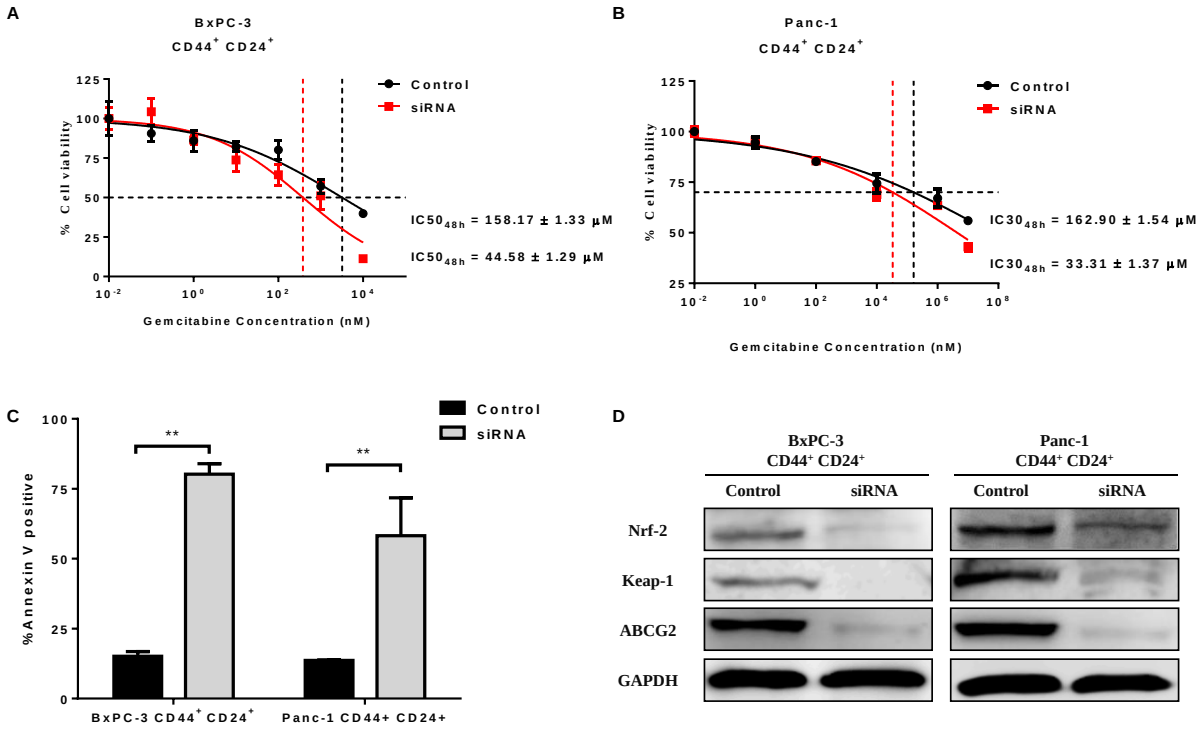



**Figure 4.9 SALL4 downregulation sensitizes PCSCs to chemotherapy.** A, IC50 shift of $CD44^+$ $CD24^+$ BxPC-3 cells after 48 h gemcitabine treatment. B, IC50 shift of $CD44^+$ $CD24^+$ PANC-1 after 48 h gemcitabine treatment. C, FACS annexin V analysis of $CD44^+$ $CD24^+$ pancreatic cancer cells after 48h gemcitabine treatment. D, Western blotting of drug resistant genes in lysates transfected with control siRNA vs SALL4 siRNA. *P value* < 0.05 is considered as significance.

**SALL4 is involved in drug resistance in PCSCs**

One of typical characteristics in PCSCs is drug resistance. This leads to high dose of drugs during treatment and causes severe side effects to cancer patients (Bao et al., 2013; Liu, H. et al., 2015). Furthermore, PCSCs are responsible for tumor recurrence due to their capacities of tumor generation (O'Brien et al., 2010; Vidal et al., 2014). Previous studies showed the regulations of drug resistant marker genes by SALL4 in other types of cancer (Zhang, X. et al., 2015). In this study, we examined the correlation between SALL4 and drug resistance in PCSCs. Results showed that downregulation of SALL4 sensitized PCSCs to gemcitabine. There were IC50 shifts in both PCSC cell lines (Figure 4.9 A and B). FACS analysis result showed the significant increases of percentage of cells positive with Annexin V in groups treated with SALL4 siRNA as compared to control siRNA (Figure 4.9 C). Further analysis indicated that there were the downregulations of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and ATP-binding cassette sub-family G member 2 (ACBG2) while upregulating Kelch-like ECH-associated protein 1 (Keap1) when SALL4 siRNA introducing (Figure 4.9 D). These data suggested that SALL4 expression dominates to drug resistance in PCSCs.

**SALL4 regulates Notch1 signaling in PCSCs**

Activities of CSCs are governed by various signaling pathways that are shared with normal stem cells (Matsui, 2016). Recently, Notch signaling is emerging as one of the main signaling pathways during carcinogenesis and becomes potential target therapy (Espinoza and Miele, 2013; Ranganathan et al., 2011). In the pancreatic cancer, Notch signaling is suggested as a key factor which have failed recent anti-cancer therapies cause its important roles to CSC maintenance, drug resistance (Abel et al., 2014; Espinoza and Miele, 2013; Gao et al., 2017; Guo et al., 2014).

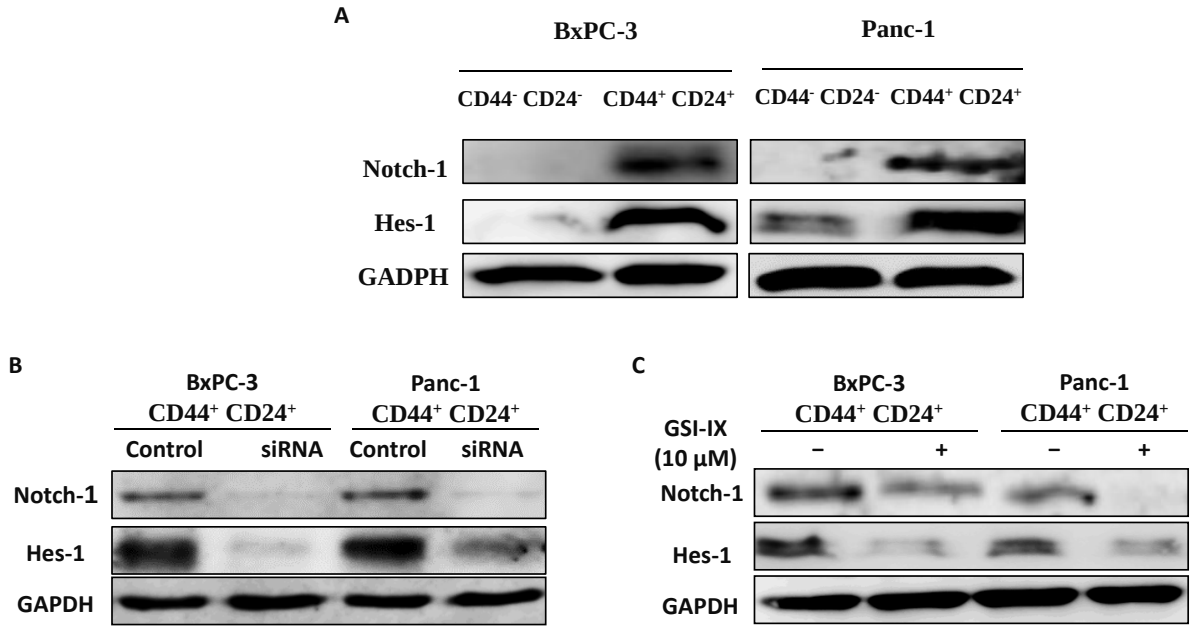



**Figure 4.10 SALL4 regulates Notch1 signaling in PCSCs**. A, Western blotting of Notch1/Hes1 in lysates double positive versus double negative cells. B, Western blotting of lysates as silencing SALL4. C, Western blotting of lysates with and without γ-secretase inhibitor IX (GSI-IX) (10μM, 48h treatment).

In this study, we found the regulation of SALL4 to Nrf2 /Keap1 axis, which get involved tightly to drug resistance (Hong et al., 2010; Ma, 2013; No et al., 2014) (Figure 4.9). Another evidences indicated that Notch signaling and Nrf2 signaling have a crosstalk and NICD drives Nrf2 expression via binding to the promoter of this gene (Wakabayashi et al., 2015; Wakabayashi et al., 2014). Therefore, we next tracked the link between SALL4 and Notch signaling. We have found that $CD44^+$ $CD24^+$ PCSCs display remarkable upregulation of Notch1 and its downstream gene Hairy and Enhancer of split 1 (Hes1) as compared to double negative cells (Figure 4.10 A). Results showed that downregulation of SALL4 negatively suppressed the expression of Notch1, resulting in attenuation of Hes1 level (Figure 4.10 B). Treatment with gamma-secretase inhibitor IX (GSI-IX) revealed the suppression of Notch1 signaling (Figure 4.10 C). Taken together, Notch1 expression is regulated by SALL4 activity, leading to the expressions of Nrf2 and ABCG2 and therefore enhance drug resistance in PCSCs.

**Discussion**

PDAC is extremely poor prognosis with high aggressive in metastasis and drug resistance (Adamska et al., 2017). Many approaches have been developed to combat PDAC, still ineffectively (Adamska et al., 2017; Fitzgerald and McCubrey, 2014). Besides, Notch signaling exhibits its substantial contributes to PDAC tumorigenesis and drug resistance (Abel et al., 2014; Gao et al., 2017). Therefore, the inhibition of Notch signaling may a promising approach in PCSCs treatment.

In previous studies, SALL4 is reported as a key driver to ABCA3, ABCG2 or c-Myc and therefore impinges drug resistance in leukemia, endometrial cancer and liver cancer (Zhang, X. et al., 2015). These evidences claimed that SALL4 directly binds to ABCA3 and c-Myc

promoter, resulting in excessive expressions of these genes. Furthermore, by somehow SALL4 indirectly regulates ABCG2 expression (Jeong et al., 2011). Besides, many drug resistant genes are thought to be controlled by Nrf2 which are included ABC protein family (Hong et al., 2010). Thus, it is possible that SALL4 and Nrf2 upregulates drug resistant genes independently or dependently. However, when SALL4 silencing, level of Nrf2 was also negatively modulated. This evidence connects to a thought that SALL4 might act as an upstream factor of Nrf2. Therefore, SALL4 both drives directly ABCA3 or c-Myc and indirectly via Nrf2 activity, contributing to drug resistance.

Some of evidences indicated that Notch and Nrf2 signaling are highly associated with each other (Wakabayashi et al., 2015). Nrf2 disruption would lead to the downregulation of Notch1 and Notch-responsive genes. Further analysis indicated that Notch1 promoter is also served as antioxidant response element (ARE) of Nrf2. However, Notch1 also influences Nrf2 expression (Wakabayashi et al., 2014). Notch1-ICD binds directly to Nrf2 promoter and drives transcription of this gene. Therefore, the correlation between Notch and Nrf2 is compensative.

Our data showed downregulation of SALL4 resulted in suppressions of both Notch1 and Nrf2. This might be explained that SALL4 by somehow regulates Notch1 signaling which in turns triggers the expression of Nrf2. Consequently, SALL4 redoubles drug resistance in PCSCs. These results are compromised with other study, which claimed that Nrf2 regulates drug resistance in pancreatic cancer (Hong et al., 2010). Many upstream regulators of Notch in pancreatic cancer have been identified which are included epidermal growth factor receptor (EGFR), CCN1, MEK/ERK, Aspartate β-hydroxylase (ASPH), Dominant-Negative form of Mastermind-like 1 (DNMAML) , F-box and WD repeat domain-containing 7 (FBW7) (Gao et al., 2017). Evidences showed that SALL4 expression has correlation with EGFR (Jia et al., 2016).

Other evidence showed that SALL4 can be upregulated by EGFR activation and regulates stemness traits (Du et al., 2018). Furthermore, SALL4 is believed to indirectly enhance the stability of EGFR and insulin-like growth factor 1 receptor (IGF1R) from ubiquitylation in lung cancer (Yong et al., 2016). Therefore, SALL4 might indirectly drive Notch1 expression by this pathway. One of another possibility is that SALL4 might target at Notch1 promoter and lead to the expression of Notch1. Some of evidences have tried to figure out the DNA binding motif of SALL4 and these motifs might be "ATTTGCAT" acting as consensus motif in multiple genes, or "TTGTCTACTTGGTA" for SALL4A to drive differentiation and patterning genes, or "TCGCCATA" (Tatetsu et al., 2016). However, we have retrieved promoter sequence of Notch1 (-1000 to -1) and blasted with such DNA motif, result showed that motif "ATTTGCAT" matches 100 scores to Notch1 promoter at (-416 to -423). This primarily suggested a new hypothesis for SALL4-Notch1 interlink in tumorigenesis.

Taken together, SALL4 regulates the stemness genes to maintain self-renewal and drives Notch1/ Nrf2 axis, leading to drug resistance in PCSCs. Inhibition of SALL4 might enhance the chemotherapies efficiency and prevent PCSCs from self-renewal.

**Conclusion**

In pancreatic cancer stem cells, Notch signaling is very important to maintain self-renewal, drug resistance. SALL4 governs the expressions of stemness genes and influences drug resistance of PCSCs and SALL4 regulates Notch1 signaling, resulting in modulation of Nrf2/Keap1 axis. Therefore, therapies via targeting SALL4 can sensitize PCSCs to chemo-drugs and strengthen treatment efficiency.

## CHAPTER 3

### The inhibition of SALL4 activities in pancreatic cancer stem cells by natural extract


### Abstract

Many attempts have been made to invent an efficient therapy for pancreatic cancer. However, it is still incurable owing to silent symptoms, drug resistance, and a high level of metastasis. Previous studies indicated that Sonic hedgehog signaling directs drug resistance and metastasis in pancreatic cancer stem cells. Therefore, this pathway becomes a promising target in pancreatic cancer treatment. Previous studies also showed the inhibitory effect of BRM270 on lung adenocarcinoma and glioma stem cells *in vitro* and *in vivo*, contributing to alternative anti-cancer therapies based on plant extracts. In this study, we aimed to evaluate the effect of BRM270 on $CD133^+$ and $CD44^+$ cancer stem cells derived from the pancreatic body and tail. These cells are believed to be more metastatic than malignant cells from the pancreatic head and neck. Results showed inhibitory effects on proliferation, self-renewal capacity, metastatic properties *in vitro*. BRM270 prevented CSCs maintenance mediated by SALL4 and Shh activities. Furthermore, BRM270 helped restrain pancreas body-derived CSCs induced tumor growth. These data consolidate the potential of BRM270 in anti-cancer treatment and direct a new approach in pancreatic cancer elimination.



**Keywords:** BRM270, pancreatic body-derived cancer stem cells, SALL4, Sox-2, Sonic hedgehog signaling

## Introduction

### Sonic hedgehog signaling pathway in pancreatic cancer

Many signaling pathways have been implicated in gene regulation in CSCs (Matsui, 2016; Zhan et al., 2015). These pathways help maintain proliferation, invasion, metastasis, or drug resistance, leading to poor prognosis in cancer patients. The Sonic hedgehog (Shh) signaling pathway is believed to play an important role in all the steps involved in the progression of tumorigenesis, including tumor initiation, promotion, or metastases in skin, lung, brain, and gastrointestinal cancers and leukemia (Xie et al., 2013). Therefore, the Shh signaling pathway increasingly becomes a great concern in cancer treatment.

Hedgehog signaling pathway is activated by three well-known ligands: Sonic hedgehog (Shh), Indian hedgehog and Dessert hedgehog. In normal cases, receptor of Hh ligand named as Patched 1 (PTCH1) located at the plasma membrane is able to suppress transmembrane protein Smoothened (SMO) and leads to the ubiquitin process of Gli proteins. As a result, cleaved Gli protein is unable to transcript downstream genes. In the presence of Hh ligand, the suppression from PTCH1 is released and driving SMO activates full length-Gli proteins and consequently upregulates target genes (Dosch et al., 2010).

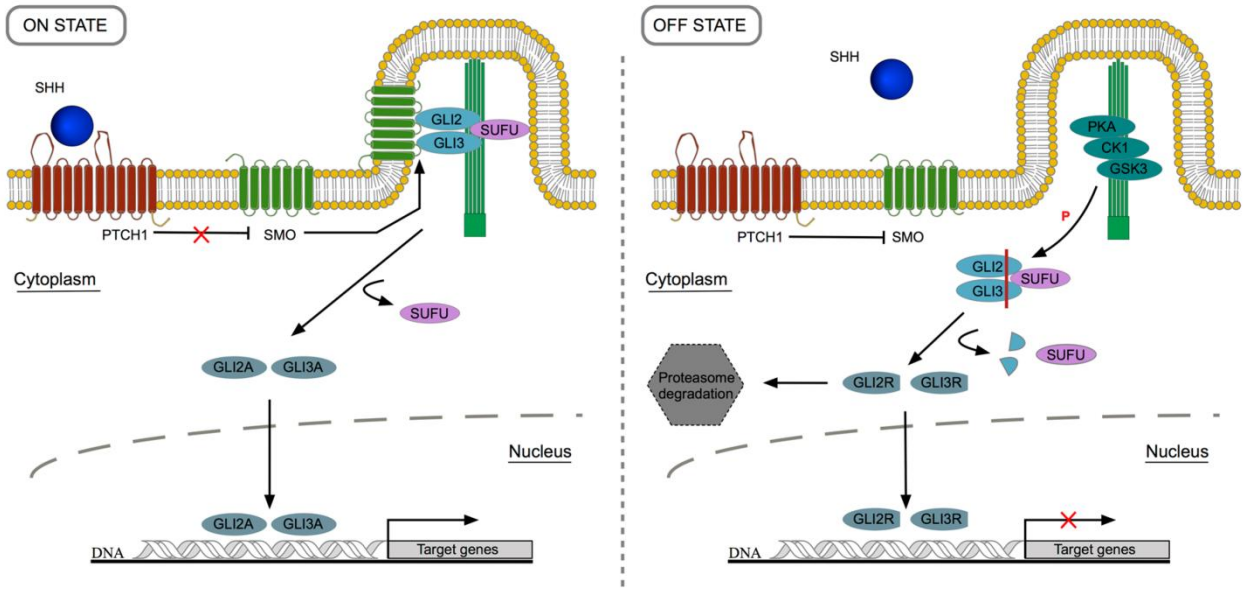



**Figure 5.1 Sonic hedgehog signaling pathway at "ON state" and "OFF state".** At "ON state", Shh protein engages to PTCH1 receptor, facilitate SMO mobility to activate Gli proteins via SUFU detachment. This event creates the expressions of target genes. At "OFF state", without Shh engagement, PTCH1 suppresses SMO activity and therefore forming the phosphorylated Gli protein which in turns translocate into nucleus to repress target genes or are degraded by proteasome (Fernandes-Silva et al., 2017).

In pancreatic cancer, Shh signaling pathway is essential to early stages of pancreatic tumorigenesis which are called as Pancreatic intraepithelial neoplasias (PanINs) (Pasca di Magliano et al., 2006). Hh signaling pathway is also found in primary human pancreatic cancer, clinical samples and cancer cell lines (Dosch et al., 2010). To pancreatic cancer stem cells (PCSCs), cells positive with $CD44^+$ $CD24^+$ $ESA^+$ shows high expression of Shh as compared to non-tumorigenic population (Dosch et al., 2010; Kelleher, 2011). This subpopulation is believed to show high tumorigenesis, drug resistance and metastasis traits in pancreatic cancer (Onishi and Katano, 2014; Tang et al., 2012). Many target genes such as transcription factors of pluripotency (Oct-4, Sox-2, Nanog, c-Myc) or epithelial-mesenchymal transition (EMT) genes (MMP-9, CXCR4, Snail-1, N-cad) are upregulated by Shh stimulation, resulting in metastatic phenotypes and drug resistance, or even tumor relapse (Mimeault and Batra, 2010; Nagai et al., 2008).

Some reports indicated that the cell surface markers for PCSCs, CD133 and CD44, impart a self-renewal capacity to the cells and may be responsible for drug resistance (Banerjee et al., 2014; Fitzgerald and McCubrey, 2014; Hermann et al., 2007; Hong et al., 2009). Hermann *et al.* indicated that CD133 is indispensable for tumorigenesis and chemoresistance, while CD44 is responsible for gemcitabine resistance; it acts as a CSC marker and directs metastatic behaviors in PDACs (Hong et al., 2009; Yan et al., 2016). In addition, compared to PDACs from the pancreatic head, PDAC tumors from pancreatic body-tail seem to be more frequent with a higher metastatic ability (Kanda et al., 2011; Lau et al., 2010). Thus, targeting the subpopulation of pancreatic body-tail-derived CSCs (bPCSCs) with CD133 and CD44 might be a new strategy in combatting pancreatic tumor metastasis.

**BRM270, an extract from herbal plants and its application in cancer treatment**

BRM270 is herbal extract from seven plants including: *Aloe vera, Arnebia euchroma, Citrus unshiu Markovich, Portulaca oleracea, P.vulgaris var. lilacina, Saururus chinensis, Scutellaria baicalensis* which are believed to prevent tumorigenesis and inflammation (Huynh et al., 2017; Hwang et al., 2013; Lee et al., 2015; Li-Weber, 2009; Park et al., 2014; Xiong et al., 2009; Yonehara et al., 2015; Zhao et al., 2013). These plants are widely grown in North East Asia including China, Korea, Japan and have been used as traditional medicine for long time.

Recently, BRM270 showed itself effects in anti-cancer proliferation. Evidences showed that BRM270 has inhibitory effects to osteosarcoma, glioblastoma, chemoresistant lung adenocarcinoma (Jeon et al., 2017; Kwon et al., 2018; Mongre et al., 2015; Mongre et al., 2016). BRM270 also demonstrated itself in ani-tumor growth (Jeon et al., 2017; Kwon et al., 2018; Mongre et al., 2016). These evidences create new prospect in anti-cancer therapies based on herbal plants.

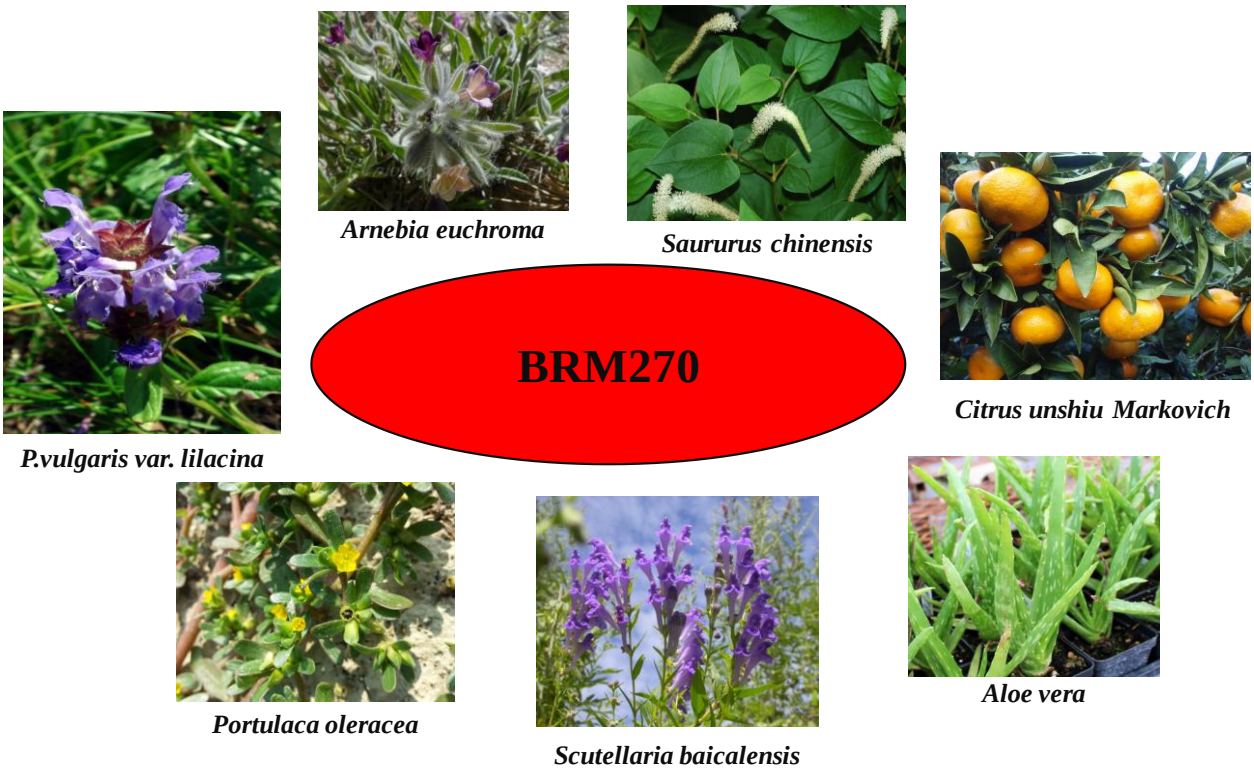



**Figure 5.2 Herbal plants compromise the effects of BRM270.** Seven plants constitute of BRM270 including *Aloe vera, Arnebia euchroma, Citrus unshiu Markovich, Portulaca oleracea, P.vulgaris var. lilacina, Saururus chinensis, Scutellaria baicalensis* These herbal plants are used for therapies many years ago in Northeast Asia (China, Korea, Japan), which are believed to inhibit inflammation, carcinogenesis.

## Materials and methods

### Reagents

BRM270 supplied by BRM institute (Seoul, Korea), was extracted using methanol/ethanol, followed by rotary concentration. The pellet was dissolved in DMSO (Sigma-Aldrich, MO, USA) and stored at −20 °C for further analysis.

### Cell culture

BxPC-3 and PANC-1 cells were cultured in DMEM (Gibco, CA, US) supplemented with 10 % FBS (Welgene, Korea) and 1% anti-mycotic agent and maintained in a humidified atmosphere of 5% $CO_2$ in the incubator at 37 ℃; the cells were passaged twice per week. After isolated by Magnetic activated cells sorting (MACS, Miltenyi Biotec, Germany), BxPC-3 CSCs were maintained in DMEM/F12 supplemented with 10 ng/ml hEGF, (Sigma-Aldrich, MO, US) and 10 ng/ml bFGF (KOMA biotech, Seoul, Korea) and 1X B27 supplement (Thermofisher scientific, MA, US).

### Cell viability

Cells ($5 \times 10^3$) were seeded onto a 96-well plate and incubated for 24 h before treatment with the indicated BRM270 concentrations. After 48 h, cytotoxic effects were measured using an EZ-cytox kit (Daeil lab, Seoul, Korea) according to the manufacturer's protocol. The cell viability results are presented as the ratios of the optical densities of the treated groups and the vehicle control group at 450 nm ($OD_{450}$); this was calculated using the following formula: (%) cell viability = (OD treatment groups or control groups/ OD vehicle control group) × 100%.

**Isolation of CSCs in pancreatic body-derived cancer cells**

Cells in the log phase were used for MACS with CD133 and CD44 microbeads (Miltenyic biotech, Germany), as per the manufacturer's protocol. The isolated subpopulations of $CD133^-$ $CD44^-$ and $CD133^+$ $CD44^+$ were subjected to further analysis.

**FACS annexin-V apoptosis assay**

After isolation by MACS, the cells were bound to the CD133-PE and CD44-APC antibodies (Miltenyic biotech, Germany) and subjected to FACS analysis, as per the manufacturer's protocol. For the apoptosis assay, after 48 h treatment with BRM270, the cells were resuspended in 100 µl of binding buffer containing 5 µl Annexin V-FITC-conjugated antibody and 5 µl propidium iodide for exactly 10 min in the dark at room temperature. Cells were then analyzed using the BD Accuri C6 cytometer (BD Biosciences, NJ, US).

**qPCR analysis**

Total RNA of cells was extracted, followed by a reverse transcription process. The cDNA obtained was subjected to qPCR analysis using the StepOne Applied Biosystems machine and the Evagreen dye (Biotium, CA, US). Data were analyzed by the method suggested by Schmittgene and Livak (Schmittgen and Livak, 2008). Primers for quantitative PCR are as follow human CD133 forward: GTC CAA CAG GGC TAT CAA TC; human CD133 reverse: TAG GAA GAC GCT GAG TTA CA; human CD44 forward: CAG GAG ACC AAG ACA CAT TC; human CD44 reverse: CAG CCA TTC TGG AAT TTG GG; human Beta-2-Microglobulin forward: GTC TTT CAG CAA GGA CTG G; human Beta-2-Microglobulin reverse: ACA TGT CTC GAT CCC ACT.

**Immunocytochemical staining**

Cells after treated with BRM270 were fixed using 3.7% paraformaldehyde (PFA). Before overnight staining with the primary antibodies of interest (CD133, CD44, c-Myc, CXCR4, Nanog, Oct4, SALL4, Sox-2), cells were blocked by PBST 1X with 3% BSA. After washing twice with PBST 1X, the secondary antibodies were added, followed by a 2-h incubation. Cells were stained for 10 min with 4', 6-diamidino-2-phenylindole (DAPI) before microscopic observation.

**Western blotting**

The protein content of the cell lysates was determined using the BCA assay. Proteins were separated on by 12% SDS-PAGE, transferred electrophoretically (Bio-Rad, CA, USA) onto a polyvinylidene fluoride (PVDF) membrane and blocked with 5% non-fat milk powder (w/v) in phosphate-buffered saline 0.1% Tween-20 (PBST) for 1 hour at room temperature; this was followed by incubating the membrane with primary antibodies or with anti-GADPH mouse monoclonal antibody as an internal control overnight at 4 ℃, and with appropriate HRP-conjugated secondary antibodies for 4 h at room temperature or overnight at 4 $^{0}$C. The bands were captured using an ImageQuant™ LAS 4000 mini Fujifilm camera.

**Table 3: List of antibodies for Western blotting**

| No. | Antibodies | Company | Catalogue number | Source |
|---|---|---|---|---|
| 1 | CD133 | Proteintech | 18470-1-AP | Rabbit polyclonal |
| 2 | CD44 | Santa cruz | sc-7297 | Mouse monoclonal |
| 3 | CD24 | Bioss | bs-4890R | Rabbit polyclonal |
| 4 | SALL4 | Abfrontier | YF-MA11603 | Mouse monoclonal |
| 5 | c-Myc | Santa cruz | sc-40 | Mouse monoclonal |
| 6 | CXCR4 | Abfrontier | YF-MA16239 | Mouse monoclonal |
| 7 | GADPH | Abfrontier | LF-MA0038 | Rabbit polyclonal |
| 8 | Nanog | Santa cruz | sc-33759 | Mouse monoclonal |
| 9 | N-cadherin | Abfrontier | LF-MA50067 | Mouse monoclonal |
| 10 | Oct-4 | Santa cruz | sc-9081 | Rabbit polyclonal |
| 11 | Sox-2 | Santa cruz | sc-20088 | Mouse monoclonal |
| 12 | Bcl2 | Santa cruz | Sc-492 | Rabbit polyclonal |
| 13 | BxL-CL | Santa cruz | Sc-7195 | Rabbit polyclonal |
| 14 | P53 | Abfrontier | LF-PA0050 | Rabbit polyclonal |
| 15 | Caspase-3 | Santa-cruz | Sc-7148 | Rabbit polyclonal |
| 16 | PCNA | Abfrontier | yf-ma10672 | Mouse monoclonal |
| 17 | Shh | Abcam | ab135240 | Rabbit polyclonal |
| 18 | Gli1 | Abcam | ab151796 | Rabbit polyclonal |
| 19 | SNAI 1 | Santa cruz | sc-271977 | Rabbit polyclonal |
| 20 | N-cadherin | Abfrontier | LF-MA50067 | Mouse monoclonal |
| 21 | MMP-9 | Santa-cruz | Sc-21733 | Mouse monoclonal |

**Clonogenicity assay**

Cells ($1 \times 10^3$) were seeded onto 6-well plates at 37 $^0$C/ 5% $CO_2$. After 7 days of incubation in DMEM supplemented with 10% FBS, cells were fixed with 3.7% PFA and stained with 0.05% Crystal violet, followed by washing with 1X PBS before observation.

**Sphere formation**

Cells ($1 \times 10^3$) in serum-free medium (DMEM/F12 supplemented with 2% B27, 10 ng/ml hEGF and 10 ng/ml bFGF and 1% antimycotic) were seeded onto ultralow attachment 6-well plates (Corning, NY, US) with and without BRM270 treatment. After 7 days of incubation, the spheres were viewed using a microscope. For a single sphere formation, cells were seeded onto 96-well ultralow attachment plate (Corning, NY, US) such that there was 1 cell/well, with and without BRM270 treatment. The growth of cells was observed after day 7.

***In vitro* cell migration and invasion assays**

A cell migration assay was performed using 8-µm pore-sized hanging cell-inserts (Merck Millipore, MA, US). Cells ($1 \times 10^5$) in 0.5% FBS-DMEM were seeded in the upper chamber while the lower chamber was filled with 20% FBS. After 48 h of incubation, the migrating cells were stained with 0.05% crystal violet (w/v). The number of migrated cells in the lower surface of the membrane was counted under a microscope in five random fields at 100 ×. For the cell invasion assay, all procedures carried out were the same as that in the migration assay, except that in this case, a Matrigel matrix growth factor reduced basement (BD Biosciences, NJ, US) (3.5 mg/ml) was coated on the upper chamber, according to the manufacturer's protocol.

**Wound healing assay**

Cells were used for the wound healing assay as per the manufacturer's protocol. Briefly, cells in the log phase were seeded onto a 96 well plate, reaching 98%–100% confluence overnight. The monolayer of cells was scratched using wound-maker and imaged real-time by IncuCyte system (Essen Bioscience, MI, US).

***In vivo* evaluation**

Tumors were induced by subcutaneously injecting $1 \times 10^6$ cells into the flanks of 6-week-old nude female BALB/c-nu mice. The tumor sizes were measured every 7 days using calipers. BRM270 was supplied orally to the mice every day at a dosage of 5 mg/kg. The tumor volume ($V = W \times L \times H/2$) was evaluated by length (L), height (H), width (W). Mice were sacrificed after day 35 of cell injection.

**Statistical analysis**

Statistical analysis was performed using the Graphpad Prism software (Version 6.02). Data were expressed as mean ± standard deviation (SD). Experimental differences were examined by ANOVA and Student's *t*-tests, as appropriate. *P* values of <0.05 were considered to indicate statistically significant data.

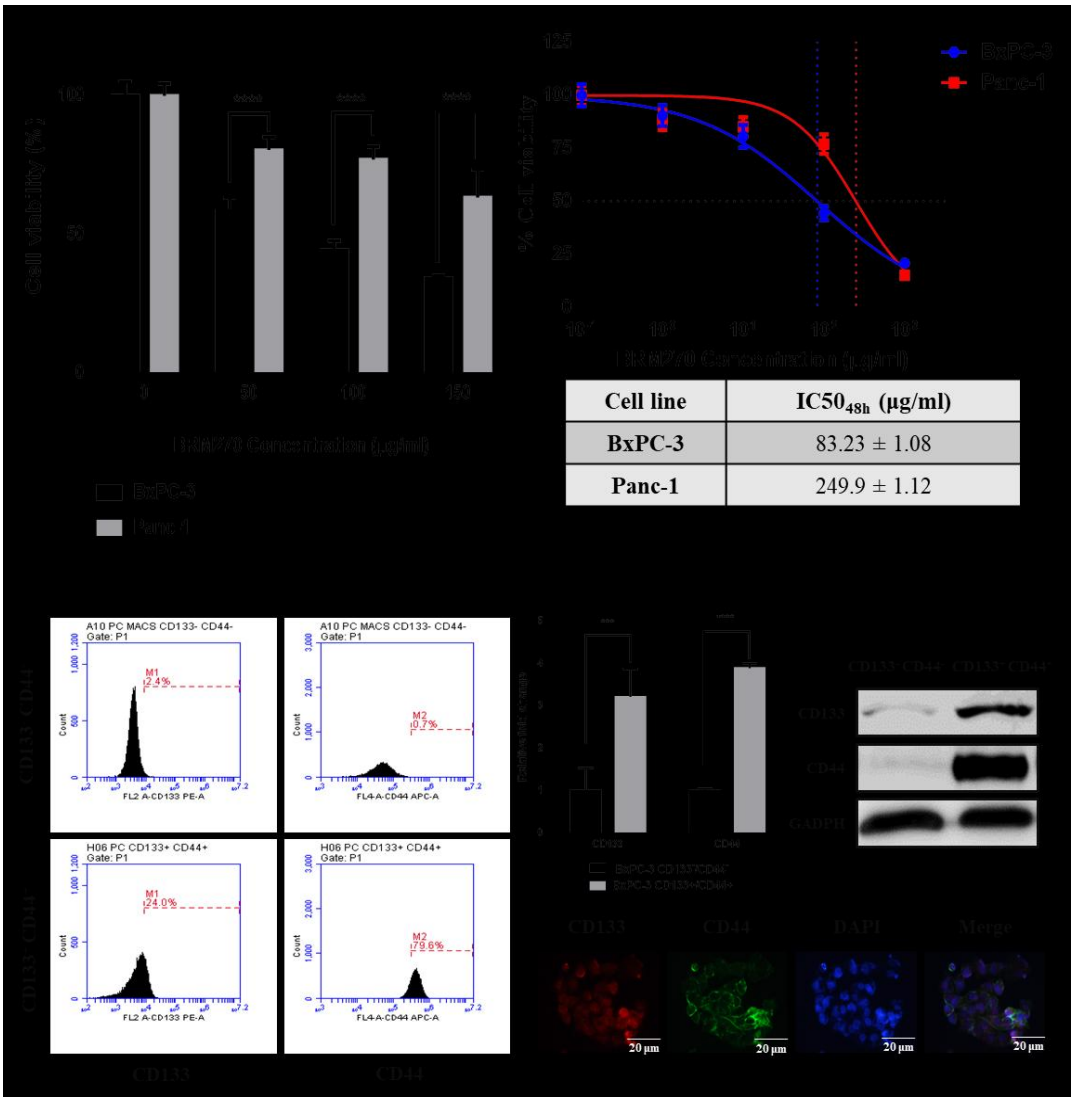
## Results

### Isolation of pancreas body-derived CSCs

BRM270 shows its anti-cancer effects on lung adenocarcinoma, and glioma cell lines *in vitro* and *in vivo* (Jeon et al., 2017; Mongre et al., 2016). In this study, we evaluated the inhibitory effects of BRM270 on the PDAC cell lines, PANC-1 (head of pancreas) and BxPC-3 (body and

tail of pancreas) (Deer et al., 2010). After 48 h treatment, the BRM270 extract had different inhibitory effects on BxPC-3 and PANC-1 cells (Figure 5.3 A) and the IC50 of BRM270 at 48 h for BxPC-3 and PANC-1 cells were 83.23 μg/ml and 249.9 μg/ml, respectively (Figure 5.3 B). Therefore, PDAC cells from the pancreatic body are more sensitive to BRM270, than the cells from the pancreatic head.

The use of CSCs for cancer treatment is a recent development. Among the CSCs markers that have been defined, $CD133^+$ and $CD44^+$ found in the subpopulation of bPCSCs are the ones that concern our study. Next, we examined whether BRM270 can inhibit bPCSCs, and attempted to elucidate the signaling pathway it interferes with. Among CSCs markers have been found, we concern about $CD133^+$ $CD44^+$ subpopulation of PCSCs. CD133 surface marker is responsible for CSC marker in many types of cancer and play important role in renewal capacity in CSCs (Banerjee et al., 2014; Fitzgerald and McCubrey, 2014; Hermann et al., 2007). Furthermore, CD44 marker recently becomes increasing concern in pancreatic cancer treatment due to its involvement to drug resistance and stemness maintenance (Hong et al., 2009; Yan et al., 2016). Therefore, we isolated $CD133^+$ $CD44^+$ subpopulation bPCSCs from the BxPC-3 cell line.




| Cell line | $IC50_{48h}$ (μg/ml) |
|---|---|
| BxPC-3 | 83.23 ± 1.08 |
| Panc-1 | 249.9 ± 1.12 |

**Figure 5.3 Isolation of pancreas body-derived CSCs**. (A) Inhibitory effects of BRM270 on pancreas body adenocarcinoma cells (BxPC-3) and pancreas head adenocarcinoma cells (PANC-1). (B) $IC50_{48h}$ of BxPC-3 and PANC-1 under BRM270 treatment. (C) Isolation of $CD133^{+}$ $CD44^{+}$ subpopulation by MACS; cells after MACS were analyzed by FACS CD133-PE and CD44 FITC. (D) qPCR analysis of CD133 and CD44 between double positive and negative (Left top), Western blotting of CD133 and CD44 after MACS separation (Right top), Immunocytochemistry staining of CD133 and CD44 in $CD133^{+}$ $CD44^{+}$ BxPC-3 (bottom). ****P< 0.001; ****P< 0.0001*

As shown in Figure 5.3 C, FACS analysis with CD133-PE and CD44-APC antibodies indicated that histograms for CD133 and CD44 were shifted. Data for qPCR analysis and western blotting showed that the expression levels of CD133 and CD44 in the double-positive subpopulation were significantly higher than those in the double-negative subpopulation (Figure 5.3 D, top). The presence of CD133 and CD44 in the $CD133^+$ $CD44^+$ subpopulation was also confirmed by immunocytochemical staining (Figure 5.3 D, bottom). These data suggested that $CD133^+$ $CD44^+$ subpopulation in pancreas body-derived cancer cells is successfully isolated.

**BRM270 induces apoptosis in pancreas body-derived CSCs**

Next, we have examined whether BRM270 can induces apoptosis in bPCSCs. FACS annexin-V data showed that the percentage of apoptotic bPCSCs had increased dose-dependently after 48 h BRM270 treatment (Figure 5.4 A and B).

In addition, Western blotting data showed the activations of p53 and Caspase-9, leading to apoptosis in bPCSCs (Figure 5.4 C). Image J measurement indicated that significant increases in both activated caspase-9 and p53 as compared to negative control (Figure 5.4 D). These data revealed that BRM270 is able to induce apoptosis in the $CD133^+$ $CD44^+$ subpopulation of bPCSCs.

**BRM270 inhibits the self-renewal capacity of bPCSCs**

CSCs share common features with normal stem cells such as self-renewal capacity or differentiation ability (Hadjimichael et al., 2015; Lobo et al., 2007). In cancer treatment, prevention of self-renewal in CSCs becomes a prerequisite (Borah et al., 2015). In this study, we examined the effects for BRM270 on self-renewal capacity in bPCSCs via clonogenicity assay

and sphere formation. Figure 5.5 A showed inhibitory effects of BRM270 on bPCSCs after 7 days incubation. Number of colonies counted in treatment group is significantly lower than those in non-treatment (Figure 5.5 B). In term of sphere formation, BRM270 constrained sphere formation ability of bPCSCs in serum free medium (Figure 5.5 C, top) and minimized sizes of single sphere (Figure 5.5 C, bottom). Numbers of sphere were also reduced remarkably in presence of 50 μg/ml BRM270 (Figure 5.5 D). These data suggested that BRM270 enable inhibiting self-renewal capacity in bPCSCs.

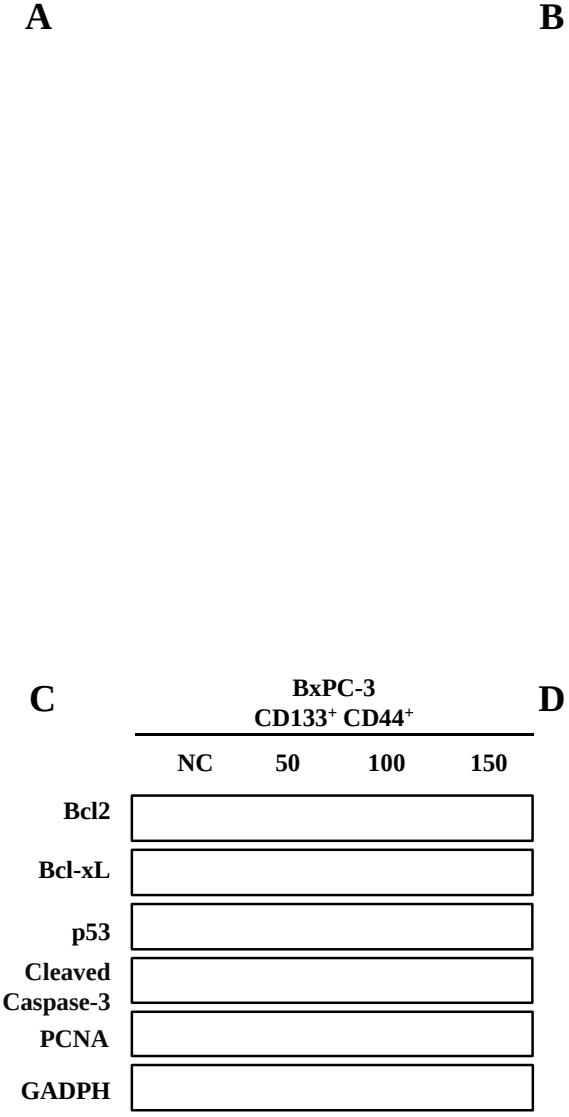



**Figure 5.4 BRM270 induces apoptosis in pancreas body-derived CSCs.** (A) FACS annexin-V analysis of pancreas body adenocarcinoma cells (BxPC-3) exposed with BRM270 (0, 50, 100, 150 μg/ml). (B) Percentage of apoptotic cells after BRM270 treatment. (C) Western blotting of apoptosis markers in lysates with and without BRM270 treatment. (D) Relative activations of Caspase-3 and p53 measured by Image J. ****P< 0.001; ****P< 0.0001*.

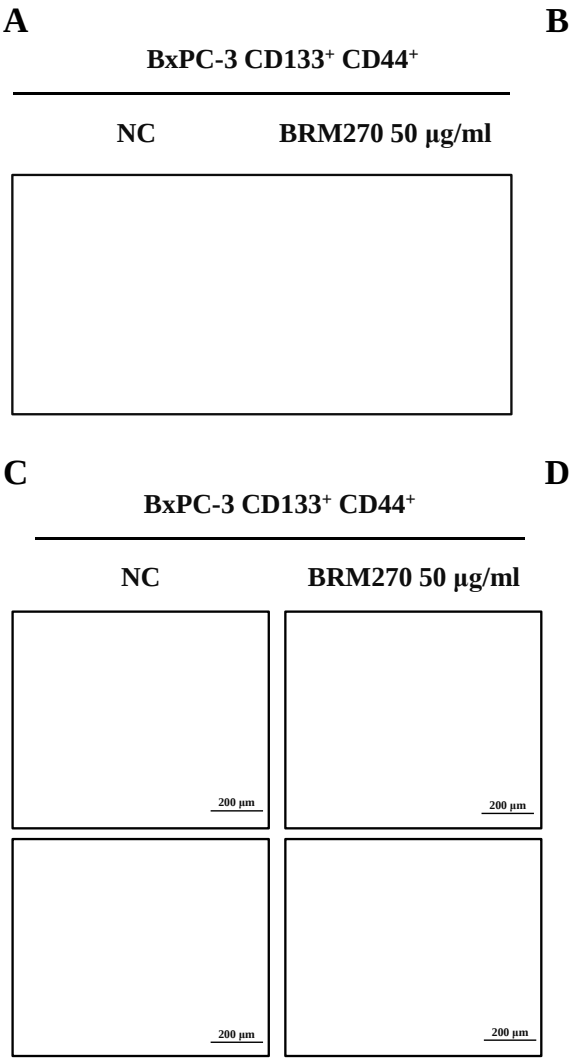



**Figure 5.5 BRM270 inhibits self-renewal capacity of bPCSCs. (**A) Clonogenicity assay of $CD133^{+}$ $CD44^{+}$ BxPC-3 with and without 50 µg/ml BRM270 treatment. (B) Colony numbers treated with and without BRM270 50 µg/ml. (C) Sphere formation assay (top) and single sphere formation (bottom) with and without 50 µg/ml BRM270 treatment. (D) Sphere numbers of bPCSCs with and without BRM270 treatment. ***P< 0.01; ****P< 0.0001*.

**BRM270 downregulates stemness genes in bPCSCs**

Stemness genes such as Sox-2, Nanog, Oct-4, c-Myc, or surface markers like CD133 and CD44 have been associated with CSCs' self-renewal capacity to maintain tumor growth (Fitzgerald and McCubrey, 2014; Herreros-Villanueva et al., 2014). Therefore, the downregulation of such targets might promote cancer treatment. Data above showed inhibitory effects on self-capacity, therefore BRM270 might target stemness genes to interfere CSCs traits. Western blotting indicated that levels of Sox-2, Nanog, Oct-4, c-Myc, C-X-C chemokine receptor type 4 (CXCR4) after exposure to BRM270 were decreased (Figure 5.6 A). Furthermore, BRM270 helped reduce expressions of CD133, CD44 and SALL4 in bPCSCs (Figure 5.6 B). Immunocytochemistry staining also indicated same trends after 48 h treatment (Figure 5.6 C). These data indicated that BRM270 interferes with the self-renewal capacity of bPCSCs via downregulation of stemness markers.

**BRM270 restrains metastasis in bPCSCs via downregulation of the Shh signaling pathway**

Evidence shows that the Shh signaling pathway is tightly involved in PCSCs (Onishi and Katano, 2014; Tang et al., 2012). Once driven by the Shh protein, downstream genes of the activated Gli-1 protein would be upregulated, leading to metastasis, drug resistance, or stemness maintenance in PCSCs. Therefore, the inhibitory effect of BRM270 on the self-renewal capacity might involve the Shh signaling pathway. Next, we examined the involvement of BRM270 in the Shh signaling pathway.

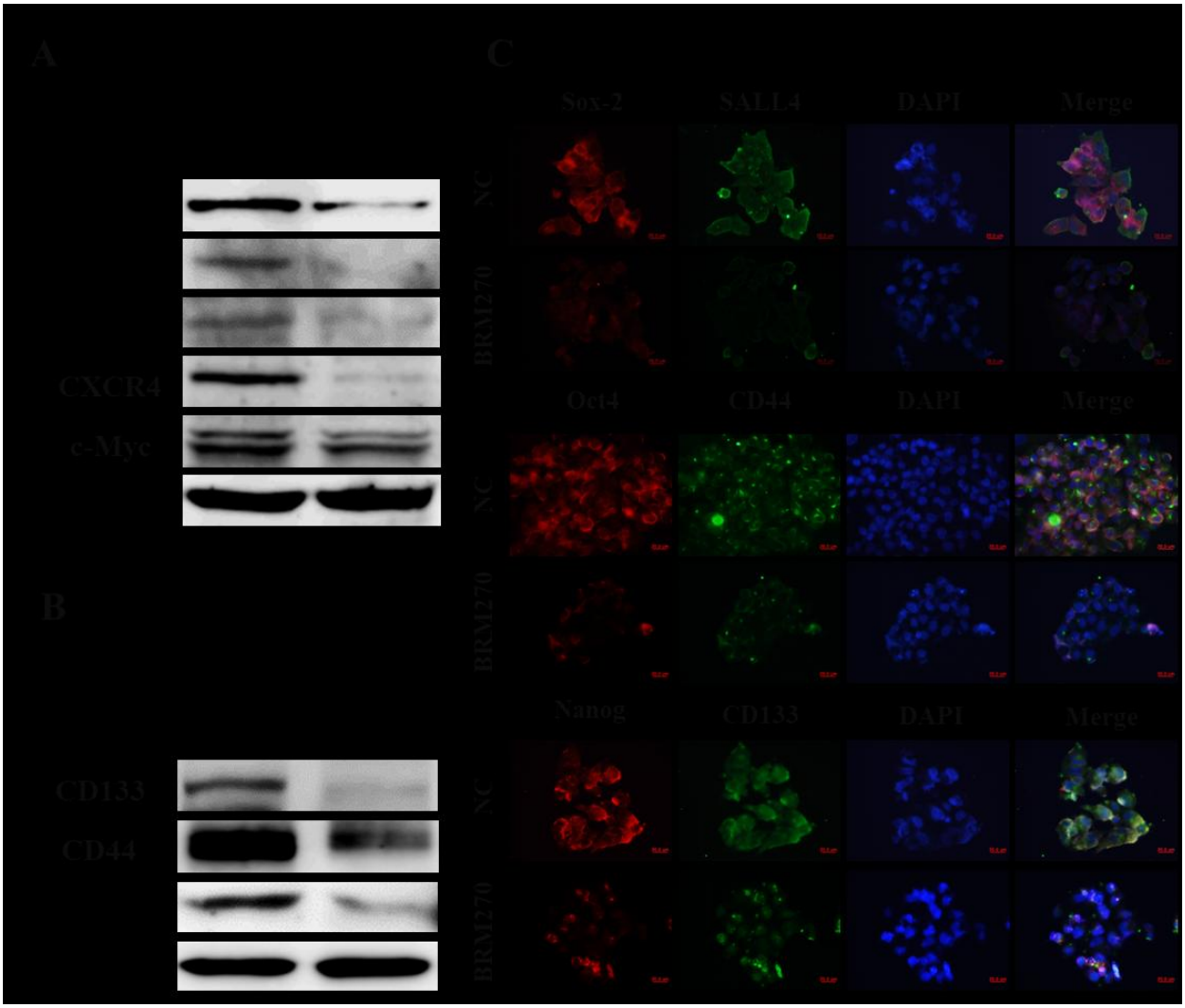



**Figure 5.6 BRM270 downregulates stemness genes in bPCSCs**. (A) Western blotting of stemness genes in $CD133^{+}$ $CD44^{+}$ BxPC-3 markers from protein lysates with and without 50 μg/ml BRM270 treatment. (B) Western blotting of CSC markers from protein lysates with and without 50 μg/ml BRM270 treatment. (C) Immunocytochemistry staining of stemness genes (Sox-2, Oct-4, Nanog) and SALL4, CD133, CD44 compared BRM270 treatment and non-treatment.

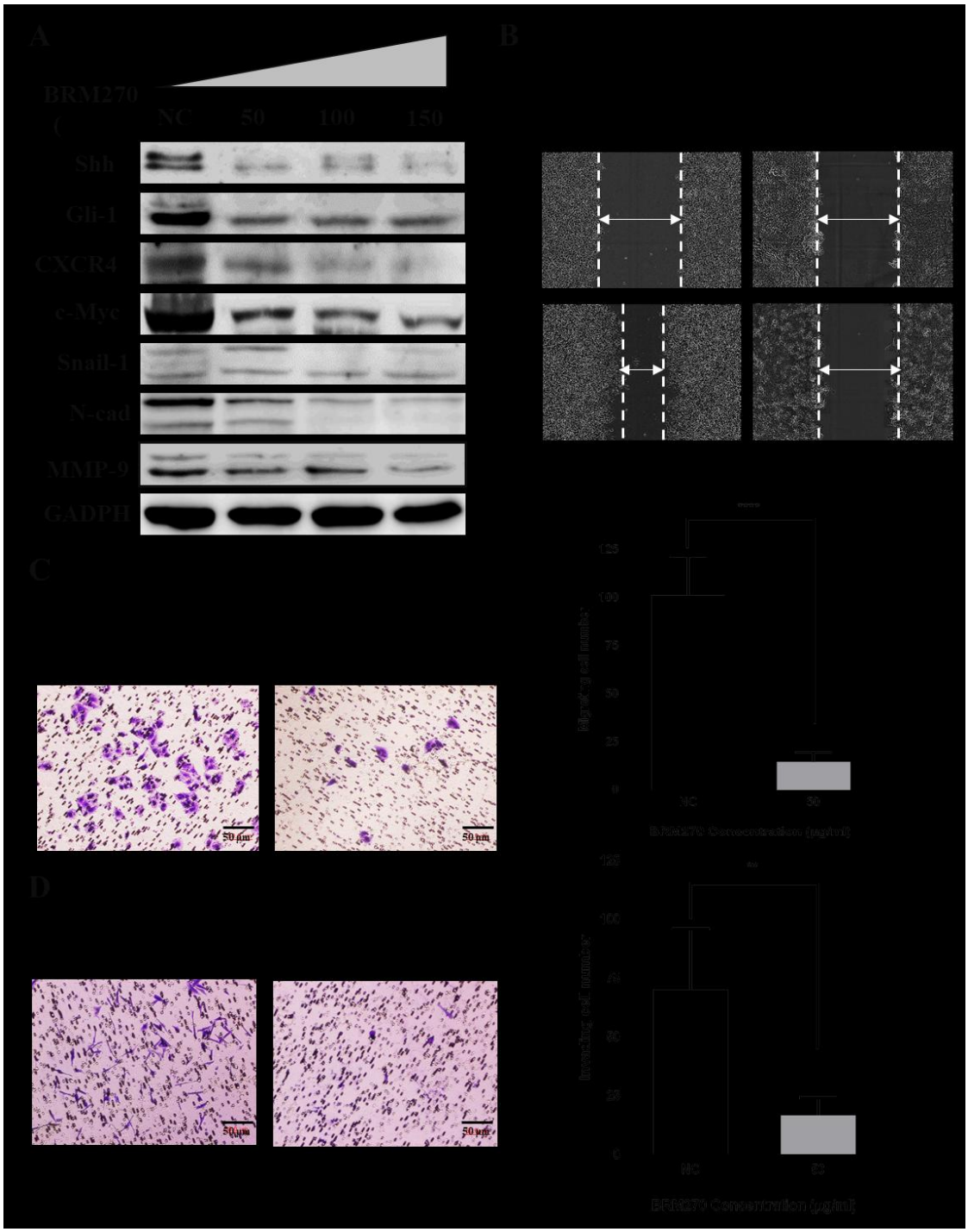



**Figure 5.7 BRM270 restrains bPCSCs metastasis via downregulation of Shh signaling pathway.** (A) Western blot analysis of metastatic markers in $CD133^{+}$ $CD44^{+}$ BxPC-3 under BRM270 treatment (0, 50, 100, 150 μg/ml). (B) Wound healing assay of $CD133^{+}$ $CD44^{+}$ BxPC-3 with and without BRM270 50 μg/ml. (C) Migration assay of $CD133^{+}$ $CD44^{+}$ BxPC-3 with and

without BRM270 50 μg/ml (left), number of migrating cells with and without BRM270 50 μg/ml. (D) Invasion assay of $CD133^{+}$ $CD44^{+}$ BxPC-3 with and without BRM270 50 μg/ml (left), number of invasive cells with and without BRM270 50 μg/ml ***p< 0.01; ****P< 0.0001*.

Western blotting data showed that BRM270 inhibits the Shh signaling pathway dose-dependently (Figure 5.7 A). As a result, the downstream genes of Gli-1, such as CXCR4, c-Myc, Snail-1, N-cad, and MMP-9, were repressed. These genes are markers of metastatic phenotypes. Therefore, we investigated the effect of BRM270 on cancer mobility. A wound healing assay revealed that at 50 μg/ml, BRM270 significantly inhibited bPCSC mobility (Figure 5.7 B), while the number of invasive and migrating cells was also reduced (Figure 5.7 C and D). These data suggested that by Shh/Gli-1 inhibition, BRM270 indirectly represses downstream genes and facilitates the inhibition of metastasis.

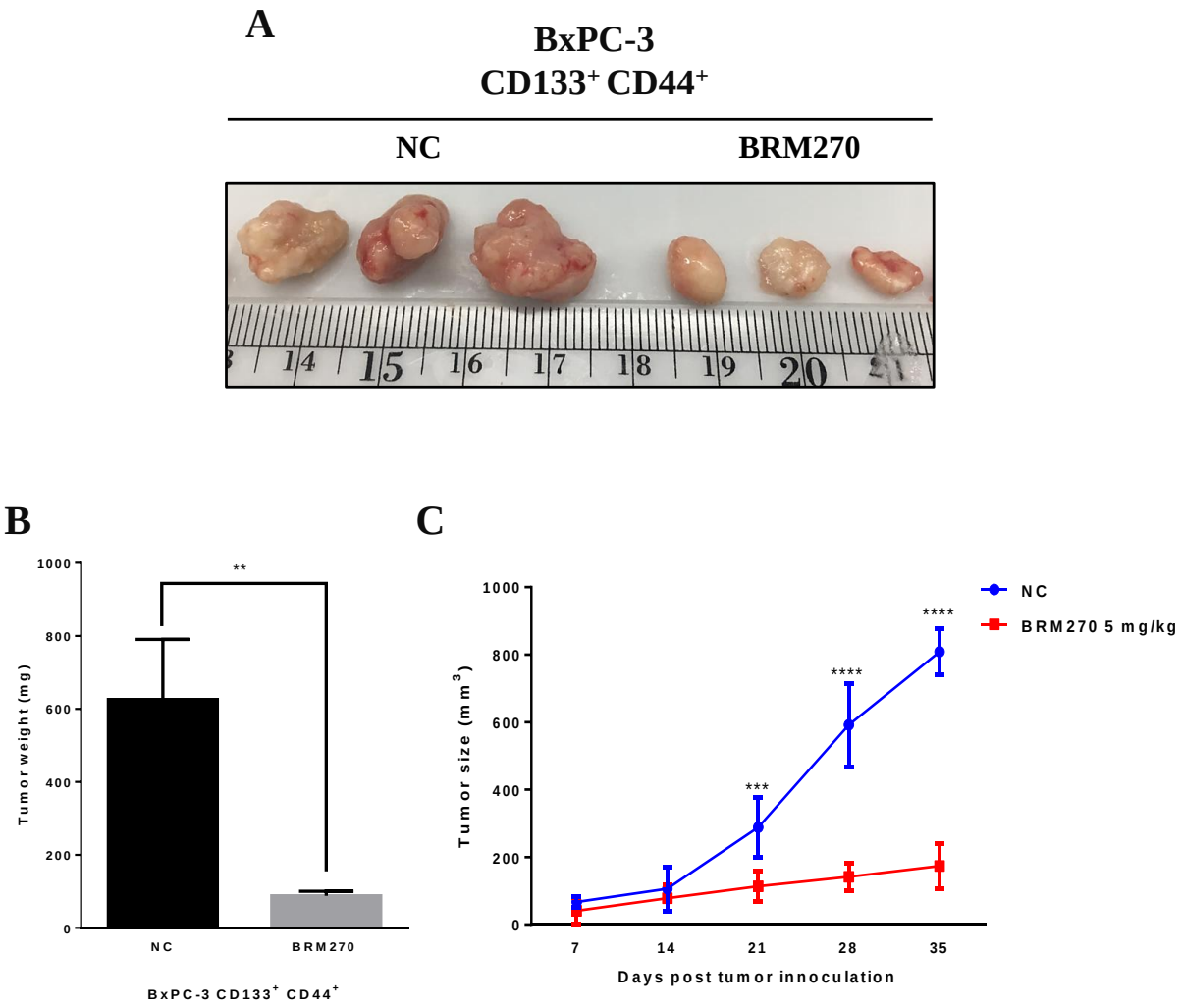



**Figure 5.8 BRM270 suppresses bPCSCs induced tumor growth.** BRM270 suppresses bPCSCs induced tumor growth. **(A)** Resected subcutaneous tumors with and without BRM270 treatment after 35 days inoculation. **(B)** Comparing of tumor weights with and without BRM270 treatment after 35 days inoculation. **(C)** Tumor sizes with and without BRM270 treatment after 35 days inoculation. ***p< 0.01; ***P< 0.001; ****P< 0.0001.*

**BRM270 suppresses tumor growth**

Our previous studies showed an anti-tumor effect of BRM270 in lung cancer and glioblastoma. BRM270 inhibited tumor growth, but there was no evidence of body weight loss or tumor recurrence (Jeon et al., 2017; Mongre et al., 2016). In this study, we examined whether BRM270 can suppress the tumorigenesis induced by bPCSCs. After 5 weeks of inoculation, the number of tumors in the 5 mg/kg BRM270-treated mice was significantly reduced as compared to that in the PBS-treated mice (Figure 5.8 A and B). In addition, tumor sizes were also decreased from week 3, and tumor weights were notably reduced (Figure 5.8 C and D). These data suggested that BRM270 inhibits bPCSC-induced tumorigenesis.

**Discussion**

Pancreatic cancer often has a poor prognosis, and PDAC accounts for 85% of pancreatic cancer-diagnosed cases (Hezel et al., 2006). Clinical reports suggested that body and tail (BT) pancreatic cancer patients have a poorer overall survival and lower rate of resectability compared to those with head-pancreatic cancer (Kanda et al., 2011; Lau et al., 2010). Therefore, the tumor site might be a prognostic factor for survival in pancreatic cancer.

Our study indicated that BRM270 has a stronger inhibitory effect on pancreas body-derived PDAC cells (BxPC-3) than on pancreas head-derived PDAC cells (PANC-1) and induces apoptosis in $CD133^{+}$ $CD44^{+}$ bPCSCs via the upregulation of p53. Furthermore, BRM270 inhibits the stemness-trait in bPCSCs. The levels of stemness genes such as Sox-2, Nanog, Oct-4, and c-Myc were attenuated after BRM270 treatment, resulting in the repression of colony- and sphere-forming ability in bPCSCs. Many studies indicated that Shh signaling regulates CSCs, and is involved in the initiation and maintenance of tumors (Barakat et al., 2010). Therefore, targeting

Shh signaling is a promising approach for cancer treatment. In this study, we investigated the effects of BRM270 on bPCSCs, and data showed that BRM270 dose-dependently inhibits the proliferation of CSCs by downregulating Shh/Gli1 signaling, leading to the suppression of target genes such as CXCR4, c-Myc, Snail-1, N-cad, MMP-9 and inhibition of their metastatic abilities. These data are consistent with those of previous reports that showed that Shh/Gli-1 signaling regulates metastatic/stemness/survival target gene expression (Mimeault and Batra, 2010; Nagai et al., 2008).

Furthermore, other reports showed that SALL4 regulates Gli-1 expression and mediates carcinogenesis in colorectal cancer (Cheng et al., 2015). Whether SALL4 indirectly or directly reactivates Gli-1 expression is undefined. However, other reports accentuated that Sox-2 activates Shh signaling via enhancing Hedgehog Acyl Transferase (HHAT) expression at its promoter site, and as a result, the level of HHAT is increased (Justilien et al., 2014). Thus, this implies that Sox-2 might be responsible for the accumulation of activated Shh, leading to tumorigenesis. Acting as a downstream gene of SALL4, Sox-2 may contribute as an intermediator of Shh/Gli-1 signaling. This might partially explain why SALL4 inhibits the Shh/Gli-1 signaling pathway in bPCSCs after BRM270 treatment.

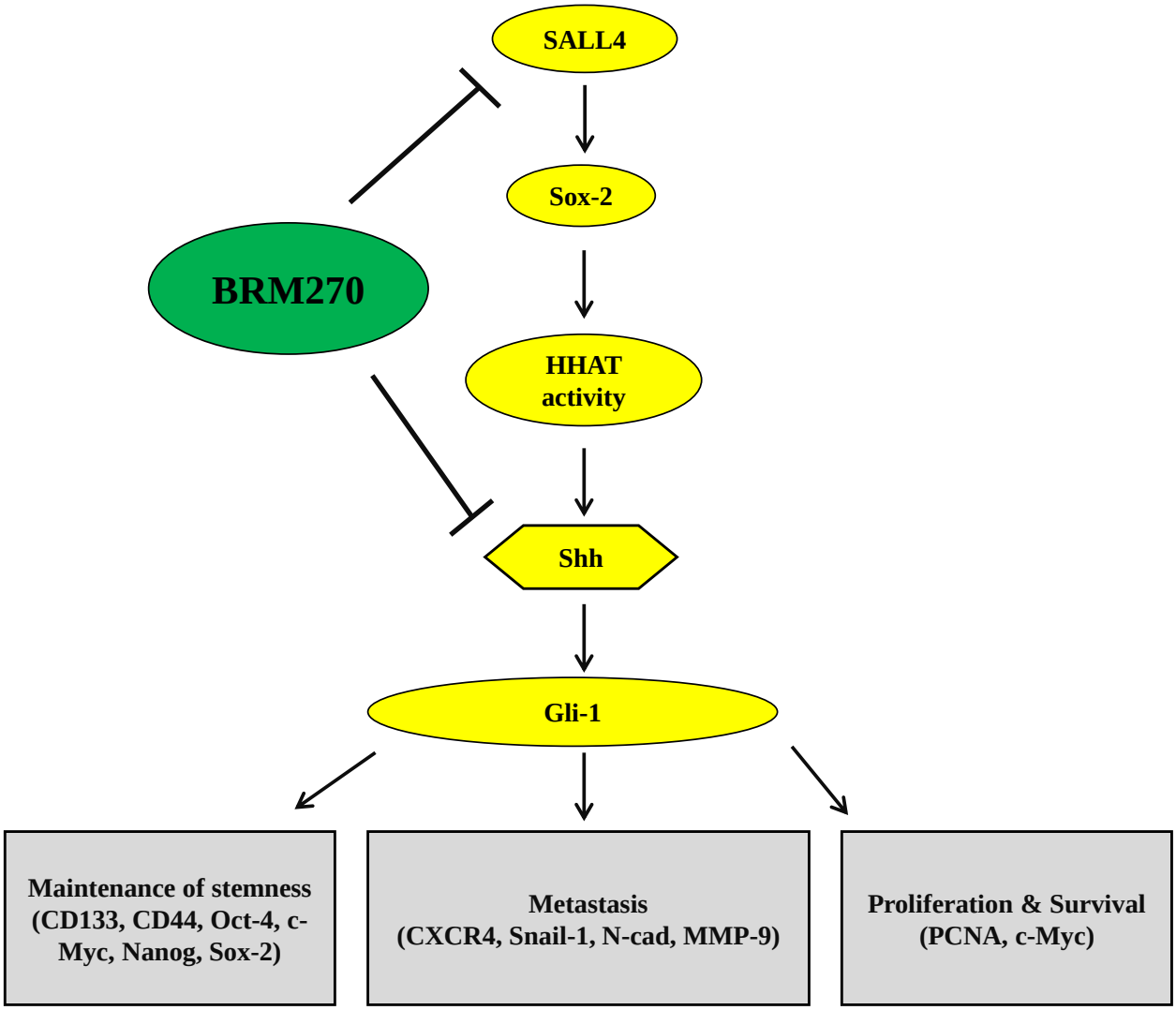



**Figure 5.9 Schematic signaling pathway of BRM270 treatment inhibits bPCSCs induced tumorigenesis.** BRM270 represses Shh expression, subsequently influencing Gli-1 expression and activation. As a result, target genes such as stemness genes or metastatic genes or genes relating to survival and proliferation are downregulated, leading to the impairment in renewal capacity, metastasis, carcinogenesis.

In conclusion, this study provides evidence that BRM270 treatment regulates Shh/Gli-1 signaling in bPCSCs via two possible pathways: one, BRM270 directly inhibits Shh/Gli-1 expression, and two, BRM270 inhibits Shh/Gli-1 expression via the inhibition of SALL4/ Sox-2 expression, thereby indirectly modulating the HHAT-mediated-Shh activation process. These effects constrain the tumor growth induced by bPCSCs. In fact, pancreatic tumor is hardly eliminated by complete resection, owing to their silent symptoms, high metastasis rate, and rare curative. Once diagnosed, i.e. at the late stage of pancreatic cancer, patients have a very short survival time (Hezel et al., 2006). Therefore, prevention of metastasis in pancreatic cancer is very important. Our findings introduce a new option for treating metastases of pancreatic tumors; it is believed to be a first-precedence in PDAC treatment (Stromnes and Greenberg, 2016). There are many aspects yet to be studied, such as the effects of BRM270 on tumor environments at early metastatic stages, its behavior amidst anti-metastasized or anti-circulating pancreatic tumor cells, and the role of the combination of BRM270 and gemcitabine in pancreatic cancer intervention. Nevertheless, our findings promote BRM270 as a therapeutic agent for treating pancreatic cancer.

**Conclusion**

The proliferation and metastasis in PDAC phenotype are great concerns in pancreatic cancer treatment. SALL4 is tightly involved in proliferation. stemness trait maintenance and metastasis in PDAC phenotype. BRM270 mixture extract showed its effect to proliferative inhibition, anti-mobility on bPCSCs and tumorigenesis via repressing Shh signaling pathway. BRM270 also negatively targets SALL4 expression, resulting in indirect inhibition in Shh signaling pathway.

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