The role of Hedgehog signaling in Cholangiocarcinogenesis: An in vitro and in vivo (mouse model) study

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LIST OF ABBREVIATIONS

ASCs	Adult stem cells
AMP	Adenosine 5'-monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
Akt	Protein kinase B (PKB)
AlbCre	Albumin-Cre
AS	Antisense
ATP	Adenosine triphosphate
BRCA1	Breast Cancer 1
Bcl-2	B-cell lymphoma 2
BRAF	v-Raf murine sarcoma viral oncogene homolog B
C. sinensis	Clonorchis sinensis
CA-19-9	Carbohydrate antigen (CA) 19-9
cDNA	complementary Deoxyribonucleic acid
CI	Confidence interval
COX-2	Cyclooxygenase-2
C-Terminal	Carboxyl-terminus
CK-19	Cytokeratin 19
CSCs	Cancer stem cells
CC	Cholangiocarcinoma
DNA	Deoxyribonucleic acid
DAB	3,3′-Diaminobenzidine
DBP	D site of albumin promoter (albumin D-box) binding protein
DMEM	Dulbecco's Modified Eagle Medium
DHH	Desert Hedgehog
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
ddH2O	Double-distilled water
DYRK	dual-specificity tyrosine phosphorylation-regulated kinase
DAPI	4, 6-diamidino-2-phenylindole
ELISA	Enzyme-Linked Immunosorbent Assay
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor
EBW	Excess body weight
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal–regulated kinases
ES	Excretory/Secretory
EH-CC	Extrahepatic cholangiocarcinoma
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FGFR2	Fibroblast growth factor receptor 2
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GPCR	G-protein coupled receptor
GLI1	Glioma-associated oncogene homolog 1
HRP	Horseradish peroxidase
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDF	Human dermal fibroblast
HMW	High molecular weight
HNRNPM	Heterogeneous Nuclear Ribonucleoprotein M HR Hazard Ratio
Hh	Hedgehog
HIF	Hypoxia-inducible factors
HIF-1α	Hypoxia-inducible factor 1-alpha
HIF-1β	Hypoxia-inducible factor 2-beta
IH-CC	Intrahepatic cholangiocarcinoma
ICH	Immunohistochemistry
IDH	Isocitrate dehydrogenase
IGF-1	Insulin-like growth factor 1
IgG(H+L)	Immunoglobulin G (Heavy chain, light chain) IL Interleukin
IP	Intraperitoneal
IRS	Immunoreactive score
IL6	Interleukin 6
IHH	Indian Hedgehog
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
KRAS	Kirsten RAt Sarcoma virus proto-oncogene L/L Homologous loxP
LKB1	Liver kinase B1
LMW	Low molecular weight
LSL	LoxP-STOP-LoxP
MEK	Mitogen-activated protein kinase kinase
MAPK	Mitogen-activated protein kinase
Min	Minute
miR	microRNA
MMP2	Matrix metalloproteinase-2
MMP9	Matrix metalloproteinase-2
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
MRI	Magnetic resonance imaging
MET	Mesenchymal-to-epithelial transition
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
N-terminal	Amino-Terminal
NCCN	National Comprehensive Cancer Network
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency (NOD/SCID)
O. viverrini	Opisthorchis viverrini

OD	Optical density
Oct-4	Octamer-binding transcription factor 4
PBS	Phosphate-buffered saline
pCC	Perihilar cholangiocarcinoma
Pen/Strep	Penicillin/Streptomycin
PI	Propidium iodide
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PPARα	Peroxisome proliferator-activated receptor alpha
PSC	Primary sclerosing cholangitis
PTEN	Phosphatase and tensin homolog
RIA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640 Medium
RR	Relative Risk
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SEM	Standard Error of Mean
SFM	Serum-free medium
SFPQ	SFPQ Splicing Factor Proline and Glutamine Rich
siRNA	siRNA Small interfering RNA
SSM	Serum-supplemented medium
SOX2	SRY-Box 2
SMO	Smoothened
TBS-T	Tris-buffered saline Tween 20 buffer
TMA	Tissue microarray
TNF-α	Tumour necrosis factor alpha
TRIM28	Tripartite motif-containing 28
VGFR	Vascular endothelial growth factor A

1. INTRODUCTION

1.1.Cholangiocarcinoma

1.1.1. *Epidemiology and Characterization of Cholangiocarcinoma*

Cholangiocarcinoma (CC) is a malignant tumor arising from the epithelial cells of the bile ducts. Anatomically, distinction is made between intrahepatic cholangiocellular carcinoma (IH-CC) and the extrahepatic cholangiocellular carcinoma (EH-CC), which is further divided into distal cholangiocellular carcinoma (dCC) and perihilar cholangiocellular carcinoma (pCC). Based on the perihilar longitudinal extension, latter can be characterized into four different types according to the Bismuth-Corlette classification. The morphology-based groups of IH-CC are: mass-forming type, the periductal infiltrating or the intraductal-growth type; whereas dCC contains macroscopic mass-forming or mostly a periductal-infiltrating type. The pCC is further characterized into three subtypes: the periductal-infiltrating (or sclerosing) subtype, nodular subtype and the least common papillary subtype (1-3).

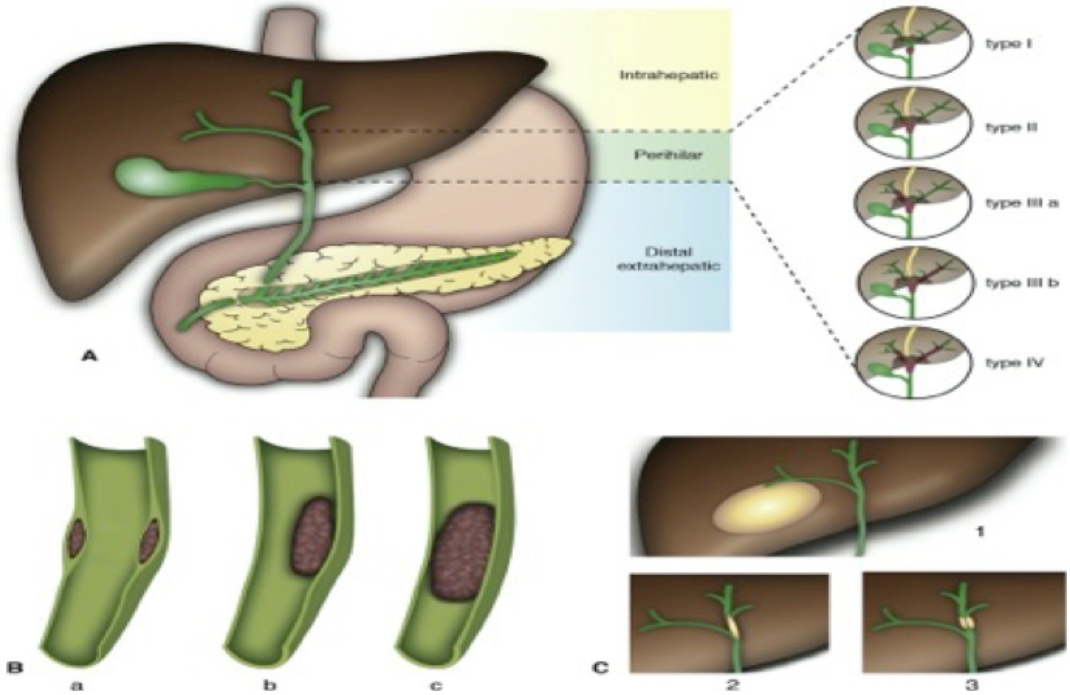


Figure 1.1: Anatomic classification and macroscopic subtypes of CC. (A) CC is classified as intrahepatic, when located proximally to the second-order bile ducts; perihilar, when it is extrahepatic and above the cystic duct; distal, when located between the origin of the cystic duct and the ampulla of Vater. Bismuth-Corlette classification for pCC: type I, in the common hepatic duct; type II, confined to the bifurcation of the common hepatic duct without involvement of the secondary intrahepatic ducts; type III, located in the bifurcation of the common hepatic duct involving the right (IIIA) or the left (IIIB) confluence, while type IV affects also the secondary intrahepatic ducts on both sides. (B) Morphologic subtypes of pCC: (a) periductal infiltrating type (sclerosing); (b) nodular (mass forming) into the common hepatic duct; (c) polypoid (papillary) subtype, with an intraductal growing pattern. (C) Macroscopic IH-CC types: (1) mass-forming subtype, (2) periductal infiltrating subtype, (3) intraductal-growth subtype. (Source: Giovanni Brandi *et al.,* 2015)

CC is the second most commonly occurring primary liver tumor worldwide after hepatocellular carcinoma (HCC), which is responsible for 10-20% of all hepatobiliary neoplasia. Specifically, intrahepatic CC comprises 6–8% of the cases, perihilar accounts for 50–67% and distal extrahepatic comprises 27-42 % of the cases (4-6).

CC incidence and mortality rates evaluated in epidemiological studies seem to be increasing for IH-CC and falling for EH-CC in many Western countries, though the pattern is not consistent. The incidence of age-adjusted CC rate is reported to be the highest in Asian population (2·8–3·3 per 100000) and lowest in black and non-Hispanic white people (2·1 per 100 000). Moreover, the incidence of CC has been reported more in men (1·2–1·5 per 100000 vs one per 100000 population), with the exception in Hispanic female population, where IH-CC is high (1·5 per 100 000) compared to the male population (0·9 per 100 000) (7-8). According to the WHO database, age standardized mortality rates (ASMR) for IH- CC has increased in almost all countries, through all continents at different rates except Denmark, where incidence rates has decreased from 1.3 to 0.5 per 100,000 people, whose reason is unknown (9-10). The mean estimated annual percent change (EAPC) of ASMR for males is 6.9 ± 1.5 and for females is 5.1 ± 1 (10). The average age of diagnosis for CC is >50, but in Western countries, most cases of CC are found in patients over 65 years of age; while it is unusual in children (4).

1.1.2. Risk factors

The causes of CCs are not well understood; however, a number of factors have been recognized in many patients like primary sclerosing cholangitis (PSC), choledochal cysts, choledocholithiasis, liver cirrhosis, parasitic biliary infections, alcohol consumption and diabetes mellitus and obesity increase a person's risk of developing this malignancy. In United States and Europe, primary sclerosing cholangitis (PSC) and fibro polycystic liver disease (e.g. choledochal cysts) are the main factors. Chronic liver disease (cirrhosis and viral infection) is also now known as a risk factor, especially for intrahepatic CC. Lynch syndrome, BRCA-associated protein-1 (BAP1) tumor predisposition syndrome, cystic fibrosis, and biliary papillomatosis are some of the genetic conditions which have been documented to increase the risk for CC (11-14).

1.1.2.1.Primary sclerosing cholangitis (PSC)

It is a cholestatic liver disease characterized by inflammation of the biliary tree, which leads to the structuring and obliterative fibrosis of bile ducts and hepatic failure in most of the cases (15,16). PSC is also associated with the inflammatory bowel disease, particularly ulcerative colitis (UC). Approximately, 90% of PSC patients have colitis, whereas 40-50% patients have symptomatic colitis. The relation between PSC and CC is well established, where 30% of patients are diagnosed with PSC, with or without ulcerative colitis; moreover, annual incidence rate of CC patients with PSC has been assessed to be between 0.6 and 1.5 % per year, with a 5-15% of life risk. The average age of diagnosis for CC with PSC is between 30-50 years. The reason behind the development of CC with PSC patients is unclear. Alcohol consumption, certain genetic polymorphisms could be implicated risk factors for CC with PSC (17-20).

1.1.2.2.Cholelithiasis

Cholelithiasis is a robust risk factor for gallbladder cancer, where hard, crystal-like lumps known as gallstones trigger inflammation that increase the risk of cholangiocarcinoma (21,22). However, the relationship between gallstones and CC is not well established.

1.1.2.3.Liver cirrhosis

Long-term continuous damage to the liver induces cirrhosis, which leads to the formation of scars and is recognised as fibrosis. Nodule formation in the liver transforms the smooth liver tissue into hard liver tissue; and fibrosis together with nodule formation is termed as cirrhosis. It takes many years to develop cirrhosis, which develops without any noticeable symptoms, unless there is a high degree of damage. The fibrosis in the liver tissues interferes with the blood flow, which interrupts the proper functioning of the liver and can cause liver failure. The patients with cirrhosis are at an increased risk of having CC (23, 24). A few studies have revealed that HCC patients with cirrhosis is associated with reduced long-term survival [25,26), whereas study from Zhang et al. has reported that cirrhosis has no influence in diagnosis of patients with IH-CC (27). Consequently, the exact relationship between liver cirrhosis with the diagnosis of CC is not well established and needs to be further clarify.

1.1.2.4.Chronic liver disease

Hepatitis B virus (HBV) and hepatitis C virus (HCV) have been recognised as risk factors for IH-CC (28-30). The influence of HCV and HBV on tumor development varies in western

countries. A link between HCV infection and CC was first suggested in 1991. Subsequently, numerous reports have shown a higher rate of HCV-associated cirrhosis in cardiovascular disease (CVD) patients, whereas risk is significantly low in hepatocellular cancer. In some of the Asian countries, HCV is more predominant, and HBV is endemic (31,32).

Studies from Japan, USA and Europe have shown HCV as a risk factor for IH- CC induction. Correspondingly, a cohort study of adults from United States was associated with an increase in the risk of both EH- CC and IH- CC. Studies from China and South Korea have shown HBV as a risk factor strongly associated with IH- CC (33-37).. However, no correlation has been found between the existence of cirrhosis and viral hepatitis, and the development of CC in these patients

1.1.2.5.Parasitic infection

In 2009, WHO's International Agency for Cancer Research categorized the liver flukes as a carcinogen for CC. Apparently 40% of CC are associated with flukes are IH-CC (38).Liver fluke infections occur in some of the Asian countries, specifically North- eastern Thailand, but is rare in United States. The main cause of liver fluke infection is consumption of poorly cooked fish containing tiny parasitic worms, or raw meat. In humans, liver fluke inhabits the bile ducts causing irritation, and can induce bile cancers. *Clonorchis sinensis* and *Opisthorchis viverrini* are the two main types of liver flukes. These parasites cause inflammation in the proximal biliary tree, which can lead to malignant transformation of the epithelial lining (39).

1.1.3. Molecular pathogenesis of Cholangiocarcinoma

CC is receiving medical importance because of its rising number of incidences, poor diagnosis and substandard response to the therapy (40, 41). It is associated not only with genetic alterations, but other essential modifications of tumour microenvironment, which lead to the activation of various signaling pathways involved in tumour induction and progression. A better understanding of the underlying mechanisms that trigger the process of cholangiocarcinogenesis may help to develop more specific diagnostics, and preventive strategies to improve the outcome of this disease.

The following section describes some of the signaling pathways involved in driving tumour onset and progression of CC.

1.1.3.1.Microenvironment and inflammation- related pathways

1.1.3.1.1. Interleukin-6 (IL-6) / Signal transducer and activator of transcription (STAT3) pathway

Inflammation has been a known risk factor for IH- CC induction. IL-6 is a critical oncogenic player and helps in the development of malignant cholangiocytes. It plays an important role during the acute phase response upon liver injury and inflammation. The secretion of IL-6 in the tumour environment takes place by various cells such as kupffer cells, cancer- associated fibroblasts (CAF), tumour- associated macrophages (TAM) and CC cells, leading to cellular stress, inflammation and proliferation.

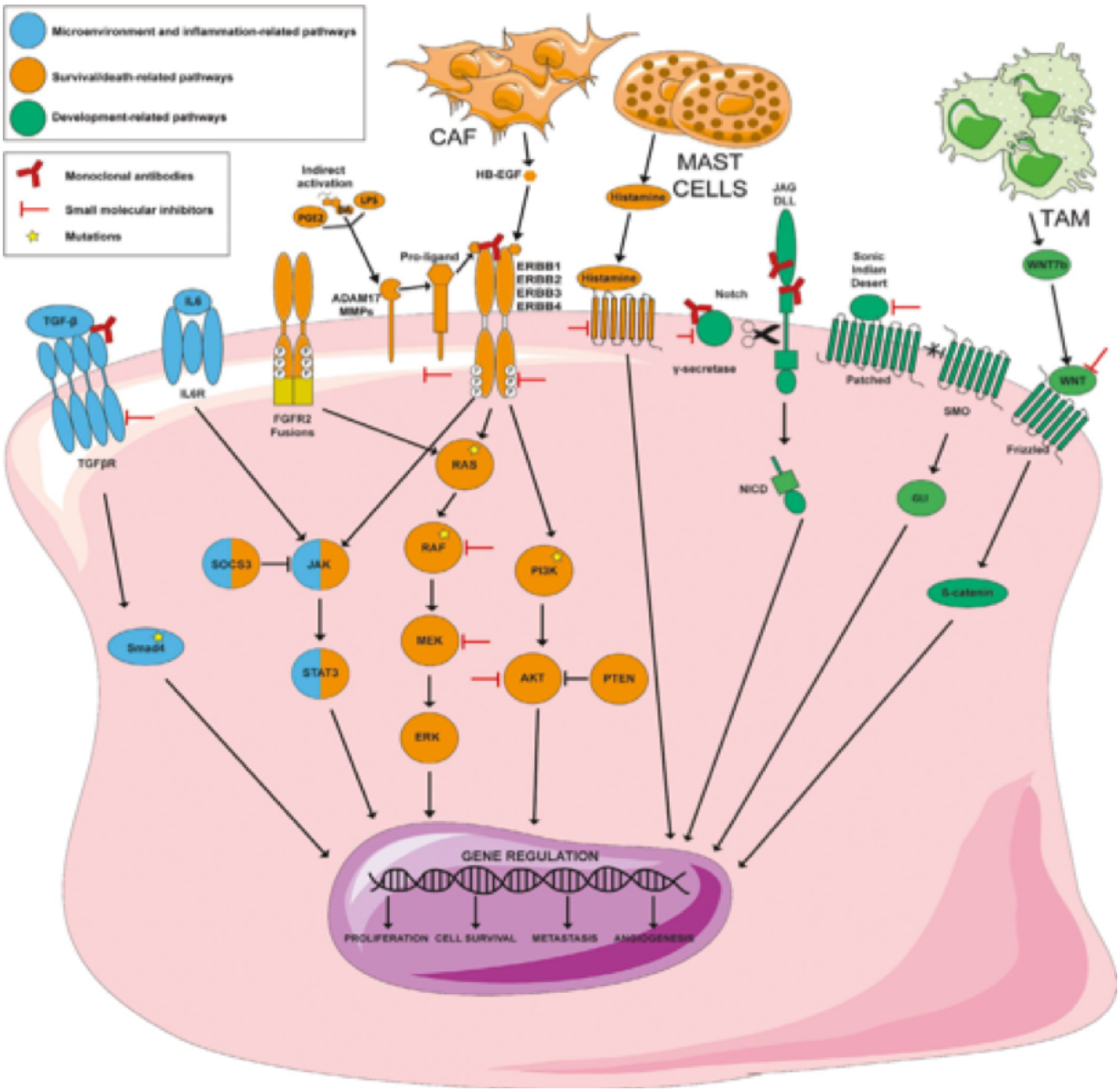


Figure 1.2: Major signaling pathways involved in cholangiocarcinoma (CC).The signaling pathways involved in CC progression can be classified into three main types: (i) microenvironment and inflammation-related pathways, including TGF-β and IL-6 signaling pathways; (ii) proliferation/ survival/death-related pathways ignited by constitutive activation of receptor tyrosine kinases such as FGFR2 and ERBB receptors or components of downstream signaling modules, such as JAK/STAT, RAS/RAF/MEK/ERK and PI3K/; (iii) development-related pathways, including Notch, Hedgehog and WNT/β-catenin. Note that membrane receptors displayed by CC cells may be activated by ligands provided by the tumour microenvironment including CAFs, mast cells and TAMs, that produce HB-EGF, histamine and WNT7b, which in turn activate EGFR, histamine receptor, Frizzled/β-catenin respectively. In addition, ERBB1/EGFR can be indirectly activated by other molecules, such as PGE2, BA and LPS. Several components of these signaling pathways can be targeted by monoclonal antibodies or small molecule inhibitors, as indicated. Stars indicate signaling molecules that may be affected by recurrent pathogenic mutations in CC and are candidates for therapeutic targeting. Abbreviations: ADAM17, ADAM metallopeptidase domain 17; BA, bile acids; CAF, cancer-associated fibroblast; CC, cholangiocarcinoma; DLL, delta-like ligand; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FGFR2, fibroblast growth factor receptor 2; GLI, glioma-associated oncogene; HB- EGF, heparin-binding EGF-like growth factor; IL-6, interleukin 6; IL6R, IL-6 receptor; JAK, janus kinase; JAG, jagged; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NICD, NOTCH intracellular domain; PGE2, prostaglandin E2; PI3K, phosphatidylinositol 3-kinase; PTCH, patched receptor; PTEN, phosphatase and tensin homologue; SMO, smoothened; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; TAM, tumour-associated macrophage; TGF-β, transforming growth factor-β; TGF-βR, transforming growth factor-β receptor. (Source: Laura Fouassier *et al.* 2019)

IL-6 signaling gets activated by binding of IL-6 to the IL-6 receptors via gp130, which further activates the Janus kinases (JAK)- STAT pathway. This pathway has been found to be activated in 50% of the CC patients. Various studies have demonstrated the role of IL-6 by promoting STAT3 overexpression in proliferation and induced CC cell line survival potential in xenograft mice model (42,43). The IL-6 /STAT3 induces the expression of myeloid cell leukaemia-1 (MCL-1), which belongs to the anti-apoptotic BCL-2 family and inhibits cell death. Subsequently, increased expression of IL-6 in CC also deregulates control over cell cycle by activating p38 MAPK, which is known for decreasing the expression of the cell cycle regulator p21 (44, 45).

1.1.3.1.2. Transforming growth factor β (TGFβ) / SMAD pathway

TGFβ is a pleiotropic cytokine which is involved in deciding the fate of cells. Mostly, all cell types can produce and respond to multiple TGFβ family members. TGFβ induces the activation of perisinusoidal cells, also known as hepatic stellate cells (HSC), which in turn can drive liver fibrosis. Cytostatic and tumour promoting effects of TGFβ through stimulation of liver epithelial cells could affect CC pathogenesis in a complicated manner. Studies have shown that TGFβ provokes epithelial-to-mesenchymal transition (EMT) in CC cell lines by increasing mesenchymal features (decreasing E-Cadherin and CK-19 expression levels, increasing vimentin and N-cadherin levels), which enhances the migration and invasiveness of CC (46,47). Increase in nuclear presence and immunoreactivity of Snail, concomitant with a decrease in

CK19 and increase in vimentin levels, is responsible for lymph node metastasis and poor survival.

SMAD4/TGF-β signaling pathway plays a pivotal role in signal transduction from cell membrane to the nucleus and is involved in proliferation, differentiation of the cells, apoptosis and various other cell processes, including tumor progression (48). SMAD4 is a tumour suppressor and a known mediator of TGF-β signaling and is explicitly inactivated in many cancers. TGFβ/SMAD4 signaling pathway supresses tumor induction by cell cycle arrest (through G1/S checkpoint activation) and apoptosis induction. Besides, mutations in SMAD4 abolish the tumour suppressor activity of TGFβ and promote tumour induction. In many studies, *SMAD4* mutations have been reported in CC, which includes 45% of IH- CC cases with TGFβ associated gene expression and are being correlated with the patient survival (49).

1.1.3.2.Cell survival/death-related pathways

1.1.3.2.1. Pathways associated to FGFR2 fusions

The fibroblast growth factor receptor (FGFR) pathway plays a pivotal role in proliferation, differentiation, cellular migration and survival. It has also been involved in various physiological processes such as embryogenesis, angiogenesis and wound healing. Recent studies have uncovered the fusion of FGFR2 gene with multiple partners in CC patients, as well as in other cancers (50).

Extracellular signal-regulated kinase (ERK)1/2 activation is one of the major oncogenic pathways which gets activated by FGFR2 fusions (FFs). The FGFR2-BICC1 gene fusion is also observed in several cases of CC cohorts, where one case of novel FHFR gene fusion, FGFR2-AHCYL1, was also identified. There are other cases also where FGFR2-MGEA5 and FGFR2-TACC3 gene fusions have been described in IH- CC (51, 52). However, the FF signaling, which plays an important role in maintaining the oncogenic phenotype in IH- CC, is still not fully understood.

1.1.3.2.2. Pathways associated to BRAF, KRAS and TP53 mutations

B-Raf is a protein encoded by the BRAF (v-raf murine sarcoma viral oncogene homolog B1) gene, and is known for regulating cellular growth and division. BRAF, which is upstream of

MEK/MAPK signaling pathway, is the main effector of KRAS oncogenic activity and is found to be mutated in around 21% of IH- CC patients (53). Thus, BRAF mutants initiate cell transformation through MEK/ERK module activation. The mutations of BRAF mostly occur at V600 position by generating class 1 mutants i.e. BRAF oncoproteins. However, class 2 (eg. K601E, G469A and F595L) and class 3 mutants (eg. G469E) have also been observed in IH-CC (54).

KRAS is a member of the RAS/RAC family, known to propagate growth signals via downstream effectors such as RAF and PI3K. KRAS activation has been found in subsets of CC patients, ranging from 3% to 100% (55, 56). The incidence of KRAS mutation shows 40-50% of consistency among IH-CC, which suggests that the RAF/MAPK signaling pathway activation is the key player in majority of cancers. KRAS and TP53 mutations occur in both IH- CC and EH- CC (57). Studies in mice models have determined the role of KRAS mutations in the induction of IH- CC in association PTEN and p53 mutations, and in EH- CC with the ablation of Cdh1 and Tgfbr2 (58).

1.1.3.2.3. Epidermal Growth Factor Receptor gene (EGFR) pathway

The ErbB/HER family of receptor tyrosine kinases involve EGFR/ ERBB1 (HER1), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). Mutations in ERBB family members are not so frequently found in CC, however ERBB1-4 overexpression has been broadly described in IH-CC and EH- CC. This overexpression has been commonly associated with a poor prognosis (59). Many studies have described the influence of EGFR in CC proliferation and invasion through activation of various downstream pathways i.e. JAK/STAT, RAS/MEK/ERK and PI3K/AKT. Many compounds such as conjugated bile acids, lipopolysaccharide and prostaglandin E2 are known to activate EGFR pathway indirectly, which leads to the activation of metalloproteinases and release of ERBB ligands. Aberrant phosphorylation of EGFR activates MAPK/ERK and p38, which subsequently increases cyclooxygenase-2 (COX-2) levels (60, 61). Over expression of COX-2 further initiate and promote carcinogenesis. In Cholangiocarcinogenesis, the ErbB receptor family are known as a compelling mediator which interacts with various other factors such as COX-2, IL- 6, vascular endothelial growth factor (VEGF), etc. They can activate themselves and are also capable of enhancing the expression of other genes thereby helps in cancer progression (62).

1.1.3.2.4. PI3K/AKT pathway

PI3K/AKT pathway is involved in multiple cellular processes such as apoptosis, proliferation and cytoskeletal rearrangement. Aberrant activation of PI3K/AKT signaling pathway and its role in human cholangiocarcinogenesis have been comprehensively evaluated in *in-vitro* and *in-vivo* studies (63, 65). Functional gain in mutated PI3K promotes CC and has been associated with poor prognosis, whereas increase in expression of AKT2 is found predominantly in EH-CC which is associated with phosphor- mTOR, loss of PTEN and shorter survival of patients (66). In human Biliary tract carcinomas (BTCs) tissue samples, positive immunostaining of AKT and PI3K has been reported as 46–100% and 34–100% respectively in neoplastic cells, compared to the surrounded normal cells (67).

1.1.3.3.Development- related pathways

1.1.3.3.1. NOTCH pathway

NOTCH signaling pathway plays a critical role during embryogenesis and is also associated with differentiation of hepatoblasts towards cholangiocyte lineage. It also plays an important role in cellular communication and cell fate determination. There are four NOTCH transmembrane protein receptors (Notch 1, Notch 2, Notch 3 and Notch 4) which possess epidermal growth factor (EGF)-like repeats, an intracellular domain (NIC) containing six ankyrin-cdc10 motif and a nuclear localization signal whereas five ligands, Jagged (JAG1, 2) and Delta- like (DLL1, 3 and 4) are present. Upon activation of NOTCH signaling resulting from proteolytic cleavage by γ-secretase complex, NOTCH intracellular domain translocates from the plasma membrane to the nucleus, causing subsequent activation of cell fate determining target genes by Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ) (68). On the other hand, deregulation of NOTCH pathway induces developmental defects and postnatal pathologies like CC (69, 70).

In CC, NOTCH has been associated with an increased rate of proliferation and survival of CC cells, upregulation of MCL-1 and BCL-XL (a known pro-survival protein) and EMT induction.

1.1.3.3.2. Wnt/β- catenin pathway

The Wnt/β-catenin signaling pathway is required in both physiological and pathophysiological processes, plays a critical role during hepatobiliary development and supports cell survival in CC (71). Wnt ligands associate with their corresponding receptors and activate the signaling pathway. The ligands undergo palmitoylation by the Wnt acyl-transferase porcupine, followed

by transportation of ligands to the cell membrane from Golgi, where they signal in an autocrine and paracrine manner (72).

β-catenin plays a central role in the Wnt signaling cascade. The association between LEF/TCF transcription factor and nuclear β-catenin regulates the expression of target genes (such as CCND2, CDKN2A, BIRC5) involved in cell differentiation, proliferation, migration and apoptosis (73, 74). Studies in rat model and patient derived CC tumours have demonstrated the presence of WNT7B, co-localised with $CD68^{+}$ macrophages neighbouring tumour cells. These macrophages have been recognized as a source of WNT signals that augment CC cell proliferation through β-catenin (73).

1.2.Hedgehog pathway

The SHH pathway plays a pivotal role in early embryonic development and numerous other important regulatory processes of cell fate decisions, including proliferation, apoptosis, migration, and differentiation (75). Additionally, SHH pathway augmentation has also been observed in various human malignant neoplasms, where increased auto secretion of Hedgehog ligands has been found to play an important role in the induction of cancers like HCC, pancreatic cancer, gastric carcinoma and CC (76-78). Sonic ligand, which belongs to the Hedgehog family, binds to Patched (a 12-pass trans-membrane receptor) and activates the pathway by terminating the inhibitory effect of Patched on Smoothened (SMO) protein, resulting in the activation of growth regulating SHH target genes like GLI1. Various studies suggest that SHH pathway deregulation plays an important role in cholangiocarcinogenesis [79]. Aberrant activation of SHH pathway has also been implicated in the induction of cancer stem cell (CSC) phenotype. Presence of aberrant activated SHH pathway has been reported in various cancer cells such as, breast CSCs, glioblastoma stem cells (80, 81), CD34+ leukemic cells (82, 83).

1.2.1. Hedgehog signal transduction

The hedgehog proteins are the secreted proteins of the Hedgehog gene, which was discovered in 1980 by genetic analysis of the fruit fly *Drosophila melanogaster.* The underlying mechanisms of Hedgehog protein secretion and signaling are more or less the same in Drosophila and vertebrates; however, a few differences still exist. Only one Hedgehog gene exists in Drosophila, whereas there are three Hedgehog homologues in vertebrates: Sonic

Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH), which are differentially expressed in different tissues and have specific functions. DHH and IHH play an important role in the development of normal tissues such as in testis and pancreas organogenesis and bone formation, whereas SHH is highly expressed during embryonic development and is usually found in all mammalian tissues, where it regulates tissue patterning (84-86).

SHH can signal through a canonical or a non-canonical pathway. In the canonical Hedgehog pathway, the secreted SHH glycoprotein binds and inactivates the Patched protein (PATCH1), which in turn is known to suppress the activity of the 7-transmembrane protein Smoothened (SMO), a G-protein coupled receptor (GPCR). This suppression leads to the prevention of SMO translocation into the primary cilium. During the presence of SHH ligand, the inhibition process of PATCH1 on SMO is abrogated, and SMO translocate to the primary cilium. The translocation of SMO activates the glioma associated (GLI) transcription factors, and these activated GLIs enter into the nucleus to regulate the expression of target genes involved in proliferation (*Cyclin-D1*, *MYC*), apoptosis (*Bcl-2*), angiogenesis (*ANG1/2*), epithelial-to-mesenchymal transition (*SNAIL*), and stem cell self-renewal transcription factors (*NANOG*, *SOX2*) (88-90). There are three GLI transcription factors (GLI1, GLI2 and GLI3) present in vertebrates, which are the terminal effectors of SHH signaling. Gli1 behaves as an activator and does not possess an N-terminal repressor domain, while GLI2 and GLI3 encompass both activation and repression domains. The PATCH2 receptor shares about 54% of homology with PATCH1, has significant differences in the expression level and signaling pattern in different tissues and is found to be highly expressed in spermatocytes, where it acts as a mediator of DHH activity during germ cell development. In the absence of ligand or active SMO in the ciliary membrane, Suppressor of Fused (SUFU) act as a negative regulator and inhibits SHH signaling pathway by directly binding and degrading the GLI family of latent zinc-finger transcriptional mediators (91-93). Besides, SUFU, DYRK1A, which is a member of the dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) family, also regulates GLI-1. DYRK1A enhances the activity of GLI-1 by phosphorylating at multiple serine/threonine sites (94).

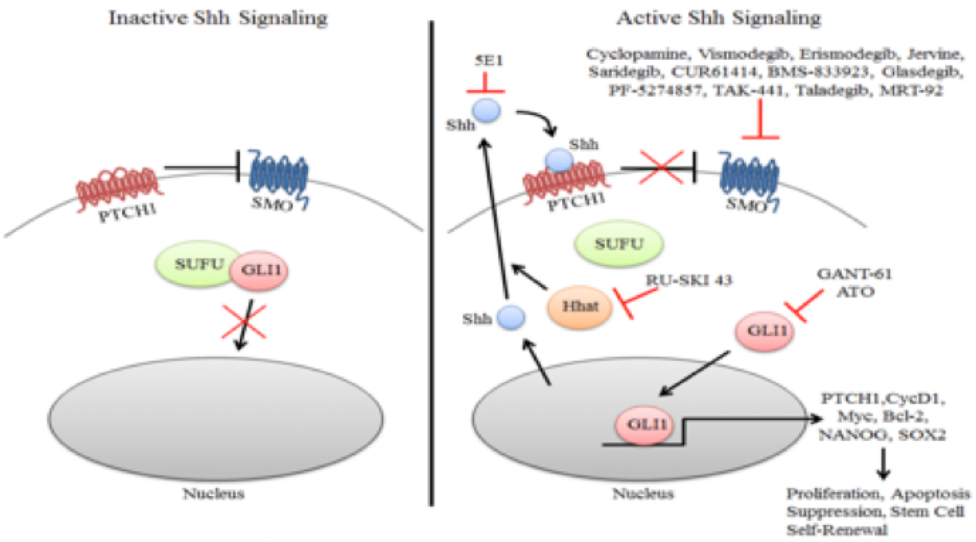


Figure 1.3: Inhibition of components of the SHH Pathway in cancer. Inactive signaling (left) occurs in the absence of SHH ligand wherein PTCH1 inhibits SMO resulting in GLI1 sequestration in the cytoplasm by SUFU. In the presence of SHH (right), PTCH1 suppression of SMO is abrogated resulting in the nuclear accumulation of GLI1 and activation of target genes that promote several oncogenic properties to tumor cells. Inhibition of the SHH pathway is primarily directed at inhibition of SMO and GLI1, with many of these compounds in clinical trials for solid cancers. More recently attempts have been made to inhibit the SHH signaling pathway by using the monoclonal antibody 5E1 or the SHH at inhibitor RU-SKI 43 to inhibit SHH directly. SHH at is an *O*-acyltransferase that catalyses the palmitoylation of SHH, which is critical to its function (Source: Tadas K. Rimkus *et al.* 2015)

The non-canonical signaling activation takes place in two ways: 1) The signaling gets activated from PATCH1/SMO but independent of GLI; or 2) activation of GLI, independent of SHH ligand or PTCH1/SMO. There are many oncogenic pathways, which enhance GLI activity. GLI has been shown to be positively regulated by K-RAS, TGF-β, PI3K-AKT, and PKC-α. Studies have shown that K-RAS pathway is capable of activating GLI-1 independently, regardless of SHH pathway activation. In addition, p53, pKA and PKC-δ are also known to regulate GLI-1 in a negative manner (95-98).

1.2.2. The Hedgehog pathway in healthy adult liver

The Hedgehog pathway plays a very important role in the liver and biliary tract. During embryogenesis, the ventral foregut endoderm has high expression of SHH, which augments endodermal differentiation to gives rise to the liver, pancreas and lung buds. The Hedgehog ligand expression by ventral endoderm eventually gives rise to hepatic progenitors. Expression of the SHH disappears after formation of liver bud but is induced transiently in hepatoblasts later during development (99, 100).

In healthy adult livers, the Hedgehog pathway is inactive due to very low secretion of ligands by immature cholangiocytes, and also due to the secretion of Hedgehog inhibitors e.g. Hhip, by

the liver sinusoidal cells (e.g., endothelial cells and quiescent hepatic stellate cells) which binds with Hedgehog ligands and prevents them from interacting with PATCH receptor.

The activity of Hedgehog pathway is also silenced during liver epithelial cell maturation process, where ptc1 expression is exponentially low in healthy mature hepatocytes than in bipotent hepatic progenitors, and similarly reduced as compared to the multipotent endodermal progenitors (101).

1.2.3. The Hedgehog pathway in injured adult liver

Hedgehog ligand production and pathway activation occurs when major re-construction of liver is required in an adult (e.g. after an acute liver insult with hepatic necrosis or after partial hepatectomy or chronic liver regeneration/repair), where the level of Hedgehog expression associates with the severity and duration of liver injury. For instance, an increase in SHH and IHH expression level has been observed during liver regeneration after 70% of partial hepatectomy (PH) (102).

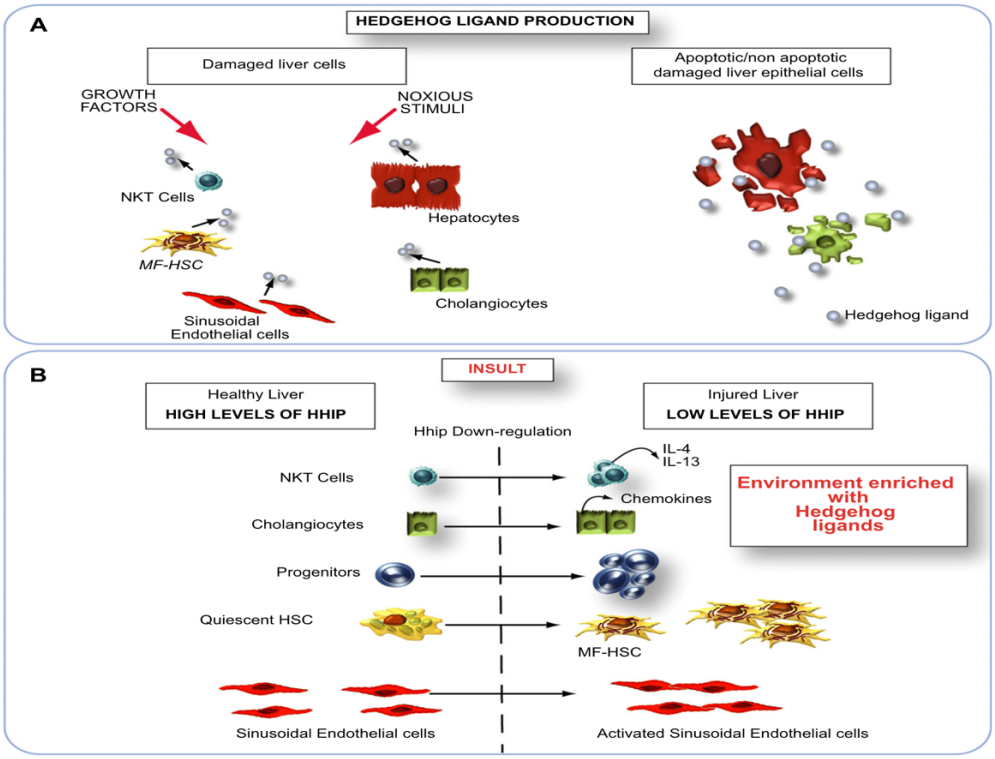


Figure 1.4: (a–b) Liver progenitor cells giving rise to hepatocytes or cholangiocytes. Both hepatoblasts and hepatoblast derivatives express Hedgehog ligands and Hedgehog target genes. (a) In uninjured livers, mature cholangiocytes have a weak expression of both Hedgehog ligands and Hedgehog target genes. On the other hand, these factors are not expressed in mature hepatocytes. (b) In injured livers, cholangiocytes secrete Hedgehog ligands and over express hedgehog target genes. In addition to that, hepatocytes release Hedgehog ligands (Source: Omenetti *et al.,* 2011).

Lethal stimuli, which provoke re-construction of the liver, also stimulate the production of Hedgehog ligands in the liver. Studies have also shown the presence of biologically active Hedgehog ligands in the fragments released from apoptotic and non-apoptotic liver epithelial cells. Histological analysis of diseased human livers with chronic viral hepatitis, alcoholic liver disease, or non-alcoholic fatty liver disease (NAFLD) endorse that GLI-2 transcription factor co-localizes with the activated specific endothelial marker marker (e.g., CD31), alpha-smooth muscle actin (MF), and immature liver epithelial cells (e.g., Ker7) and confirms that the Hedgehog-responsive cell numbers are closely associated with the level of Hedgehog ligand production (103, 104).

1.2.4. Hedgehog pathway in cancer

Aberrant activation of SHH signaling has been considered as a hallmark of many cancers. There are four models, which represent the abnormal Hedgehog pathway activity in cancer. *Type I* cancer includes mutation- driven, ligand independent hedgehog signaling such as in Gorlin's syndrome. Gorlin's syndrome is an autosomal dominant disorder that presents itself with craniofacial and skeletal abnormalities and a notably increased risk of advanced basal cell carcinoma and medulloblastoma. The role of dysregulated SHH signaling in cancer was first characterized by studies on Gorlin's syndrome or basal cell nevus syndrome. These tumours mostly had inactivating mutations in PATCH (85%) or activating mutations in SMO (10%). *Type II* cancers exhibit an autocrine, ligand dependent aberrant Hedgehog pathway activation rather than possessing somatic mutations in the pathways like BCC tumors. Such cells have been detected in a wide variety of tumours such as lung, prostate, breast, stomach, liver and brain. In type I (low grade) or II (high grade) tumours, the expression is primarily induced within the epithelial compartment. *Type III* cancer involves ligand dependent aberrant Hedgehog pathway activation, in which tumour cells work in a paracrine manner. The SHH ligand secreted by tumour cells is received by the stroma in the microenvironment, which further stimulates cells to produce secondary growth factors to support cell proliferation, angiogenesis and survival. During the growth and maintenance of many epithelial organisations like small intestines, paracrine Hedgehog signaling plays a very important role. The Hedgehog ligands secreted by the epithelium are further received by the mesenchymal stroma to sustain proliferation in the mesenchyme.

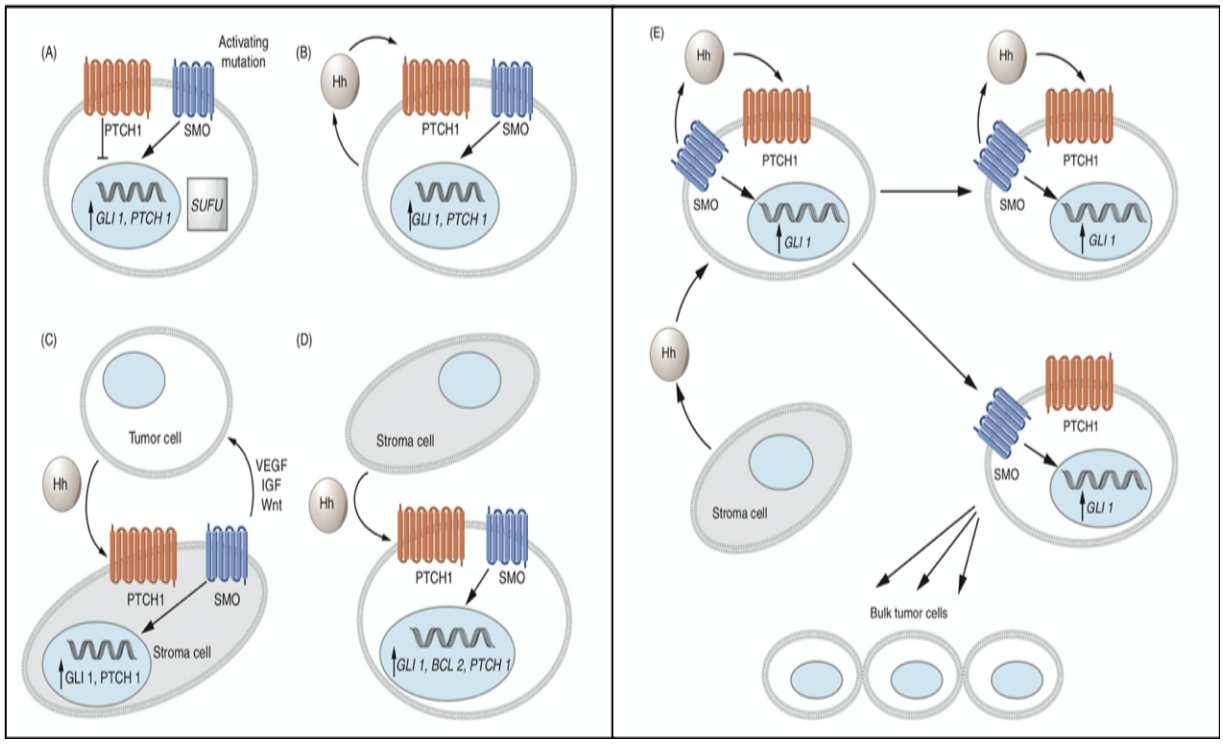


Figure 1.5: **Different models of Hedgehog pathway signaling.** (A) Type I ligand-independent cancers harbour inactivating mutations in Patched 1 protein (PATCH) or activating mutations in SMO leading to constitutive activation of the Hedgehog pathway even in the absence of the Hedgehog ligand. (B) Type II ligand-dependent autocrine cancers both produce and respond to the Hedgehog ligand leading to support tumor growth and survival. (C) Type III ligand-dependent paracrine cancers secrete the Hedgehog ligand which is received by the stromal cells leading to pathway activation in the stroma. The stroma in turn feeds back various signals such as IGF, Wnt, VEGF to the tumor tissue leading to its growth or survival. (D) Type IIIb reverse paracrine tumors receive Hedgehog secreted from the stroma leading to pathway activation in the tumour cells and upregulation of survival signals. (E) Cancer stem cells (CSCs): Hedgehog signaling occurs only in the self-renewing CSCs, from the Hedgehog ligand produced either by the CSCs or by the stroma. CSC will give rise to more Hedgehog pathway dependent CSCs or possibly may differentiate into Hedgehog pathway negative tumour cells comprising the bulk of the tumour. (Source: Trends Pharmacol Sci 30: 303-312)

1.2.5. Hedgehog pathway in cancer stem cells

Stem cells are known for their self-renewal and pluripotent capacity, which remain in the body throughout life. It is believed that a subset of tumour initiating cells, which helps to maintain tumour growth and tumour progression, share the same signaling molecules as the normal stem cells to maintain a self-renewing reservoir (105, 106).

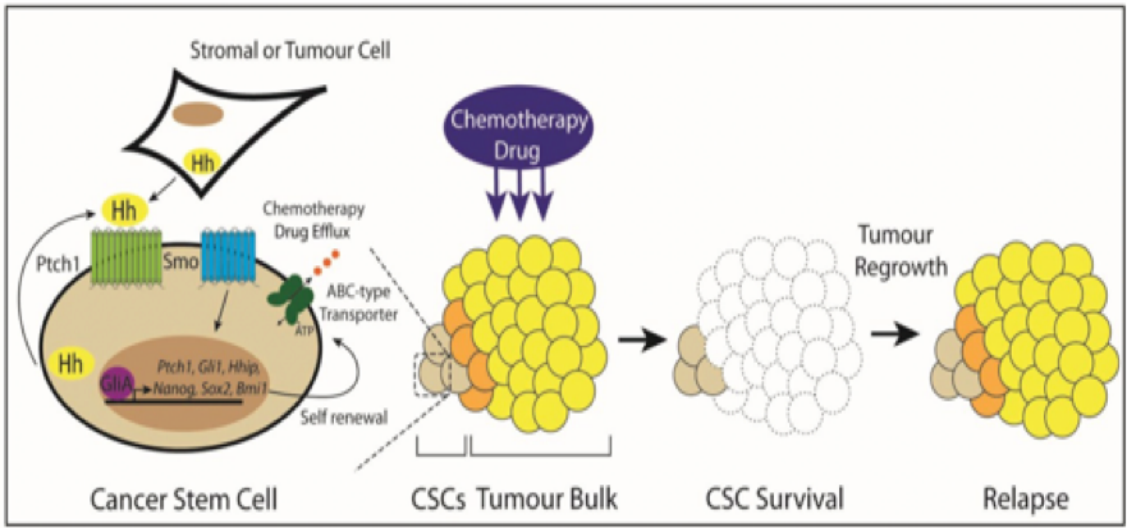


Figure 1.6: Hedgehog signaling in cancer stem cells (CSCs). CSCs respond to the Hedgehog ligand, secreted by adjacent stromal cells, tumour cells or the CSCs themselves, to maintain a stemness signature by the regulation of pluripotency genes, including *Nanog*, *Sox2* and *Bmi1*. CSCs signature by the regulation of pluripotency genes, including Nanog, Sox2 and Bmi1. CSCs are resistant to conventional chemotherapeutics, surviving treatment before expanding and deriving the heterogeneous tumour bulk population, resulting in disease relapse. (Source: Catherine R. Cochrane et al., 2015)

Many experimental evidences propose that the abnormal formation and progression of cancer is due to the aberrant activation of multiple signaling pathways including Hedgehog, wingless-related integration site (Wnt), NOTCH and bone morphogenic protein (BMP). Presence of aberrantly activated SHH pathway has been reported in various cancer stem cells such as breast CSCs, glioblastoma stem cells, CD34+ leukemic cells (83); however, its impact on CSCs in CC is still unclear. Studies have implicated the Hedgehog signaling pathway for initiating the CSC phenotype by regulating stemness-determinant genes. Nanog, Oct-4 and Sox-2 CSC transcription factors are a master element for the self-renewal of embryonic stem cells and are a direct target of Hedgehog singling pathway (107-110). While Hedgehog-driven CSCs have been validated for numerous haematological malignancies, their existence in solid tumours remains more controversial. Tumours are known to harbour a small population of CSCs, which can proliferate and give rise to more CSCs in addition to non-tumorigenic cancer cells. Many studies have suggested that the activation of SHH signaling in CSCs inside solid tumours endorse metastatic progression and induce EMT (111, 112).

1.3.Hypoxia

A reduced oxygen level is termed as hypoxia, which is quantitatively associated to the cells, tissue and type of organ. Hypoxia appears with the continuous lack of oxygen, and could be

transient, acute or chronic. On an average, an individual tissue utilizes 5–6 mL of oxygen per decilitre of blood delivered during the resting phase of body. An imbalance in oxygen level in tissues results in the compromise of its baseline function and disturbs the homeostasis by affecting energy metabolism, gene expression, hormone secretion and responses, apoptosis and behaviour of organisms (113).

The presence of hypoxia in many solid tumours is very well known, A solid tumor is comprised of three different regions in the tissue microenvironment: normoxic, hypoxic and necrotic.

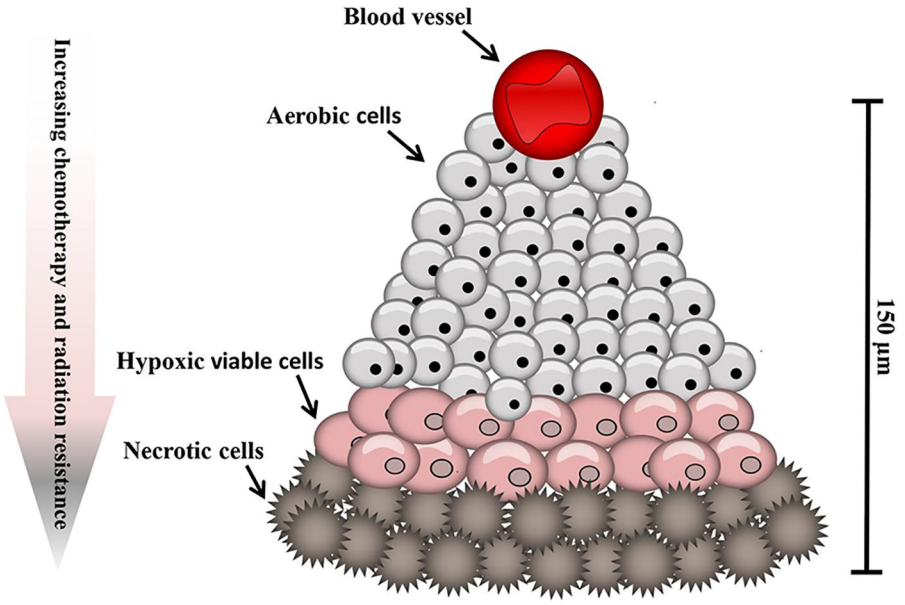


Figure 1.7: Hypoxic regions of solid tumours. Tumours contain regions of oxygenated cells situated near to blood vessels, becoming increasingly hypoxic with increased distance from a functional blood supply. (Source: Wafaa Al Tameemi *et al.,* 2019)

The cells in the normoxic regions are well oxygenated with well-established surrounding blood vessels, whereas the anoxic regions lack blood vessels. The distance of 150 µm from the blood vessels may cause anoxic region inside the tissue, leading to patches of necrosis. The hypoxic environment in the solid tumours induces many genetic changes to help tumour cells to acclimatize to the hostile environment with poor nutrition and remain viable. Additionally, to overcome the limited proliferation rate, they encourage formation of new blood vessels by secreting VEGF, which creates chaotic vasculature such as, twisted and blind-ended blood vessels, dilated and elongated blood vessels, and poor endothelium leading to poor oxygen delivery with inadequate blood flow (114-115).

Hypoxia is also known for its contribution in providing stem-ness to cancer cells and supporting their survival in the tumour microenvironment, helping them in maintaining their undifferentiated state. Many *in-vitro* studies have shown that maintenance of stem cells under atmospheric oxygen conditions (21%) may lose their stemness characteristics, while adult stem cells (ASCs) retain their stemness features under 1% hypoxic condition (116).

Because of drop-in blood oxygen, the cells induce cellular signaling by HIF-1 activation and nuclear translocation, which affects and regulates the expression of many genes. HIF is a heterodimer composed of two subunits: HIF-1α or HIF-2α dimerizing with a HIF-1β subunit. HIF-1 binds to the promoter sequence in the DNA, causing increased transcription of hypoxic response genes. There are studies showing the involvement of HIFs in the activation of specific signaling pathways such as NOTCH and many others. HIF-1 stabilization augments NOTCH function resulting in the inhibition of differentiation, alteration of stem cell signaling pathways (Wnt and SHH), and induction of c-Myc expression (117-120). Hypoxia also causes downregulation of certain cell adhesion molecules, facilitating tumour cell detachment and EMT, which is an acute event in the course of cancer metastasis. Various underlying mechanisms are associated with the malignant progression of cancer cells under hypoxic microenvironment; SHH pathway is one of them, though its involvement in cholangiocarcinogenesis is still unclear.

1.4.Epithelial–mesenchymal transition (EMT)

EMT is a process of transition between epithelial and mesenchymal states, which usually occurs during embryogenesis in a highly dynamic manner. This allows the cells to acquire migratory and invasive behaviour after alterations in the expression of the adhesion molecules, cytoskeleton, and changes in cell–matrix. Tumour cells also have the potential to activate EMT, resulting in an increase in their aggressive behaviour and tumour progression with metastatic expansion. It is also associated with enhanced stem cell properties, which plays a pivotal role in resistance to cancer treatment. However, the process of EMT is still unclear in many types of tumours. Most of the tumour cells do not endure a full EMT, rather acquire some of the mesenchymal qualities and preserve features of epithelial cells. There is also a reversible process of EMT i.e. mesenchymal-to-epithelial transition (MET) by which cancer cells develop a secondary tumour after reaching at a desired niche. Metastasis is the most lethal risk for cancer patients, where more than 90% of deaths take place due to this, rather than by primary tumours. Many studies have demonstrated that induction of EMT causes tumour cells to lose cell-cell

junctions, degrade basement membrane by activating degrading enzymes, thereby allowing their migration as single cells.

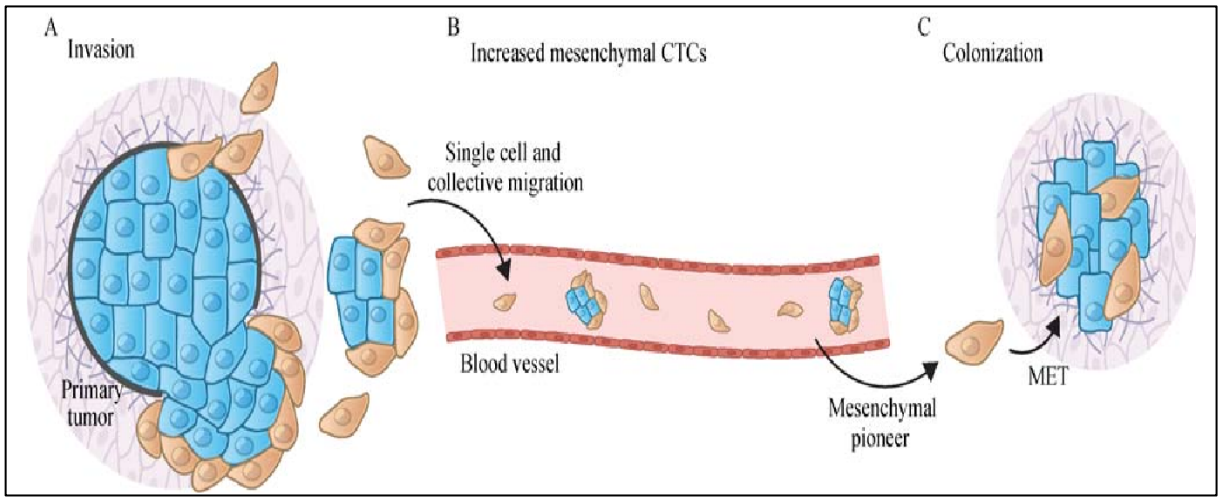


Figure 1.8: The EMT program facilitates multiple steps of the invasion-metastatic cascade. (A) At the primary tumor site, induction of an EMT program allows carcinoma cells to lose cell-cell junctions, degrade local basement membrane via elevated expression of various matrix-degrading enzymes and supports cancer cell dissemination in both the "single cell" and "collective migration" modes. (B) Many circulating tumor cells (CTCs), representing carcinoma cells that have entered into the vasculature and may thereafter be capable of seeding new metastatic colonies at distant anatomical sites, display partial EMT activation with co-expression both epithelial and mesenchymal markers. Moreover, mesenchymal CTCs have been found to be significantly enriched in cancer patients with refractory or progressive disease. (C) At the colonization step, robust outgrowth of macro-metastases, at least in some destination organ sites, requires the reversion of EMT program and the associated gain of epithelial characteristics. (Source: Yun Zhang *et al.,* 2018)

Various signaling mechanisms are associated with this process, such as TGF-β, SHH, and WNT pathways. For instance, activation of the SHH pathway to promote EMT process have been observed in many cell lines such as lung cancer cell lines, renal cell cancer, and gastric cancer (121-123).

1.5.Theraputic modalities

1.5.1. Surgery

Surgical treatment is the only curative option for patients with CC; however, it is highly dependent on the clinical condition of patients, functionality of the liver after severe cholestasis, tumor expansion to the local areas including involvement of the vasculature and the metastatic condition. One in five patients with CC is entitled for surgical resection during the time of presentation; most of the CC patients with metastatic and advanced disease are ineligible for

surgical resection (124). The main objective of the surgery is thorough (R0) resection with minimum postoperative mortality of the patients. Complete surgical resection, regardless of the tumor localization, extensive resection of the liver including removal of lymph nodes and metastasis are critical for long term survival in CC patients (125-127).

The results of post-surgical resection of CC remains relatively guarded. The recurrence incidence has been reported in 50%- 60% of CC patients, with 26 months of median disease-free survival (128, 129). Various factors have been associated with the relapse of this disease like multiplicity of tumors, metastasis to the lymph nodes and vascular invasion (7). For patients who are undergoing resection, particularly individuals with N1 disease, adjuvant therapy would be beneficial.

1.5.2. Systemic therapy

Patients with IH- CC and EH- CC are often diagnosed at advanced stages, as the disease symptoms do not appear at early stages. Nearly 10,000 new cases are diagnosed annually in the United States with a 5-year survival rate below 20%, whereas 11.2 new cases per 100,000 people are diagnosed annually in Korea with a 5-year survival rate of 29.2 %. Surgical resection is the only curative option; however, CC has a high rate of relapse even after curative surgery. Therefore, chemotherapy is considered to be the most important modality for treating patients with CC, although there are very limited chemotherapeutic options available for advanced CC. The National Comprehensive Cancer Network (NCCN) proposes adjuvant chemotherapy after surgical resection of cholangiocarcinoma after analysing lymph node and margin status (130). After receiving encouraging results from randomized trials held in UK and Japan (131), the combination of Gemcitabine and Cisplatin chemotherapy has been recommended for an inoperable case according to the latest treatment guidelines. This regimen has become a standard treatment option for first line (1L) chemotherapy for patients with advanced and metastatic CC. Among Gemcitabine-based chemotherapy combinations, many studies have taken place with a combination of gemcitabine with cisplatin with one-armed phase II trial, and they testified with effective efficacy and good tolerability of this combination (response rate from 72 to 81%; $p = 0.049$).

During the time of disease development under 1L chemotherapy, 15% to 40% of patients with advanced biliary track cancers persist in good condition, and consequently may get successive lines of therapy (132-136). There are very few studies which have been reported regarding

second line (2L) chemotherapy for CC, though there is a lack of standard 2L chemotherapy regimen for the patients with advanced stage of CC which is refractory to gemcitabine/cisplatin 1L chemotherapy. Active symptom control (ASC) is the current standard of care after development of resistance to 1L chemotherapy. Studies have revealed that Oxaliplatin could be a promising therapeutic agent for treating advanced CC as compared to cisplatin-based regimens (137, 138). FOLFOX is also another chemotherapy regimen consisting of three therapeutic agents i.e. 5-FU, leucovorin (LV), and oxaliplatin, has significantly improved the survival rate of patients with metastatic gastrointestinal caners (139, 140). Initially, FOLFOX regimen was used to treat colorectal cancer and was found to have positive impact in the phase II study in Korea (141). To evaluate the effect of 2L chemotherapy in CC patients who received cisplatin/ gemcitabine (CisGem) as 1L chemotherapy, a randomized phase III ABC-06 study has been conducted in such patients to compare the efficacy of ASC with and without the addition of FOLFOX to the treatment. Data from the trial showed that ASC+mFOLFOX improved the overall survival after 2L treatment, with a clinically meaningful increase in 6 months and 12 months overall survival rate (142). The study indicates that there is a benefit from this 2L chemotherapy and that ASC+mFOLFOX should become the new standard of care for suitable CC patients.

Additionally, next-generation sequencing technology has considerably played an important role in unrevealing the molecular complexity appearing events in CC. Mutations in the genes of *IDH1* and *2* and fusions of *the FGFR2* have been detected in CC (143, 144). Alterations in the IDH and FGFR are currently considered as a main modern target with a significant therapeutic impact and also the most innovative progress in clinical trials. The phase II study trial of pan-BGJ398 (a pan- FGFR inhibitor) with 14% overall response rate and progression-free survival of 5.8 months are quite promising (145). Targeting mutations in IDH in iCC is also becoming a promising approach. In one of the studies, wherein 73 iCC patients with IDH1-mutation were enrolled to receive AG-120 (an oral inhibitor of IDH1) after gemcitabine-based chemotherapy, the disease control rate was found 56% and median progression free survival was 3.8 months (146). The assessment of tyrosine kinase receptor inhibitors are also in phase II clinical studies with advanced CCs (147).

1.6.Cyclopamine

Cyclopamine is a naturally occurring chemical isolated from the plant Veratrum californicum. It belongs to the steroidal alkaloids family and has been identified as the teratogen responsible

for craniofacial birth defects, including cyclops, in the offspring of sheep grazing on mountain ranges in the western United States. Cyclopamine is a specific antagonist of Smoothened (SMO) and has been found to inhibit the hedgehog signalling pathway (148-150). It has been considered as a treatment agent in various cancers eg. basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma, which exhibit an excessive SHH activity. Treatment with Cyclopamine has been shown to inhibit tumor growth in a mouse medulloblastoma allograph model (151), genetic mouse medulloblastoma model (152) and mouse xenograft models with pancreatic cancer (78, 153, 154) , human glioma (107), colon cancer (155), melanoma (156), small cell lung cancer (76) and prostate cancer (157) without any noticeable side effects. Studies on human patients revealed tumor regression after application of a topical cream comprising Cyclopamine when applied directly onto tumors, with no adverse effects (77). Since aberrant Hedgehog signalling has been associated with several types of cancers, inhibitors of the Hedgehog signalling pathway, including Cyclopamine derivatives, could be used as a potential treatment.

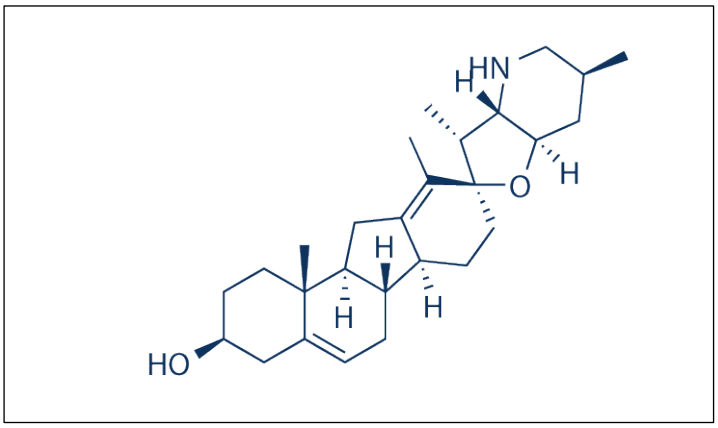


Figure 1.9: Structure of Cyclopamine

1.7.Aims of the study

The aim of the study is to elucidate the role of Hedgehog pathway in the the process of cholangiocarcinogenesis.

The objectives designed to achieve the above-mentioned aim are as follows:

1. To study the role of Hedgehog pathway during cholangiocarcinogenesis in a genetic mouse model (*Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$)*

2. To study the effect of Cyclopamine on cholangiocarcinogenesis and repopulating potential of CSCs in a genetic mouse model (Alb-Cre/KRAS^{G12D}/p53$^{L/L}$)

3. To study the involvement of Hedgehog pathway in tumour induction in xenograft models of implanted immune- sorted cells

4. To investigate the effect of hypoxia on Hedgehog pathway activation and cancer stemness during cholangiocarcinogenesis

2. Materials and Methods

2.1.Materials

2.1.1. Cell Lines

In vitro studies were carried out in well-established and characterized malignant cell lines of human origin. TFK-1, an intrahepatic CC cell line, was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany and HUCCT-1, an extrahepatic CC cell line, was kindly provided by Prof. Bitzer, UKT.

2.1.2. Experimental mouse models

1. *Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$*

The mouse model with activating mutations of Kras (KrasG12D) and deletion of p53, as a model of intra- hepatic CC, has been reported previously (158). In this study, Alb-Cre/ p53$^{L/L}$ (male) and LSL-KrasG12D, p53L/L (female) mouse strain were intercrossed and genotyped to acquire the Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mouse strain, while the Alb-Cre$^{-/-}$ /LSL-KRAS $^{G12D-/-}$/p53$^{L/L}$ strain was used as the negative control. The mice were housed in pathogen free conditions within Interdisciplinary Center for Clinical Research (IZKF) Transgenic Facility Tübingen FORS/HI, Tübingen, Germany, with free access to standard laboratory murine diet and water *ad libitum*.

2. *NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG)*

A pair of male and female NSG mice was purchased from Charles River Laboratory, USA. They were bred inhouse and maintained in IVC caging systems in pathogen free conditions within the IZKF, Tübingen animal facility, with free access to standard laboratory murine diet and water *ad libitum*. The obtained offspring were acclimatized for 1 week prior to the experiments and used for developing the xenograft models.

2.1.3. Chemicals and Bio-chemicals

Cyclopamine: Cyclopamine was purchased from Selleck Chemicals, Houston, TX, USA.

GANT61: GANT61 (InSolution™ Hedgehog/Gli Antagonist) was purchased from Calbiochem, CA, USA.

A list of general chemicals and bio- chemicals used in the study is given in table1

2.1.4. Cell culture materials

Roswell Park Memorial Institute (RPMI) - 1640 medium and Phosphate-buffered saline (abbreviated PBS) were procured from Gibco. Penicillin-Streptomycin solution was procured from Lonza, Belgium. Fetal Bovine Serum (FBS) was procured from Gibco/BRL, Gaithersburg, MD, USA.

Table 1: General Chemicals and Bio-chemicals used in the study

Chemicals	**Company**
Acrylamid-solution (30%) 37,5:1	Carl Roth, Karlsruhe, Germany
Agar	BD Biosciences, Heidelberg, Germany
Albumin FrAktion V	Carl Roth, Karlsruhe, Germany
Ammonium persulfate	Sigma-Aldrich, Schnelldorf, Germany
Ammonium sulfate	AppliChem, Darmstadt, Germany
Antigen Unmasking Solution	Vector, Eching, Germany
Boric acid	AppliChem, Darmstadt, Germany
Calcium chlorid	AppliChem, Darmstadt, Germany
Cesium chloride 99%	AppliChem, Darmstadt, Germany
Crystal violet	Sigma-Aldrich, Schnelldorf, Germany
DMF (Dimethylformamide)	Sigma-Aldrich, Schnelldorf, Germany
DMSO	AppliChem, Darmstadt, Germany
EDTA	AppliChem, Darmstadt, Germany
EGTA	AppliChem, Darmstadt, Germany
Ethanol	AppliChem, Darmstadt, Germany
FBS	Biochrom, Berlin, Germany
Glycine	AppliChem, Darmstadt, Germany
HEPS	AppliChem, Darmstadt, Germany
Hydrogen Peroxide Solution	Merck, Darmstadt, Germany
Magnesium chloride	AppliChem, Darmstadt, Germany
Methanol	AppliChem, Darmstadt, Germany
Mounting medium	Vector, Eching, Germany
Nonfat dried milk powder	AppliChem, Darmstadt, Germany

Nonidet® P40	AppliChem, Darmstadt, Germany
Paraformaldehyde	Sigma-Aldrich, Schnelldorf, Germany
Penicillin-Streptomycin solution	Lonza, Bornem, Belgium
Ponceau S solution	Roth, Karlsruhe, Germany
Potassium chlorid	AppliChem, Darmstadt, Germany
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Propidium iodide	Sigma-Aldrich, Schnelldorf, Germany
Protease and phosphatase Inhibitor Cocktail Tablets	Roche, Mannheim, Germany
SDS	AppliChem, Darmstadt, Germany
Sodium azide	Sigma-Aldrich, Schnelldorf, Germany
Sodium chloride	VWR, Darmstadt, Germany
Sodium dihydrogen phosphate	Merck, Darmstadt, Germany
Sodium fluoride	AppliChem, Darmstadt, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
Sodium pyruvate	AppliChem, Darmstadt, Germany
ß-Mercaptoethanol	AppliChem, Darmstadt, Germany
TEMED	AppliChem, Darmstadt, Germany
Toluidine blue	Sigma-Aldrich, Schnelldorf, Germany
Tris base	Sigma-Aldrich, Schnelldorf, Germany
Triton X 100	AppliChem, Darmstadt, Germany
Trypsin/EDTA solution	Lonza, Bornem, Belgium
Tween-20	AppliChem, Darmstadt, Germany
WST-1 reagent	Roche Diagnostics, Mannheim, Germany

2.1.5. Kits

Detergent compatible (DC) Protein Assay kit was purchased from Bio-Rad, München; ELISA (SHH and MMP-9) kits were purchased from R & D systems, Minneapolis; Alanine Transaminase (ALT) and Aspartate Transaminase (AST) assay kits were purchased from Abcam, Cambridge, UK; Annexin V Apoptosis Kit was purchased from BD Pharmingen, Heidelberg; VECTASTAIN Elite ABC kit ; DAB substrate Kit was purchased from Vector, Eching, Germany; RNeasy protect Mini Kit (50) was purchased from QIAGEN, Hilden, Germany.

Table 2: List of antibodies used in the study

Primary antibodies	**Catalog number**	**Company**
SHH	sc-9024	Santa Cruz Biotechnology, Heidelberg, Germany
SMO	sc-13943	Santa Cruz Biotechnology, Heidelberg, Germany
GLI1	2534	Cell Signaling Technology, Heidelberg, Germany
HIF-1α	610958	BD Biosciences, Heidelberg, Germany
Vimentin	sc7557	Santa Cruz Biotechnology, Heidelberg, Germany
N-cadherin	EPR1792Y	Millipore
Oct 04	2840	Cell Signaling Technology, Frankfurt, Germany
NANOG	3580	Cell Signaling Technology, Frankfurt, Germany
SOX2	2748	Cell Signaling Technology, Frankfurt, Germany
β-actin	AC74	Sigma Aldrich Corporation
E-Cadherin	3195	Cell signaling, Technology, Frankfurt, Germany
N-Cadherin	13116	Cell signaling, Technology, Frankfurt, Germany
PARP	9542	Cell signaling Technology, Frankfurt, Germany
Vimentin	sc-7557	Santa Cruz Biotechnology, Heidelberg, Germany
Ki-67	NCL-L-Ki67-MM1	Leica, Wetzlar, Germany
CD133	Ab19898	Abcam, Berlin, Germany
Secondary antibodies	**Catalog number**	**Company**
Anti-mouse IgG, Horseradish Peroxidase linked antibody (from sheep)	Amersham, NA931	GE Healthcare
Anti-Rabbit IgG, Horseradish Peroxidase linked antibody (from donkey)	Amersham, NA934	GE Healthcare

Flowcytometery Antibodies	**Catalog**	**Conjugate**	**Company**
CD133	17-1338-42	APC	eBioscience
CD44	11-0441-82	FITC	eBioscience
EPCAM	25-9326-42	PE-Cyanine7	eBioscience

Table 3: Glassware and Plasticware used in the study

Products	Company
6,12,24,96-well plate	Falcon, Wiesbaden, Germany
15 ml culture tube	Falcon, Wiesbaden, Germany
24-well plate	Falcon, Wiesbaden, Germany
50 ml culture tube	Falcon, Wiesbaden, Germany
6-well plate	Falcon, Wiesbaden, Germany
96-well plate	Falcon, Wiesbaden, Germany
Cell culture dishes 1000mm	Sarstedt, Nümbrecht, Germany
Cell culture dishes 60mm	Sarstedt, Nümbrecht, Germany
Centrifuge tubes	Eppendorf, Hamburg, Germany
Cover slip	Menzel-Glaeser, Darmstadt, Germany
Filter Tips 0,1-10 ul	Sarstedt, Nümbrecht, Germany
Filter tips 1000 ul	nerbe plus, Winsen, Germany
Filter tips 20 ul	Sarstedt, Nümbrecht, Germany
Filter tips 200 ul	nerbe plus, Winsen, Germany
Freezing tube	Thermo Fisher Scientific, Darmstadt, Germany
Hyperfilm TM ECL	GE Healthcare, Darmstadt, Germany
Microscope slides	R. Langenbrinck GmbH, Emmendingen, Germany
Tips 20-200 ul	Sarstedt, Nümbrecht, Germany

Table 4: Composition of various regents/buffers used in the study

Buffer	Composition
Radioimmunoprecipitation assay	1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8.0,
(RIPA) Buffer	80 mM NaCl, 50 mM NaF, 20mM Na4P2O7, 1 mM EDTA, 1 mM EGTA, H2O
Laemmli buffer (2X)	0.125M Tris HCl (pH 6.8), 4 % (w/v) SDS, 10 % (v/v) 2- mercaptoethanol, 20 % (v/v) glycerol and 0.004 % (w/v) bromophenol blue
SDS- PAGE running buffer (1X)	25mM Tris base, 250 mM glycine (electrophoresis grade), 0.1% SDS
Transfer Buffer (1X)	25mM Tris base; 192mM Glycine; Methanol 20%
10 % neutral buffered formalin	10 % (w/v) formaldehyde in 1X PBS
4 % PFA (fixative)	4 % paraformaldehyde (w/v) in 1X PBS
TBST	20mM Tris,150mM NaCl, 0.2% Tween-20
Blocking Buffer	5 % (w/v) dry non- fat milk or 3% (w/v) BSA in 1X PBST

10X Red Blood Cell (RBC) lysis buffer	8.02g Ammonium chloride, 0.84g Sodium bicarbonate, 0.37g Disodium EDTA, 100ml milli-Q water

Table 5: Equipments used in the study

Equipments	**Company**
Centrifuge	Thermo Scientific, Darmstadt, Germany
Fluorescence microscope	Olympus, München, Germany
Gel electrophoresis systems	Bio-RAD, München, Germany
Hypoxia chamber	Invivo2 300, Baker Ruskinn, Sanford, USA
Incubator	Thermo Scientific, Darmstadt, Germany
Water Bath	GFL, Burgwedel, Germany
Mixer 5432	Eppendorf, Hamburg, Germany
Vortex-Genie	Scientific Industries, Darmstadt, Germany
Heater	Heidolph Instruments, Schwabach, Germany
Nanodrop 2000 spectrophotometer	Thermo Scientific, Darmstadt, Germany
LightCycler 480	Roche, Indianapolis, US

Table 6: Primers used in the study

Primer name	**Primer sequence**
SHH S (Human)	GATGTCTGCTGCTAGTCCTCG
SHH AS (Human)	CACCTCTGAGTCATCAGCCTG
SMO S (Human)	GTTCTCCATCAAGAGCAACCAC
SMO AS (Human)	CGATTCTTGATCTCACAGTCAGG
GLI-1 S (Human)	CTCCCGAAGGACAGGTATGTAAC
GLI-1 AS (Human)	CCCTACTCTTTAGGCACTAGAGTTG
NANOG S (Human)	CCGCGCCCTGCCTAGAAAAGAC
NANOG AS (Human)	AGCCTCCCAATCCCAAACAATACG
OCT-4 S (Human)	CCCGCCGTATGAGTTCTGTGG
OCT-4 AS (Human)	CCGGGTTTTGCTCCAGCTTCTC
SOX-2 S (Human)	GGAGGGGTGCAAAAGAGGAGAG
SOX-2 AS (Human)	TCCCCCAAAAAGAAGTCCAGG
CD133 S (Human)	GCCCCCAGGAAATTTGAGGAAC
CD133 AS (Human)	GCTTTGGTATAGAGTGCTCAGTGATTG
Actin S (Human)	ACCCGCCGCCAGCTCACC
Actin AS (Human)	GGGGGGCACGAAGGCTCATC
Gapdh S (Human)	TTGATTTTGGAGGGATCTCG
Gapdh AS (Human)	GAGTCAACGGATTTGGTCGT
Genotyping	
p53 AS	CACAAAAACAGGTTAAACCCAG

p53 S	AGCACATAGGAGGCAGAGAC
KRAS S	CTAGCCACCATGGCTTGAGT
KRAS AS	TCCGAATTCAGTGACTACAGATG
AlbCre S	GGAAATGGTTTCCCGCAGAAC
AlbCre AS	ACGGAAATCCATCGCTCGACC
Gabra 12	CAATGGTAGGCTCACTCTGGGAGATGATA
Gabra 70	AACACACACTGGCAGGACTGGCTAGG

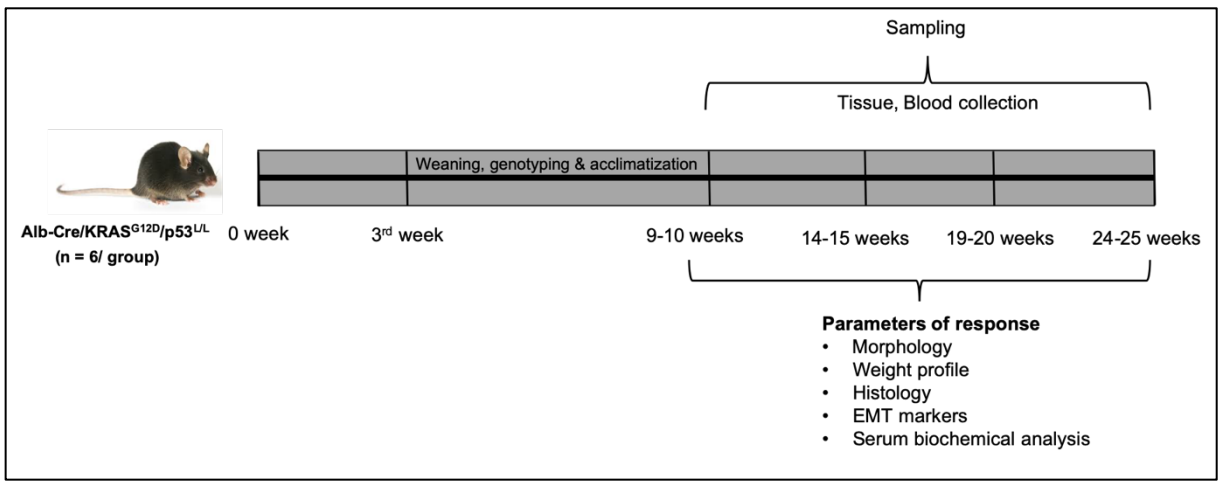
2.2. Methods

2.2.1. Experimental design

2.2.1.1. To study the role of SHH pathway during cholangiocarcinogenesis in a genetic mouse model (*Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$*)

In this study, tissue sampling was done at 4 different time points for characterization of the Hedgehog pathway. At each time point, n=4 mice were euthanized and macroscopic and microscopic evaluation of tumours, precursor lesions and metastases were done. Further, immunohistochemistry, Western blotting and RT-PCR were performed to assess Hedgehog pathway activation at each time point. Mice were monitored until they displayed signs of sickness such as poor grooming, weight loss and abdominal bloating. The body weight of the animals was also recorded every alternate day. At each time point, the tumours (if present), liver, spleen and lungs were excised, photographed, weighed, and immediately snap frozen or fixed in formaldehyde for further studies. Blood was also drawn for serum biochemical analysis.

Animals were treated as per the following scheme.



2.2.1.2.To study the effect of Cyclopamine on cholangiocarcinogenesis and repopulating potential of CSCs in a genetic mouse model (Alb-Cre/KRAS^{G12D}/p53$^{L/L}$)

a) Prevention study

In the prevention study, AlbCre, LSL-KRASG12D, p53L / L mice cohorts (n=6) were treated with intraperitoneal (i.p.) injection of Vehicle or Cyclopamine every alternate day, starting from 14th to 20th week of age. The body weight of the animals was recorded every alternate day for studying the effect of Cyclopamine. At the end of treatment (20th week of age), mice were sacrificed and macroscopic and microscopic evaluation of tumors, precursor lesions and metastases were done; while Hedgehog pathway activation in liver and tumor tissue was assessed by immunohistochemical staining, Western blotting and RT-PCR.

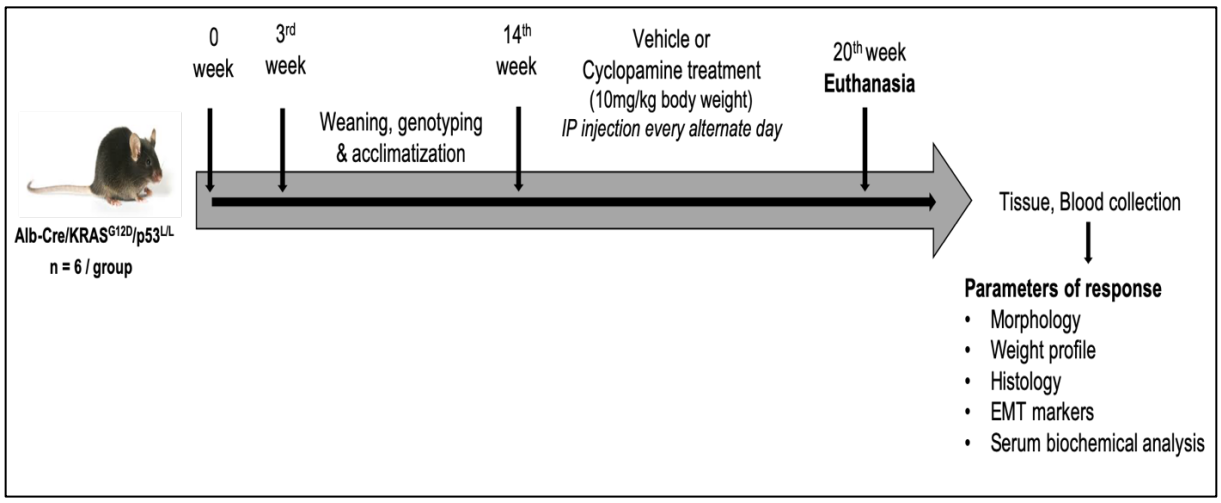
Age matched animals were randomly assigned into the following experimental groups.

i) Control, where animals with Alb-Cre-/- /KRAS G12D-/-/p53L/L genotype did not receive any treatment (n=3)

ii) Vehicle, AlbCre, LSL-KRASG12D, p53L / L mice received combination of 10% DMSO + 30% PEG300 + 5% Tween 80 + 55% Water (n=6)

iii) Drug, AlbCre, LSL-KRASG12D, p53L / L mice were administered freshly prepared Cyclopamine i.p. at a dose of 10mg / kg body weight (n=6)

Animals were treated as per the following scheme.



a) Survival study

In this study, AlbCre, LSL-KRASG12D, p53L/L mice were treated with Vehicle or Cyclopamine (as described before) from the 14th week of age until they showed signs of sickness such as poor grooming, weight loss and abdominal bloating. The body weight of the animals was recorded every alternate day. At the time of sacrifice, macroscopic and microscopic evaluation of tumors, precursor lesions and metastases were done; while Hedgehog pathway activation in liver and tumor tissue was assessed by immunohistochemical staining, Western blotting and RT-PCR.

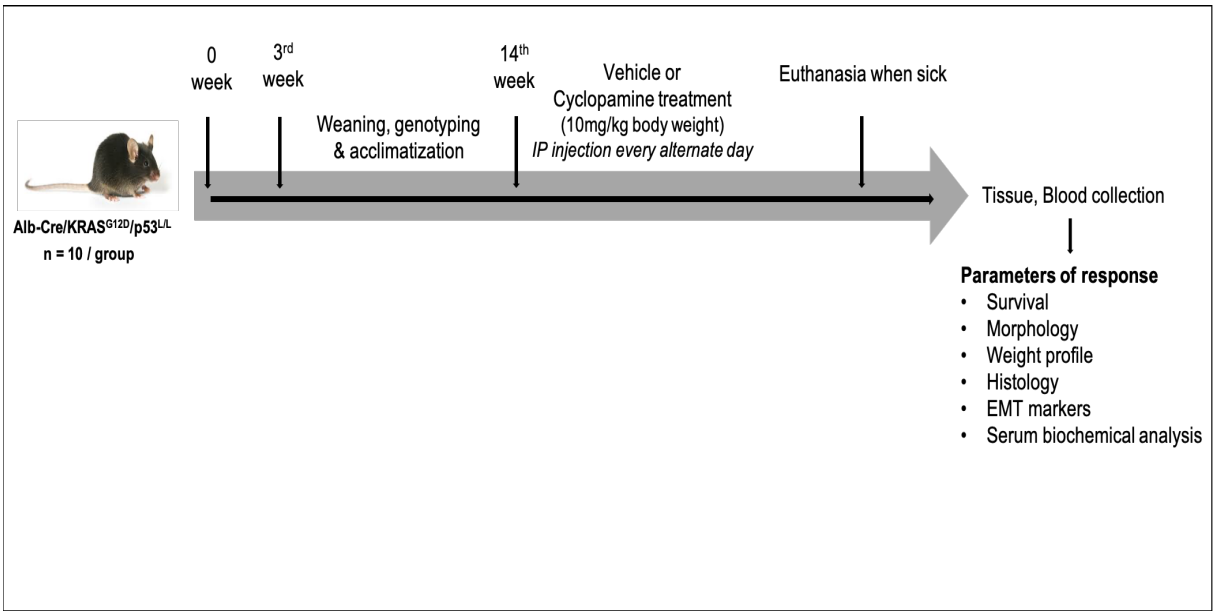
Age matched animals were randomly assigned into the following experimental groups.

i) Control, where animals with Alb-Cre-/- /KRAS G12D-/-/p53L/L genotype did not receive any treatment (n=3)

ii) Vehicle, AlbCre, LSL-KRASG12D, p53L/L mice received a combination of 10% DMSO + 30% PEG300 + 5% Tween 80 + 55% Water (n=10)

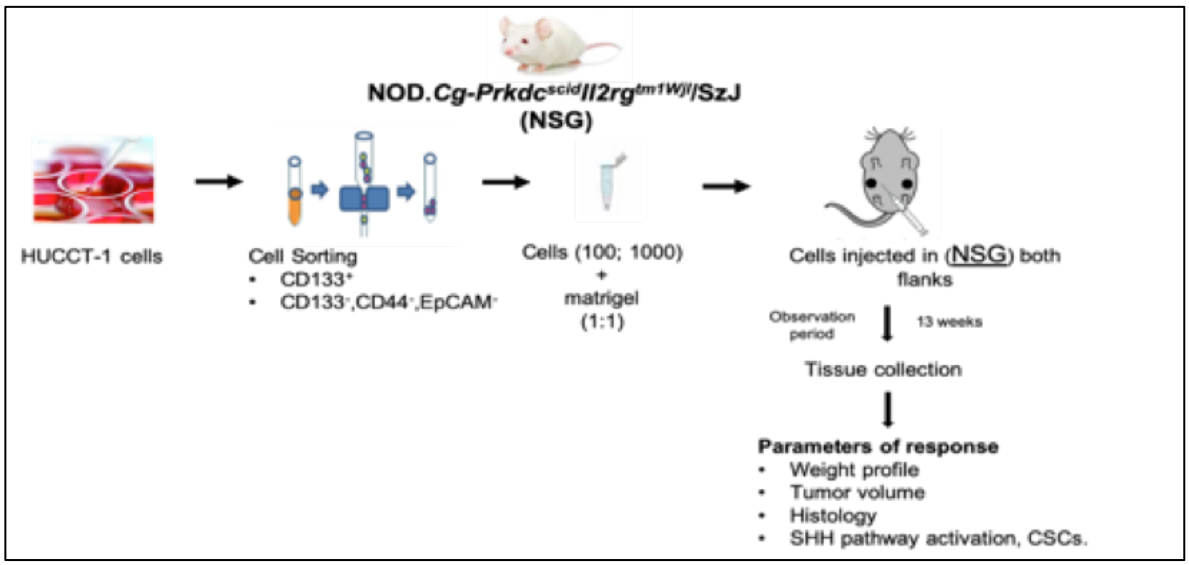
iii) Drug, AlbCre, LSL-KRASG12D, p53L/L mice were administered with freshly prepared Cyclopamine at a dose of 10mg / kg body weight (n=10)

Animals were treated as per the following scheme.



2.2.1.3.To study the involvement of Hedgehog pathway in tumor induction in xenograft models of implanted immune- sorted cells

Animals were treated as per the following scheme.



2.2.2. Genotyping

For genotyping of AlbCre, LSL-KRASG12D and p53L/L mice, ear punched material was collected from the weaned murine pups into 1.5ml centrifuge tubes using forceps. The tissue was then digested with proteinase K at 56°C overnight with continuous agitation. DNA extraction was done using isopropanol and 70% ethanol. Thereafter, an independent PCR reaction was carried out using the PCR primers listed in Table 6.

Table 7: Genotyping protocol

Genotyping p53								
No.	**Reaction Component**	**Final Con.**	**Reaction**	**V (µl)**	**Reaction**	**Total V (µl)**	**Protocol**	
1	Qiagen 10X Buffer + 15 mM MgCl2	1X; 1,5 mM	1	2	10	**20**	95 C x 15'	

2	25 mM MgCl2	0,17 mM	1	0,13	10	**1,3**	**94 C x 30"**	
3	10 mM dNTPS	0.20 mM	1	0,4	10	**4**	**58 C x 30"**	
4	20 uM Int 1F	0,67 uM	1	0,5	10	**5**	**72 C x 50"**	30 cycles
5	20 uM Int 1R	0.67 uM	1	0,5	10	**5**	72 C x 6'	
6	Qiagen HotStar Taq		1	0,1	10	**1**	Hold @ 10 C	
7	ddH2O		1	15,87	10	**158,7**		
	Total		1	**19,5**	10	**195**		
	DNA			**0,5**				

Genotyping KRAS								
No.	**Reaction Component**	**Final Con.**	**Reaction**	**V (µl)**	**Reaction**	**Total V (µl)**	**Protocol**	
1	Qiagen 10X Buffer + 15 mM MgCl2	1X; 1,5 mM	1	2	10	**20**	95 C x 15'	
2	25 mM MgCl2	0,20 mM	1	0,15	10	**1,5**		
3	10 mM dNTPS	0.20 mM	1	0,4	10	**4**	**94 C x 30"**	
4	20 uM Gabra 70		1	0,34	10	**3,4**		
5	20 uM Gabra 12		1	0,34	10	**3,4**	**60 C x 1'**	
6	20 uM 5' Kozak 3'LoxP1	0,67 uM	1	0,34	10	**3,4**	**72 C x 1'**	**33 cycles**
7	20 uM 5' Flank 3'LoxP1	0.67 uM	1	0,34	10	**3,4**	72 C x 6'	
8	Qiagen HotStar Taq		1	0,1	10	**1**	Hold @ 10 C	
9	ddH2O		1	15,5	10	**155**		
	Total		1	**19,51**	10	**195,1**		
	DNA			**0,5**				

Genotyping Alb Cre								
No.	**Reaction Component**	**Final Con.**	**Reaction**	**V (µl)**	**Reaction**	**Total V (µl)**	**Protocol**	
1	Qiagen 10X Buffer + 15 mM MgCl2	1X; 1,5 mM	1	3	10	**30**	95 C x 15'	
2	25 mM MgCl2	0,20 mM	1	0,2	10	**2**		
3	10 mM dNTPS	0.20 mM	1	0,6	10	**6**	**94 C x 30"**	
4	10 uM Gabra 70		1	0,5	10	**5**		
5	10 uM Gabra 12		1	0,5	10	**5**	**60 C x 1'**	
6	10 uM Cre 1	0,67 uM	1	0,5	10	**5**	**72 C x 1'**	**33 cycles**
7	10 uM Cre 2	0.67 uM	1	0,5	10	**5**	72 C x 6'	
8	Qiagen HotStar Taq		1	0,2	10	**2**	Hold @ 10 C	
9	ddH2O		1	23,5	10	**235**		
	Total		1	**29,5**	10	**295**		
	DNA			**0,5**				

Ethics statement

All the experimental protocols were reviewed and approved by the UKT institutional and Baden Württemberg Committee on Ethics of Animal Experimentation, in accordance with the Guidelines for the Care and Use of Laboratory Animals (animal proposal numbers: M2/16 and M3/16) before starting the experiments.

2.2.3. Drug preparation

For *in vivo* studies, Cyclopamine was first dissolved in 10% DMSO (until clear), following which 30% PEG-300 and 5% Tween 80 were added to the drug solution. After homogeneous mixing, the drug was further diluted with 55% of water. For *in vitro* studies, Cyclopamine was dissolved in 100% DMSO and diluted in the culture media to achieve the desired concentrations.

2.2.4. Maintenance of cell lines

TFK-1 and HUCCT-1 cell lines were cultured in RPMI-1640 containing Glutamax, supplemented with 10% fetal calf serum and 100 U/mL penicillin and streptomycin. For normoxic conditions (21% oxygen), cell lines were maintained in a humidified atmosphere at 37°C in a 5% CO_2 incubator and for hypoxia treatment, cells were maintained in 1% O_2, 5% CO_2 and 94% N_2 using Invivo2 300 Baker Ruskinn chamber for different time intervals (3, 6, 9, 24 and 48hr) in the presence or absence of different concentrations (5, 10 and 15 μM) of Cyclopamine or GANT61.

2.2.5. Fluorescence-activated cell sorting (FACS)

HUCCT-1 cells were harvested using trypsin, washed with PBS and then blocked with 5% BSA for 10 minutes at room temperature. The cells were then washed with FACS buffer and incubated with APC conjugated monoclonal anti-CD133 antibody (6 μL / 6.4x 10^6 cells in 100 μl staining buffer), 10 minutes at 4°C), FITC conjugated monoclonal anti-CD44 antibody (0,6μg / 6.4x 10^6 cells in 100 μl staining buffer) or PE-Cyanine7 conjugated monoclonal anti-EpCAM antibody (0.0072 μg / 6.4x 10^6 cells in 100 μl staining buffer) for 30 minutes on ice, followed by fractionation using BD FACS Aria-IIIu cell sorter.

2.2.6. Development of tumor xenograft models

Male and female NOD/SCID mice of 6 to 8 weeks old age were maintained according to the standard conditions and used for developing xenografts. Two different cell densities of CD133+ cells (100 and 1000 cells) and 1000 CD133- CD44- EpCAM- cells were mixed with Matrigel in a 1:1 ratio, and injected subcutaneously (n= 6/group) in both flanks (100μl/ flank) of a NOD/SCID mouse using a 25-guage needle. Tumor incidence, tumor latency and tumor growth were assessed by macroscopic inspection, while tumor volume was measured every two days using a digital Vernier Caliper. Mice were euthanized when tumor volume reached a maximum of 2000 mm3. At this point, the tumors, liver, spleen and lungs were excised, photographed and weighed; and snap frozen or fixed in formaldehyde for further studies. Blood was also drawn for serum biochemical analysis.

2.2.7. Blood collection and serum isolation

Whole blood was collected from the euthanized mouse by cardiac puncture in a 1.5ml centrifuge tube using a 25G needle at the terminal stage of the study. The blood was allowed to clot for serum isolation by keeping it undisturbed at room temperature for 1 hour, followed by centrifugation at 13,000 rpm for 25 minutes at 4^0C. The supernatant was collected and immediately stored at -80^0C for further studies.

2.2.8. ALT and AST activity assays

ALT and AST activities in the serum were assessed using ALT and AST kits, according to the manufacturer's protocol.

2.2.9. Western blotting

For *in vitro* studies, total protein from cell lines was extracted immediately after termination of each experimental time point by sonication, using Radioimmunoprecipitation assay buffer (RIPA) buffer containing protease and phosphatase inhibitors. For *in vivo* studies, snap frozen tissues were weighed and homogenized mechanically in ice cold RIPA buffer (containing protease and phosphatase inhibitor cocktail) using a pestle, and incubated on ice for 50 minutes with gentle agitation after every 10 minutes. Following incubation on ice, tubes were centrifuged at 13000 rpm for 25 minutes at 4oC and supernatants were collected. Total extracted protein was quantified using DC protein assay kit according to manufacturer's instruction and absorbance was measured at 650-750 nm using a microplate reader (Gen5™ Microplate Reader). The lysate containing 20µg protein was mixed with an equal volume of sample buffer, denatured by boiling at 95oC for 5 minutes, and then separated on a 12% polyacrylamide mini gel. The proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes, and then blocked with 5% (w/v) nonfat dry milk or BSA for 1 hour at room temperature. After washing with TBST for 30 minutes, the membranes were incubated overnight with primary antibodies against SHH (1:1000) , SMO (1:1000), GLI1 (1:1000), HIF-1α (1:1000,), Vimentin (1:1000), N-cadherin (1:15000), E-cadherin (1:1000), CD133 (1:1000), Oct4 (1:1000), NANOG (1:1000), SOX2 (1:1000) and Actin (1:10000), followed by incubation with horseradish peroxidase conjugated secondary antibodies (1:10,000) for 1 hour at room temperature. Finally, the blots were incubated with chemiluminescent substrate solution for 5

minutes and exposed to Amersham hyper film for visualization of protein bands. For densitometric analysis, band intensity of target protein was normalized to β- actin or Vinculin using Image J software.

2.2.10. Histology and Immunohistochemistry (IHC)

After euthanizing the mice, the harvested tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin according to the standard protocol. For IHC, sections were deparaffinized using 100% xylene and rehydrated in a series of decreasing concentrations of ethanol. Antigen retrieval was done using Antigen Unmasking Solution in a pressure cooker. Peroxidase activity was blocked by incubating the sections with 1% H_2O_2 for 10 minutes. The tissue sections were then washed with TBST and blocked with 5% or 10% (for CK-19) goat serum containing 0.3% Triton X-100 for 1 hour at room temperature. Further, the sections were incubated with primary antibodies overnight at 4 °C in a humidified chamber. Immunodetection was performed using the mouse specific VECTASTAIN Elite ABC kit as per manufacturer's instructions. The sections were washed with TBST and incubated with biotinylated secondary antibody (provided in the kit), followed by streptavidin- peroxidase conjugate, and visualized using DAB as the chromogen. Finally, the slides were counterstained with Hematoxylin, dehydrated in a series of increasing concentrations of ethanol and mounted with VectaMount permanent mounting medium for examination under a bright field microscope.

2.2.11. Evaluation of Immunohistochemistry staining

The immunoreactive score (IRS) system was firstly introduced in 1987 by Remmele and Stegner (159). After IHC staining of human TMAs, the staining intensity was assigned as negative, weak, moderate or strong, and scored as 0, 1, 2, 3 and 4 points respectively. Moreover, in each TMA spot, the proportion of each staining intensity level was evaluated and categorized into: ≤10%, 11-50%, 51-80% and ≥81%. The final IRS was calculated as per the scoring criteria given in table 8. Based on the final IRS, the overall staining of each sample was defined as negative, weak, moderate and strong if the IRS was 0 - <1, 1 - ≤4, 4 - ≤8 and >8 respectively.

Table 8: Staining intensity evaluation criteria

Percentage of positive cells	**Stain intensity**			
	Negative (0)	Weak (1)	Moderate (2)	Strong (3)
Negative	0	0	0	0
≤10%	0	1	2	3
11-50%	0	2	4	6
51-80%	0	3	6	9
≥81%	0	4	8	12

2.2.12. Measurement of MMP-9 concentration in culture medium

Immediately before performing ELISA, serum samples were thawed and prepared according to the manufacturer's protocol for the detection of MMP-9 concentration in the hypoxia treated cell culture medium. MMP-9 concentration was measured and calculated using a standard curve generated using known concentrations (0- 10, 000 pg/ml) of MMP-9.

2.2.13. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using RNeasy protect Mini Kit (50) and quantified using a Nanodrop 2000 spectrophotometer 1µg of total RNA was then reverse transcribed to cDNA using Revert Aid First Strand cDNA Synthesis Kit. For qRT-PCR, primers mentioned in table 6 were used for amplification. Reactions were run with iQ SYBR Green Supermix on LightCycler 480 at the cycling parameters: 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60- 62°C for 1 min. The amount of each target gene was normalized to β-actin or GAPDH respectively.

2.2.14. Immunofluorescence microscopy

Cells were plated in a 4- well chamber slide at a density of 3 x10^4 per well for 48 hours. Cells were then treated with Cyclopamine and placed under hypoxic or normoxic conditions for 24 hours. Cells were fixed with 2% formaldehyde for 15 min at room temperature, washed with PBS for 10 min, and then permeabilised with 1% BSA supplemented with 0.2% Triton X-100 for 5 mins on ice. After washing with PBS containing 1% BSA for 5 mins, cells were blocked with 3% BSA for 1 hr at room temperature followed by overnight incubation with primary

antibodies (diluted in PBS containing 1% BSA and 0.5% Tween-20) against GLI1(1:250), NANOG (1:800), Oct4 (1:400. The cells were then washed and incubated with anti-rabbit Alexa Fluor 594 labeled secondary antibody, diluted 1:300 in PBS containing 1% BSA and 0.5% Tween-20 for 30 mins in dark at room temperature. Finally, the preparations were embedded in Vectashield Mounting medium containing DAPI as the DNA-specific counterstain. Slides were stored at 4 °C until image acquisition with a Leica fluorescence microscope.

2.2.15. Measurement of SHH and MMP-9 level in cell culture medium

After 48hr of seeding 3 x10^5 cells in a 60 mm dish, the dishes were placed under hypoxia for 3hr, 6hr, 9hr, 24hr, 48hr and 72hr. At each time point, the culture supernatant was collected, centrifuged at 1000 rpm for 5 minutes at 4 °C and stored at −80 °C until analysis. The levels of SHH and MMP-9 in the cell culture medium were quantified using commercially available sandwich ELISA kits according to the manufacturer's instructions. SHH and MMP-9 concentration was measured and calculated using a standard curve generated using known concentrations (0 to 10, 000 pg/ml) of SHH/MMP-9.

2.2.16. Small interfering RNA (siRNA) knockdown of HIF-1α

Cells were seeded at a density of 5 x10^4 cells/ ml in a 6-well plate. After 48hr, cells were transfected with 25nM of HIF-1 siRNA (ON-TARGET plus SMART pool, L-003896) using Dharma-FECT transfection reagent for 24hr according to the manufacturer's protocol, following which the media was replaced with fresh media. Cells were further incubated for 24hr for mRNA and 48hrs for protein expression analysis and later on transferred to the hypoxia chamber for 3hr, 6hr, 9hr and 24hr respectively.

2.2.17. Metabolic viability assay

The effect of Cyclopamine or GANT61 on the metabolic viability of TFK-1 and HUCCT-1 cells was assessed by WST assay. Cells were seeded at a density of 1x10^4 cells/ml in a 96- well plate for 48 hours, followed by treatment with DMSO (Vehicle) or various concentrations of Cyclopamine or GANT61 (5, 10 and 15 μM). The plates were then incubated under normoxic or hypoxic conditions for 24, 48, 72 and 96hrs. 20 μL of WST-1 reagent was added to each well at each time point and incubated for 2hr in a humidified chamber with 5% CO_2 at 37ºC.

Absorbance was read at a wavelength of 490 nm using 650 nm reference wavelengths in a plate reader.

2.2.18. Apoptosis assay

Induction of apoptosis was studied using the Annexin V-FITC assay kit. Cells were seeded at a density of $1x10^4$ cells/ml in a 96- well plate for 48 hours, followed by treatment with DMSO (Vehicle) or various concentrations of Cyclopamine (5, 10 and 15 μM). The plates were then incubated under normoxic or hypoxic conditions for 24, 48 and 72hrs. After completion of the treatment, cells were washed twice with PBS and harvested by trypsinization. The cells were then stained with FITC- Annexin V according to the manufacturer's protocol. Sample acquisition was done with BD LSR Fortessa and analysis was carried out using FlowJo 8.7 software (Tree Star Inc., Ashland, USA). The percentages of Annexin-V +ve/-ve and PI +ve/-ve cells were estimated by applying appropriate gates.

2.2.19. Invasion assay

Cells were seeded (TFK-1, $0.1x10^6$ /ml; HUCCT-1, $0.04 x10^6$ /ml) into each well of a 12- well BD BioCoatTM Matrigel TM Invasion Chamber in serum free medium. Seeded cells were then treated with Cylopamine (15 μM) or GANT61 (10 μM) or DMSO and then placed in BD Falcon TC Companion 24 well plate containing 10% FCS, followed by treatment with normoxia or hypoxia for 48hrs. The invaded cells were fixed with 100% methanol and stained with 1% toluidine blue in 1% borax. Stained cells were counted under a bright field microscope and invasion index calculated using the following formula:

$$\text{Invasion Index} = \frac{\%\ \text{Invasion Test Cell}}{\%\ \text{Invasion Control Cell}}$$

2.2.20. Clonogenic assay

500 cells/ well were seeded in triplicates in a 6- well plate. After 24 hours, cells were treated with Cyclopamine (15μM) and placed under hypoxia or normoxia for 24hr, 48hr, 72hr and

96hr, and then transferred to a humidified chamber with 21% O_2 and 5% CO_2 at 37 °C for another 11 days. Colonies were washed once with PBS to remove media, fixed with 2% PFA for one hour followed by staining with 1% crystal violet for one hour. Plates were then washed with water and air dried. Colonies of at least 50 cells (5 to 6 generations of proliferation) were scored as survivors. Colony formation efficiency was calculated using the following formula:

Colony formation efficiency = (Number of colonies counted / Number of cells plated) x100

2.2.21. Statistical analysis

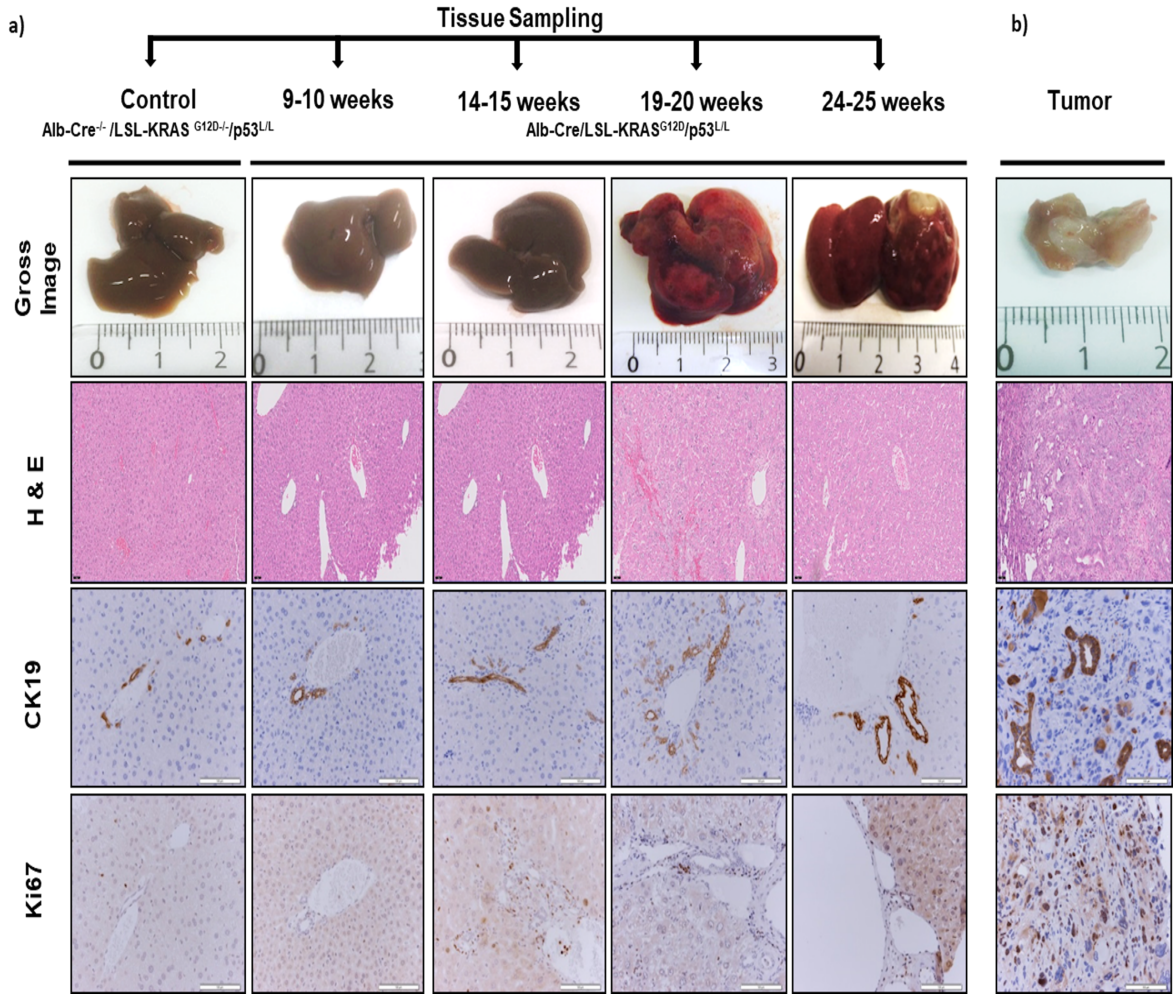
Data was analyzed using Graph Pad Prism (version 8.0) and the experimental results expressed as mean ± SEM. One-way or two-way ANOVA followed by the Bonferroni post hoc multiple comparison test was used to test the significance of any differences between groups. Data of survival studies was analysed using the Kaplan-Meier method followed by Mantel-Cox (log-rank) and Gehan-Breslow-Wilcoxon tests for assessment of significant differences. Results were considered significant at $p < 0.05$.

3. Results

3.1. Role of Hedeghog pathway during cholangiocarcinogenesis in a genetic mouse model (*Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$*)

3.1.1. Albumin-Cre synergized with K-Ras^{G12D} and p53$^{L/L}$ promote CC development in mice

To evaluate the role of Hedgehog pathway towards the induction and progression of CC, we used a mouse model with Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ genotype, specifically harboring Alb-Cre, Kras^{G12D} and p53$^{L/L}$ mutations. These mice cohorts were regularly monitored for signs of morbidity, and genotyping was done at various time points.



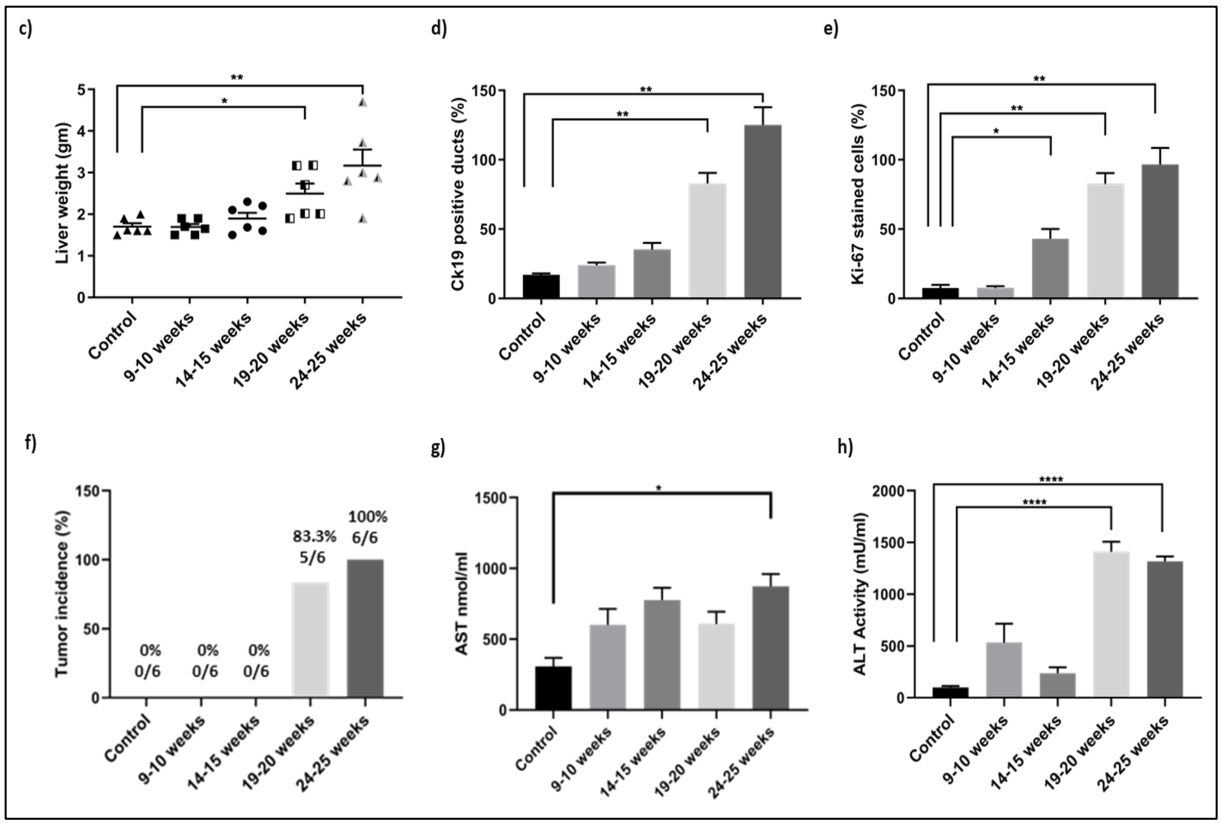


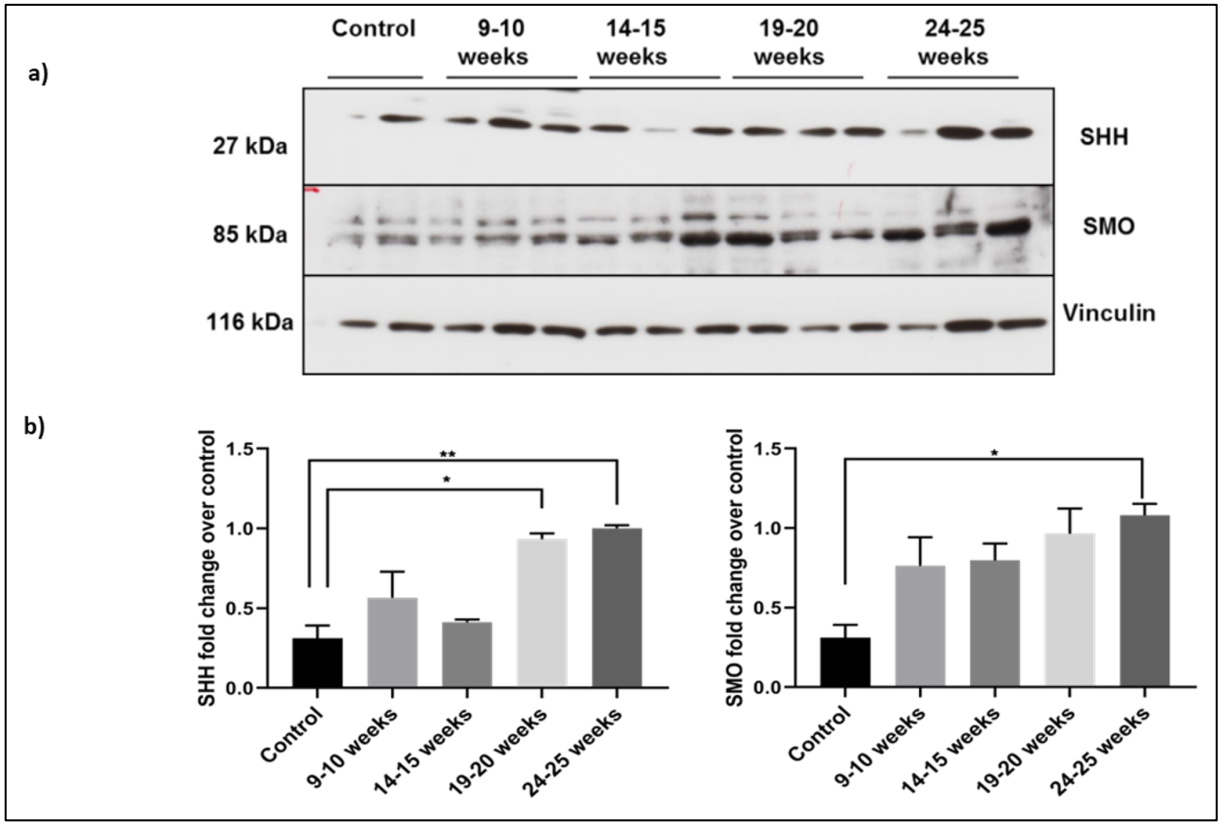
Figure 3.1 AlbCre, LSL-Kras^{G12D} and p53$^{L/L}$ mutations in the hepatic epithelium concur to trigger CC in mice. (a) Representative macro- and microscopic images of mice liver tissues at the indicated time points. Mice were sacrificed at various time points and H&E (50 µm) and IHC for CK19 and Ki67 (100 µm) was performed to study tumorigenesis. Tumor appeared only at 19-20 weeks of age of the mouse, and no tumor formation was observed, either macroscopically or microscopically, before. **(b)** Macro- and microscopic images of tumor: H&E stained tumor sections (50 µm) and IHC for CK19 and Ki67 (100 µm) (**c)** liver weight (**d** & **e**) Quantification of IHC staining for Ck19 positive ducts and the proliferation marker Ki67 **(f)** Tumor incidence rate (**g & h)** ALT & AST activity in serum. Densitometric quantification of target proteins. Data represents as mean ± SEM with n=6 per group. ∗p < 0.05; ∗∗p < 0.01; ∗∗∗ p < 0.001; ∗∗∗∗ p < 0.0001.

Macro- and microscopic evaluation of liver tissue showed that the mice were tumor free up to the age of 14-15 weeks, but they developed solid tumours in the liver at the age of 19-20 weeks (Fig. 3.1a, b). The tumor incidence rate at this age was observed to be 83.3%, which further increased to 100% at 24-25 weeks of age (Fig. 3.1f). IHC staining for CK19, a marker for cholangiocarcinoma, was found to significantly increase in the liver tissue of mice from 19-20 weeks onwards ($p< 0.01$) (Fig. 3.1d). Furthermore, a significant increase in Ki-67 positivity ($p< 0.01$) (Fig. 3.1e), indicating proliferation of cholangiocytes, was also observed in these liver tissues, that followed the same pattern as CK19 staining. Moreover, liver derived tumors also stained positive for CK19 and Ki67 (Fig. 3.1b). Histological examination of liver tissue from Alb-Cre-/- /LSL-KRAS G12D-/-/p53L/L mice, which were used as a control in the study, showed that the mice were healthy, and no carcinogenesis appeared in the mouse liver.

At the biochemical level, serum ALT and AST liver enzyme activities were significantly elevated ($p < 0.05$, 0.0001) at 19-20 weeks of age (Fig. 3.1g, h), which corroborated with significantly increased liver weights in the respective age group mice cohorts ($p < 0.01$; Fig. 3.1c).

3.1.2. CC cells acquire metastatic potential with the development of Hh signaling activity

To investigate the role of Hh signaling in cholangiocarcinoma progression, we studied the Hh pathway activation by examining its pathway elements SHH, SMO and GLI1 in liver tissues. The SHH and SMO protein levels were significantly upregulated at 19-20 weeks age of mice ($p< 0.01$) (Fig. 3.2 a, b). GLI1 activation was observed by immunohistochemical staining, wherein a time dependent increase in GLI-1 expression was visualised from 19-20 weeks till 24-25 weeks of age (Fig. 3.2 e). However, no significant difference in the expression level of SHH and SMO was observed between the tumor and the liver tissue (Fig. 3.2c, d).



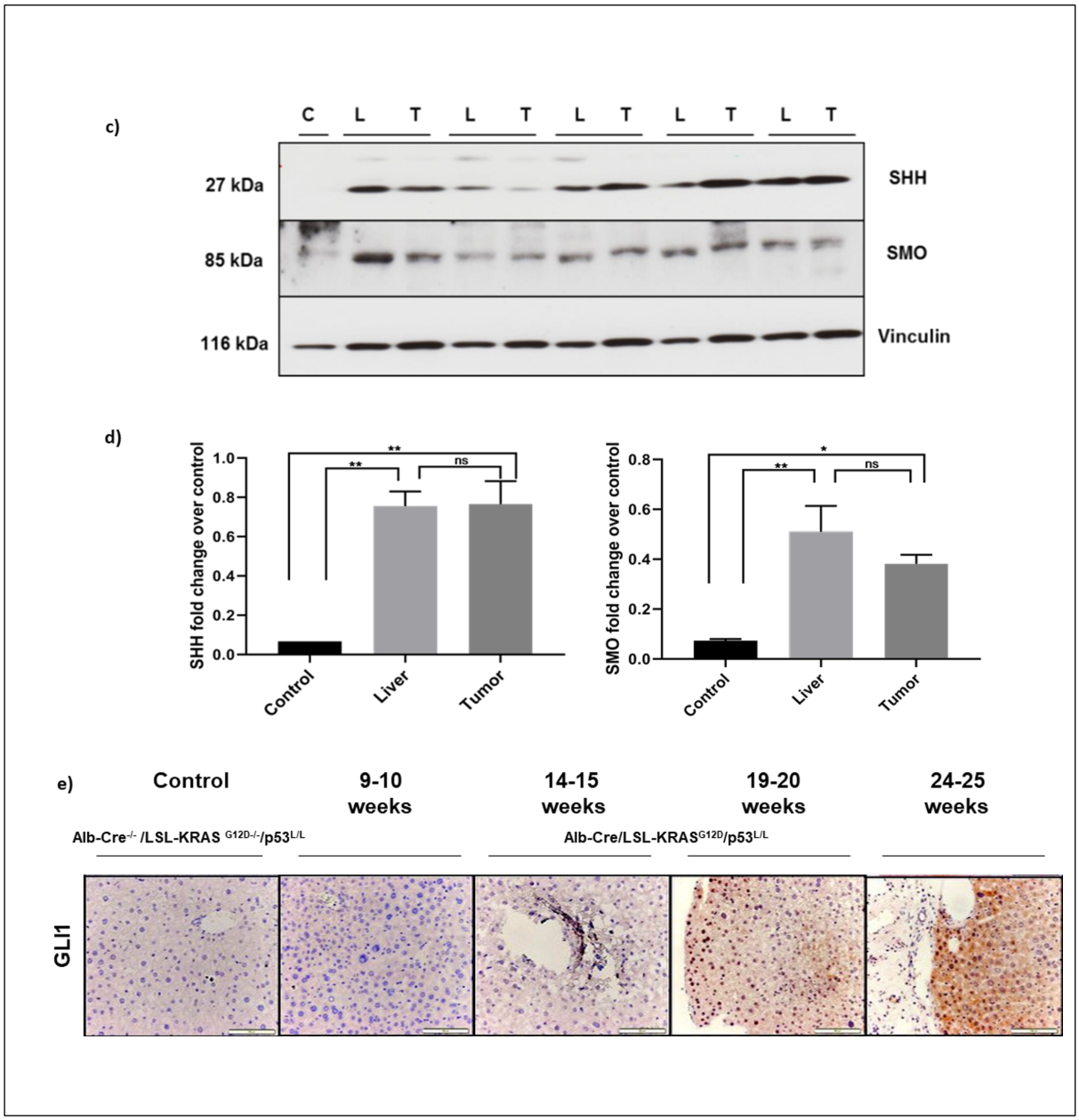


Figure 3.2 Hh pathway activation promotes development of CC in mice. (a) Western blot analysis and **(b)** Densitometric quantification of SHH and SMO expression in liver tissue (n=3 per group) **(c)** Western blot analysis and **(d)** Densitometric quantification of SHH and SMO expression in tumor and liver tissue **(e)** Immunohistochemical analysis of GLI1 nuclear translocation in liver tissues at the indicated time points (100 μm in x20). Data represents as mean ± SEM. ∗p < 0.05; ∗∗p < 0.01; **L**: Liver **T**: Tumor

Further, growth of cholangiocarcinoma in distant organs following metastasis was also investigated. Gross assessment identified frequent lung metastases with an incidence rate of 50% at 19-20 weeks of age, which increased to 66.6% at 24-25 weeks of age (Fig. 3.3a, b). Concurrently, serum MMP-9 concentrations were significantly elevated in both these cohorts (Fig. 3.3c) ($p < 0.0001$).

Subsequently, the expression of N-cadherin and its molecule partner α-SMA, which are involved in the malignant behavior of cancer through EMT, were measured in the mice liver tissues.

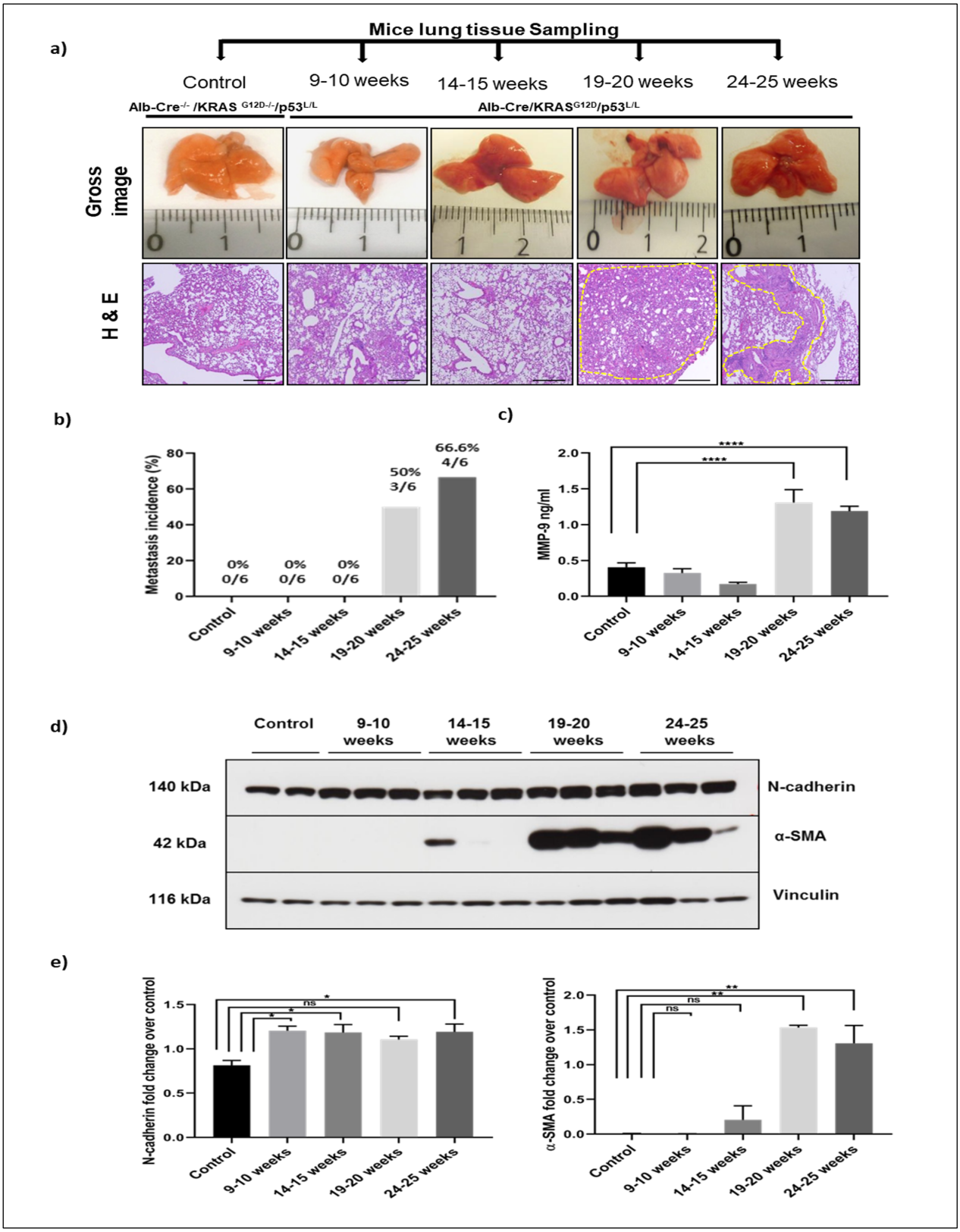


Figure 3.3 Hedgehog pathway activation promotes metastasis in the lungs during CC progression. **(a)** Representative images of gross lung morphology and H&E stained lung sections of Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mice at various time points (500 µm in x4) **(b)** Metastasis incidence (n=6 per group) **(c)** Serum MMP-9-protein level (n=3-4 per group) **(d)** Western blot analysis and **(e)** Densitometric quantification of N-cadherin and α-SMA in liver tissues (n=3 per group). Data represents as mean ± SEM. ∗p < 0.05; ∗∗p < 0.01; ∗∗∗∗ p < 0.0001.

Western blot analysis showed that the expression of N-cadherin was higher in all the groups as compared to the control group, whereas α-SMA protein level was found to be elevated only in19-20 and 24-25-week-old mice (Fig. 3.3 d, e).

3.1.3. Effect of activated Hedgehog signaling on cancer stemness

To assess the effect of Hedgehog signaling on cancer stemness, we analysed the protein expression of CSC transcription factors (SOX-2, Oct-4, NANOG) as well as CSC marker (CD133) by Western blotting in the mice liver and tumour tissues. An age dependent increase in the protein level of all the three transcription factors (SOX-2, Oct-4, NANOG) was observed in the liver tissue (Fig. 3.4a).

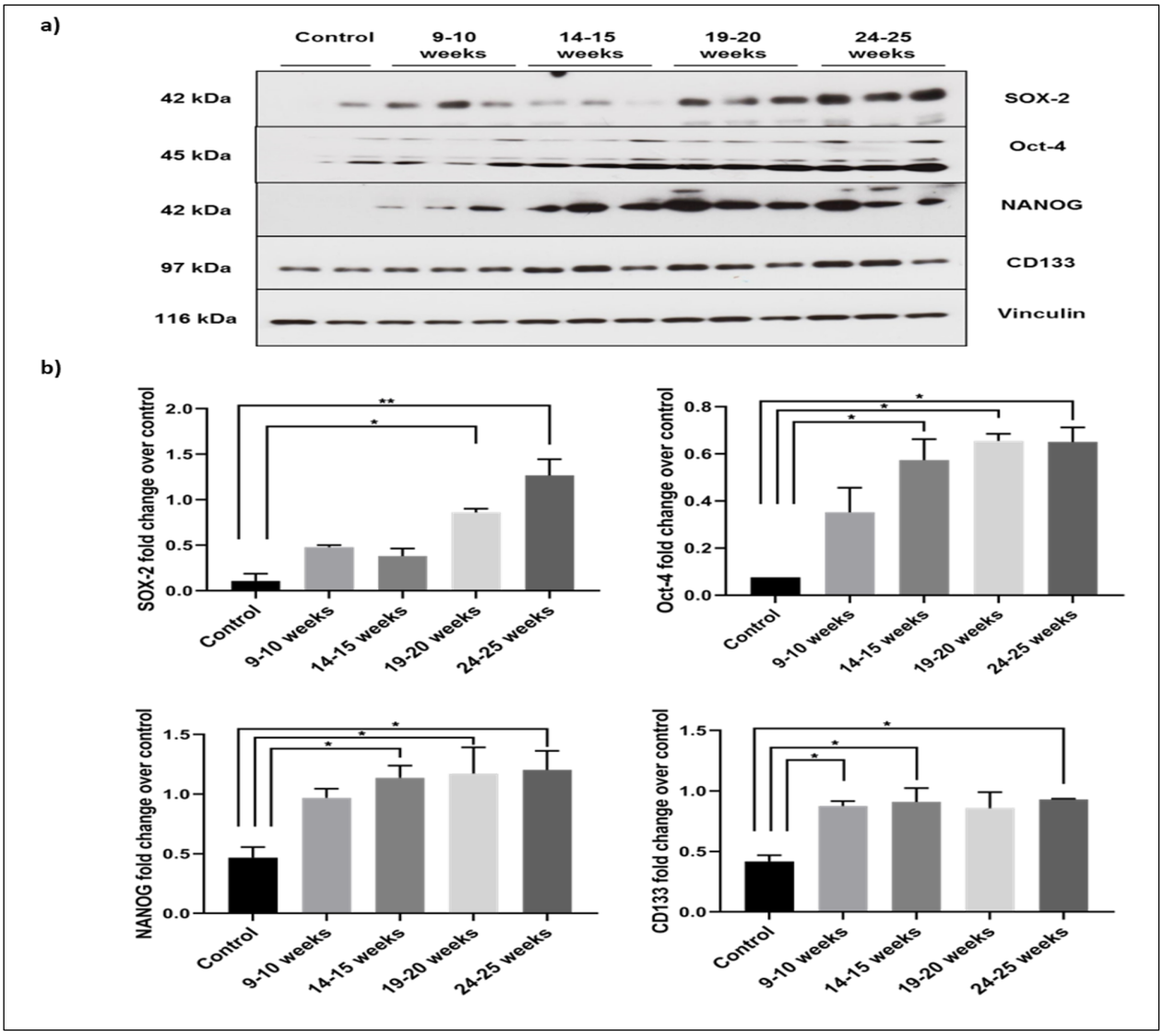


Figure 3.4 Hedgehog pathway activation leads to an increase in stemness related markers. (a) Protein expression of SOX2, Oct-4, NANOG, CD133 observed by western blot in liver tissue at various time points (n=3 per group) **(b)** Densitometric quantification of target proteins. Data represents as mean ± SEM. ∗p < 0.05; ∗∗p < 0.01.

SOX-2 levels were significantly high, from 19-20 weeks onwards of age ($p < 0.01$), whereas protein expression of Oct-4 and NANOG were found elevated in mice of all the age groups ($p < 0.05$). Similarly, CD133 protein expression also showed an increasing pattern with the progression of the disease with age ($p < 0.05$) (Fig. 3.4 b). Concurrently, the protein expression level of SOX-2, Oct-4, NANOG and CD133 was also analysed in the adjacent tumour tissues. SOX-2 protein level was found significantly increased in liver, as compared to the tumour tissue, whereas no significant difference was found in Oct-4, NANOG and CD133 protein expression in between liver and tumour tissues (Fig. 3.5a, b).

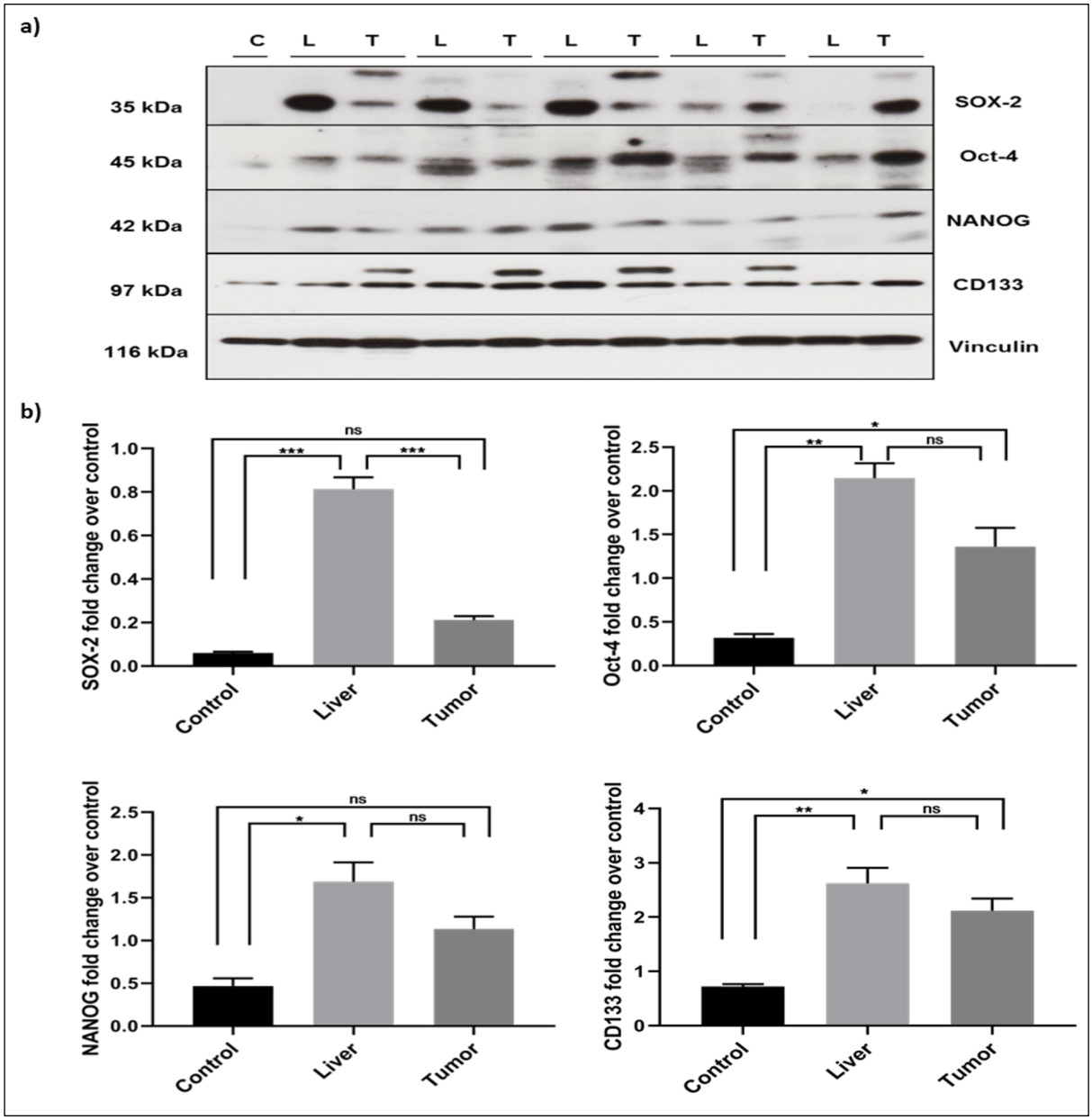


Figure 3.5: Hedgehog pathway activation maintains stemness signature. (a) Western blot analysis of SOX2, Oct-4, NANOG, CD133 in liver and tumor tissue (n=3 per group) **(b)** Densitometric quantification of target proteins. Data represents as mean ± SEM. ∗p < 0.05; ∗∗p < 0.01; ∗∗∗ p < 0.001. **C**: Control **L**: Liver **T**: Tumor

3.2. To study the effect of Cyclopamine on cholangiocarcinogenesis and the repopulating potential of CSCs in a genetic mouse model (Alb-Cre/KRAS^{G12D}/p53$^{L/L}$)

3.2.1. Hedgehog pathway inhibition prevents tumour growth in transgenic mice

To assess the tumour preventive potential of Hedgehog pathway inhibition, the established K-Ras-driven CC mouse model was treated with Cyclopamine (10mg/kg body weight) or Vehicle for 7 weeks, beginning from 14 weeks of age. Interestingly, after 7 weeks of alternate day treatment with Cyclopamine, no tumour formation was observed in the livers of these mice, whereas the tumour incidence rate in the Vehicle treated group was significantly high at 83.3% (Fig. 3.6 a, d). IHC staining for CK19 positive ducts and Ki-67, was found significantly decreased in Cyclopamine treated group as compared with Vehicle administered mice cohorts ($p < 0.05$, $p < 0.0001$) (Fig 3.6b, c).

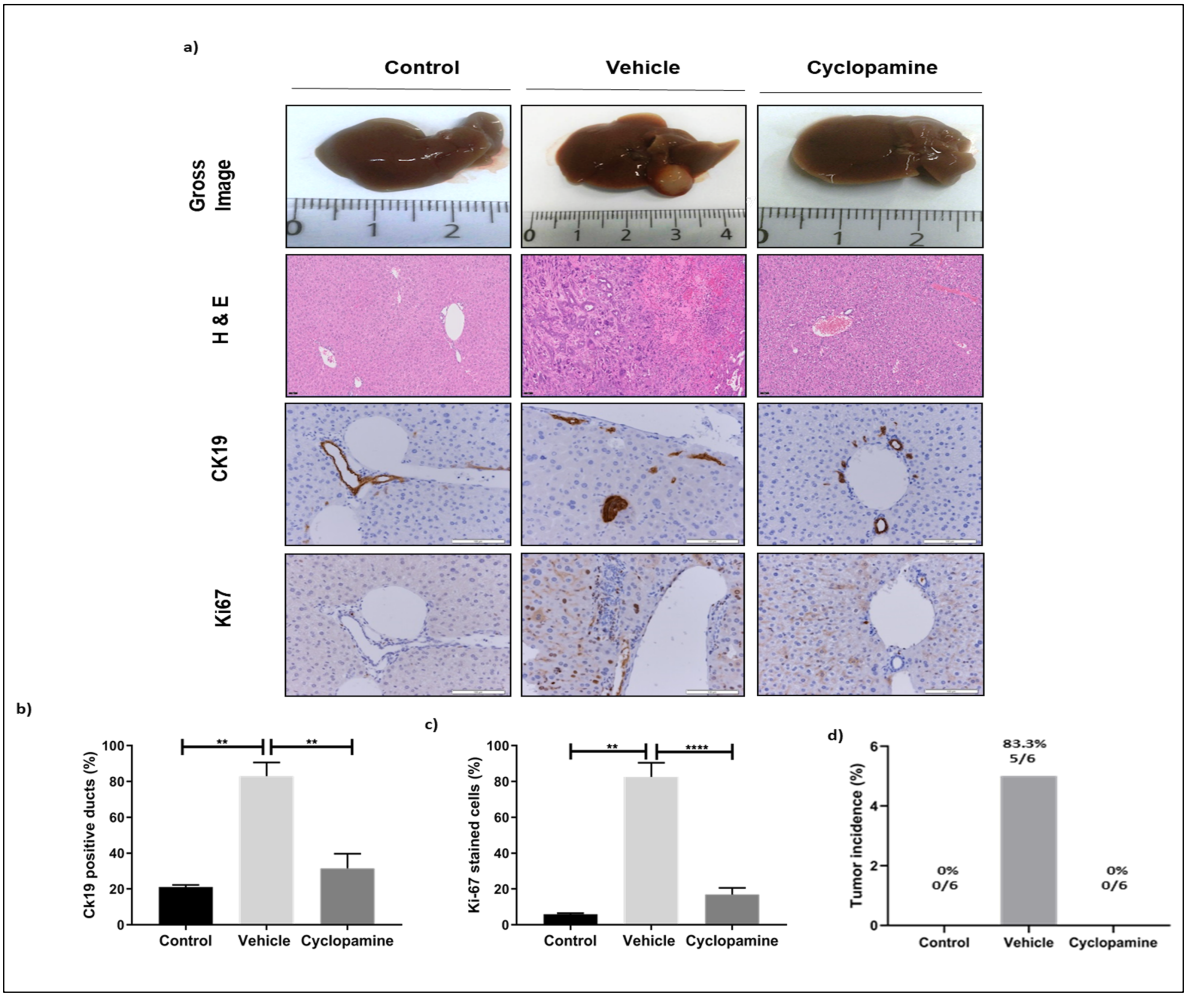


Figure 3.6 Cyclopamine inhibits tumorigenic potential of Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mouse. **(a)** Gross images and histological analysis (H & E staining, 50 µm IHC staining, 500 µm) of Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mouse liver from control, Vehicle and Cyclopamine treated groups **(b & c)** Quantification of IHC staining for CK19 positive ducts score and the proliferation marker Ki 67 (n=6 per group) **(d)** Tumor incidence. Data represents as mean ± SEM. $^{**}p < 0.01$; **** $p < 0.0001$.

Furthermore, an increase in the body weight of Vehicle treated group was observed in comparison to the Cyclopamine administered cohort (Fig. 3.7a). The treated mice exhibited a significantly higher total liver weight ($p < 0.0001$) and splenomegaly ($p < 0.001$) than the Cyclopamine treated group ($p < 0.001$), indicating increased CC tumor burden (Fig.3.7 b, c). Consistent with these results, a significant increase in ALT and AST ($p < 0.01$, $p < 0.0001$) activities was observed in Vehicle treated mice as compared to the Cyclopamine treated cohort ($p < 0.05$), indicative of compromised liver function (Fig.3.7d, e).

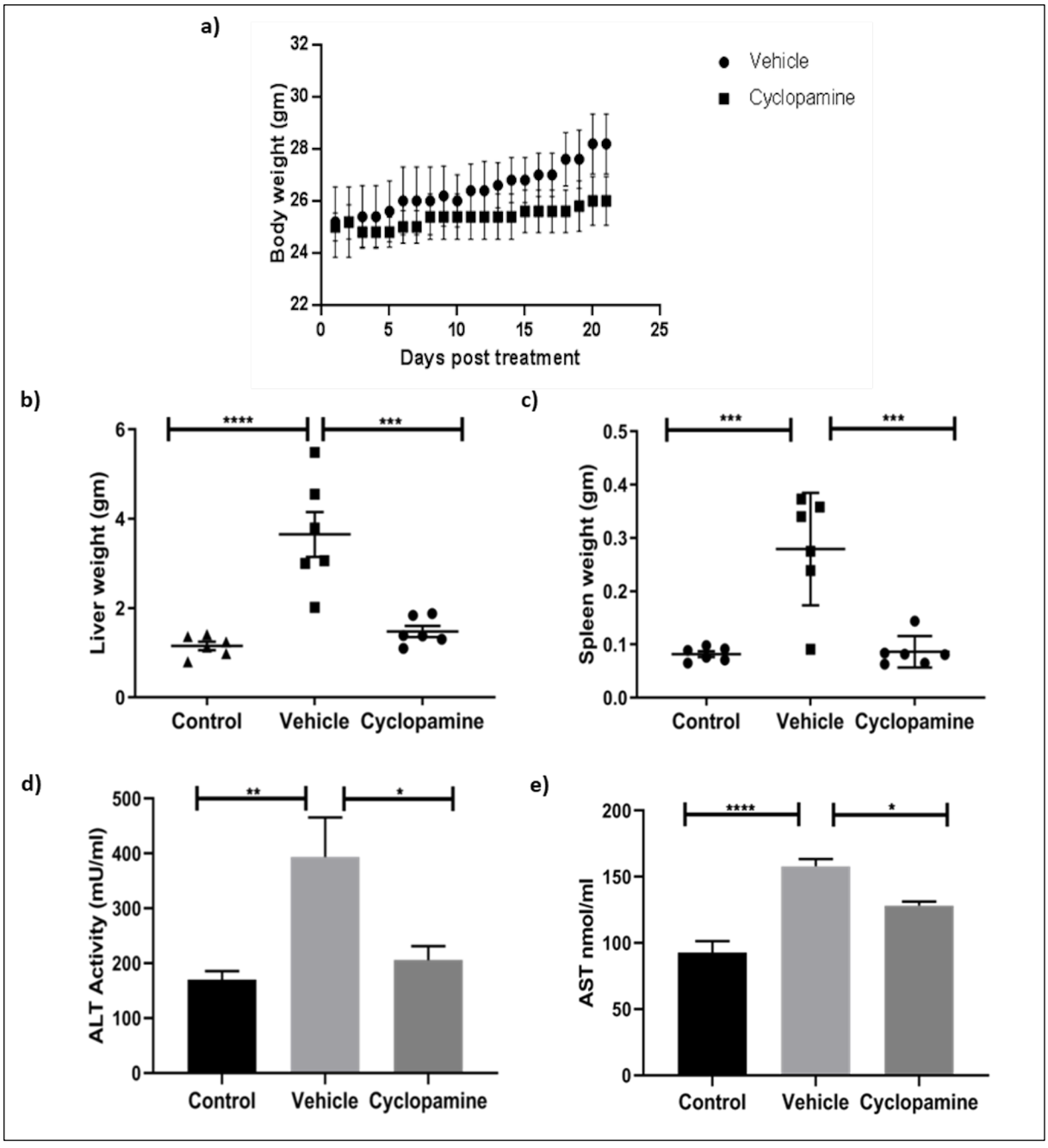


Figure 3.7 Cyclopamine restores abnormal physiological parameters in Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mouse. (a) Body weight **(b** & **c)** Liver & spleen weight **(d** & **e)** ALT & AST activity in liver (n=6 per group). Data represents as mean ± SEM. ∗p < 0.05; ∗∗∗ p < 0.001; ∗∗∗∗ p < 0.0001.

3.2.2. Cyclopamine inhibits Hedgehog signaling pathway in Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mouse model

To check the specificity and modulatory effect of the SMO inhibitor Cyclopamine towards Hh signaling pathway, we assessed the expression level of SHH and SMO, and GLI1 by Western blot and immunohistochemistry respectively. The SHH protein expression was significantly increased in Vehicle ($p < 0.001$) and Cyclopamine ($p < 0.05$) treated group as compared to the control, whereas no significant difference was observed in between Vehicle and Cyclopamine treated cohort. Furthermore, treatment with Cyclopamine significantly suppressed the protein expression of SMO ($p < 0.005$) and GLI1, whereas their expression was significantly upregulated in the Vehicle treated group, suggesting that SMO and GLI1 are involved in the Hedgehog pathway activation (Fig. 3.8a, b, c).

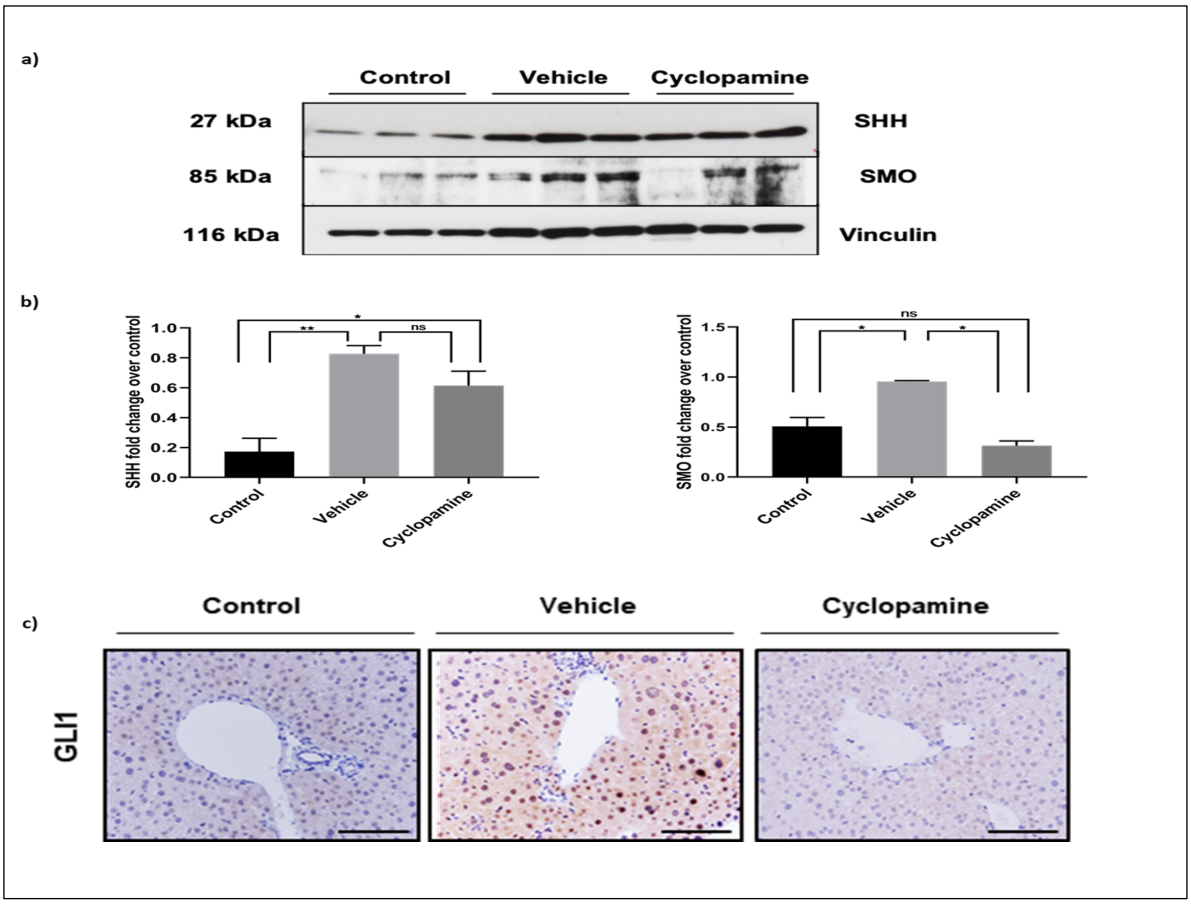
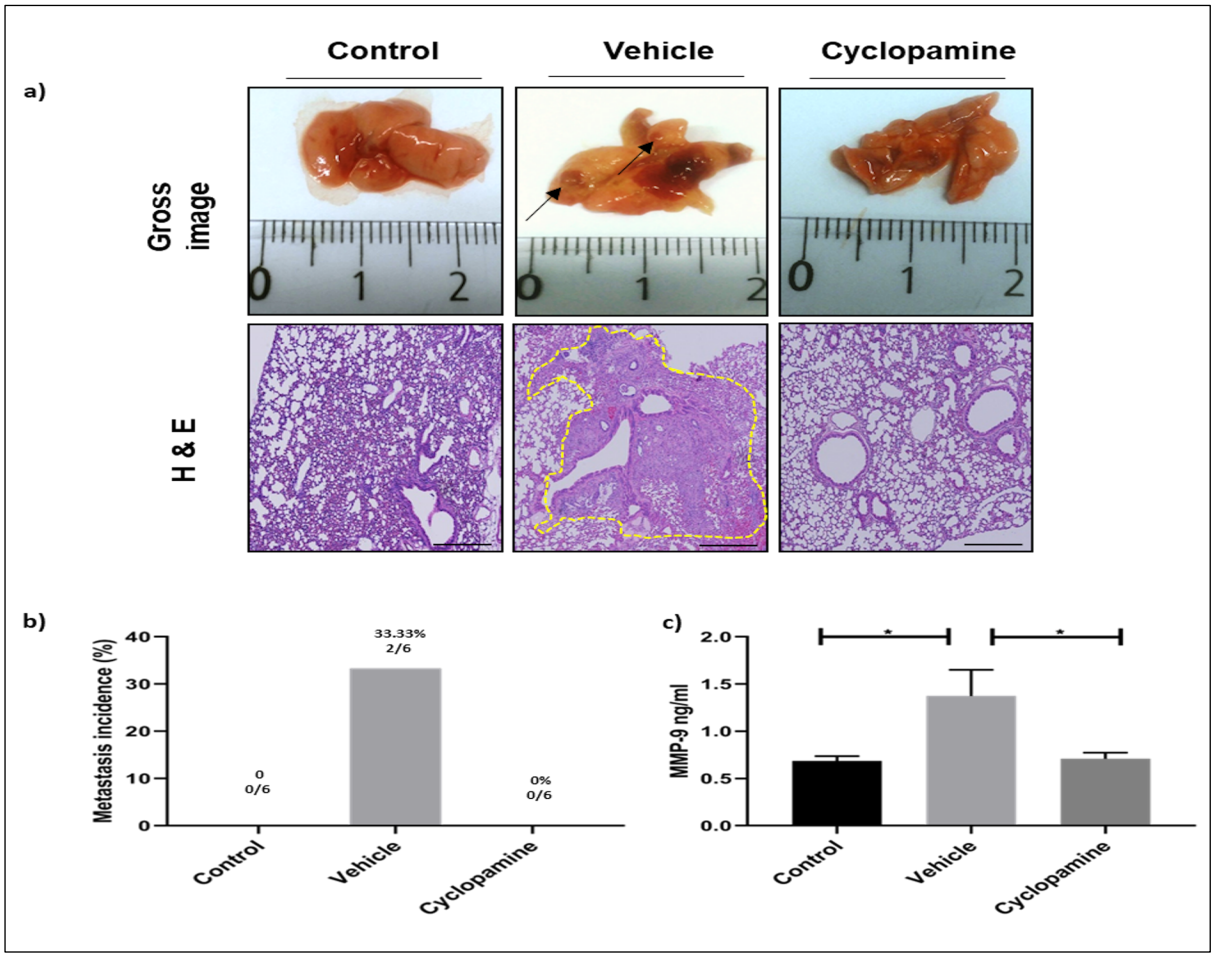


Figure 3.8 Cyclopamine attenuates SHH pathway activation. (a) Western blot analysis of SHH and SMO expression in liver tissue from control, Vehicle and Cyclopamine treated groups **(b)** Densitometric quantification of SHH and SMO (n=3 per group) **(c)** Immunohistochemical analysis of GLI1 nuclear translocation in liver tissues (100 µm in x20) (n=3 per group). Data represents as mean ± SEM. ∗p < 0.05; ∗∗p < 0.01.

3.2.3. Hedgehog signaling is critical for CC progression and metastasis

Growth of CC following metastasis to the distant organs leads to an irrepressible disease (160, 161). Also, patients with pulmonary metastasis often have poor prognosis and a short survival rate. Therefore, we assessed the effect of Hedgehog pathway inhibition on lung metastasis. Lungs were harvested after completion of the treatment regime, following which pathological examination of lung metastases was done macroscopically. The representative images of the lungs are shown in (figure 3.9 a). The Vehicle treated group had a metastatic incidence of 32.33%, whereas as Cyclopamine completely inhibited lung metastasis with 0% metastatic incidence rate (Fig 3.9 b).

MMPs play a pivotal role in controlling cell migration and invasion of tumour cells (162, 163). To examine the efficacy of Cyclopaminne treatment on tumour cell invasion, we measured MMP-9 levels in the serum of Cyclopamine and Vehicle treated mice. MMP-9 levels were significantly high in Vehicle treated group ($p < 0.05$), whereas no change was observed in MMP-9 level in Cyclopamine-administered group as compared with the control group (Fig. 3.9c).



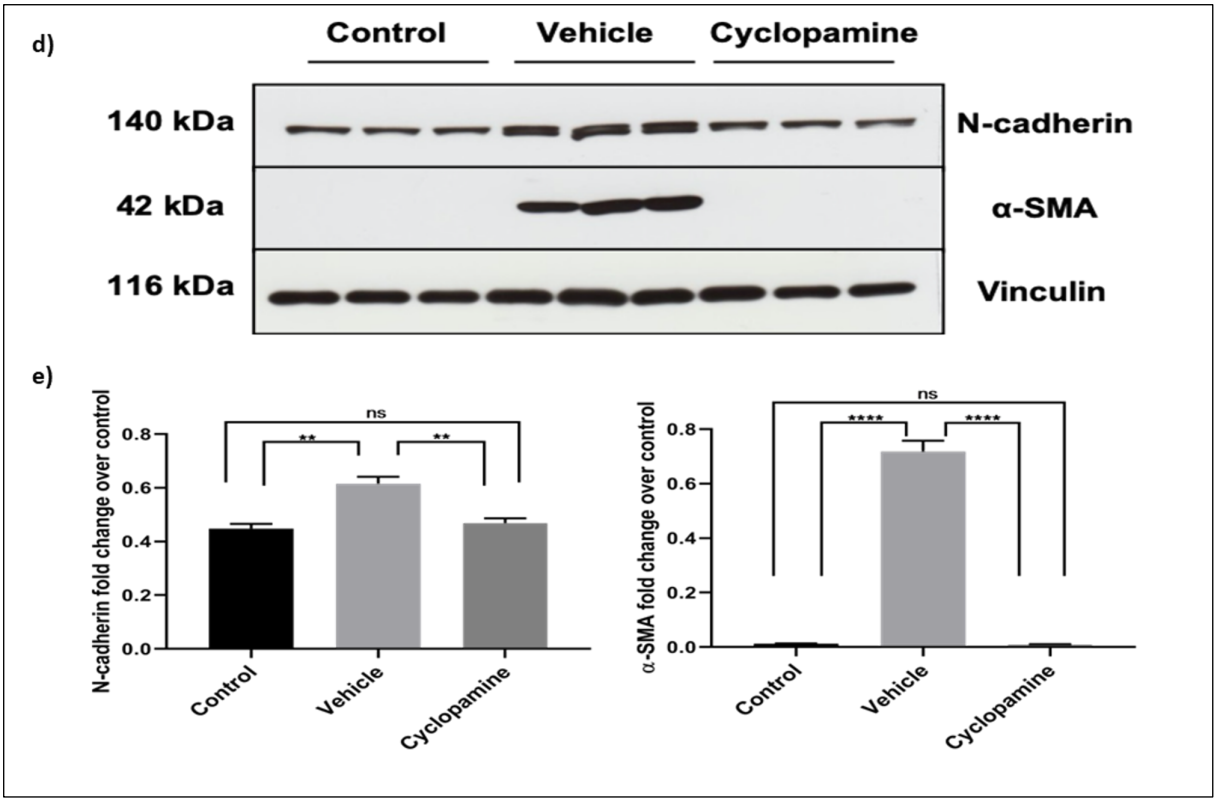


Figure 3.9 Hedgehog pathway inhibition suppresses lung metastasis. (a) Representative gross images and H&E stained sections of mouse lung from control; Vehicle and Cyclopamine treated groups (500 µm in x4) **(b)** Metastasis incidence **(c)** Serum MMP-9 protein level (n=6 per group) **(d)** Western blot analysis of N-cadherin and α-SMA in liver tissues **(e)** Densitometric quantification of target proteins (n=3 per group). Data represents as mean ± SEM. ∗p < 0.05; ∗∗p < 0.01; ∗∗∗ p < 0.001; ∗∗∗∗ p < 0.0001.

EMT plays an important role in inducing invasion of tumor cells to secondary sites due to loss of cell-cell adhesion. To investigate the effect of Hedgehog pathway inhibition on EMT, the liver tissue from Vehicle and Cyclopamine administered mice cohorts were assessed for changes in N-cadherin and α-SMA (mesenchymal protein markers) expression level. Hedgehog pathway inhibition by Cyclopamine led to a significant reduction in the expression of both mesenchymal markers ($p < 0.01$) evidencing *in-vivo* EMT inhibition (Fig. 3.9d, e)

3.2.4. Hedgehog pathway is activated in tumours of Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mice

The results of the prevention study, in which Cyclopmaine was given every alternate day for 7 weeks starting from 14 weeks of age, revealed 100% inhibition of CC development by 20 weeks of age in Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mice. For further characterization of CC tumours, liver tissue was excised and stained for tumour- specific markers. CK19, a cholangiocyte marker, was found to be increased in the CC tumour sections. Further, high positive staining

for the proliferation marker Ki-67 indicated that the tumours were highly proliferating (Fig. 3.10).

To implicate the role of Hedgehog pathway activation in cholangiocarcinogenesis, tumour sections were immunoassayed for SHH, SMO and GLI1 proteins. An increase in the expression of SHH, SMO and GLI1 was observed by IHC staining of the tumour sections (Fig. 3.10), thus confirming the role of Hedgehog pathway in the induction and development of CC in Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mice.

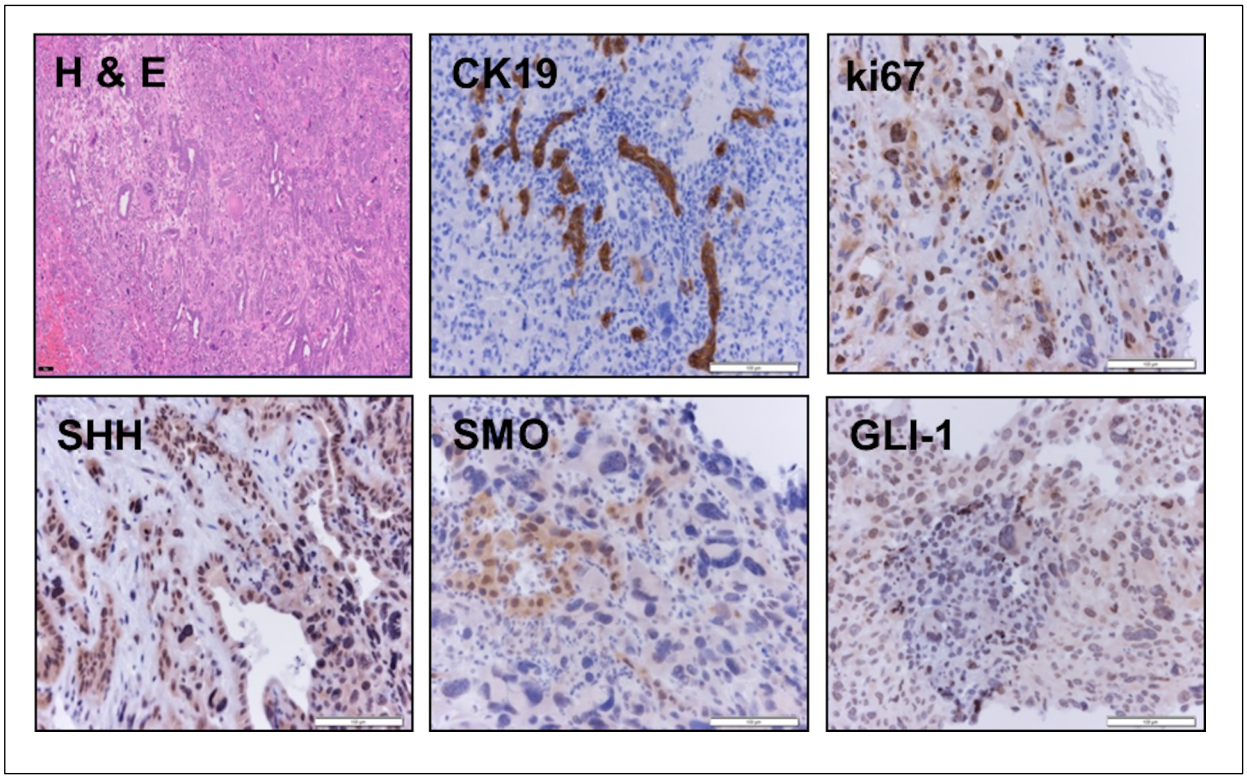


Figure 3.10 Characterization of Hedgehog signaling pathway in liver derived tumor. H&E staining (50 µm) of tumor; Immunohistochemical identification of CK19; Ki67; SHH, SMO, GLI1 (100 µm in x20)**.**

3.2.5. Hedgehog pathway inhibition prolongs the survival of Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mice

Based on the efficacy of Cyclopamine towards tumour inhibition in the short- term prevention study, a long term Cyclopamine treatment study was conducted to evaluate its effect on tumour regression. Animal survival was assessed in order to test the effect of Cyclopamine as an extended treatment. Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mice were administered Vehicle or Cyclopamine (10mg/kg body weight) every alternate day starting from 14 weeks of age and monitored for signs of morbidity and mortality. The survival time for Vehicle treated group

was 162.5 days, while it was 183 days with a median survival time of 20.5 days for Cyclopamine- administered mice ($p < 0.01$), demonstrating significant extension of survival. This *in-vivo* experimental data shows that Hedgehog pathway inhibition suppresses CC progression and improves the overall survival of mice (Fig. 3.11).

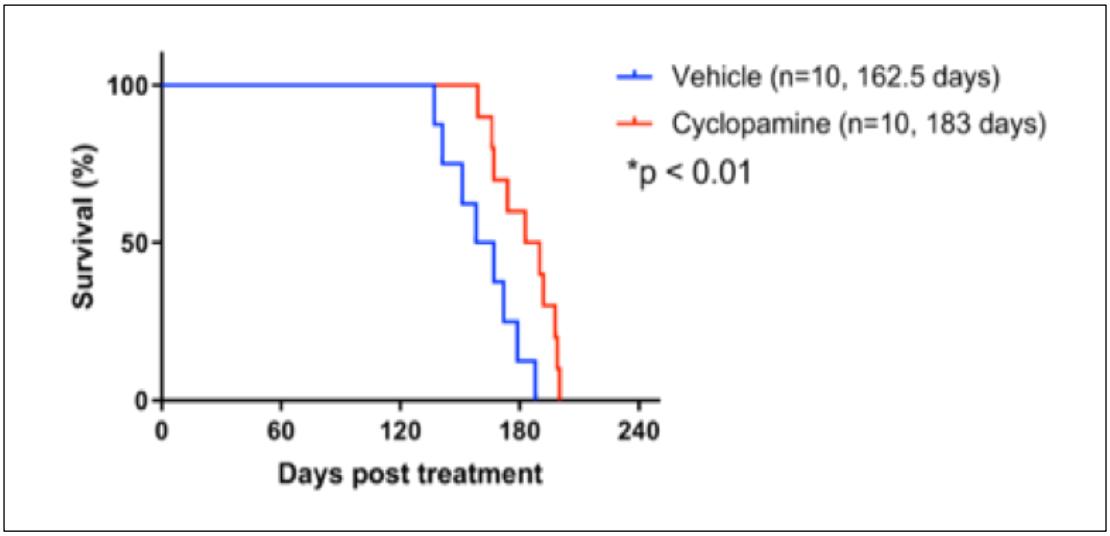


Figure 3.11 Cyclopamine prolongs the survival of tumor bearing Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mice (n=10 per group).

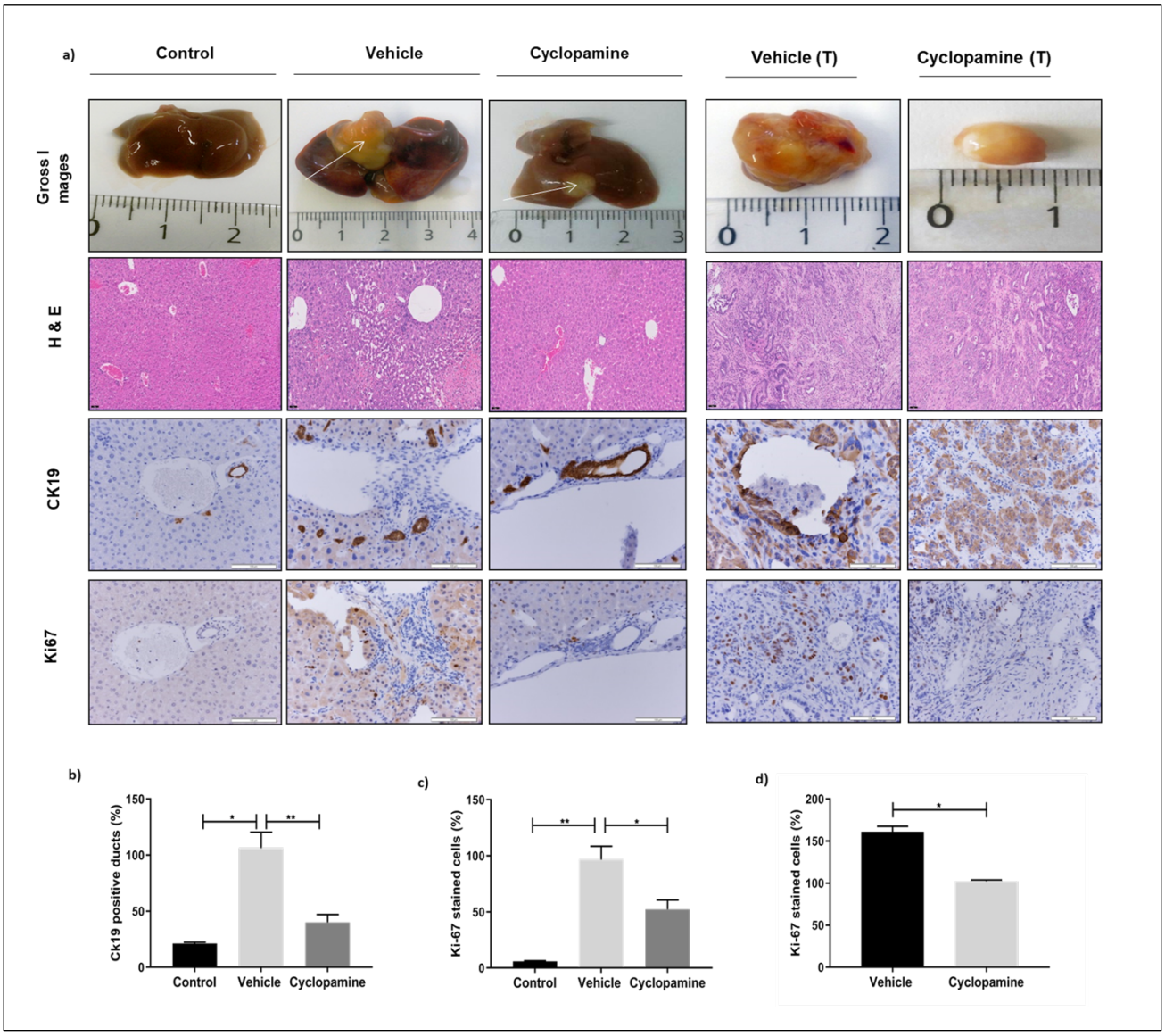
3.2.6. Long-term efficacy of Cyclopamine treatment

To understand the mechanism underlying the enhanced survival observed in long term Cyclopamine treated mice, we analysed various parameters related to the Hedgehog pathway-mediated effects on tumorigenesis. Gross morphological examination of the moribund mice showed the presence of tumours in the liver of both groups of mice. The histological analysis of liver and tumour tissue showed that Vehicle as well as Cyclopamine treated group appeared almost similar (Fig.3.12a). Additionally, all tumour cells were positive for CK19. CK19 positive ducts were quantified in Vehicle and Cyclopamine treated mouse liver tissues and the results showed that larger area was stained positive for CK19 protein in Vehicle treated groups ($p < 0.01$) than Cyclopamine administered group (Fig. 3.12b).

We next looked for histopathologic signs of tumour regression like necrosis which is associated with inflammatory reaction in tumours. Furthermore, IHC staining for Ki-67, a proliferation marker, showed that cholangiocyte proliferation was significantly decreased in Cyclopamine administered mice ($p < 0.05$), in comparison to the Vehicle treated cohort (Fig. 3.12c). We also

determined the effect of Cyclopamine in liver derived tumours of both the groups and interestingly found a significant reduction of Ki-67 staining ($p < 0.05$) in Cyclopamine treated mice cohorts as compared with the Vehicle group.

Tumour incidence rate was found to be 100% in both Vehicle and Cyclopamine administered group (Fig. 3.12e), although there was an increase in percent survival in Cyclopamine treated group (Fig. 3.11). Total liver weight was measured as an indicator of tumour burden. Cyclopamine treated mice showed a significant reduction in total liver weight ($p < 0.05$) as compared to the Vehicle treated mice cohorts. The Vehicle treated mice developed enlarged spleens and the spleen weight was found to be significantly increased in these mice. On the other hand, Cyclopamine treated mice had normal spleens with the spleen weight significantly lower ($p < 0.05$) (Fig.3.12f, g) than the Vehicle treated mice, suggesting that Cyclopamine could



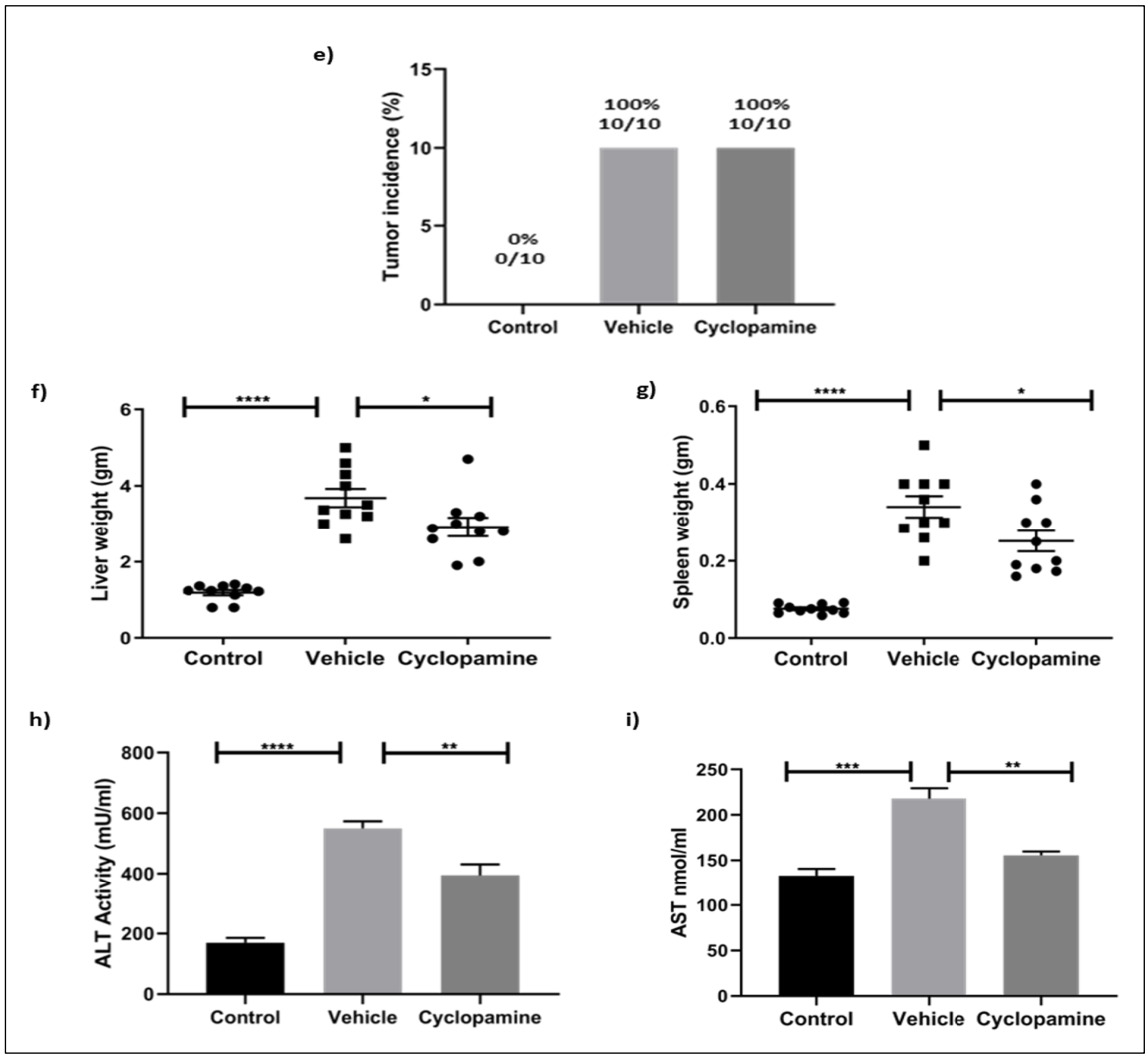


Figure 3.12 Cyclopamine attenuates tumorogenic potential of Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mouse in long term treatment. (**a**) Gross images and histological (H & E staining, 50 µm; IHC staining, 100 µm in x20) analysis of Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mouse liver and tumor tissue from control, Vehicle and Cyclopamine treated groups (**b** & **c**) Quantification of IHC staining for Ck19 positive ducts and the proliferation marker Ki67 (**d**) Difference in Ki67 staining of tumor sections of both Vehicle and Cyclopamine groups (**e**) Tumor incidence (**f** & **g**) Liver & spleen weight (n=10 per group) (**h** & **i**) ALT & AST activity in serum (n=6 per group). Data represents as mean ± SEM. ∗p < 0.05; ∗∗p < 0.01; ∗∗∗ p < 0.001; ∗∗∗∗ p < 0.0001.

have an impact on the host- tumour interactions. Additionally, the effect of long-term Cyclopamine treatment on the functionality of liver was assessed by measuring ALT and AST liver enzyme activities in the serum long term Cyclopamine treated mice had significantly lower ALT and AST enzyme activities ($p < 0.01$) as compared to the Vehicle treated cohort (Fig. 3.12h,i), indicating that Cyclopmanine reduced cancer- related liver dysfunction.

Additionally, we assessed the effect of Hh pathway inhibition on lung metastasis. The Vehicle treated group had a metastatic incidence of 60%, whereas as Cyclopamine suppressed the lung

metastasis with 40% metastatic incidence rate. MMP-9 levels were also found significantly decreased in Cyclopamine-administered group ($p < 0.05$). Cyclopamine treatment correspondingly led to a significant reduction in the protein expression of N-cadherin ($p < 0.01$) and α-SMA ($p < 0.05$), evidencing in-vivo EMT inhibition (Fig. 3.13).

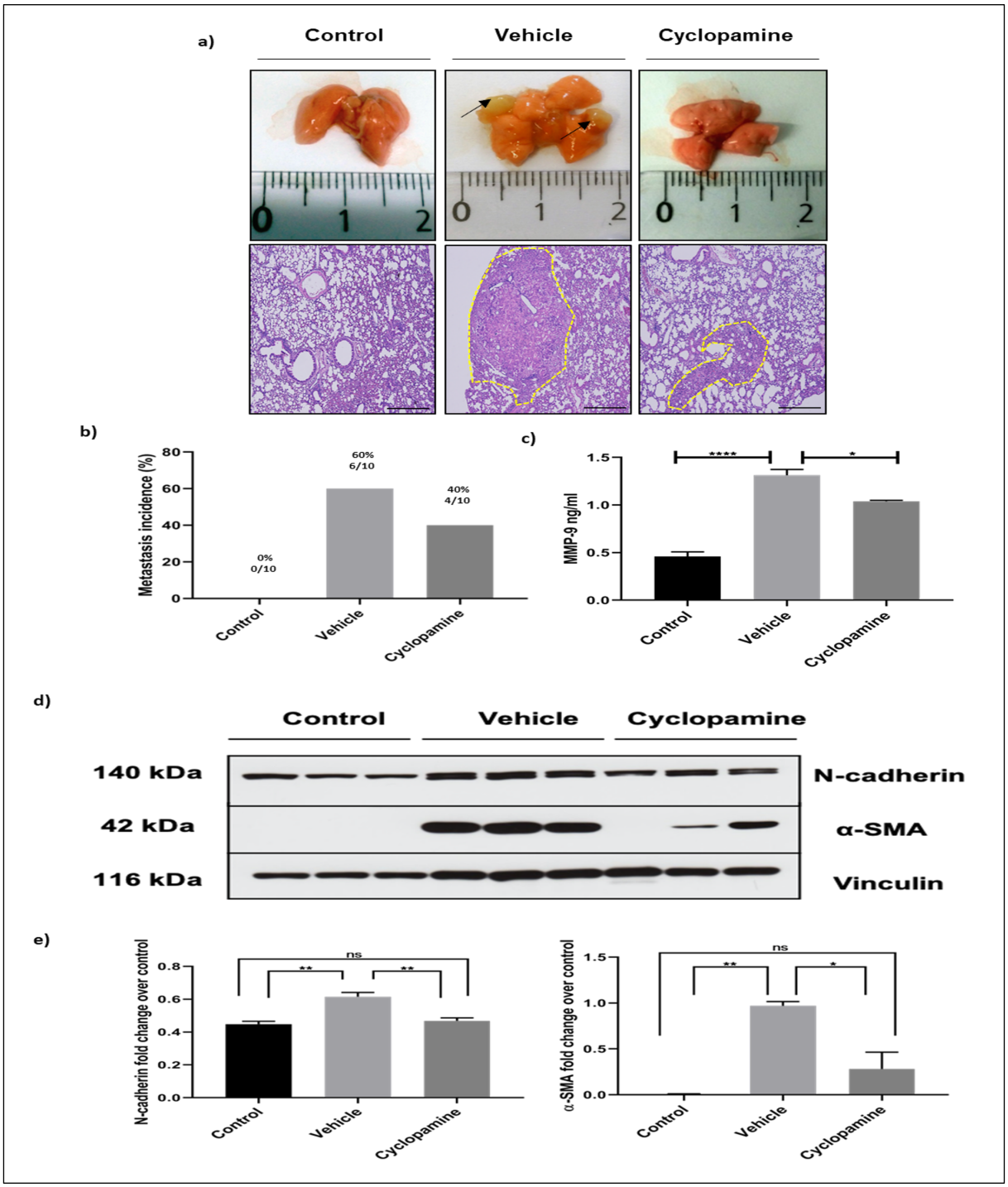


Figure 3.13: Hedgehog pathway inhibition reduces lung metastasis. (**a**) Representative images of gross lung morphology and H&E stained sections of mouse lung from control, Vehicle and Cyclopamine treated groups (500 µm in x4) (**b**) Metastasis incidence (n=10 per group) (**c**) MMP-9 protein level in serum measured by ELISA (n=3 per group) (**d**) Western blot analysis of N-cadherin and α-SMA in liver tissues (**e**) Densitometric quantification of N-cadherin and α-SMA (n=3 per group). Data represents as mean ± SEM. ∗$p < 0.05$; ∗∗$p < 0.01$; ∗∗∗ $p < 0.001$; ∗∗∗∗ $p < 0.0001$

3.2.7. Hedgehog pathway inhibition suppresses cancer stem cell transcription factors and CD133 expression

Transcription factors such as OCT4, NANOG, and SOX2 are known to bind to the particular DNA sequences at the promotor or enhancer regions of their target genes and contribute in cancer progression (164-166). Based on our previous *in-vivo* study in Alb-Cre/LSL-$KRAS^{G12D}/p53^{L/L}$ mice model, where increased expression of cancer stem cell transcription factors was observed during CC development, we tested the efficacy of short-term and long-term Cyclopamine treatment on their expression using Western blot. All three transcription factors, together with CD133, were found significantly upregulated in both Vehicle and Cyclopamine treated mice cohorts compared to the control group in the short-term treatment ($p < 0.01$) (Fig. 3.14 a, b).

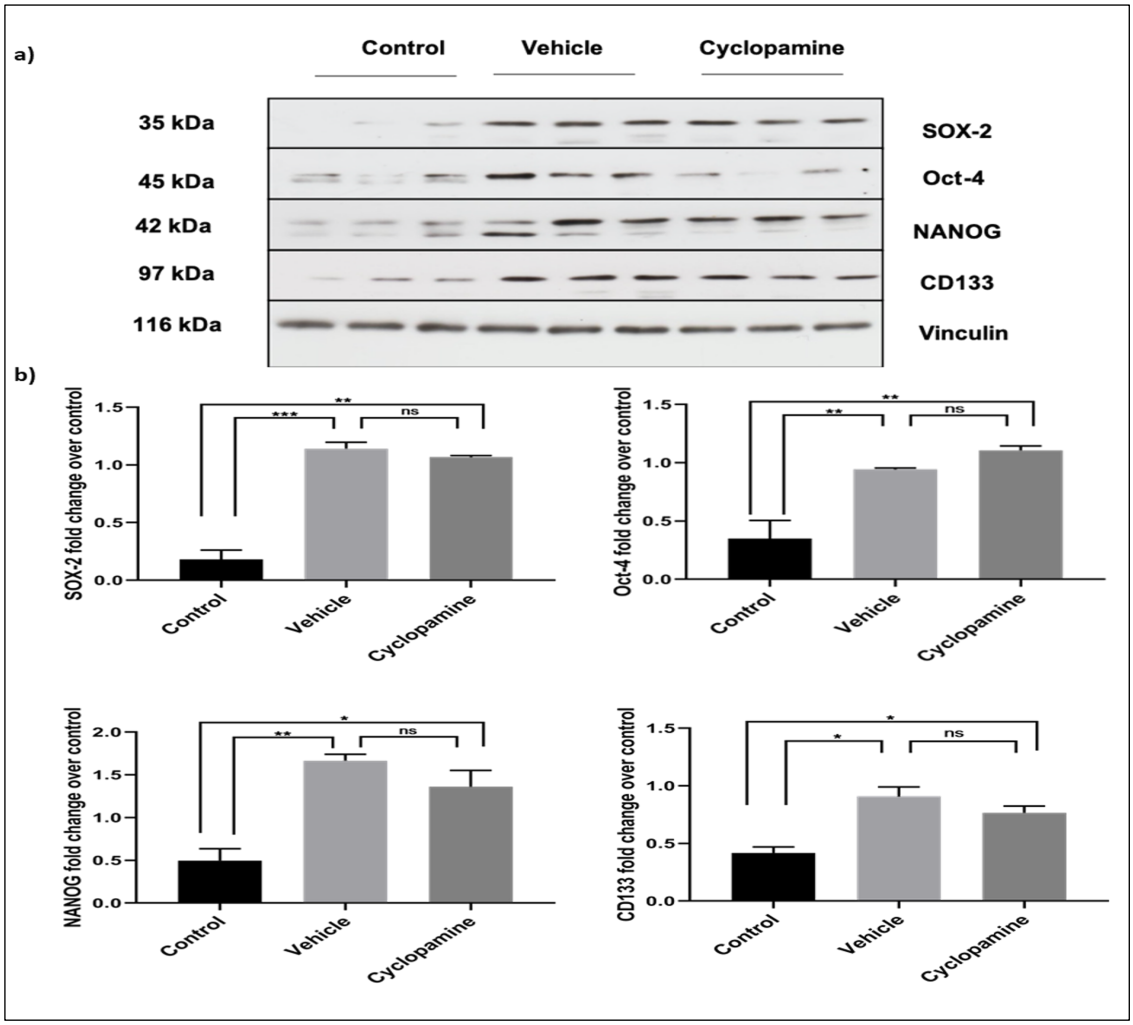


Figure: 3.14: Cyclopamine has no effect on cancer stemness in a short-term preventive treatment regime. **(a)** Protein expression of SOX2, Oct-4, NANOG, CD133 analysed by western blot in liver tissue from control, Vehicle and Cyclopamine treated groups (**b**) Densitometric quantification of target proteins (n=3 per group). Data represents as mean ± SEM. $*p < 0.05$; $**p < 0.01$; $*** p < 0.001$.

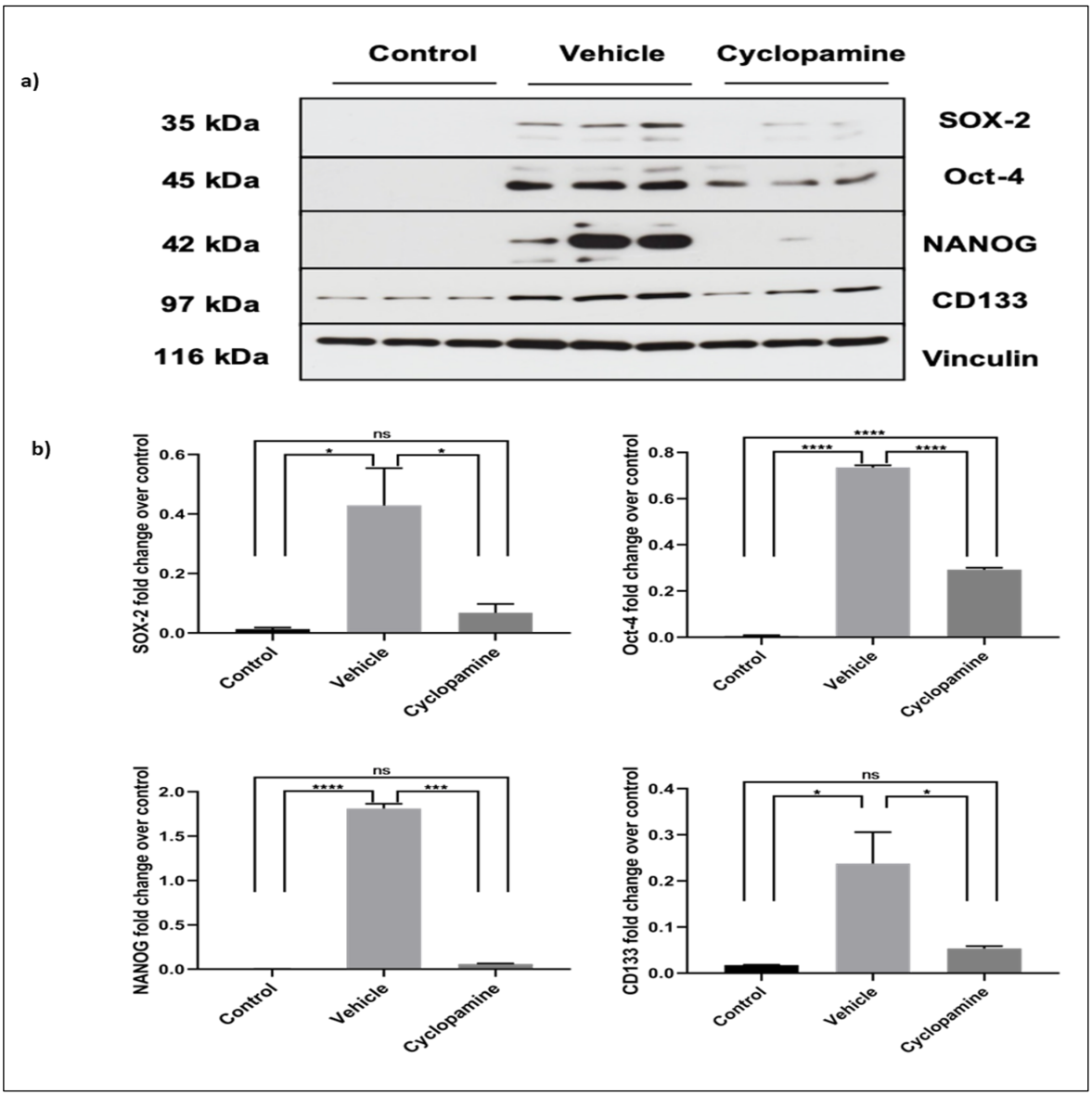


Figure 3.15: Cyclopamine abolishes cancer stemness signature in the long-term treatment study. **(a)** Western blot anaylsis of SOX2, Oct-4, NANOG, and CD133 protein expression in liver tissue of control, Vehicle group and Cyclopamine treated groups (**b**) Densitometric quantification of target proteins (n=3 per group). Data represents as mean ± SEM. ∗p < 0.05; ∗∗p < 0.01; ∗∗∗ p < 0.001; ∗∗∗∗ p < 0.0001

Moreover, no difference in their protein expression between Vehicle and Cyclopamine group was observed in the short-term treatment strategy, whereas significant reduction ($p < 0.05$; $p < 0.001$; $p < 0.0001$) in their expression was observed in the long-term treatment mice cohorts (Fig. 3.15a, b). These results suggest that Cyclopamine has a modulatory effect on the expression of cancer stem cell transcription factors in the long-term treatment regime, while it does not affect their expression in the short-term treatment.

3.3. To study the involvement of Hedgehog signaling pathway in tumor induction in xenograft models of implanted immune- sorted cells

3.3.1. Tumorigenic potential of human CC CD133$^+$ stem cells in NOD/SCID mice

A small population of distinct, highly tumorigenic CC cells are accountable for tumour formation (167). In our study, human CC cell line, HUCCT-1, was used to evaluate the tumorogenic potential of CD133$^+$, CD133$^-$CD44$^-$EpCAM$^-$ or unsorted cells in xenograft tumor models. Sorted cells were resuspended in a solution of Matrigel and RPMI-1640 media (1:1) and injected subcutaneously into the flanks of male and female NOD/SCID mice, and evaluation of tumour incidence and tumour volume was done. Tumour growth was monitored for 5 weeks, following which mice were sacrificed for histopathological examination of tumor development. HUCCT-1 cells have 1-2 % of CD133$^+$ population, whereas 99% of the cells are CD44$^+$ and EpCAM$^+$ (Fig. 3.16).

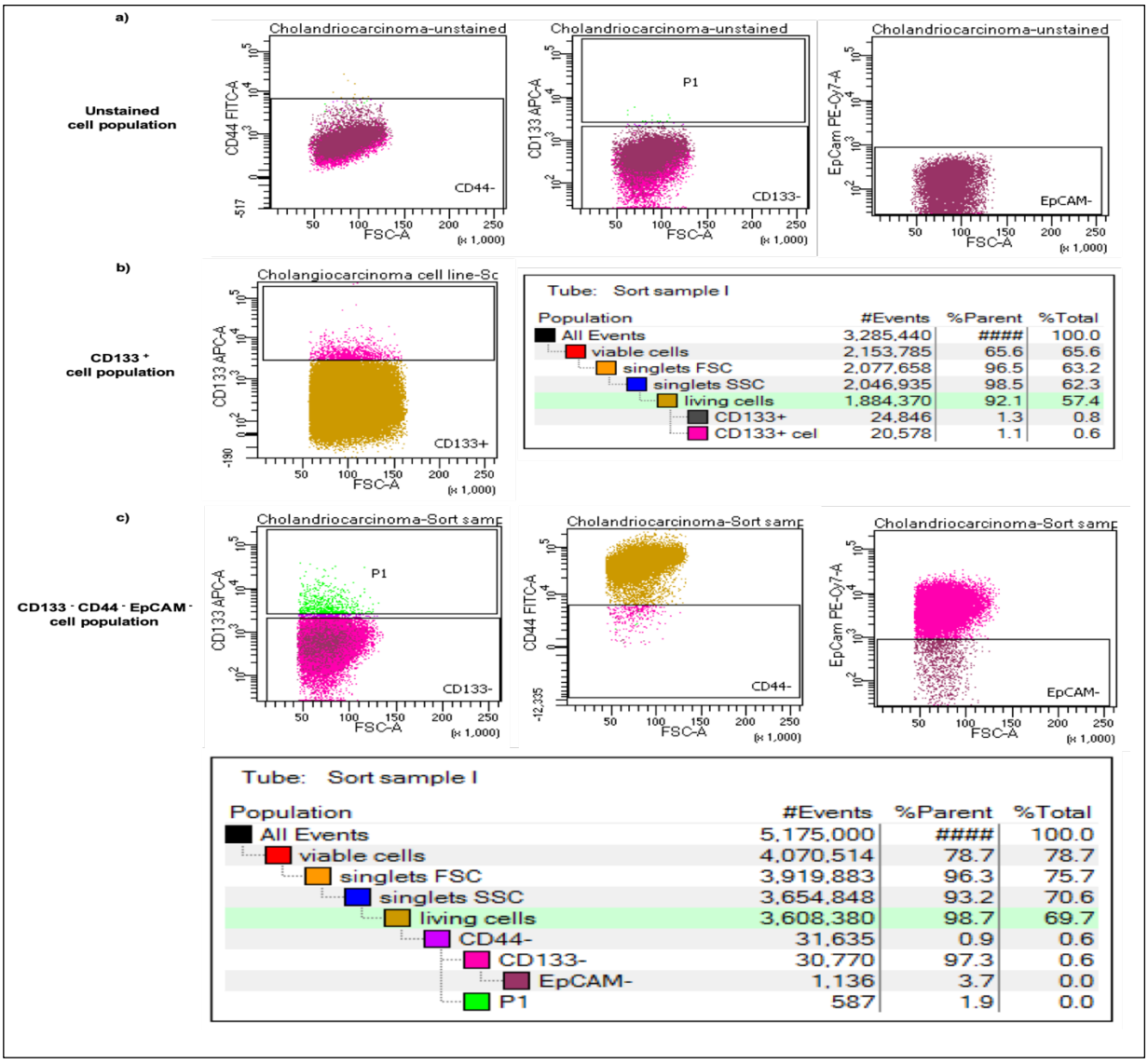
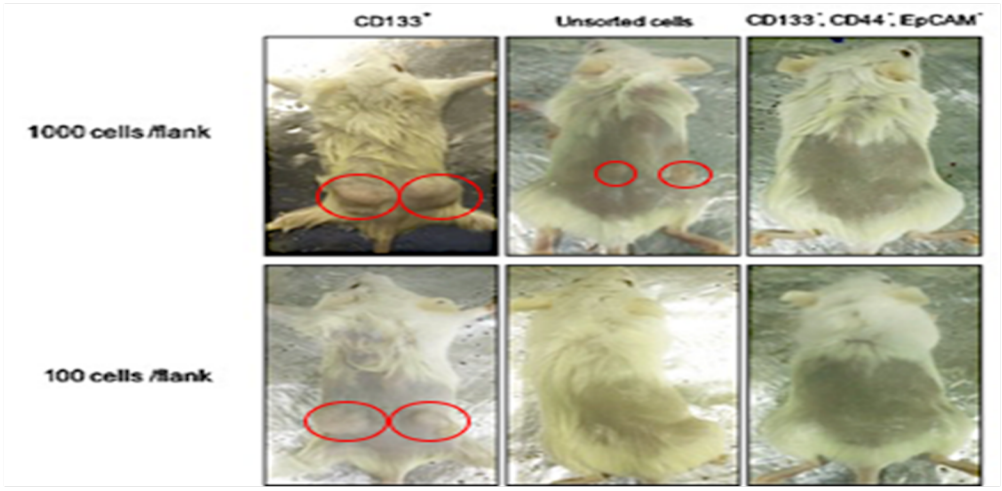


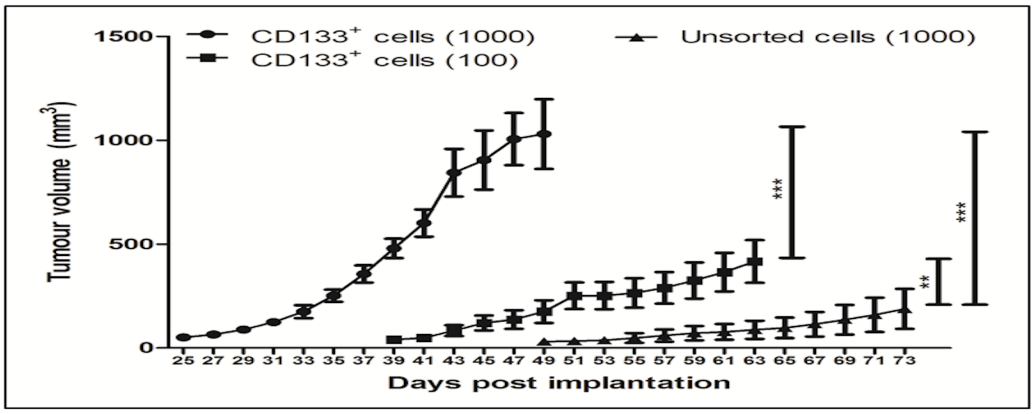
Figure 3.16: Isolation of CD133$^+$ and CD133$^-$ CD44$^-$EpCAM$^-$ cell population from HUCCT-1 cell line. Representative dot plot with cell events of **(a)** Unstained cells **(b)** CD133$^+$cells and **(c)** CD133$^-$ CD44$^-$EpCAM$^-$ cells.

The sorted cells were injected at two different cell densities of 100 or 1000/100µl into the mouse flank to compare the tumour take rates. In mice injected with $CD133^+$ cells at a density of 1000 cells, tumour node appeared after 3.5 weeks, whereas it appeared 2 weeks later at 5.5 weeks in mice injected with 100 $CD133^+$ cells per flank (Fig.3.17a, b). However, tumour incidence was similar at 100 % in both of the groups (Fig.3.17c). For unsorted group, tumour node appeared after 7 weeks of injection of 1000 cells with a tumour incidence rate of 75%, whereas, no tumour appeared when 100 unsorted cells were injected into the flanks. Further, no tumour formation was evident when $CD133^-$ $CD44^-$ $EpCAM^-$ cells were injected at either of the cell densities (Fig. 3.17 c).

a)



b)



c)

Tumor incidence		
Groups	**100 cells/flank**	**1000 cells /flank**
$CD133^+$	12/12 (100%)	12/12(100%)
Unsorted cells	0/12 ((0%))	9/12 (75%)
$CD133^-$, $CD44^-$, $EpCAM^-$	0/12 (0%)	0/12 (0%)

Figure 3.17: Tumor forming potential of immunosorted CC cells. (a) Representative images of tumor bearing mice **(b)** Tumor volume and latency **(c)** Tumor forming ability of sorted CC cells (n=12 per group). Data represents as mean ± SEM. $**p < 0.01$; $***p < 0.001$.

3.3.2. Upregulation of Hedgehog pathway in CSC- derived xenografts

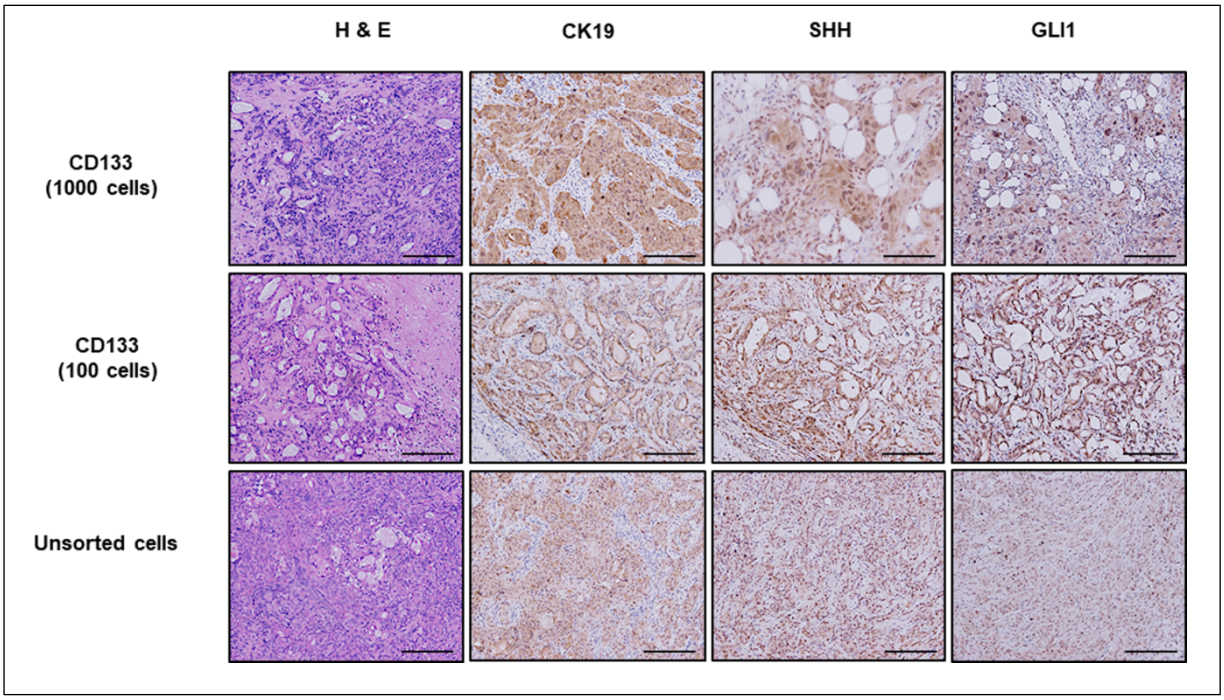


Figure 3.18: Hedgehog pathway activation in stem cells promotes tumor progression: Histological analysis of tumor: H&E stained tumor sections (200 µm in x10) and IHC stained tumor sections (200 µm in x10) for CK19, SHH & GLI1.

IHC staining for SHH and GLI1 proteins was done in the xenografted tumors samples. We found that the expression of SHH and GLI1 was markedly upregulated in $CD133^{+}$ derived tumors as compared with the tumors developed from unsorted cells (Fig. 3.18).

3.4. Hedgehog-signaling pathway components are overexpressed in patient-derived tumor samples

Histopathological examination of human TMAs prepared from CC tumors resected from German patients was done to confirm our previous findings, implicating the role of Hedgehog pathway in cholangiocarcinogenesis. The TMAs were constructed after careful pathological examination of resected tissues by the pathologists. To study Hedgehog pathway activation in patient-derived tumor samples, immunohistochemical staining for SHH and GLI1 proteins was done in the TMAs. The analysis was done according to the Immunoreactive score (IRS) of Remmele and Stegner (159), and protein expression was calculated as staining intensity multiplied by the positive cell percentage (Table: 8). An IRS of 1-4 was considered as weak, 6-8 as medium and 9-12 as strong protein expression.

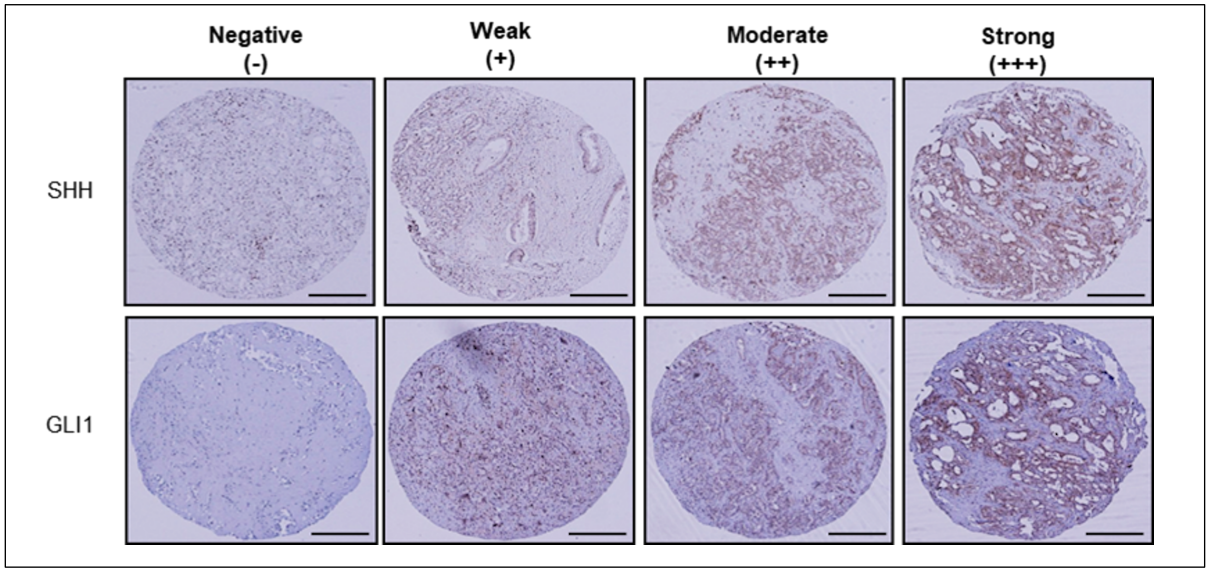


Figure 3.19: Staining patterns and intensities for SHH and GLI1 expression in human CC TMAs (500 µm in x4)

Out of the 49 CC tumour samples, 45 were positive for SHH expression, 16 were weakly stained, 20 were moderately stained and 10 were found strongly stained, whereas staining was found negative in 3 samples. Moreover, 41 samples were positive for GLI1 protein expression, out of which 25 were weakly stained, 8 were moderately stained and 8 were found strongly stained, while 8 samples were found negative for GLI1 staining (Figure 3.20a, b).

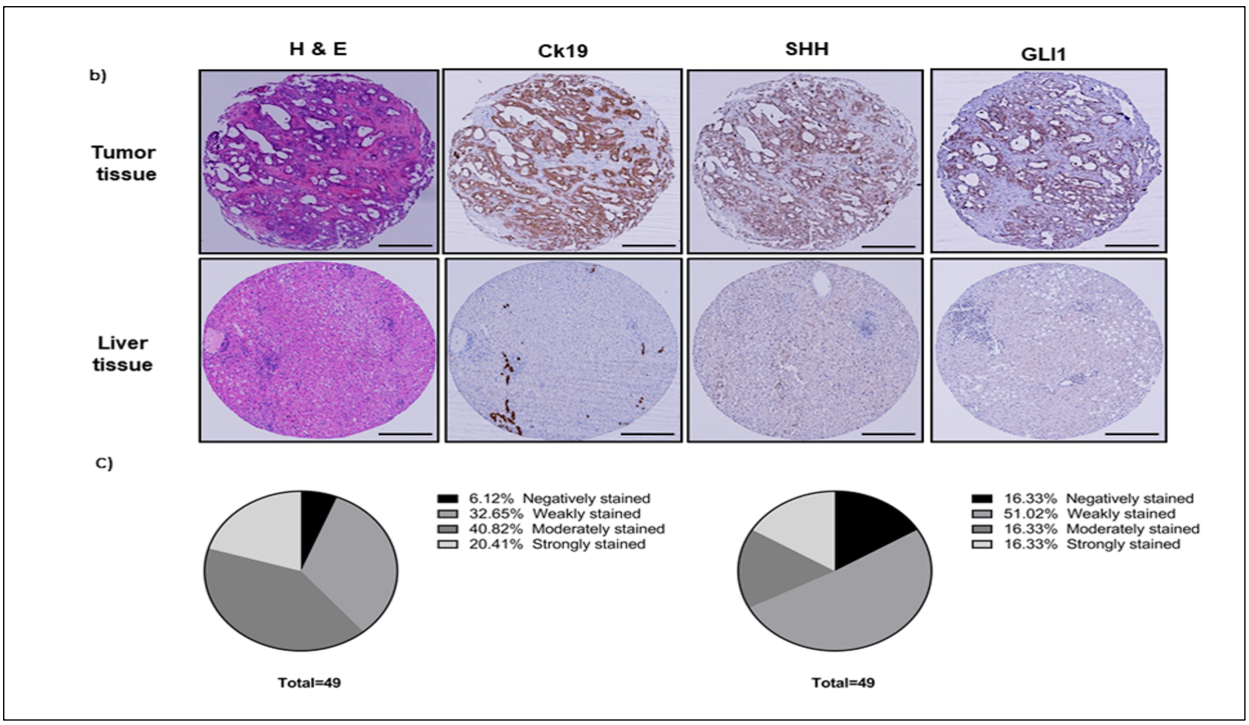


Figure 3.20 Hedgehog pathway is highly expressed in human CC tissues. (**a**) Histological analysis of tumor and normal liver (H&E stained sections and IHC for CK19, SHH and GLI1) (**b**) Percentage of SHH and GLI1 staining in human CC tissue (500 µm in x4).

3.5. To investigate the effect of hypoxia on Hedgehog pathway activation and CSCs during cholangiocarcinogenesis.

(The results have already been published: ***Reference****: 242*)

3.5.1. Hypoxia induces HIF-1α expression and activates SHH pathway

The level of HIF-1α, a transcriptional factor which responds to hypoxia, was assessed after hypoxia treatment of 3hr, 6hr, 9hr, 24hr and 48hr in TFK-1 and HUCCT- 1 cells. The protein level of HIF-1α showed a peak at 6hr of treatment ($p < 0.001$), following which its level decreased temporally till 24hr, finally returning to baseline levels at 48hr; whereas it was undetectable under normoxic conditions (Fig. 3.21a, b). Next, we determined the effect of hypoxia on SHH pathway activation by examining its effect on the transcription and translation of the SHH gene and its pathway elements SMO and GLI1. Hypoxia led to the transcriptional activation of SHH, SMO and GLI1 where the mRNA level of SHH and GLI1 was maximum at 6hr in TFK-1 ($p < 0.01$) and HUCCT-1 cells ($p < 0.05$) and mRNA level of SMO was maximum at 3hr in TFK-1 and HUCCT-1 ($p < 0.01$), which decreased thereafter in a time dependent manner (Fig. 3.22c).

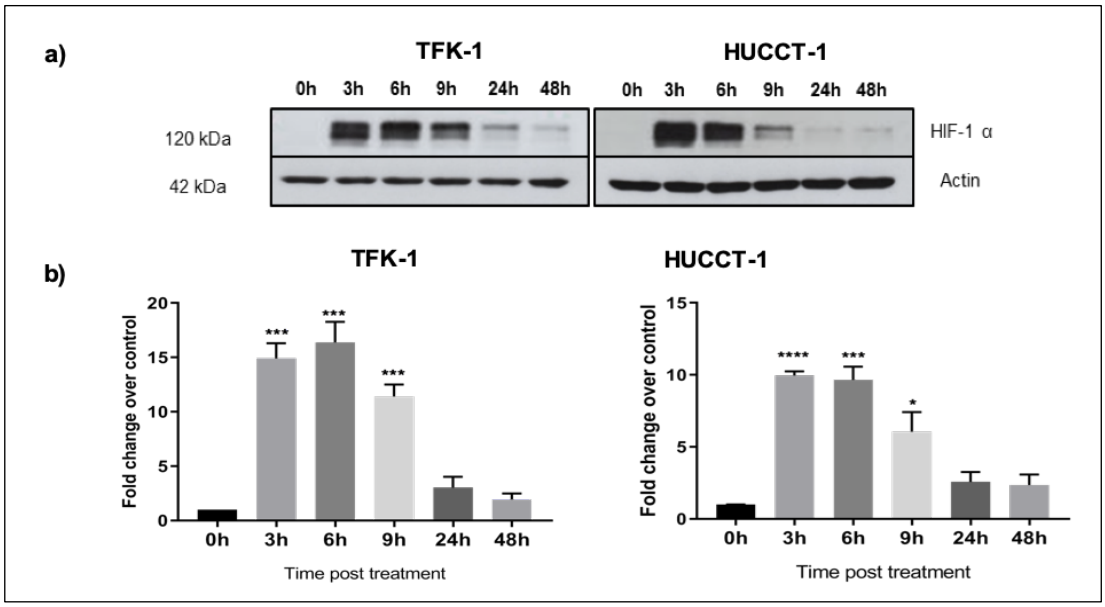


Figure 3.21: Hypoxia induces HIF-1α expression. (**a**) Western blot showing the effect of hypoxia on HIF-1α expression (**b**) Densitometric quantification of HIF-1α after normalizing with β-actin. (**242**)

The results were confirmed by immunoblotting, in which the level of SHH was found to peak at 3hr of hypoxia treatment in both TFK-1 ($p < 0.01$) and HUCCT-1 ($p < 0.05$) cell lines, followed by a decrease till 48hr, whereas the protein expression of SMO was found to peak at 9hr and 24hr ($p < 0.01$) in TFK- 1 and HUCCT-1 respectively (Fig. 3.22a, b). Additionally, we

measured the secreted level of SHH in cell culture medium of hypoxia treated cells by using ELISA, as SHH is known to be a secreted glycoprotein. SHH level was found to be increased significantly at 72hr in both TFK-1 ($p < 0.05$) and HUCCT-1 ($p < 0.05$) cells, whereas no significant change was found at early time points (Fig. 3.22d). Besides these parameters, nuclear translocation of GLI1 observed by immunofluorescence microscopy in both the cell lines at 24hr, further lent support to the previous observation of SHH pathway activation in CC under hypoxia (Fig. 3.22e).

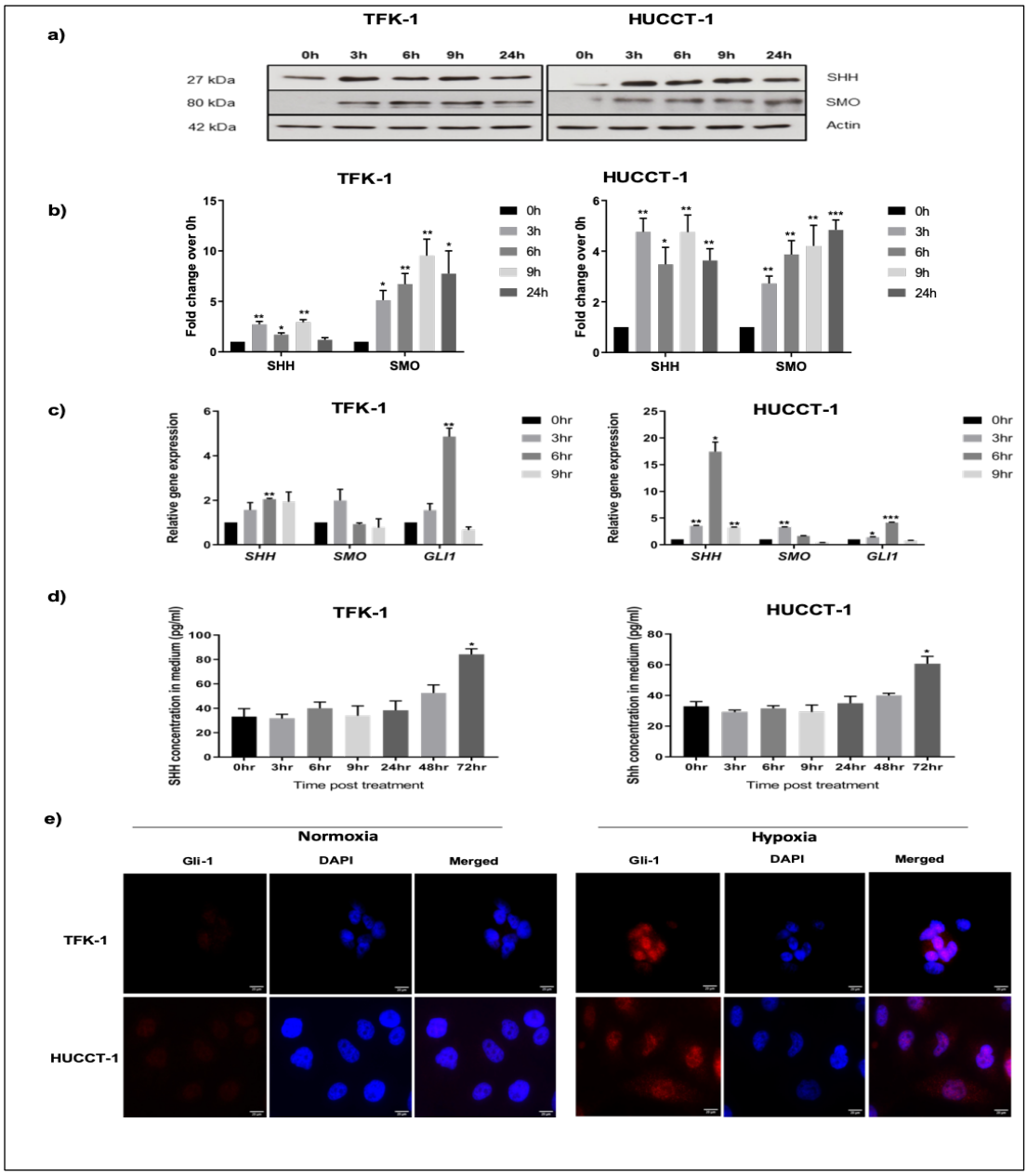


Figure 3.22: Hypoxia activates SHH pathway. (a) Western blot analysis showing the effect of hypoxia on SHH and SMO expression **(b)** Densitometric quantification of SHH and SMO after normalizing with β-actin **(c)** RT-qPCR analysis showing the effect of hypoxia on SHH,SMO and GLI1 gene expression **(d)** SHH concentration in the culture medium of TFK-1 and HUCCT-1 cells after hypoxia treatment as measured by ELISA **(e)** GLI1 induction and nuclear translocation observed at 24hr post hypoxia treatment by immunofluorescence staining (63x magnification. Scale bar 20 μm). Data represents mean ± SEM of three independent experiments. ∗$p < 0.05$; ∗∗$p < 0.01$; ∗∗∗ $p < 0.001$; ∗∗∗∗ $p < 0.0001$. (**242**)

3.5.2. Silencing of HIF-1α attenuates hypoxia-induced SHH pathway activation

To confirm the role of hypoxia in the induction and activation of the SHH pathway, we silenced HIF-1α gene expression using HIF-1α siRNA and co-treated the cells with hypoxia for 3hr, 6hr and 9hr. HIF-1α siRNA significantly suppressed HIF-1α expression as compared to the control treatment at all the time points ($p < 0.001$) (Fig. 3.23a, b). Interestingly a significant decrease in the protein expression of SHH was observed in the HIF-1 α siRNA treated cells in comparison to the untreated control ($p < 0.001$) (Fig. 3.23c, d), which subsequently led to impaired GLI1 nuclear translocation (Fig. 3.24).

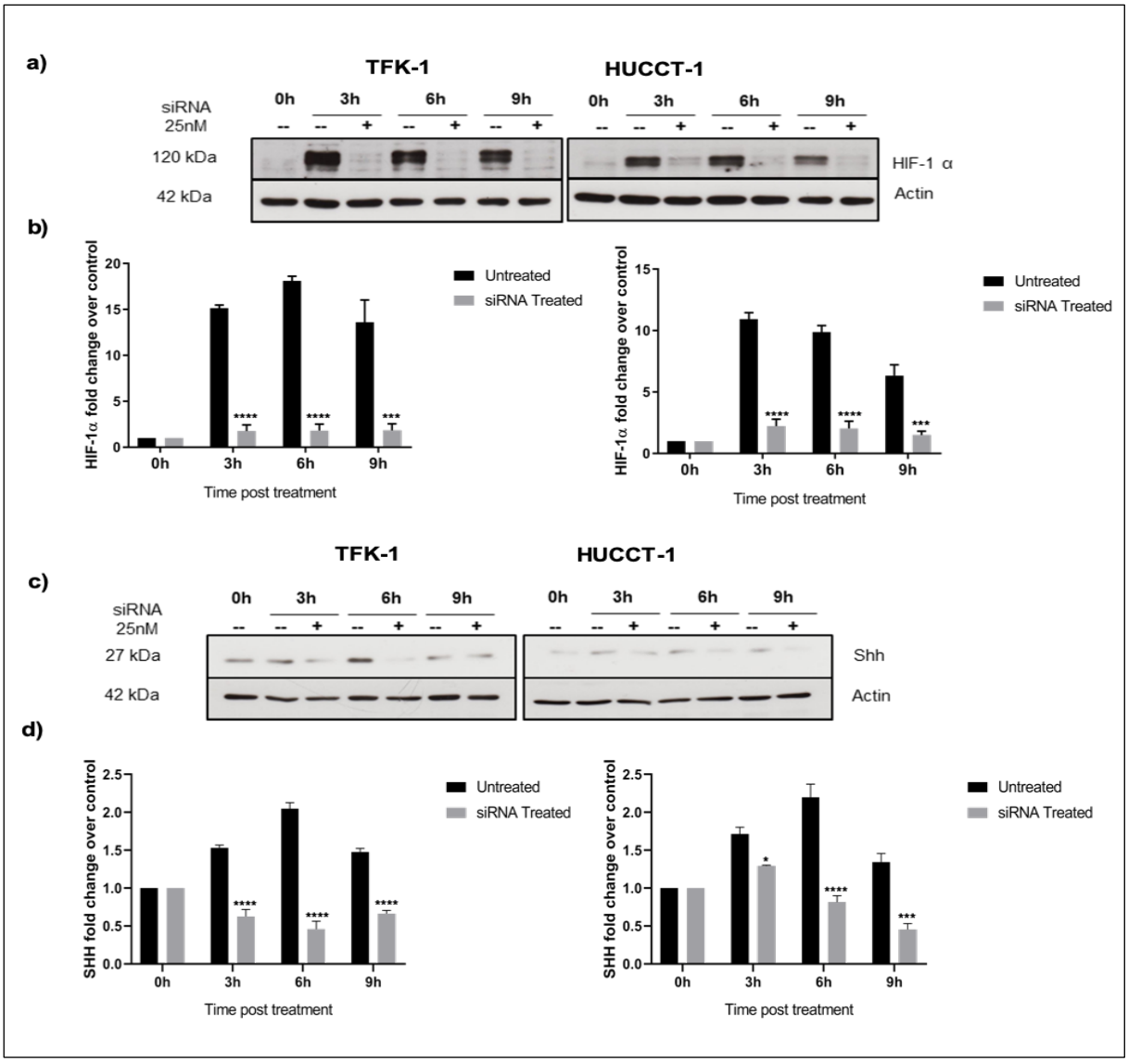


Figure 3.23: Silencing of HIF-1α attenuates hypoxia-induced SHH pathway activation. (**a**) Western blot analysis of HIF-1α expression after using HIF-1α-siRNA under hypoxic conditions (**b**) Densitometric quantification of HIF-1 α after normalizing with β-actin (**c**) Western blot analysis of SHH expression after silencing HIF-1α under hypoxia (**d**) Densitometric quantification of SHH protein expression after normalizing with β-actin. Data represents mean ± SEM of three independent experiments. ∗$p < 0.05$; ∗∗∗ $p < 0.001$; ∗∗∗∗ $p < 0.0001$. (**242**)

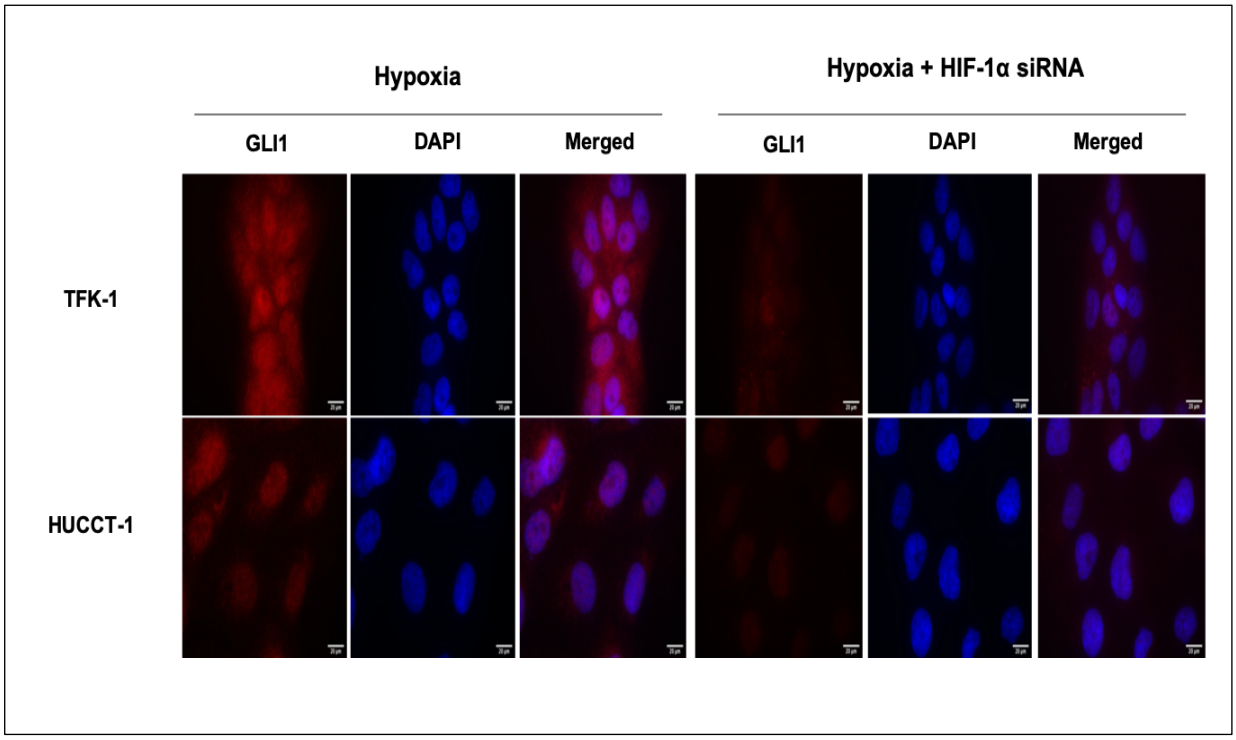


Figure 3.24: HIF-1α siRNA attenuates hypoxia-induced GLI1 nuclear translocation. GLI1 induction and nuclear translocation observed at 24hr post co-treatment with HIF-1 α siRNA and hypoxia by immunofluorescence staining (63x magnification. Scale bar 20 μm). (**242**)

3.5.3. Cyclopamine attenuates SHH pathway activation under hypoxic conditions

Hypoxia was found to have a stimulatory effect on the SHH pathway, as the expression of the SHH cascade proteins, SMO and GLI1, was found to be increased after hypoxia treatment (Fig. 3.22a, e). To study the modulatory effect of Cyclopamine on hypoxia induced activation of the SHH pathway, the expression level of SMO was assessed after 3hr, 6hr, 9hr, and 24hr; and nuclear translocation of GLI1 was assessed at 24hr of Cyclopamine and hypoxia co-treatment in both the cell lines. Cyclopamine treatment significantly suppressed the protein expression of SMO ($p < 0.01$; Fig. 3.25a, b) and GLI1 nuclear translocation (Fig. 3.25c) under hypoxic conditions, reiterating the positive correlation between hypoxia and SHH pathway activation.

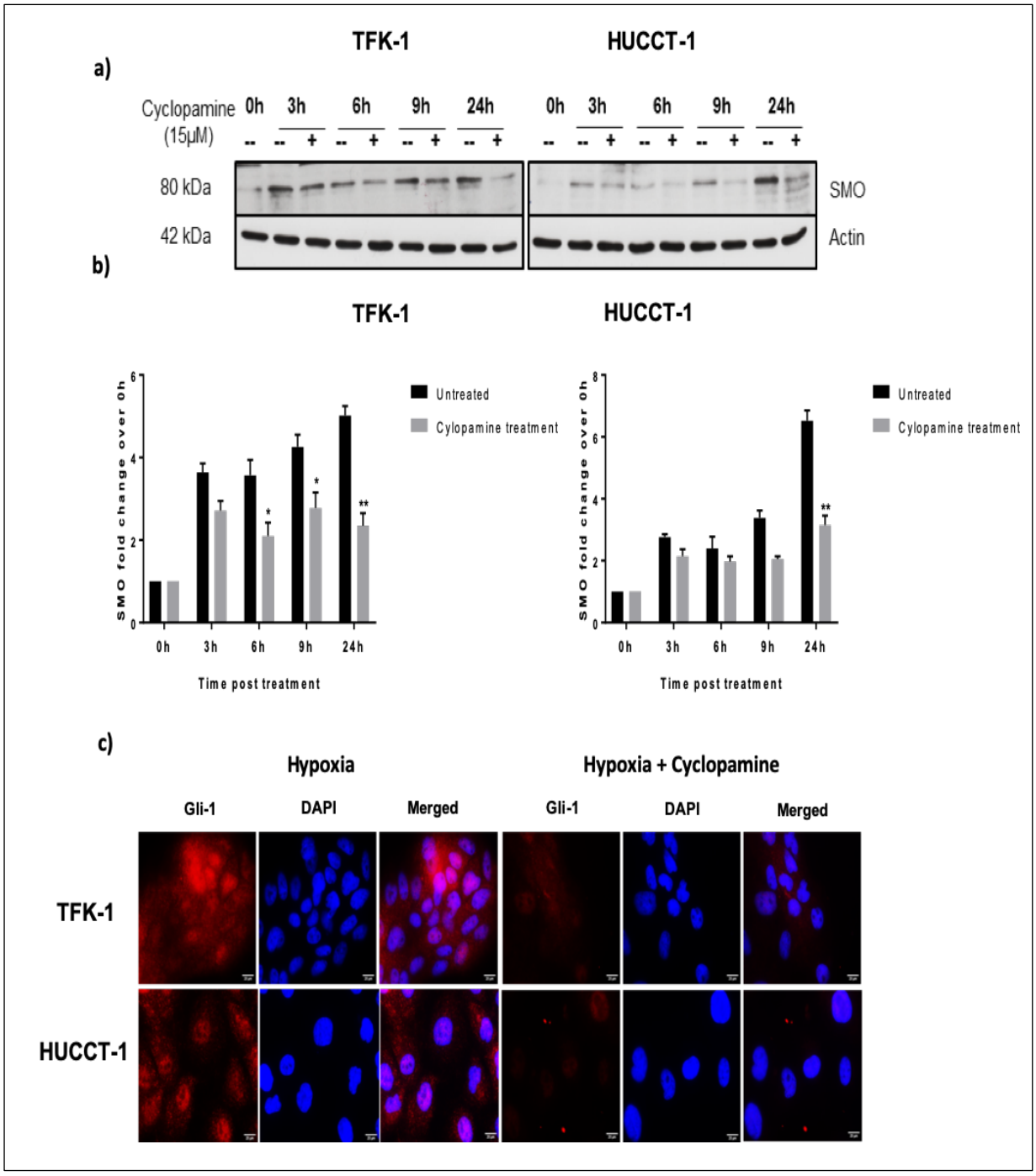


Figure 3.25: Cyclopamine attenuates SHH pathway activation under hypoxic conditions. (a) Western blot analysis of SMO expression after Cyclopamine treatment **(b)** Densitometric quantification of SMO protein expression after normalizing with β-actin **(c)** Immunofluorescence microscopy showing the effect of Cyclopamine on GLI1 induction and nuclear translocation (63x magnification. Scale bar 20 μm). Data represents mean ± SEM of three independent experiments. ∗p < 0.05; ∗∗p < 0.01. (**242**)

3.5.4. Modification of hypoxia induced cellular response by Cyclopamine

To determine the influence of constant hypoxia treatment on the cellular metabolic viability, TFK-1 and HUCCT-1 cells were exposed to hypoxia for 24hr, 48hr and 72hr. Hypoxia led to an increase in the metabolic viability of both the cell lines as compared to normoxia at 24hr,

48hr and 72hr ($p < 0.05$) (Fig. 3.26a). Further, to study the effect of Cyclopamine on hypoxia induced increase in metabolic viability; cells were co-treated with Cyclopamine and hypoxia for 24hr, 48hr and 72hr and assessed for changes in viability. Cyclopamine significantly attenuated hypoxia induced enhanced metabolic viability of both the cell lines in a concentration dependent manner (5 μM, 10 μM and 15 μM) as compared to DMSO (Vehicle) (Fig. 3.26b), wherein the maximum suppression of cellular viability was observed after 72hr in TKF-1cells ($p < 0.001$) and at 48hr in HUCCT-1 cells ($p < 0.05$).

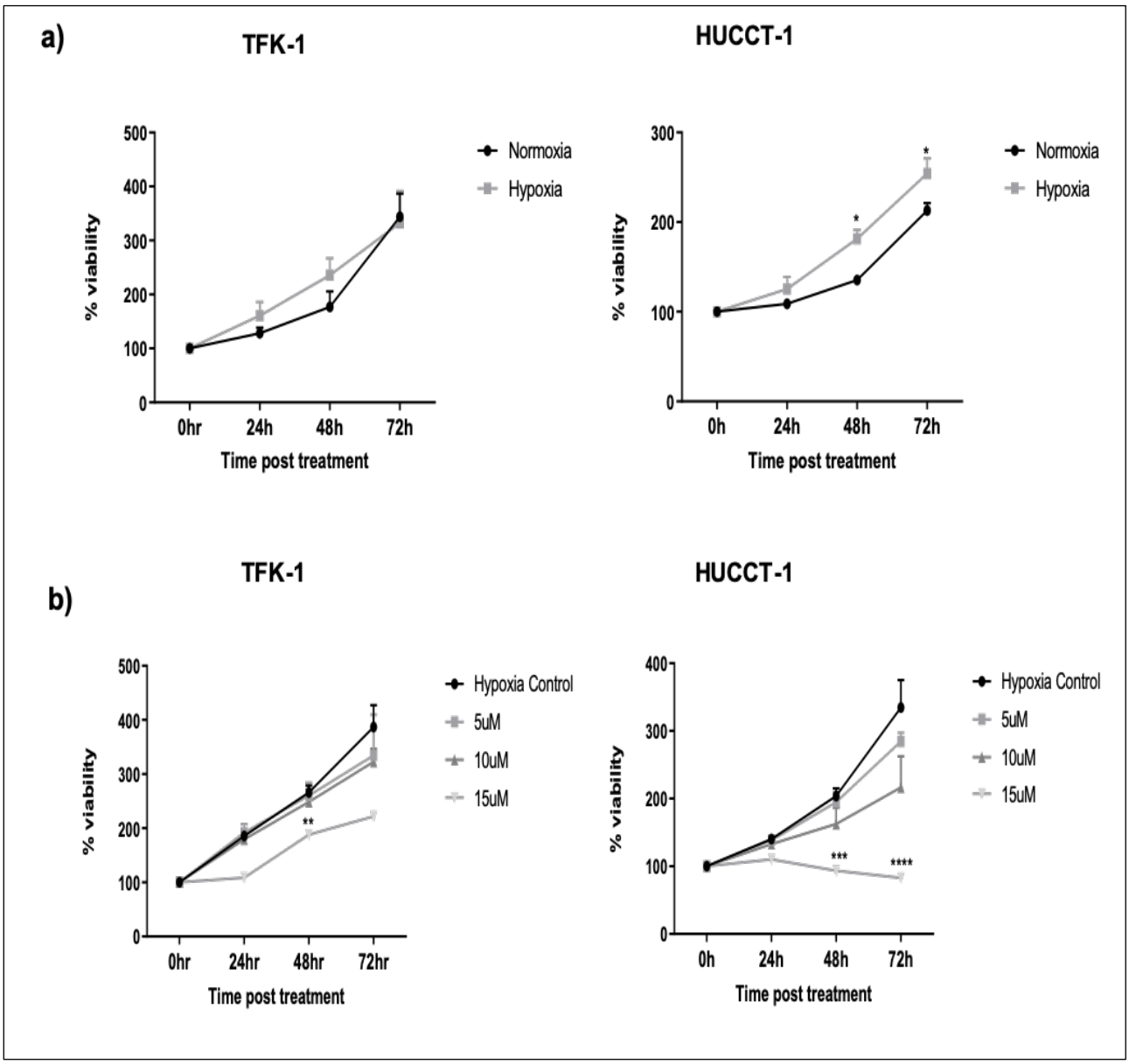


Figure 3.26: Cyclopamine attenuates hypoxia induced enhanced metabolic viability. (a) Changes in the metabolic viability of TFK-1 and HUCCT-1 cell lines after hypoxia treatment **(b)** Effect of different concentrations of Cyclopamine on the metabolic viability of hypoxic treated TFK-1 and HUCCT-1 cells. Data represents mean ± SEM of three independent experiments. Data represents mean ± SEM of three independent experiments. ∗$p < 0.05$; ∗∗$p < 0.01$; ∗∗∗ $p < 0.001$; ∗∗∗∗ $p < 0.0001$. (**242**)

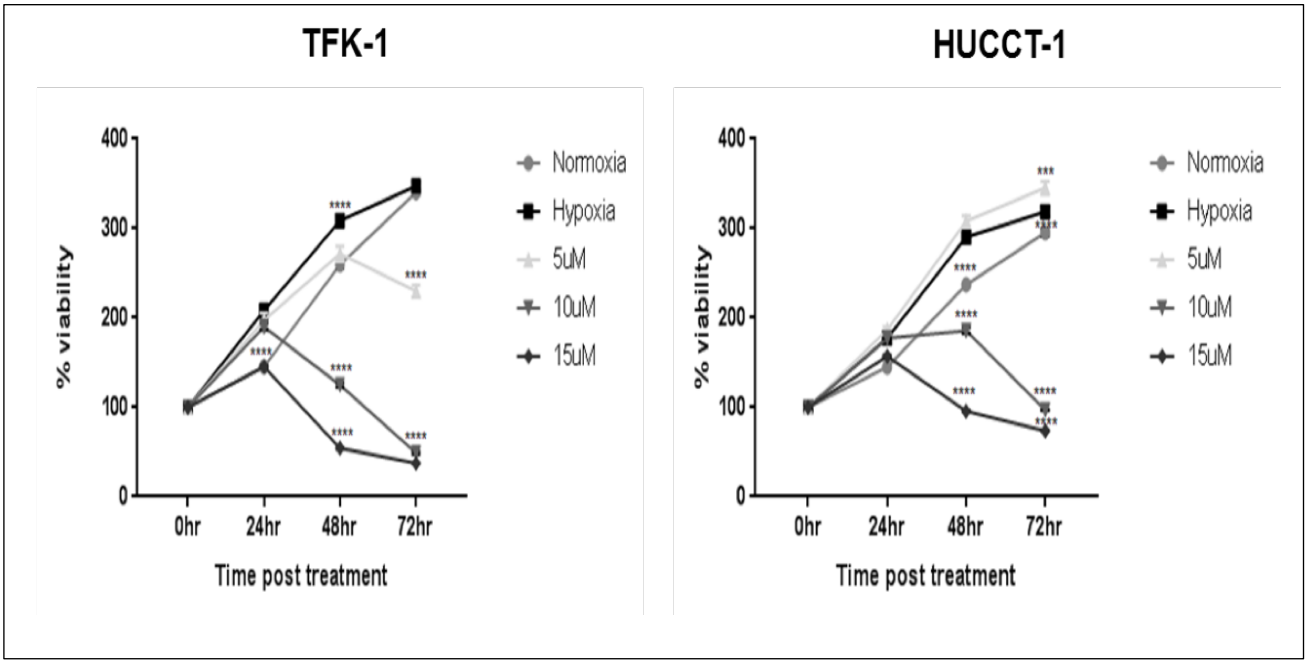


Figure 3.27: GANT61 attenuates metabolic viability under hypoxic conditions. Effect of different concentrations of GANT61 on the metabolic viability of hypoxia treated TFK-1 and HUCCT-1 cells. Differences were considered as statistically significant when P-value was. *** $p < 0.001$; **** $p < 0.0001$. (**242**)

These results were further confirmed by using a known GLI inhibitor GANT61, where GANT61 was also found to significantly attenuate hypoxia induced enhanced metabolic viability in a concentration dependent manner (5 µM, 10 µM and 15 µM) (Fig. 3.27). Under the hypoxic microenvironment, some cells may suffer permanent damage and die, while others adapt themselves to the stress and become resistant to the injury. Therefore, we wanted to assess the effect of SHH pathway on the induction of apoptotic cell death in Cyclopamine and hypoxia co-treated cells. Cyclopamine significantly augmented apoptosis in hypoxia treated cells as compared to the cells cultured in normoxia at 48hr and 72hr ($p < 0.001$), suggesting that SHH pathway has regulatory effects on tumor resistance against apoptosis under hypoxic conditions (Fig. 3.28; 3.29a, b).

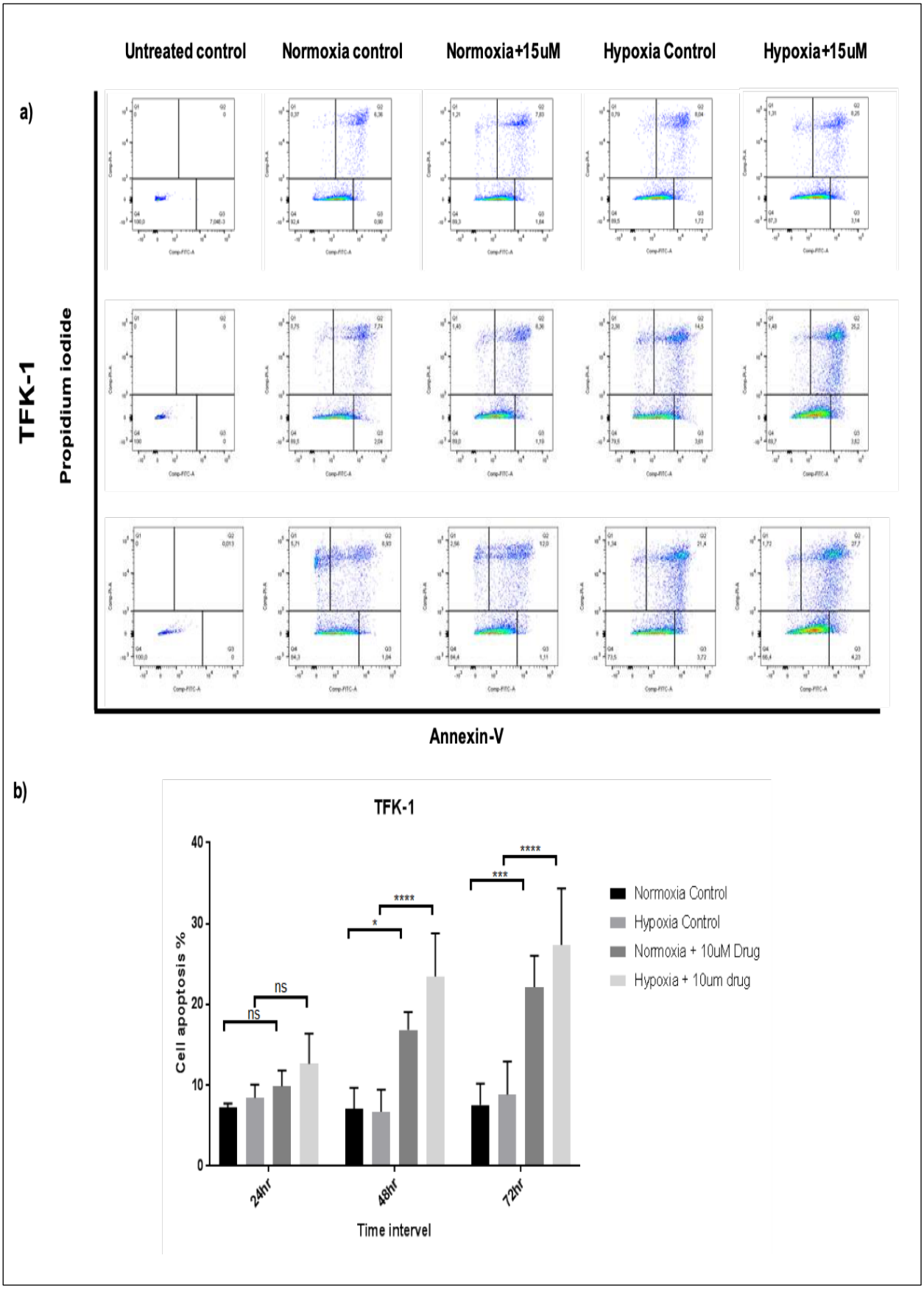


Figure 3.28: Cyclopamine augments apoptosis under hypoxic conditions. (**a**) Flowcytometric dot plots showing the effect of Cyclopamine on hypoxia induced apoptosis in TFK-1 cell line **(b)** Quantitative analysis of apoptotic cell population at different time intervals. Data represents mean ± SEM of three independent experiments. ⁎p < 0.05; ⁎⁎⁎ p < 0.001; ⁎⁎⁎⁎ p < 0.0001. (**242**)

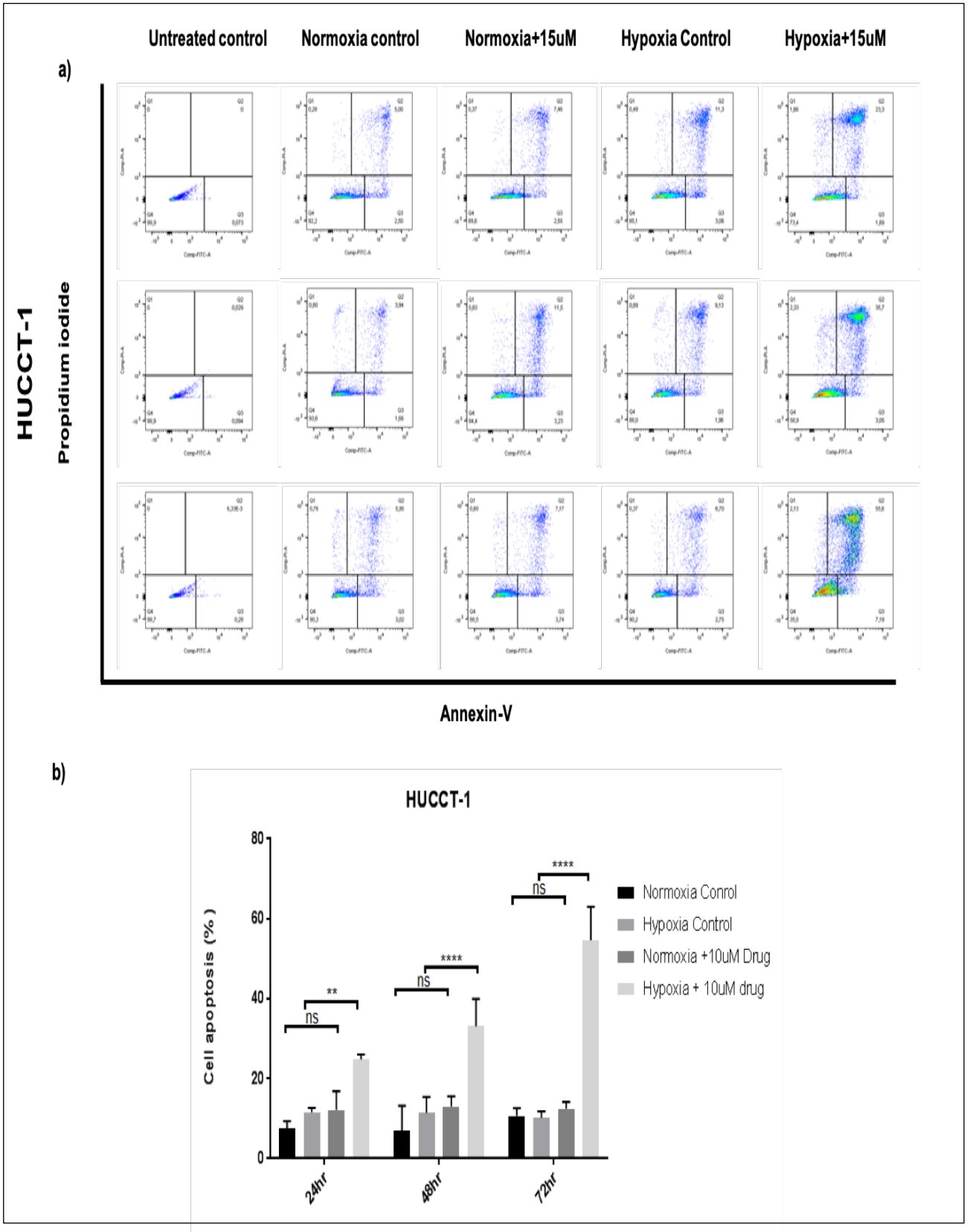


Figure 3.29: Cyclopamine augments apoptosis under hypoxic conditions. (**a**) Flowcytometric dot plots showing the effect of Cyclopamine on hypoxia induced apoptosis in HUCCT-1 cell line **(b)** Quantitative analysis of apoptotic cell population at different time intervals. Data represents mean ± SEM of three independent experiments. ∗p < 0.05; ∗∗∗ p < 0.001; ∗∗∗∗ p < 0.0001. (**242**)

3.5.5. Hypoxia treatment increases the colony formation capability of CC cells

To investigate the effect of hypoxia induced SHH pathway activation on clonogenicity, an important feature of stem cells, TFK-1 and HUCCT-1 cells were seeded at a density of 500 cells in a 6- well plate and incubated in a humidified incubator at 5% CO2 for 24hr. The plates were then exposed to 1% hypoxia for 48hr followed by transfer to normoxic conditions for another 11–14 days. Interestingly, an increased number of colonies was observed after 48hr of hypoxia treatment in HUCCT-1 cells ($p < 0.001$), whereas no significant difference in the number of colonies was found in TFK-1 cells, compared to the normoxia group (Fig. 3.30a, b). Co-treatment with Cyclopamine significantly inhibited the colony forming capability of CC cells ($p < 0.0001$) (Fig. 3.30a, b), which was consistent with the effect of Cyclopamine on metabolic viability and cell apoptosis.

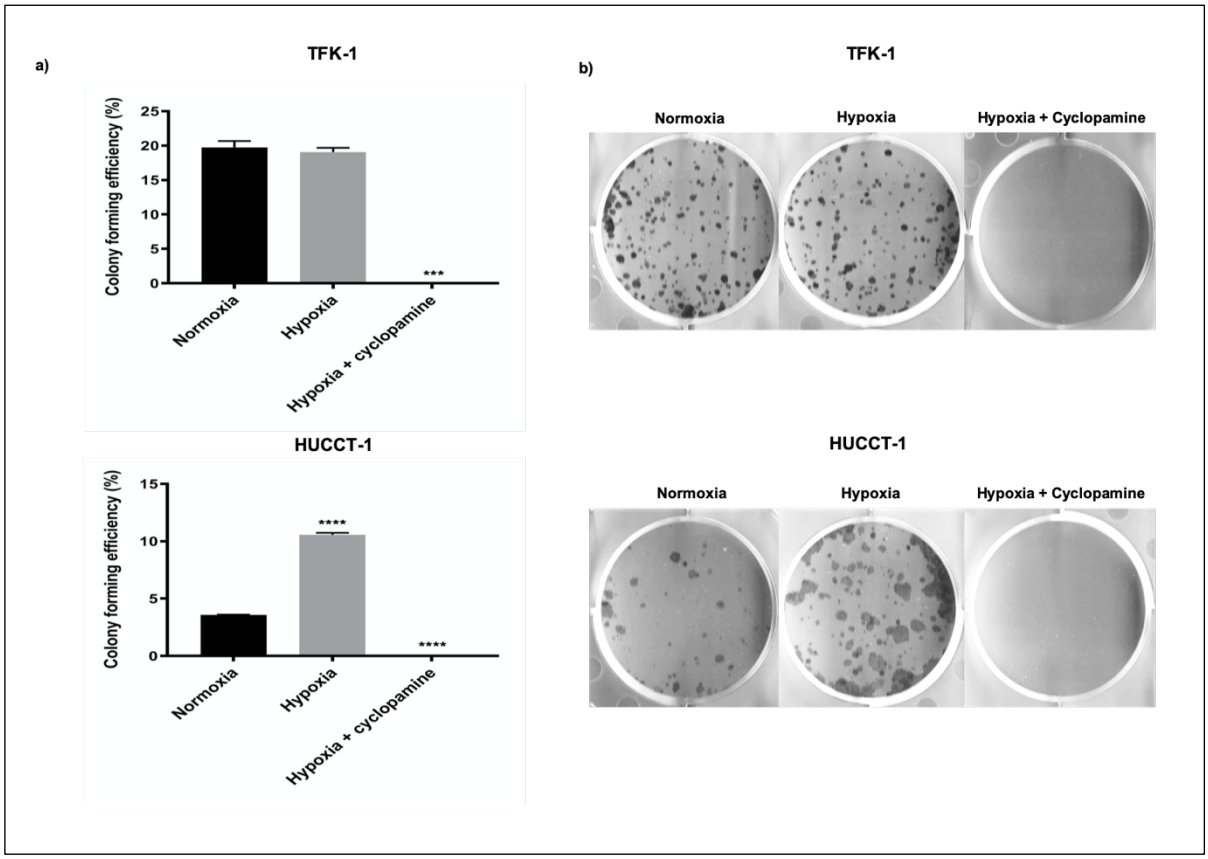


Figure 3.30: Cyclopamine inhibits clone forming ability of hypoxia treated CC cells. (a) Quantitative analysis of the clone forming ability of TFK-1 and HUCCT-1 after hypoxia +/- Cyclopamine treatment **(b)** Representative images of crystal violet stained colonies in the indicated treatment groups. Data represents mean ± SEM of three independent experiments. *** $p < 0.001$; **** $p < 0.0001$. (**242**)

3.5.6. SHH pathway inhibition diminishes hypoxia induced expression of stem cell transcription factors and CD133 expression

The effect of hypoxia on the stem cell population was evaluated by studying the changes in the expression of the CSC transcription factors (NANOG, Oct4 and SOX2) after 3hr, 6hr, 9hr, 24hr and 48hrs of hypoxia treatment by RT-qPCR and immunoblotting.

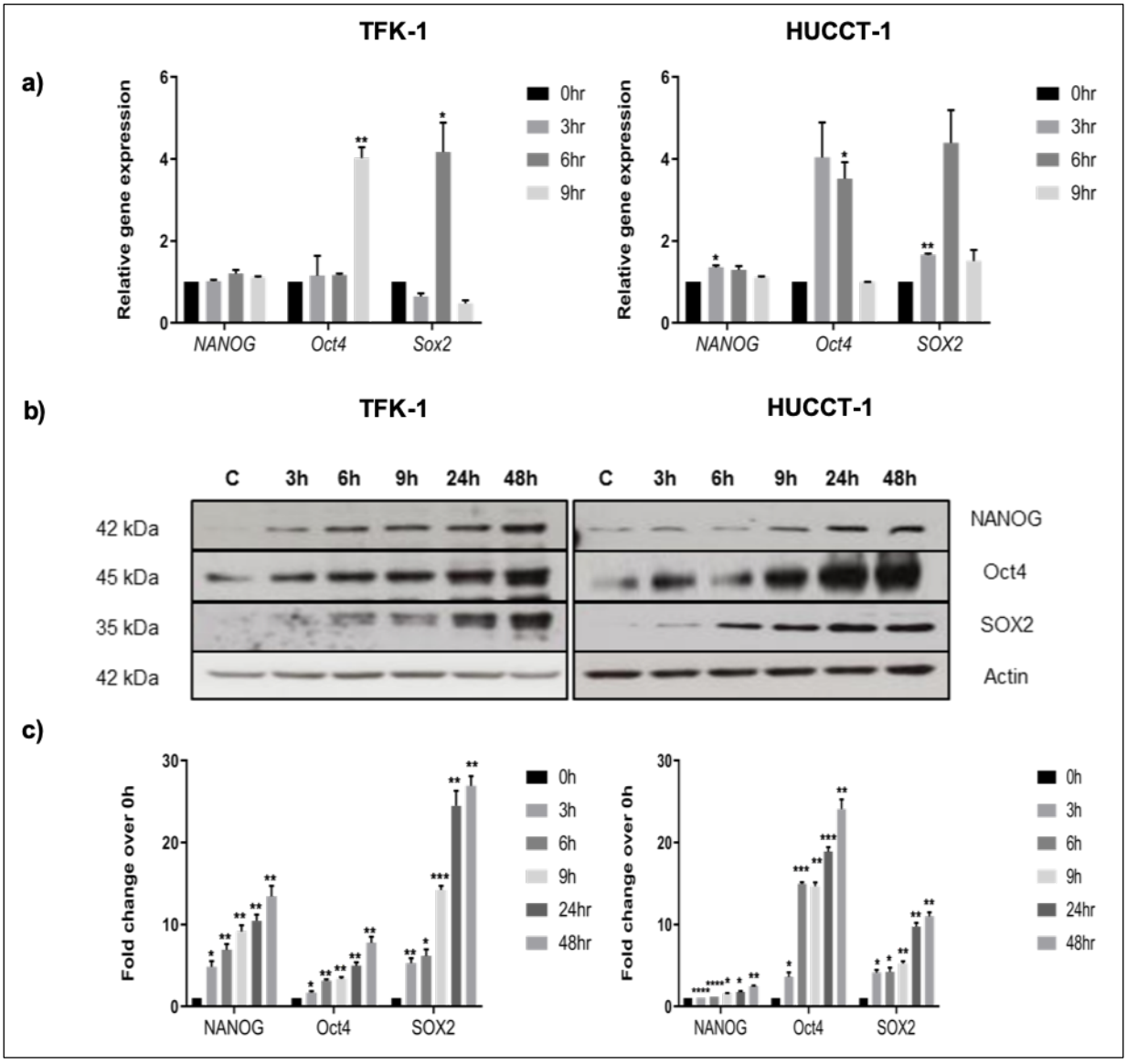


Figure 3.31: Hypoxia induces expression of stem cell transcription factors (a) RT-qPCR analysis of NANOG, Oct4, SOX2 in TFK-1 and HUCCT-1 cells under hypoxic conditions **(b)** Protein expression of NANOG, Oct4, SOX2 observed by Western blot under hypoxic conditions **(c)** Densitometric quantification of NANOG, Oct4, SOX2 after normalizing with β-actin. Data represents mean ± SEM of three independent experiments. $*p < 0.05$; $**p < 0.01$; $*** p < 0.001$. (**242**)

A time-dependent increase in the mRNA level of all the three transcription factors was observed in both cell lines. NANOG and Oct4 were maximally expressed at 9hr in TFK-1 cells ($p < 0.01$), whereas Oct4 mRNA level was found to peak at 3hr in HUCCT-1 cells.

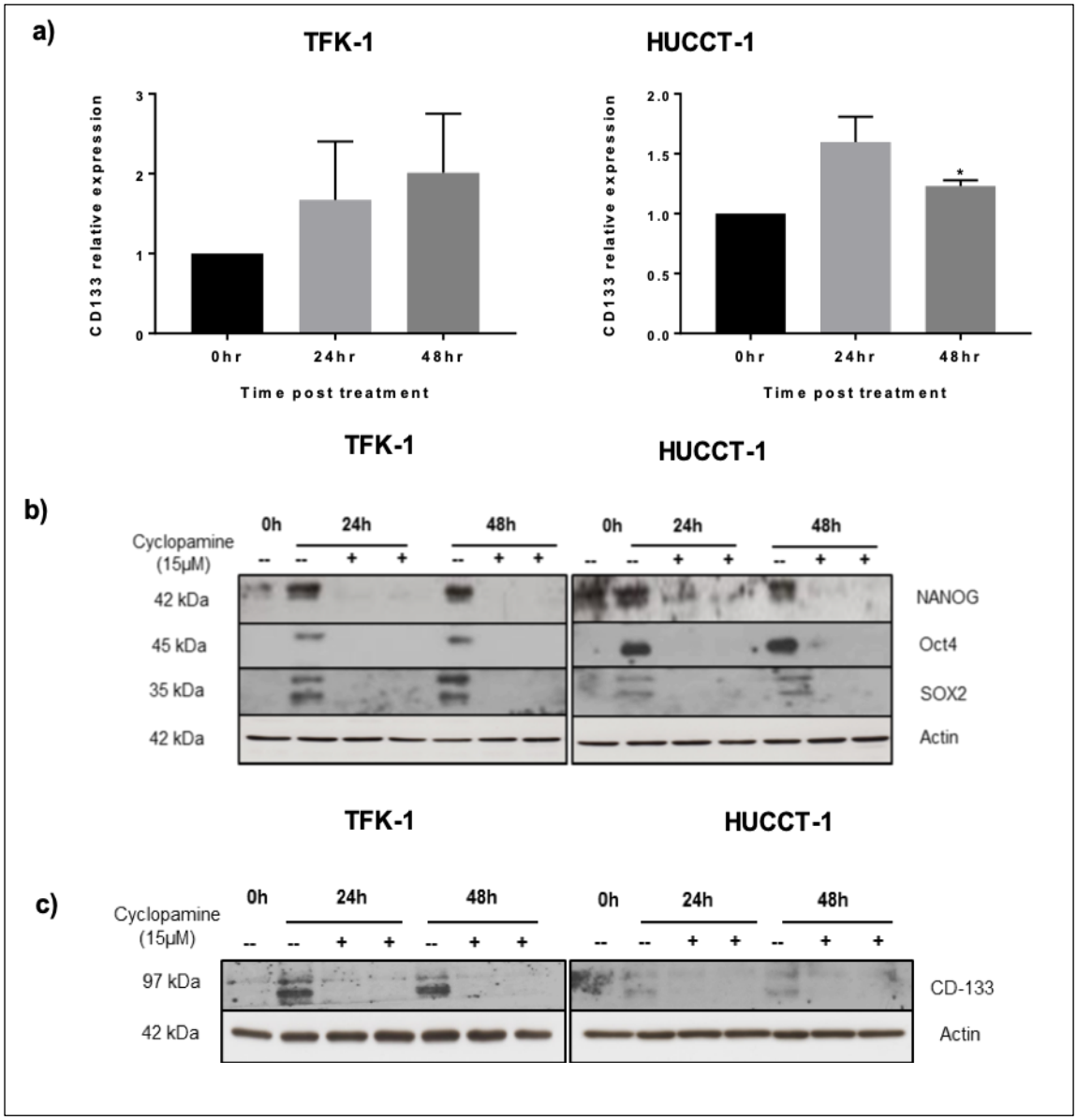


Figure 3.32: Cyclopamine diminishes hypoxia induced expression of stem cell transcription factors and CD133 expression. (**a**) RT-qPCR analysis of CD133 gene expression after 24hr and 48hr hypoxia treatment **(b)** NANOG, Oct4, SOX2 protein expression after co-treatment with Cyclopamine and hypoxia **(c)** Protein expression of CD133 after Cyclopamine and hypoxia co-treatment. Data represents mean ± SEM of three independent experiments. ∗$p < 0.05$. (**242**)

However, highest expression of SOX2 was observed at 6hr of hypoxia treatment in both cell lines ($p < 0.01$ for TFK-1) (Fig. 3.31a). A similar pattern of time dependent increase was observed in the protein expression of the three transcription factors in both the cells ($p < 0.05$; $p < 0.01$, $p < 0.001$) (Fig. 3.31b, c). The mRNA expression of CD133, a known stem cell

marker, was also found to be increased in hypoxia-treated cells, with peak expression at 48hr and 24hr in TFK-1and HUCCT-1 ($p < 0.05$) cell lines respectively (Fig. 3.32a). Interestingly, SHH pathway inhibition by Cyclopamine abrogated the hypoxia induced increase in the expression of NANOG, Oct4, SOX2 and CD133, as assessed by western blotting at 24hr and 48hr (Fig. 3.31b, c). Furthermore, GANT61 treatment also led to significant translational suppression of NANOG, Oct4 and SOX2, with substantial abrogation of CD133 expression (Fig.3.33a, b). These results were further confirmed by immunofluorescence staining after 24hr of hypoxia treatment in TFK-1 and HUCCT-1 cells, where Oct4 and NANOG were highly expressed and Cylopamine treatment diminished the expression of these transcription factors (Fig. 3.34, 3.35)

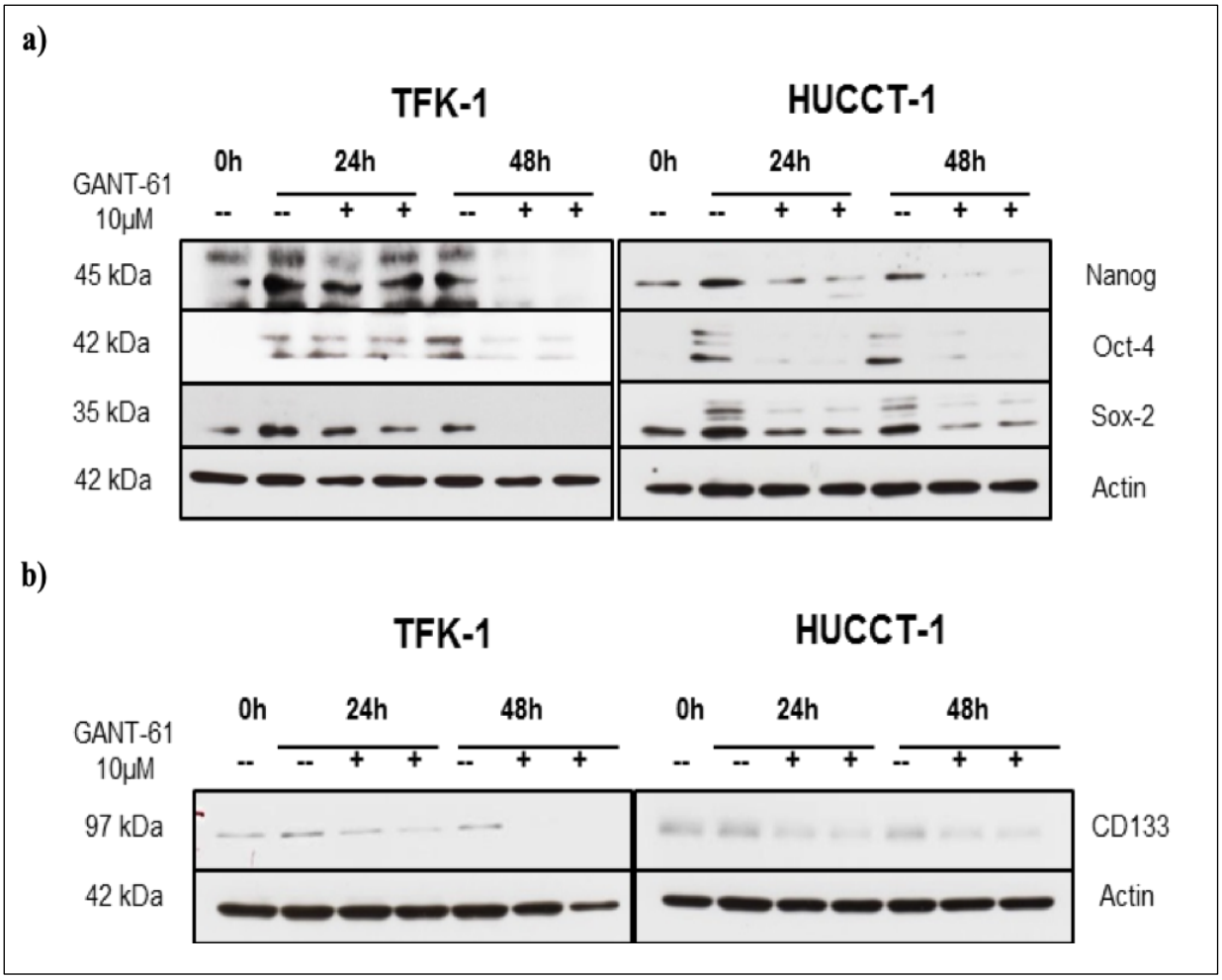


Figure 3.33: GANT61 diminishes hypoxia induced expression of stem cell transcription factors and CD133 expression. (a) NANOG, Oct4, SOX2 protein expression after co- treatment with GANT61 and hypoxia **(b)** Protein expression of CD133 after GANT61 and hypoxia co- treatment. (**242**)

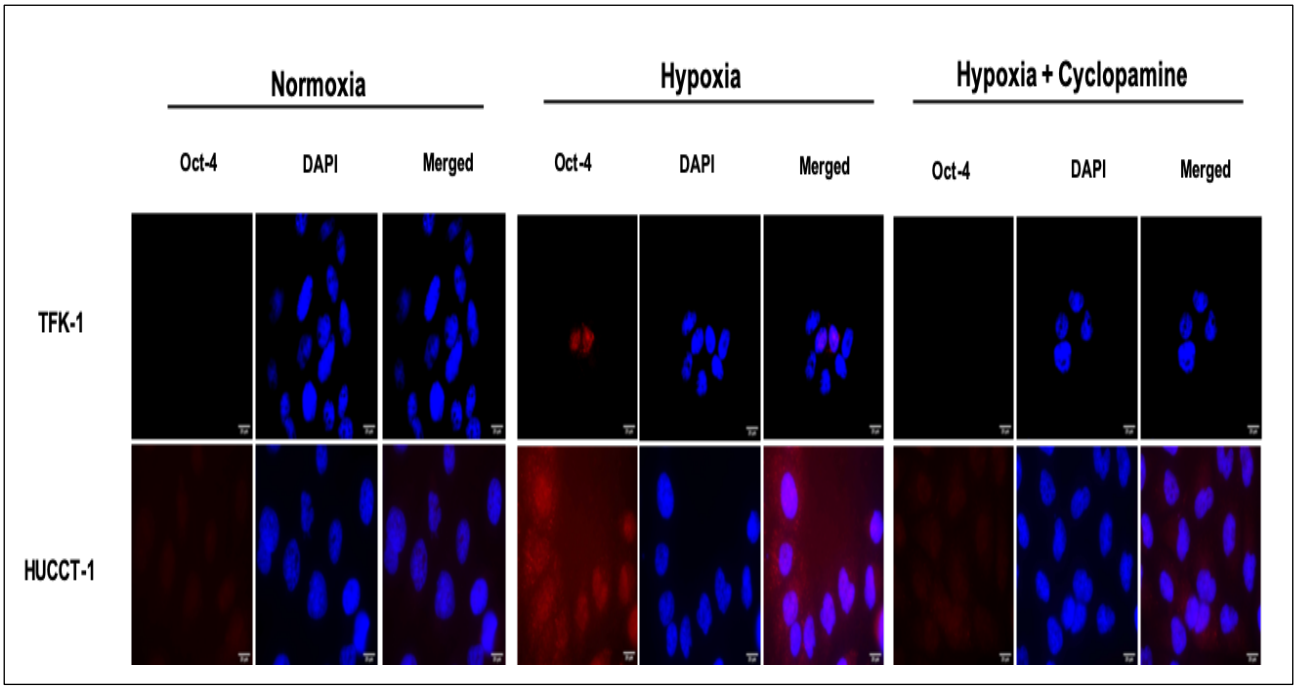


Figure 3.34: Immunofluorescence staining showing the effect of Cyclopamine on hypoxia induced upregulated stem cell transcription factors. Oct4 expression in TFK-1 and HUCCT-1 cell lines (63X magnification; Scale bar 20 μm). (**242**)

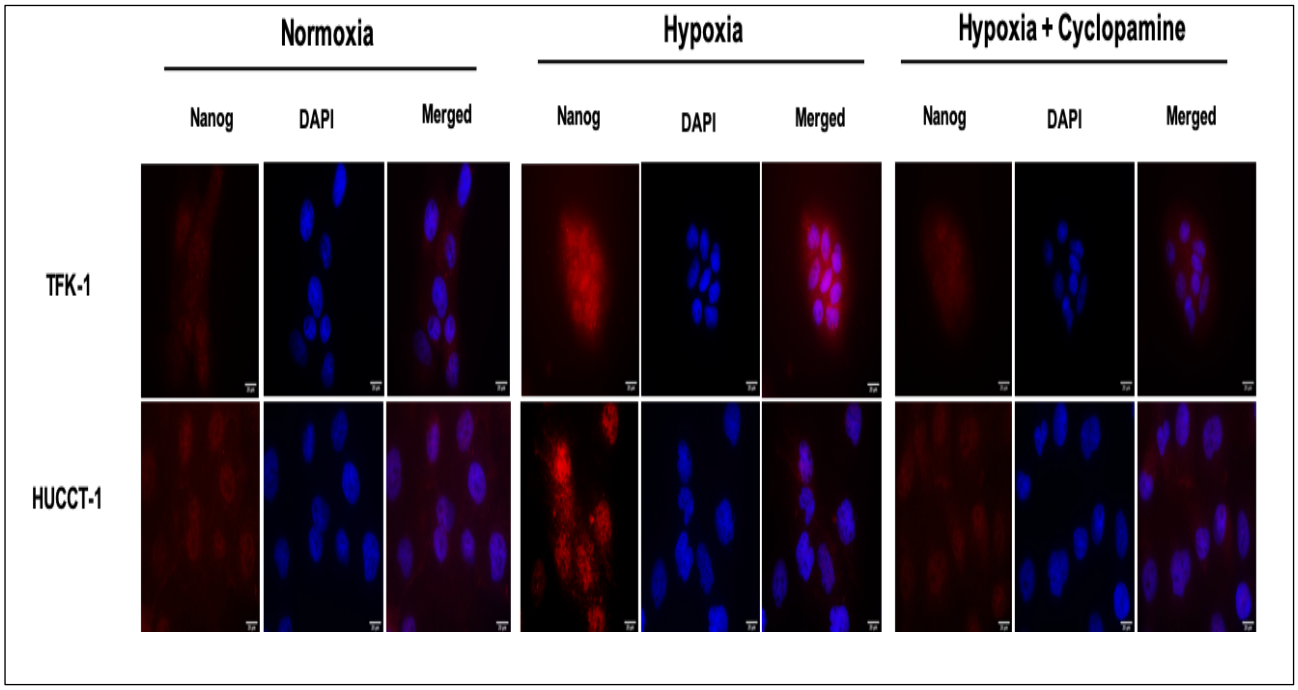


Figure 3.35: Immunofluorescence staining showing the effect of Cyclopamine on hypoxia induced upregulated stem cell transcription factors. NANOG expression in TFK-1and HUCCT-1 cell line (63x magnification. Scale bar 20 μm). (**242**)

3.5.7. SHH signaling modulates hypoxia induced EMT and invasion

Exposure to hypoxia, facilitated by HIF-1α, triggers many target genes which are involved in various cellular processes associated with malignant progression such as angiogenesis and proliferation. Hypoxia is also known to induce EMT through HIF-1α; therefore, to examine the effect of SHH pathway on hypoxia induced EMT in CC, we investigated the expression of EMT markers after Cyclopamine or GANT61 and hypoxia co-treatment. The results showed that hypoxia upregulated the expression of N-cadherin and Vimentin, whereas no significant change was observed in E-cadherin level (Fig. 3.36a, b). On the other hand, SHH pathway inhibition by Cyclopamine led to a decrease in the expression of all the mesenchymal markers (Fig. 3.37a, b). Hypoxia also enhanced the invasive potential of TFK-1 and HUCCT-1 cells after 48 h of treatment ($p < 0.05$), whereas Cyclopamine treatment significantly abolished this phenotype ($p < 0.001$) (Fig. 3.38).

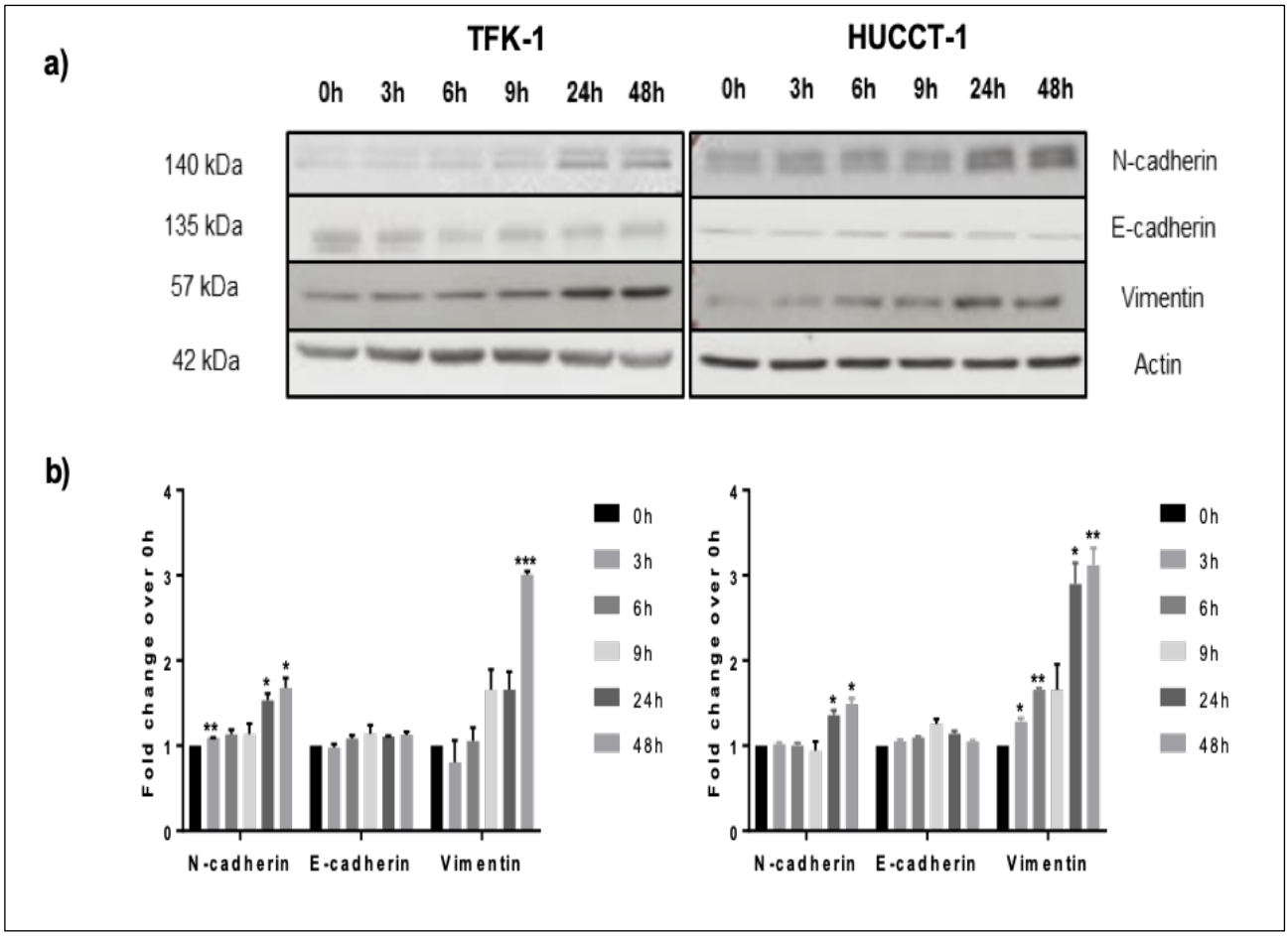


Figure 3.36: Hypoxia induces EMT in CC cell lines. (**a**) Western blot analysis of EMT markers after 3hr, 6hr, 9hr, 24hr and 48hr of hypoxia treatment **(b)** Densitometric quantification of N-cadherin, E-cadherin and Vimentin protein expression after normalizing with β-actin. Data represents mean ± SEM of three independent experiments. ∗$p < 0.05$; ∗∗$p < 0.01$; ∗∗∗∗ $p < 0.0001$. (**242**)

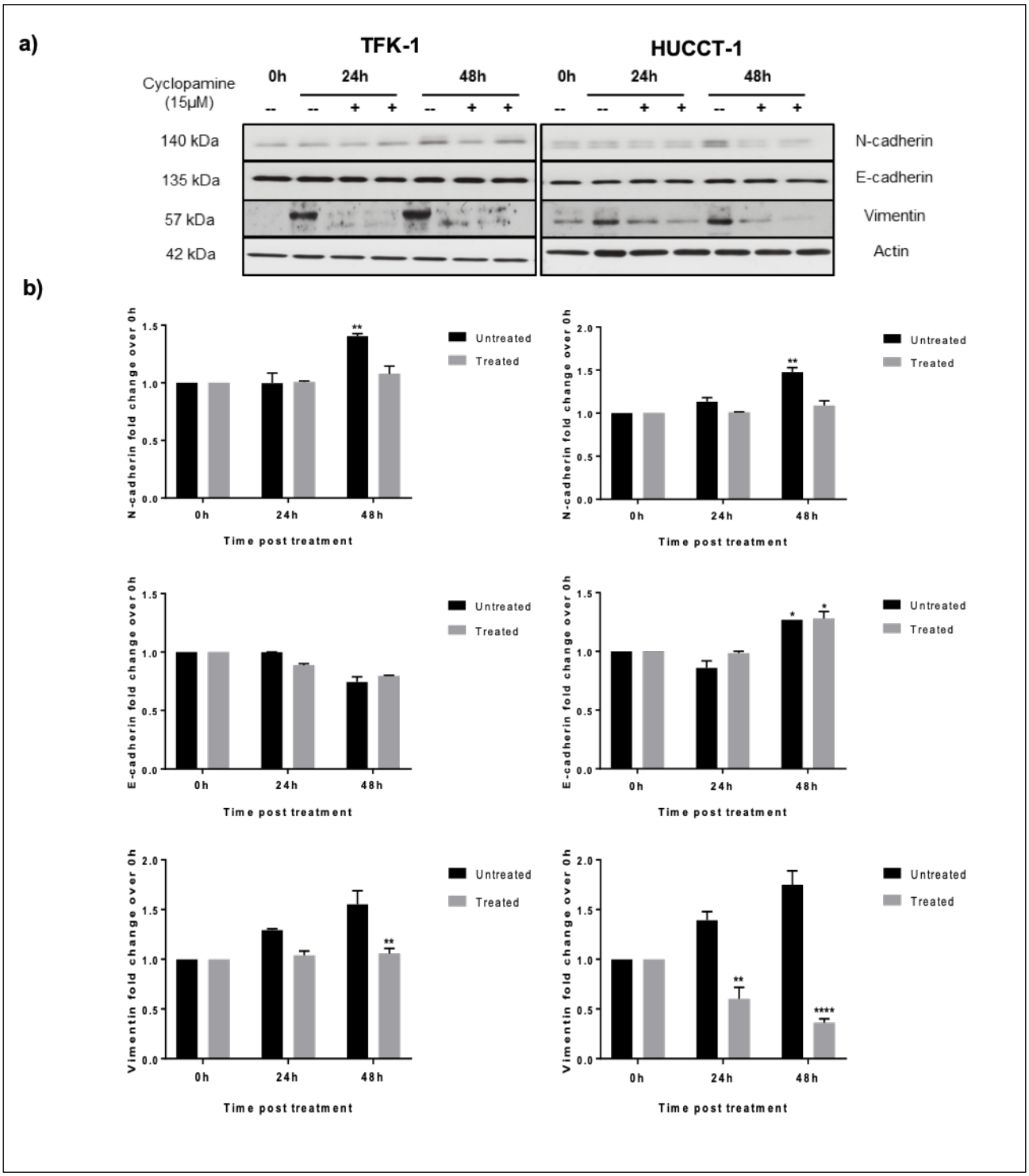


Figure 3.37: Cyclopamine abolishes hypoxia induced EMT in CC cell lines. (a) Western blot analysis showing the effect of Cyclopamine on hypoxia induced EMT markers **(b)** Densitometric quantification of N-cadherin, E-cadherin and Vimentin after normalizing with β-actin. Data represents mean ± SEM of three independent experiments. ∗p < 0.05; ∗∗p < 0.01; ∗∗∗∗ p < 0.0001. (**242**)

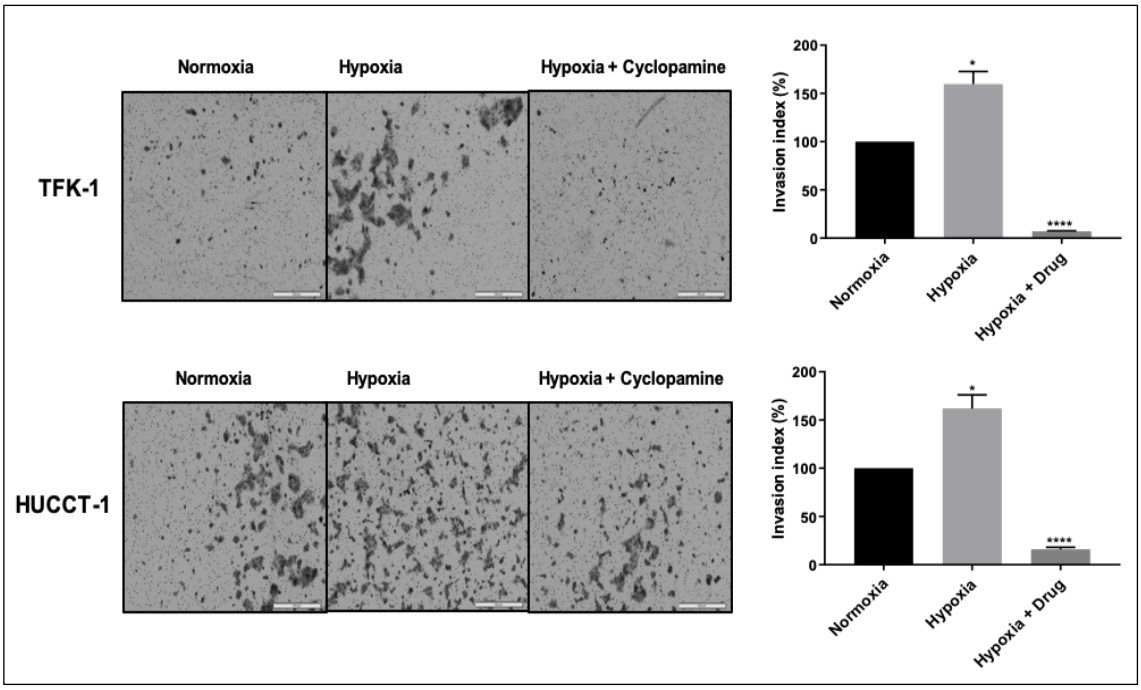


Figure 3.38: Cyclopamine inhibits hypoxia induced invasion in CC cell lines. Effect of hypoxia and/or Cyclopamine on the invasiveness of TFK-1 and HUCCT-1 cells. Data represents mean ± SEM of three independent experiments. ∗p < 0.05; ∗∗∗∗ p < 0.0001. (**242**)

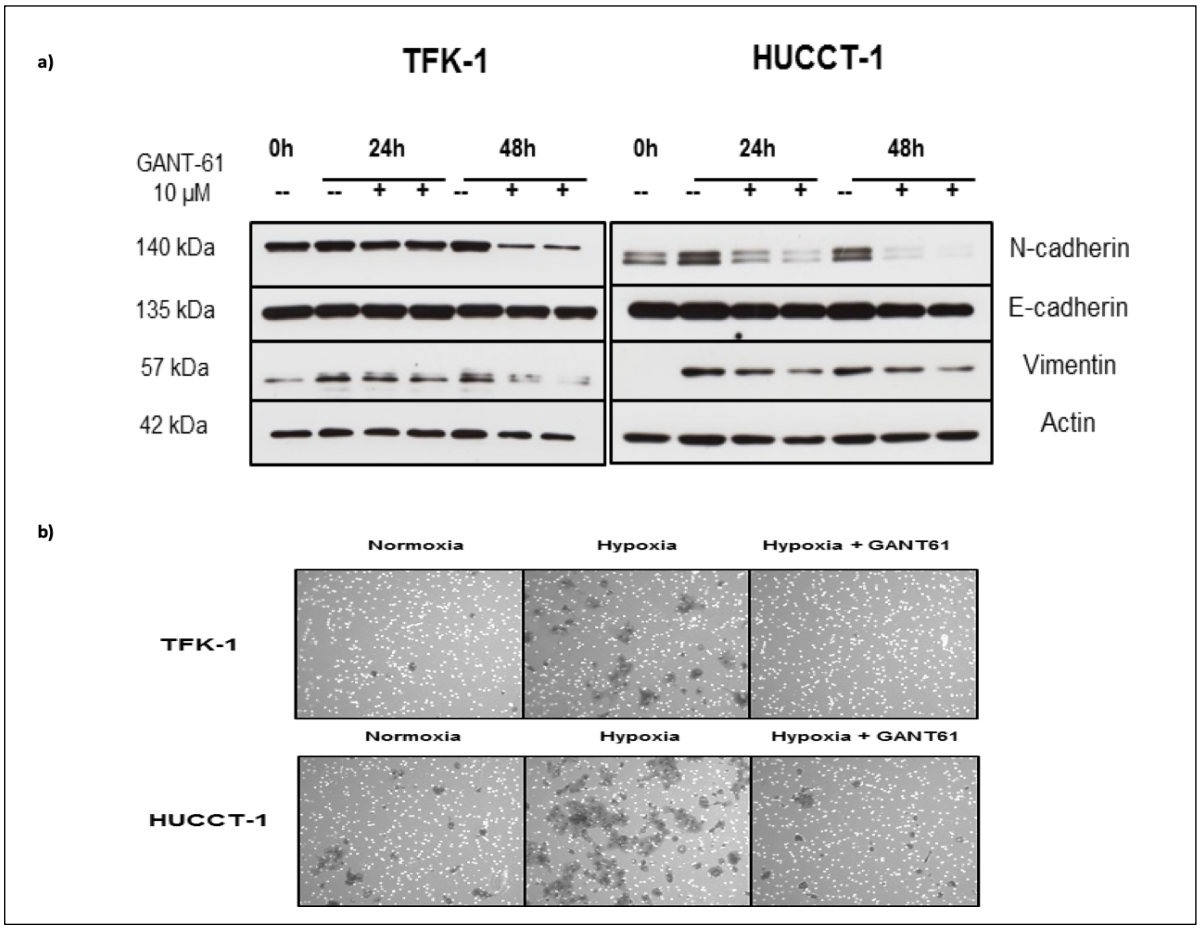


Figure: 3.39: GANT61 abolishes hypoxia induced EMT in CC cell lines (a) Western blot analysis showing the effect of GANT61 on hypoxia induced EMT markers **(b)** Effect of hypoxia and/or GANT61 on the invasiveness of TFK-1 and HUCCT-1 cells. (Scale bar 200 μm). (**242**)

Inhibition of the SHH pathway using GANT61 also resulted in the suppression of N-cadherin and Vimentin expression, leading to attenuated invasiveness (Fig. 3.39).

4. Discussion

CC represents an incurable, yet understudied primary malignancy of liver. Multiple underlying molecular mechanisms have been described previously, but none of them has proven to offer a relevant treatment till now. Therefore, to reduce mortality from CC, novel treatment strategies are urgently needed. Pre-clinical trial on pancreatic cancer using small molecule Hedgehog inhibitors have shown encouraging results. A study from our group demonstrated the role Hedgehog pathway in CC pathogenesis using human CC cell lines. To gain further insight, in the present study, we have investigated the role of Hedgehog signaling pathway activation in the initiation and progression of CC using cellular and murine models.

4.1. Role of SHH pathway in cholangiocarcinogenesis in a genetic mouse model (*Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$*)

4.1.1. Progression of CC in Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$ mouse model

CC represents a cancer entity that covers various epithelial tumors and is considered as one of the principal causes of cancer- related deaths in the western world. Thus, to overcome the morbidity associated with surgery and limited treatment options , there is a need for exploring alternate, less toxic therapies for CC. Murine models are critically important for studying the oncogenic potential of various genes and signaling pathways involved in the induction and progression of cancer and for the assessment of the therapeutic potential of novel and innovative cancer treatment strategies (168). Since human CCs are predominantly with K-Ras mutations, various murine models carrying K-Ras mutations have been developed and used for CC studies (158, 169, 170). In this study, a genetically engineered mouse model with activated K-Ras combined with p53 inactivation and Alb-Cre for CC development has been used (158), which is known for mimicking the human disease. While this mouse models have been used to support an important role of certain mutations with regard to the devolvement of CC; however, in our study this animal model has been used for characterizing and understating the Hedgehog pathway activation during cholangiocarcinogenesis

Histopathological analysis revealed CC development in our mouse model by 19-20 weeks of age, which was confirmed by CK19 expression of the epithelial tumor cells. Additionally, the mice with growing liver tumor burden had persistently high levels of ALT and AST, which is an indicator of liver injury and damaged hepatocytes. Furthermore, the maximum survival of

these mice was observed to be 25 weeks of age due to tumor associated mortality. Therefore, this murine model with high reproducibility of CC development was considered as a perfect preclinical tool to assess the importance of a signaling pathway in cholangiocarcinogenesis.

4.1.2. Development of CC is associated with distant metastasis

CC has been known to be asymptomatic in an early phase and often diagnosed when metastasized to distant organs. The 5-year survival rate of gastric cancer patients with pulmonary metastasis without metastasectomy has been found to be less then 5% as compared to 28-33% in the patients who undergo surgery (171). Survival rate studies of hepatocellular carcinoma have also reported, in which the patients who undergo pulmonary metastasectomy have a better survival rate of 32-48%. A number of studies have shown that EMT plays an important role in the progression and metastasis of cancers, including cholangiocarcinoma, where a direct correlation has been found between the expression of MMP-9 and EMT- related proteins (such as E-cadherin, N-cadherin, α-SMA), and poor prognosis (172, 173).
In our study, upon CC development in mice harboring $Kras^{G12D}$/mutant p53, distal metastasis in the lungs was also observed. The metastasis incidence was found to be 50% by the age of 19-20 weeks, whereas it increased to 60.6% by 24-25 of age.

MMPs play an important role in tumor progression by constructing a pathway for cancer cells to move from primary tumor niche to the distant organs (174). We thus analyzed the concentration of MMP-9 in the serum samples and found a significant increase by the age of 19-20 weeks. Gain of N- cadherin is an essential event towards EMT, which promotes motility and invasion (175, 176). Consequently, mesenchymal markers N-cadherin and α-SMA were also concurrently upregulated by the same age of 19-20 weeks. Together, these findings provide an evidence of tumor aggressiveness, which can be correlated with the metastatic potential of CC tumors.

4.1.3. Hedgehog Signaling pathway activation is associated with CC progression

Activation of $Kras^{G12D}$ with Alb-Cre induces adenocarcinoma with CC features. The Raf MeK-ERK cascade is the most common signaling event downstream of K-Ras, which upon activation regulates the growth, survival and differentiation of cells (177). However, no

achievement in preventing and curing cancers has been found till date by direct targeting of K-Ras. Therefore, there is a need to explore new molecular targets with high anti- cancer efficacy.

Hedgehog signaling deregulation is known to induce cancer, including pancreatic ductal adenocarcinoma (PDA), CC and many others. Various studies have implicated the relationship between K-Ras and Hedgehog signaling in pancreatic carcinoma progression (178). In our study, we studied Hedgehog pathway activation during CC progression by analyzing time dependent changes in the expression of Hedgehog pathway elements such as SHH, SMO and GLI1 in liver tissue. We found that in cohorts of 19-20 weeks of age, the expression of SHH and its downstream element, SMO was significantly elevated in liver tissues during CC development. Additionally, no significant difference in SHH protein expression was observed between tumor and liver tissues. Thus, it indicates that Hedgehog pathway activation in liver plays a pivotal role in tumor induction. This is in line the study from Thayer et.al. which claim that aberrant activation of Hedgehog signaling has a critical role in the initiation and maintenance of pancreatic ductal adenocarcinoma (153).

Next, we analyzed the protein expression of GLI1 transcription factor as the final effector of the Hedgehog pathway in liver tissues, and its expression was found to be upregulated in the mice cohorts of 19-20 weeks of age. In the present study, we show for the first time that the Hedgehog pathway is activated in the *Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$* model of CC. Based on this observation and previously done studies in pancreatic cancer, we speculate that Hedgehog signaling pathway could be one of the important mechanism by which KRAS mutation encourages tumor formation in these mice. However, further studies are warranted to establish the relationship between them.

4.1.4. High expression of cancer stem cell markers during CC progression

Cancer stem cells (CSCs) are known to cause tumor induction, persistence and a resistance towards chemotherapy. Moreover, CSCs are also known for possessing mesenchymal characteristics, resulting in tumor metastasis (179, 180). Therefore, it makes it compelling to eradicate the CSCs in order to prevent the reoccurrence of tumor. Many investigators have tried to isolate and characterize the stem cells from human origin tumors. Furthermore, CSC markers are used as an independent prognostic factor in various cancers. Numerous clinical studies have examined the clinical outcome of CC with regard to CD133 expression, an elevated expression

of which has been associated with poor prognosis in patients (181-184). A study by Shimada and colleagues revealed that patients with poor prognosis had an increased expression of CD133 in the tumor cells; and that there was a huge difference in the 5-year survival rate of $CD133^{+}$ (8%) and $CD133^{-}$ patients (185). Transcription factors like OCT-4, SOX-2 and NANOG exhibit massive clinical potential and are known for playing a pivotal role in the reprograming processes. These act as molecular markers for controlling cell fate of CSC during cancer progression (186, 187). According to a study by Chiou et al., co-expression of OCT-4 and Nanog encouraged cancer stem cell like properties by dedifferentiation and augmentation of malignancy in lung adenocarcinoma (188). Hedgehog signaling is known to drive the CSC phenotype and support stemness signature in many cancers by regulating stemness-determining genes like *Oct4* and *Sox2* (189, 190).

Based on previous studies, we also observed stem-like properties in the liver tissues due to enhanced protein expression of CSC transcription factors. The expression of SOX-2, OCT-4 and NANOG transcription factors and CD133 was remarkably upregulated during tumor progression in the liver tissue, which is in line with previous studies. Interestingly, protein expression of OCT-4, NANOG and CD133 was also found remarkably increased in tumor tissues as compared with control liver, however, no significant difference was observed with the adjacent liver tissue. These results suggest that deregulation of Hedgehog pathway is involved in providing stemness signature to the cells by upregulating stem cell transcription factors and helping them to initiate tumor development and progression.

4.2.Effect of Cyclopamine on cholangiocarcinogenesis and the repopulating potential of CSCs in Alb-Cre/$KRAS^{G12D}$/$p53^{L/L}$ mouse model

4.2.1. Hedgehog inhibition suppresses tumor growth

Aberrant activation of SHH signaling has been observed in many cancers. Numerous studies, including ours, have demonstrated the role of SHH pathway activation in CC. To the best of our knowledge this is the first study where the immediate and later effects of Hedgehog pathway activation during CC has been studied by using Alb-Cre/$KRAS^{G12D}$/$p53^{L/L}$ mice model. In our study, we observed that increased expression of SHH activates the signal-transduction cascade by upregulating its downstream element SMO, leading to the activation of GLI1 transcription factor, a confirmation of an active pathway. Also, activation of SHH pathway could induce the metastatic nature of CC by initiating EMT.

Taken together, these findings propose that blocking the Hedgehog signaling could be an effective strategy for CC management. To test this hypothesis, we designed two different approaches to evaluate the efficacy of Hedeghog signaling pathway inhibition by Cyclopamine in this cholangiocarcinoma mouse model. The mice were treated from 14 weeks onwards for 7 weeks as a *short-term treatment*, and until the signs of sickness such as, hair fall, diarrhea and death as a *long-term treatment*.

4.2.2. Efficacy of short-term Cyclopamine treatment on cholangiocarcinogenesis.

Increased expression of Hedgehog pathway elements: SHH, SMO and GLI1 were observed in mice liver tissues during cholangiocarcinogenesis. Cyclopamine treatment significantly inhibited SMO upregulation and GLI-1 nuclear. Numerous studies, including ours, have shown that Cyclopamine can inhibit the growth of xenografted tumors such as medulloblastomas, pancreatic and CC (151, 154, 79). Our present study is an extension of these outcomes and shows that systemic Cyclopamine treatment inhibits the growth of endogenous Hedgehog pathway- dependent tumors. Deregulation of Hedgehog signaling can have modulatory effects on liver injury. Indeed, in our study, liver size was decreased in Cyclopamine treated mice as compared to the Vehicle treated cohort. Assessment of liver function further showed that Cyclopamine treatment maintained the liver functionality by decreasing the serum activities of ALT and AST.

EMT is a process by which epithelial cells lose cell-to-cell contact and acquire mesenchymal phenotype to gain invasive and metastatic character. There are numerous evidences showing that EMT induction is the result of hepatic injuries like bile duct obstructions and that Hedgehog signaling is known to regulate EMT during tumor development and metastasis (191, 192). In the present study, distant metastasis in the lungs due to upregulation of invasive markers such as MMP-9, N-cadherin and α-SMA in liver tissues supports the induction of EMT by liver injury in mice. On the other hand, blockade of Hedgehog pathway using Cyclopamine inhibited metastasis by suppressing these invasive markers. This indicates that Hedgehog pathway inhibition by Cyclopamine has a modulatory effect on EMT induction through downregulation of genes associated with invasive capacities.

4.2.3. Effect of long term Cyclopamine treatment on cholangiocarcinogenesis *in-vivo*

Based on the results of the short- term preventive treatment regime, we designed a study to assess the tumor regression potential of long term Cyclopamine treatment on

cholangiocarcinogenesis. Survival was monitored as the endpoint following the long-term treatment. The CC mouse model in our study was first reported by O'Dell *et al*. According to them, average mean survival of these mice was 19 weeks, as compared with other $KRAS^{G12D}$ or $KRAS^{G12D}$+$p53^{L/+}$ mice, where mean survival was observed to be 52 weeks (158). In our study, however, the mean survival of the control group was found to be 23.2 weeks, which is comparable to the findings from O'Dell *et al*.

Cyclopamine treatment significantly prolonged the mean survival of tumour bearing mice to 20.5 days. Though the increase in the overall survival is moderately small, it is indeed meaningful. Notably, Cyclopamine did not show any toxicity in mice even in the long- term treatment regime. These observations support our previous data on mice xenograft model and suggest that pharmacological blocking of Hedgehog pathway might have the potential to improve the prognosis of CC.

Upon gross examination of tumor formation in Vehicle and Cyclopamine group at the time of necropsy, no tumor development was observed after Cyclopamine administration in the short (prevention) treatment study, whereas tumors were present in both Vehicle and Cyclopamine treated cohorts in the regression strategy, clearly indicating the differences in latency in CC tumor induction in the short and long term treatment strategies in this mouse model. Interestingly, a significant decrease in liver weight was found in Cyclopamine group, which correlated well with the lesser tumor burden in this group. Furthermore, decreased spleen weight observed in Cyclopamine treated group as compared to the Vehicle treated group, could also be a contributing factor for the increased survival rate, as cancer associated spleen enlargement has been associated with a high mortality and morbidity. We also observed the suppressive effect of long-term Cylopamine treatment over short-term treatment on distant metastasis in the lung, which was due to inhibition of MMP-9 and EMT.

4.2.4. Cyclopamine inhibits cancer stemness in long term therapy

It has been demonstrated that Hedgehog signaling induces cancer progression by regulating cell proliferation, metastasis and cancer stem cell expansion; therefore, targeting cancer stem cells could be a novel approach for cancer therapy, particularly in CC (190). In the regression study, long term Cyclopamine treatment significantly downregulated the expression of CSC transcription factors and CD133 in the liver tissue, whereas, short-term treatment had no effect on the expression of these markers. Inhibiting the signaling pathway active in CSCs has been

considered as one of the essential strategies towards cancer therapy. For instance, Mueller *et al.* showed an upregulation of CSCs expression in concurrence with high SHH expression in gemcitabine-resistant pancreatic cancer cells, reasoning that Hedgehog pathway target genes might provoke stem- ness in the cells, increase cancer cell survival and migration potential of progenitor cells (193). Moreover, EMT has also observed in CSCs, which correlates with increased metastasis to distant organs (194).

Taken together, the results indicate that suppression of CSCs transcription factors and lung metastasis as a result of Hedgehog pathway inhibition by Cyclopamine could be the reason behind increased survival observed in the used CC mouse model.

4.3.Over expression of Hedgehog signaling pathway components in patient-derived tumor samples

To validate our findings from mouse model studies, we investigated the expression of Hedgehog pathway elements in human TMAs. Our data showed that in the study cohort of 49 patients, more than 80 % of CC samples were SHH and GLI1 positive (which supports our previously published data; (79) and that, SHH and GLI1 protein expression was increased in CC TMAs as compared to the adjacent normal liver tissue. The observed results support the importance of activated Hedgehog pathway signaling during cancer progression.

4.4. $CD133^+$ cells drive tumorigenesis in NOD/SCID mice via Hedgehog pathway regulation

In this study, we have shown the potential of CD133 cells in cancer progression using xenografted $CD133^+$ cancer stem cells in immunocompromised mice. This method is considered as a gold standard for CSCs study. Numerous studies have reported the enrichment of CD133 cell population in CC. However, CD133 is also expressed by normal stem cells residing in the biliary ducts, which is activated and expanded after chronic liver damage. These cells also possess self-renewal ability and produce differentiated progeny, including the capacity to differentiate, to regain the tumour phenotype from which they were derived (195, 196).

Based on these studies, we analysed the fraction of CD133+ population in HUCCT-1 cell line. We found that CD133+ cells had a high tumorigenic potential since as few as 100 cells could result in 100% tumor incidence as compared to unsorted cells. However, when a higher number

(1000 cells) of CD133+ cells were injected, there was a decrease in tumor latency, where tumors with larger volume appeared at a higher rate in a short time.

Aberrant activation of developmental signaling pathways, including the Hedgehog pathway, in cancer stem cell population has been shown in various studies (189, 197). To validate this finding, we evaluated the expression Hedgehog pathway elements such as SHH and GLI1 in xenografted tumors derived from implantation of various populations of CSC marker- based sorted cells. We found that the expression of SHH and GLI1 was markedly upregulated in CD133+ derived tumors as compared with the tumors developed from unsorted cells, suggesting that Hedgehog pathway is persistently activated and highly upregulated in CC CSCs whereas with lower expression in their differentiated progeny.

4.5. Effect of Hypoxia on Hedgehog pathway activation and CSCs maintenance during cholangiocarcinogenesis: an *in-vitro* study

The present study, for the first time, sheds light on the interplay between hypoxia and SHH pathway activation and its consequences on cancer-stemness and the process of EMT in CC. Our study demonstrates that hypoxia induces HIF-1α expression (master regulator of oxygen homeostasis) in cancer cells, which is involved in the transcriptional and translational activation of the SHH pathway. The triggering of SHH provokes CSCs transcription factors, influencing CD133 expression and initiating the process of EMT in CC. Silencing of HIF-1α by siRNA did not promote SHH induction after hypoxia treatment. Additionally, inhibition of the SHH pathway by Cyclopamine attenuated the expression of CSC transcription factors significantly, leading to the abrogation of CD133 expression and EMT. Similar results were obtained using GANT61, which is a GLI antagonist. This suggests that targeting the SHH pathway could be a viable therapeutic option for CC treatment and its resistance towards chemotherapy, relapse and metastasis.

CC is a heterogenous malignancy; representing a miscellaneous group of epithelial cancers with a high recurrence rate (40). It is thus, of utmost importance to understand the biological complexity of the tumor microenvironment in order to develop better treatment options. Oxygen concentration plays a crucial role in various biological processes of maintaining cell and tissue integrity. Lack of oxygen in cells stabilizes HIF-1α protein, which regulates the expression of a number of genes involved in cell apoptosis, cell survival, glucose metabolism, metastasis and in various other processes (113). Stabilized HIF-1α translocates to the nucleus, where the activation of target genes takes place by binding HIF-1β. There are several studies

where HIF-1α has been identified as an important factor in the process of tumor adaptation to hypoxia (198). Numerous studies have also shown the involvement of HIF-1 in the activation of tumor-associated inflammatory signaling, which has been strongly interconnected to CC progression via chronic inflammation of biliary tracts (199). Chronic inflammation is known to activate nuclear factor-kappa B (NF- κB), a key coordinator of inflammation, and is linked to HIF-1 induced inflammatory signaling under hypoxia. This results in the activation of tumor-promoting genes including IL-6, platelet endothelial cell adhesion mole- cule-1 (PECAM-1), COX- 2, and MMP 9 and various other pro-survival genes such as BCL2, CXCR1 and CXCR2 (200, 201). Inflammatory conditions also play a pivotal role in the enhancement of Hedgehog signaling activity (202, 203). In pancreatic cancer, production of SHH through activated NF-kB signaling occurs as a result of inflammation-stimulated monocytes, which in turn helps in the proliferation of cancer cells (204). With respect to immunological responses, Hedgehog pathway is an important factor that often overlaps with inflammation. For instance, treatment of CD4+ T cells with exogenous SHH induces production of inflammatory cytokines like IL-2, and IFN-γ. Under certain circumstances, Hedgehog pathway can have an anti-inflammatory effect, which suggests its dual role in endorsing cancer inflammation and host tolerance. Furthermore, activated HIF-1α has also been involved in the induction of various other signaling pathways, besides the SHH pathway, in cancer such as PI3K/AKT/mTOR, ERK, NF-κB; however, its role in CC induction and progression is still unclear.

4.5.1. Hypoxia upregulates HIF-1α and activates Hedgehog pathway signaling

Various studies, including ours, have demonstrated the role of SHH pathway activation in CC under normoxic conditions. In the present study, we have focused on the immediate and later effects of hypoxia on the regulation of SHH pathway in CC progression. Our results showed a transient increase in HIF-1α level in both TFK-1 and HUCCT- 1 cells, with a half-life of about 8–9 h, and a substantial decrease in its expression thereafter. This is in line with several other studies, which claim a decrease in HIF-1 α levels after prolonged exposure to hypoxia (205, 206). Furthermore, immediate induction of HIF-1α augmented SHH expression in both the cell lines, with an increased secretion into the culture medium. SHH protein expression was observed maximally at 9hr, whereas it was identified after 48hr–72hr in the culture medium post hypoxia treatment. This finding is in agreement with the results obtained previously by Wang et al. (207).

It has been shown that Dispatched, a 12- transmembrane, sterol- sensing domain (SSD) resembling patched 1 (a SHH receptor) is required for distant SHH signaling regulation (208-

210). The underlying mechanism of decrease in cytosolic SHH expression and secretion into the medium after prolonged hypoxia is still not fully understood. SMO and GLI1 are the downstream effectors of the SHH pathway, and SMO upregulation and nuclear translocation of GLI1 indicate SHH pathway activation. Our data is coherent with many other reports which showed that reduced oxygen level activates SHH pathway (211-215). Additionally, on silencing HIF-1α with siRNA, a decrease in SHH protein level was observed, confirming that hypoxia is involved in SHH pathway activation in CC.

4.5.2. Cyclopamine inhibits hypoxia induced Hedgehog pathway activation

Cyclopamine has been studied as a treatment agent in many tumors with excessive SHH activity like basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma (216-220). Co-treatment with Cyclopmanine inhibited hypoxia induced SHH activation by suppressing SMO upregulation and abrogation of GLI1 nuclear translocation, resulting in enhanced induction of apoptosis in the CC cells, further strengthening the proposition that SHH signaling pathway is necessary for the proliferation, growth, and maintenance of CC.

4.5.3. Inhibition of Hedgehog signaling pathway abrogates hypoxia regulated cancer stem cell signature

The inability to fully eradicate CSCs is a significant clinical problem and leads to tumor recurrence, therapy resistance and metastatic spread of disease. Hypoxia and HIFs are known to be involved in the maintenance of stem-like state in various normal tissues (221, 222). Many studies have shown that stem cells existing in a hypoxic environment depend on HIF-1 activity for their undifferentiated phenotype, through its role in the regulation of various signaling pathways like Hedgehog, NOTCH, SIRT-1 and Wnt (223-225). There are evidences which claim that SHH pathway maintains a subpopulation of CSCs in chronic myeloid leukemia (CML), acute myeloid leukemia (AML), glioblastoma multiforme (GBM), gastric cancer, pancreatic cancer, prostate cancer, small cell lung cancer (SCLC) and lung squamous cell carcinoma (LSCC) (226-230). Besides, SHH signaling also plays an important role in sustaining CSC phenotype by subverted regulation of stemness-determining genes like NANOG and Oct4.

In our study, we have shown that hypoxia, a strong inducer of the SHH pathway, also upregulates stem-like properties of the CC cell lines by enhancing the expression of CSC transcription factors at transcriptional and translational levels. The observed upregulation of

these factors is consistent with several other studies, where hypoxia and Hedgehog pathway have been shown to maintain a stem like state in multiple cancers (78, 231-234). NANOG, a transcription factor that acts as a master determinant of both embryonic stem cell self-renewal and the re-programming of differentiated somatic cells to pluripotency, is a direct transcriptional target of the SHH signaling pathway. We also found an increase in the expression of the stem cell marker CD133 after hypoxia treatment, as previously reported in GBM neurosphere, colon cancer and CC. We next assessed the effect of hypoxia on the clonogenic potential of CC cell lines. It was observed that exposure to hypoxia for 48 h increased the clonogenic capacity of HUCCT-1 cells, indicated by an increase in the number of large clones, as compared to cells under normoxic conditions. To confirm the role of SHH signaling pathway in CSCs maintenance, we inhibited the pathway using the drug Cyclopamine and studied its effect on hypoxia induced upregulation of CSCs transcription factors and CD133 expression. SMO and GLI1 suppression in response to Cyclopamine treatment led to significant translational suppression of NANOG, Oct4 and SOX2, with substantial abrogation of CD133 expression, leading to impaired clonogenic potential of these cells. We further confirmed this finding by using GANT61, and the results were in line with our results obtained with Cyclopamine treatment.

4.5.4. Inhibition of Hh signaling pathway attenuates invasive character of CC

EMT, a process of cellular reprogramming, is an essential step to- wards cancer progression and metastasis (235-237). It helps cells acquire invasiveness and resistance to apoptosis (238). There are evidences which show that CSCs and EMT have a strong relation, such as in head and neck squamous cell carcinoma, where increased EMT expression was observed in CSCs (239-241). Various environmental factors, including hypoxia, play an important role in the activation of EMT by regulating several pathways for activation of EMT in CSCs; however, precise underlying regulatory mechanisms are still unclear. Mesenchymal markers, N-cadherin and Vimentin were found to be upregulated after hypoxia exposure in a time dependent manner, whereas, no significant change was observed in E-cadherin. Gain of N-cadherin is an essential event towards EMT, which promotes motility and invasion regardless of E-cadherin. It could thus be postulated that HIF1α induced SHH pathway activation could be a plausible mechanism for the acquisition of a mesenchymal phenotype as well as invasiveness in CC CSCs, as observed in our study. Inhibition of the SHH pathway using Cyclopamine and GANT61 resulted in the suppression of N-cadherin and Vimentin expression, leading to attenuated

invasiveness, thereby, establishing the relationship between hypoxia induced SHH pathway activation and EMT.

Taken together, for the first time, the findings of our study have revealed the critical role of hypoxia-induced SHH pathway activation in the maintenance of CSCs in CC. Inhibition of SHH signaling using Cyclopamine promoted loss of stemness, as evidenced by a reduction in clonogenicity and pluripotency markers, thereby limiting the characteristics normally supporting chemoresistance. Therefore, combinatorial targeting of the SHH signaling pathway in CSCs and tumor bulk using SHH inhibitors, conventional chemotherapeutics and/or radiation may provide a viable and efficacious clinical option to limit tumor growth, tumor relapse, overcome resistance and maximize patient outcomes. However, a greater understanding of SHH-mediated CSC maintenance and how to best combine SHH antagonists with conventional therapies in the clinic will be required before the full potential of this possibility is realized.

4.6.Conclusion

The results from the present study indicate that activation of Hedgehog pathway induces the process of cholangiocarcinogenesis with distant organ metastasis in *Alb- Cre/LSL-KRAS^{G12D}/p53$^{L/L}$* transgenic mice. The results also highlight the importance of Hedgehog pathway activation in CSCs maintenance during CC progression. Inhibition of Hedgehog signaling pathway using Cyclopamine suppressed tumor induction, cancer stemness, and metastasis, thus prolonging the survival of tumorigenic mice. Additionally, our *in-vitro* data revealed the critical role of hypoxia- induced Hedeghog pathway activation in the maintenance of CSCs in CC. Inhibition of Hedgehog signaling using Cyclopamine promoted loss of stemness, as evidenced by a reduction in clonogenicity and pluripotency markers, and enhanced apoptosis, thereby limiting the characteristics normally supporting chemoresistance. Therefore, combinatorial targeting of the Hedgehog signaling pathway in CSCs and tumor bulk using SHH inhibitors, conventional chemotherapeutics and/or radiation may provide a viable and efficacious clinical option to limit tumor growth, tumor relapse, overcome resistance and maximize patient outcomes. However, a greater understanding of SHH-mediated CSC maintenance and how to best combine SHH antagonists with conventional therapies in the clinic will be required before the full potential of this possibility is realized.

5. Summary

Cholangiocarcinoma (CC) is the second most frequently occurring primary liver tumor worldwide and is receiving medical importance because of its rising number of incidences, poor diagnosis and substandard response to the therapy. It is associated not only with genetic alterations, but other essential modifications of tumour microenvironment, which lead to the activation of various signaling pathways involved in tumour induction and progression. Thus, further exploration of the molecular mechanisms involved in the regulation of CC phenotype is needed for a better understanding of tumor progression and designing of more effective therapies. Hedgehog pathway is one of them, and its augmentation has been observed in various human malignant neoplasms, but its involvement in cholangiocarcinogenesis is still unclear.

In this pursuit, this study was conducted in *Alb- Cre/LSL-KRAS^{G12D}/p53$^{L/L}$* mice and human CC cell lines (TFK-1 and HUCCT-1) to gain a better understanding about the underlying mechanisms by which Hedgehog pathway activation triggers and maintains the process of cholangiocarcinogenesis. The study revealed CC development by 19-20 weeks of age in our mouse model, which was followed by lung metastasis via upregulation of mesenchymal markers. The expression of Hedgehog components like SHH and its downstream elements, SMO and GLI1, was found to be significantly elevated in liver tissue during CC development. Consequently, the expression of CSC transcription factors SOX-2, OCT-4 and NANOG, and CD133 were remarkably enhanced, resulting in stem- like properties and tumor progression in the liver tissue. Blockade of Hedgehog pathway using Cylopamine inhibited the growth of endogenous Hedgehog pathway- dependent tumors by suppressing SMO protein expression and GLI1 nuclear translocation. Moreover, systemic Cyclopamine treatment also inhibited metastasis by inhibiting the expression of invasive markers, thereby prolonging the survival of these mice. Additionally, Hedgehog pathway inhibition by Cyclopamine abrogated NANOG, Oct4, SOX2 and CD133 augmentation. Further, to validate our findings from mouse model studies, we investigated the expression of Hh pathway elements in human TMAs. Our data showed that in the study cohort of 49 patients, more than 80 % of CC samples were SHH and GLI1 positive, thereby implicating the role of Hedgehog pathway in cholangiocarcinogenesis.

Based on previously obtained results, we analysed the fraction of CD133^{+} cells in HUCCT-1 cell line and assessed their tumor forming capacity by implanting 100 or 1000 CD133+ cells in NOD/SCID mice. We observed that CD133^{+} cells had a remarkably high tumorigenic potential, since as few as 100 cells could result in 100% tumor incidence as compared to unsorted cells.

To further validate our hypothesis, we evaluated the expression of Hedgehog pathway elements, SHH and GLI1, in xenografted tumors derived from implantation of CSC marker- based sorted cells. We found that the expression of SHH and GLI1 was markedly upregulated in $CD133^+$ derived tumors as compared with the tumors developed from unsorted cells.

Intra-tumoral hypoxia is known to contribute towards therapeutic resistance through modulatory effects on various pathways. In this study, we investigated the relationship between hypoxia and SHH pathway activation and the effect of this interplay on cancer stemness and EMT during cholangiocarcinogenesis. Hypoxia promoted SHH pathway activation, evidenced by upregulated SHH and SMO levels, and enhanced GLI1 nuclear translocation; whereas silencing of HIF-1α impaired SHH upregulation. Hypoxia also enhanced the expression of CSC transcription factors (NANOG, Oct4, SOX2), CD133 and EMT markers (N-cadherin, Vimentin), thereby supporting invasion. Cyclopamine treatment suppressed hypoxia induced SHH pathway activation, consequently reducing invasiveness by downregulating the expression of CSC transcription factors, CD133 and EMT. Cyclopamine also induced apoptosis in CC cells under hypoxia, suggesting that hypoxia induced activation of SHH pathway has modulatory effects on CC progression.

Taken together, the results presented here indicate that aberrant activation of Hedgehog signaling has an important early role in tumor induction, metastasis and maintenance of CC cancer stemness. Therefore, it suggests that Hedgehog signaling may hold promise for new diagnostic and therapeutic approaches for CC treatment.

6. Zusammenfassung

Das Cholangiokarzinom (CC) gehört zu den zweithäufigsten primären Lebertumoren weltweit und bekommt zunehmende medizinische Bedeutung aufgrund der ansteigenden Inzidenz, schlechten Prognose und den eingeschränkten Therapiemöglichkeiten. Das CC geht nicht nur mit genetischen Veränderungen einher, sondern auch mit Modifikationen der Tumor-Mikro-Umgebung, welche zur Aktivierung von verschiedenen Signalwegen führen, die grundsätzlich an einer Tumorinduktion und –progression beteiligt sind. Eine weitere Untersuchung der molekularen Mechanismen, welche an der Regulation des CC beteiligt sind, ist daher erforderlich, um die Progression des CC besser zu verstehen und wirksame Therapien zu entwickeln. Der Hedgehog Signalweg wurde bereits bei verschiedenen malignen Tumoren des Menschen analysiert, seine Beteiligung an der Cholangiokarzinogenese ist noch unklar.

In der vorliegenden Arbeit wurden Alb-Cre / LSL-KRASG12D / p53L / L-Mäuse und menschlichen CC-Zelllinien (TFK-1 und HUCCT-1) eingesetzt, um ein besseres Verständnis der zugrunde liegenden Mechanismen zu erlangen, durch die die Aktivierung des Hedgehog-Signalwegs beim CC ausgelöst wird und welche für die Cholangiokarzinogenese bedeutend sind. Die Mäuse entwickeln in einem Alter von 19 bis 20 Wochen ein CC und bekommen auch Lungenmetastasen, sowie eine Hochregulierung der mesenchymalen Marker. Die Expression von Hedgehog-Komponenten wie SHH und seinen nachgeschalteten Proteinen SMO und GLI1 waren während der CC-Entwicklung im Lebergewebe signifikant erhöht. Infolgedessen war die Expression von Transkriptionsfaktoren für Krebsstammzellen (CSC): SOX-2, OCT-4, NANOG und CD133 erhöht. Eine Blockade des Hedgehog-Signalwegs unter Verwendung von Cyclopamin inhibierte das Wachstum von endogenen Hedgehog durch Unterdrückung der SMO-Proteinexpression und der GLI1-Kerntranslokation. Darüber hinaus inhibierte Cyclopamin die Metastasierung, indem die Expression von invasiven Markern blockiert wurde, was zu einer Lebensverlängerung der eingesetzten Mäuse führte. Zusätzlich führte Cyclopamin zu einer Runterregulation von NANOG-, Oct4-, SOX2- und CD133. Um unsere Ergebnisse aus dem Mausmodell zu validieren, untersuchten wir auch die Expression von Hedgehog in humanen Proben. Unsere Daten zeigten, dass in eine Kohorte von 49 Patienten mehr als 80% der CC-Proben SHH- und GLI1-positiv waren, was eine Rolle des Hedgehog-Signalwegs bei der Cholangiokarzinogenese unterstreicht.

Zusätzlich analysierten wir die Fraktion von CD133 positive -HUCCT-1 Zellen und bewerteten ihre Tumorbildungskapazität nach Implantation von jeweils 100 oder 1000 Zellen (CD133 +) in NOD / SCID-Mäusen. Wir beobachteten, dass CD133 positive Zellen ein bemerkenswert hohes karzinogenes Potential aufwiesen, da bereits 100 Zellen im Vergleich zu unsortierten Zellen zu einer 100% igen Tumorinzidenz führten. Um unsere Hypothese weiter zu validieren, haben wir die Expression von SHH und GLI1 in Xenograft Tumoren untersucht, welche aus der Implantation von sortierten Zellen auf der Basis von CSC-Markern stammen. Die Expression von SHH und GLI1 war in den CD133 positiven Tumoren höher als in Tumoren aus unsortierten Zellen.

Es ist auch bekannt, dass intra-tumorale Hypoxie durch modulatorische Effekte auf verschiedenen Wegen zur therapeutischen Resistenz beiträgt. In dieser Arbeit wurde der Zusammenhang zwischen Hypoxie und SHH-Signalwegaktivierung sowie die Auswirkung dieses Wechselspiels auf CSC und epitheliale-mesenchymale Transition während der Cholangiokarzinogenese untersucht. Hypoxische Bedingungen fördern die Aktivierung des SHH-Signalwegs, was sich durch eine Hochregulation von SHH und SMO und einer zunehmenden GLI1-Kerntranslokation zeigt. Die Stummschaltung von HIF-1Alpha beeinträchtigte dabei die SHH-Hochregulation. Hypoxische Verhältnisse verstärken auch die Expression von CSC-Transkriptionsfaktoren (NANOG, Oct4, SOX2), CD133 und von EMT-Markern (N-Cadherin, Vimentin). Eine Behandlung mit Cyclopamin unterdrückte die Hypoxie-induzierte Aktivierung des SHH-Signalwegs und verringerte folglich die Invasivität durch Herunterregulieren der Expression der CSC-Transkriptionsfaktoren CD133 und der EMT. Cyclopamin induzierte auch die Apoptose in CC-Zellen unter Hypoxie, was darauf hindeutet, dass die durch Hypoxie induzierte Aktivierung des SHH-Signalwegs modulatorische Auswirkungen auf die CC-Progression hat.

Zusammengefasst bestätigen die präsentierten Ergebnisse, dass eine aberrante Aktivierung des Hedgehog-Signalwegs eine wichtige und frühe Rolle bei der Tumorinduktion, Metastasierung und Aufrechterhaltung des CC spielt. Es kann vermutet werden, dass der Hh-Signalweg für neue therapeutische Ansätze zur systemischen CC-Behandlung vielversprechend sein könnte.

7. Bibliography

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8. Publication

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9. Declaration of Contributions to the Dissertation

I hereby declare that the doctoral dissertation submitted with the title: “The role of Hedgehog signaling in Cholangiocarcinogenesis: An in vitro and in vivo (mouse model) study” was carried out at the Department of Internal Medicine I, Medical University Hospital, Eberhard Karls University of Tübingen under the supervision of Professor Ruben R. Plentz.

The study was designed by me and my supervisor, Professor Ruben R. Plentz. The experiments in the submitted dissertation have been performed by me except murine pathological diagnosis, which was done in collaboration with Professor Ludwig Wilkens, Hannover Regional Hospital, Germany. Statistical analysis was carried out independently by me under the supervision of Professor Ruben R. Plentz.

I confirm that I wrote this manuscript myself under the supervision of Professor Ruben R. Plentz and that any additional sources of information have been duly cited.

A part of the thesis has been published in the journal of “Experimental Cell Research” and has been duly cited.

Tübingen, December 2nd, 2019

Vikas Bhuria

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