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REVIEW ARTICLE

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Experimental models to unravel the molecular pathogenesis, cell of origin and stem cell properties of cholangiocarcinoma

Silvestre Vicent[1,2,3] | Ruby Lieshout[4] | Anna Saborowski[5] | Monique M. A. Verstegen[4] | Chiara Raggi[6,7] | Stefania Recalcati[8] | Pietro Invernizzi[9] | Luc J. W. van der Laan[4] | Domenico Alvaro[10] | Diego F. Calvisi[11] | Vincenzo Cardinale[12]

[1]Program in Solid Tumors, Center for Applied Applied Medical Research, University of Navarra, Pamplona, Spain

[2]IdiSNA, Navarra Institute for Health Research, Pamplona, Spain

[3]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain

[4]Department of Surgery, Erasmus MC University Medical Center, Rotterdam, The Netherlands

[5]Department of Gastroenterology, Hepatology, and Endocrinology, Hannover Medical School, Hannover, Germany

[6]Humanitas Clinical and Research Center, Rozzano, Italy

[7]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

[8]Department of Biomedical Sciences for Health, University of Milan, Milano, Italy

[9]Division of Gastroenterology and Center of Autoimmune Liver Diseases, Department of Medicine and Surgery, San Gerardo Hospita, l, University of Milano, Bicocca, Italy

[10]Department of Translational and Precision Medicine, Sapienza University of Rome, Rome, Italy

[11]Institute of Pathology, University of Regensburg, Regensburg, Germany

[12]Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Rome, Italy

Correspondence
Diego F. Calvisi, Institute of Pathology, University Clinic Regensburg, Regensburg, Germany.
Email: diego.calvisi@klinik.uni-regensburg.de
and
Domenico Alvaro, Department of Translational and Precision Medicine, Sapienza University of Rome, Rome, Italy.
Email: domenico.alvaro@uniroma1.it

Abstract

Human cholangiocarcinoma (CCA) is an aggressive tumour entity arising from the biliary tree, whose molecular pathogenesis remains largely undeciphered. Over the last decade, the advent of high-throughput and cell-based techniques has significantly increased our knowledge on the molecular mechanisms underlying this disease while, at the same time, unravelling CCA complexity. In particular, it becomes clear that CCA displays pronounced inter- and intratumoural heterogeneity, which is presumably the consequence of the interplay between distinct tissues and cells of origin, the underlying diseases, and the associated molecular alterations. To better characterize these events and to design novel and more effective therapeutic strategies, a number of CCA experimental and preclinical models have been developed and are currently generated. This review summarizes the current knowledge and understanding of these models, critically underlining their translational usefulness and limitations. Furthermore, this review aims to provide a comprehensive

Abbreviations: 3D, three-dimensional; BTSC, biliary tree stem/progenitor cell; CCA, cholangiocarcinoma; cHCC-CCA, combined hepatocellular carcinoma-cholangiocarcinoma; CLC, cholangiolocarcinoma; CSC, cancer stem cell; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DEN, diethylnitrosamine; EHBD, extrahepatic bile duct; EMT, epithelial-mesenchymal transition; ENSCCA, European Network for the Study of CCA; EpCAM, epithelial cell adhesion molecule; FAO, fatty acid oxidation; FASN, fatty acid synthase; GEMMs, geneticallyengineered mouse models; HCC, hepatocellular carcinoma; hHpSCs, human hepatic stem/progenitor cells; HK1/2, hexokinase 1/2; HTVI, hydrodynamic tail vein injection; ID3, inhibitor of differentiation 3; IHBD, intrahepatic bile duct; LPCs, liver progenitor cells; MUFAs, monounsaturated fatty acids; NICD, intracellular domain of the NOTCH1 receptor; OXPHOS, oxidative phosphorylation; PBGs, peribiliary glands; PKM1/2, pyruvate kinase M1/M2; ROS, reactive oxigen species; SBRT, stereotactic body radiation therapy; SCD1, stearoyl-CoA desaturase 1; SRY-box, Sex-determining region Y-box; TAA, thioacetamide; TSGs, tumour suppressor genes; YAP, Yes-associated protein.

overview on cells of origin, cancers stem cells and their dynamic interplay within CCA tissue.

KEYWORDS
cancer stem cells, cholangiocarcinoma, in vitro and in vivo models, tumour cells of origin

1 | INTRODUCTION

Cholangiocarcinomas (CCAs) are a heterogeneous group of cancers of the biliary tree, whose etiopathogenesis remains largely unknown. The incidence of CCA in European countries ranges from one to more than four cases/100,000.[1] However, the difficulties with classification coding for CCA, and with the varied terminology that is used, determine an underestimation of CCA burden.[2] Although mortality for primary liver cancer has become more uniform across Europe over recent years, and a decline of hepatocellular carcinoma (HCC) mortality has been observed, CCA mortality has substantially increased in most of Europe.[3] Furthermore, while 19 malignancies (comprising breast, lung, colon, etc) showed a reduction of the mortality rate from 1990 to 2009 (United States data), the mortality rate for malignancies of liver and bile ducts increase of more than 40% and 60% in female and male respectively.[4] In CCA the pronounced multilevel intertumoural heterogeneity and a scarce knowledge of the etiology are associated with a lack of early diagnosis and screening and an incomplete/heterogeneous molecular landscape. This limits target therapies and further knowledge of etiology, creating a vicious cycle which affects all the steps of CCA research and management. Furthermore, it should be considered that personalized medicine approach in rare cancers may hinder efforts for prevention, early diagnosis, still lacking usually, when it focuses the attention on target mutations irrespectively to early tissue modifications. In this era the crucial challenge is to conduct translational studies with the appropriate preclinical models and taking in mind the determinants of the CCA heterogeneity, like the cells of origin. In vitro and in vivo models of CCA should be carefully adopted to gain insight into the heterogeneity and to elucidate specific basic features, the cells of origin, mechanisms, and candidate therapeutic targets of the distinct CCA subtypes. This may allow in future to a successful translation for fighting CCA in patients. The European Network for the Study of CCA (ENSCCA) has made the preclinical challenge and the study of the CCA heterogeneity pillars of its action creating a dedicated working group for experimental models which will collaborate with other specific working groups to promote correlations with the experimental counterparts of human CCA subtypes. This review, aims to illustrate the state of the art of the field of the CCA experimental models, focusing the attention on cell culture innovations and mouse models, and face the issue of the multiple determinants of the CCA heterogeneity.

Key Points

- Cholangiocarcinoma (CCA) is an aggressive tumor with limited therapeutic options due to the incomplete understanding of its pathogenesis.
- Numerous in vitro and in vivo models have been generated to study the various aspects of cholangiocarcinogenesis, providing important information but also possessing intrinsic limitations.
- The cell of origin of CCA seems not be unique, but might depend on the nature and persistency of the oncogenic stimulus.
- An ideal model recapitulating the whole spectrum of human CCA is not feasible. Rather, models addressing specific questions of the various CCA subsets should be implemented for a better understanding of this deadly disease.

2 | IN VITRO MODELS FOR CCA

CCA is a heterogeneous cancer with rising incidence and poor prognosis because of late onset of symptoms, limited treatment options, high chemoresistance and a tendency to metastasize. The rising incidence of iCCA worldwide along with the highly dismal prognosis, highlight the urgent need for new therapeutic strategies. Thus, there is a grave need to improve our understanding on the molecular and cellular mechanisms responsible for CCA development and progression. In this regard, valid in vitro models are essential to advance our knowledge on CCA, allowing high throughput experimental approaches to quickly gain insight into biological processes and effectiveness of therapies.

Over 30 years ago, the first well-characterized CCA cell line was established from an iCCA by Yamaguchi et al.[5] In the following decades, a range of CCA cell lines was generated and applied to expand our knowledge on CCA. In more recent years, the poor clinical translational value of cell lines has encouraged researchers to explore new ways of cancer modelling. Primary cultures were established and alongside cell lines, they were employed to culture three-dimensional (3D) spheroids to better mimic the tumour architecture. In addition, an organoid-based CCA culture system was developed to be used as a 3D patient-specific model.

This review section focuses on the main characteristics, strengths and weaknesses of the major types of CCA in vitro models,

illustrated with examples of their applications in the field. A comparison of the assets and limitations of the range of different models is portrayed in Table 1.

2.1 | Cell lines

Since the establishment of the first CCA cell line in 1985,[5] over fifty cell lines have been established from intrahepatic (iCCA), perihilar (pCCA) and distal tumour (dCCA) origin. Published CCA cell lines up to 2013 were reviewed by Zach et al.[6] Several others have been established since then.[7-11] CCA cell lines used most frequently include iCCA cell lines RBE [12] and HuCC-T1,[13] pCCA cell lines KKU-100[14] and QBC939[15,16] and dCCA cell line TFK-1.[17] Key characteristics of these commonly used cell lines are portrayed in Table 2.

In general, cell lines are often well-characterized, easy to maintain and susceptible to genetic modification, leading to fast and reproducible results.[18] For example, studies on cell lines Huh-28, KKU-100, KKU-M213 and TFK-1 demonstrated the proliferative and invasive effect of 17β-estradiol via the oestrogen receptor-α in CCA,[19-21] while oestrogen receptor β-agonist KB9520 led to decreased cell viability by induction of apoptosis in Huh-28 cells, revealing their potential as promising novel therapeutics.[22] Although CCA cell lines have shown to be critical tools to investigate molecular mechanisms, there are several important drawbacks in cell line-based research. First, the establishment efficiency of cell lines is low, which may be because of stringent selection by 2D, long-term serum-based culture conditions. Moreover, their resemblance to the primary tumours is greatly diminished because of these artificial culture conditions. Genetic variation and gene expression patterns of cancer cell lines greatly differ from patient tumour tissue.[23-25] Therefore, treatment response in culture has poor translational value towards clinical practice, causing many promising compounds in vitro to fail when studied in clinical trials.[26,27]

2.2 | Two-dimensional primary cultures

Primary culture methods for CCA tumour tissue were established to overcome some of the limitations of traditional cell lines.[28-33] Derivation of primary cultures is labour-intensive, as unwanted outgrowth of competing non-cancer cell fractions can occur and needs to be eradicated. However, primary cultures are grown under serum-free growth factor-enhanced conditions, which better resemble the in vivo situation. Additionally, primary CCA cultures can be used shortly after derivation, retaining more of the patient tumour features.[26] Primary cultures have been applied to further elucidate differences between mucin- and mixed-type CCA. Stem cell marker analysis reveals more CD90 + cells in mixed-type CCA, while CD13 + and CD44 + cells predominate in mucin-CCA.[30] Furthermore, mixed- and mucin-CCA display differential drug sensitivity to the chemotherapeutic agents gemcitabine and cisplatin, which are part of the current treatment regime of CCA patients.[29] An important drawback of primary cultures is that they can only be established from surgically resected specimens, limiting the applicability to CCA patients accepted for surgery only.[34] Furthermore, in 2D systems, the cells are still under strong selective pressure and lack realistic cell-cell and cell-matrix interactions.[35]

2.3 | Spheroids

To be able to mimic in vivo tumour tissue structure and intercellular and cell-matrix interactions, cancer modelling is advancing to 3D culture systems.[36] Spheroids have been established from CCA cancer cell lines and primary cultures by stirring techniques and serum-free low-attachment culture approaches.[30,37-40] 2D cultures quickly acquire an epithelial-to-mesenchymal transition phenotype, upregulating mesenchymal markers such as vimentin, SNAIL and Twist, while downregulating epithelial markers such as CD133 and EpCAM.[30,41] In comparison, spheroids have more

TABLE 1 Overview of assets and limitations of in vitro CCA model types

In vitro CCA model types	Cell lines	Primary 2D cultures	Spheroids	Organoids
Culture initiation efficiency	–	NR	NA	++
Maintenance cost	++	++	+	+
Time-efficiency	++	++	+	+
Long-term expansion capacity	++	+	NR	++
Coverage of cancer stages	–	±	±	++
Retention of mutational variance	–	NR	NR	++
Modelling of cell-cell and cell-matrix interactions	–	–	+	++
Personal & Precision Medicine applicability	–	–	+	++

Range: – (poor) to ++ (excellent). NR, not reporteds; NA, not applicable.

TABLE 2 Key characteristics of five commonly used CCA cell lines

Name	Patient and tumour characteristics				Cell line characteristics				Ref.
	Gender	Age	CCA type	Differentiation status	Cell source	Doubling Time (DT)	Chromosome number	Tumour markers	
HuCC-T1	M	56y	Intrahepatic	Moderate	Ascites	74 h	61-80	Sup: CA19-9$^+$, CEA$^+$, CA125$^+$	13
TFK-1	M	63y	Distal	Moderate	Primary tumour tissue	37 h	72-76 (modal: 73)	Sup: CEA$^-$, CA19-9$^-$	17
RBE	F	64y	Intrahepatic	Moderate to poor	Primary tumour tissue	45 h	72-87	IC: CEA$^+$, CA19-9$^+$	12
QBC939	M	51y	Perihilar	Poor	Intrahepatic metastasis	23.6 h	54-68	-	15,16
KKU-100	F	65y	Perihilar	Poor	Primary tumour tissue	72 h	56-92 (modal: 78)	IC: CEA$^+$, CA125$^+$	14

CA125, cancer antigen 125; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; IC, immunocytochemistry;Sup, supernatant.

cancer stem cell (CSC) characteristics such as higher tumourigenicity upon xenografting, higher expression of CSC markers including CD13 and LGR5, and more resistance to several chemotherapeutics used in clinical management of CCA, including gemcitabine and cisplatin.[42] Furthermore, spheroids have been used to investigate the interaction of the tumour with its microenvironment by exploring macrophage activation and modulation [42] and co-culture of the tumour spheroids with cancer associated fibroblasts.[38] However, so far, CCA spheroids, generated from cell lines or primary cultures, were not able to overcome the limitations of selection within the CCA spectrum (confined to resectable tumours) and within the 2D culture system preceding spheroid formation.

2.4 | Organoids

Recently, an innovative 3D human CCA organoid model system was established from resected specimens[43] and core needle biopsies.[44] Organoids are self-organizing stem cell-like structures, which are cultured and expanded in a basement membrane-mimicking hydrogel (eg Matrigel^{TM}, Cultrex Basement Membrane Extract). CCA organoids are able to overcome several of the drawbacks of 2D cell culture and spheroids. They are efficiently established from resected tissue biopsies (100% successfulness [43]) and core needle biopsies (60% successfulness [44]). Moreover, organoids histopathologically resemble the patient tumour, both in culture and upon xenografting in immune deficient mice. Importantly, whole exome sequencing revealed that over 80% of the mutations were retained when organoids were derived from resected tissues,[43] while mutation retention in biopsy-derived organoids varied from 20% to 90%.[44,45] Drug screening using a 29 compound library was performed in CCA organoids derived from two different patients and showed proof-of-concept for drug testing and personalized medicine applications.[43] Furthermore, CCA organoid technology was applied to demonstrate that CCA organoids have a limited capacity to differentiate towards hepatocytes[46] using a differentiation protocol developed for healthy human liver organoids.[47]

Another approach to establish cancer organoids is by engineering genetic mutations in healthy organoids. Both viral transduction and CRISPR/Cas9 genome editing techniques have been applied to transform normal organoids into cancerous ones. This provides the opportunity for creating tailor-made CCA organoids to study the effects of specific genetic aberrations or combinations of mutations on tumourigenesis, progression and drug sensitivity. Engineered pCCA/dCCA organoids were created from murine EHBD organoids by in vitro gene recombination, leading to KRAS activation and deletion of TGFβ receptor type 2 and E-cadherin.[48] These organoids gained tumourigenic potential and demonstrated similar histological patterns compared to the murine pCCA/dCCA harboring the same mutations. Moreover, they were used to identify IL-33 as a factor in cholangiocarcinogenesis and a potential therapeutic target for pCCA/dCCA.

As CCA organoids are a novel source of in vitro cancer cells, its translational value has not yet been established. First proof of clinical predictive value of cancer organoids was recently demonstrated with gastrointestinal cancer organoids. They were able to predict chemotherapy and targeted therapy responses in patients with 100% sensitivity and 93% specificity.[49] The first clinical trial where the personalized effects of stereotactic body radiation therapy (SBRT) will be compared in patients and in patient-derived CCA organoids from tumour and adjacent liver and bile duct tissue is currently ongoing.[50] Although clinical applicability is an avenue that needs to be further explored for CCA organoids and the total number of published patient-derived organoid lines is still limited (n = 10), they are a promising new 3D in vitro model faithfully recapitulating the patient tumour.

3 | MOUSE MODELS FOR THE STUDY OF CCA

Unlike in vitro culture systems, mouse models of human cancer enable us to investigate pathophysiology and treatment response within the context of a complex environment. For CCA, several experimental in vivo models exist and have helped to shed light on different stages of CCA development, starting from the neoplastic transformation of normal liver or biliary cells, over CCA progression and metastasis, towards the response to chemo- and targeted therapies. A large compendium of mutations found in human CCA has been modelled in vivo through various genetic approaches,[51,52] highlighting their value to functionally dissect the mutational and oncogenic signalling landscape of CCA. Therefore, animal models provide the experimental framework for the implementation of personalized treatments to patients suffering from this devastating disease.

In this review section, we will focus on a brief description of select murine models of CCA, including transgenic genetically-engineered mouse models (GEMMs), allograft and carcinogen-based approaches, as well as transposon models. Xenotransplant mouse models of human CCA cell lines have been extensively reviewed elsewhere.[53,54]

3.1 | GEMMs

GEMMs of CCA developed to date (Table 3) mimic several of the most frequent oncogenic alterations observed in humans, such as TP53, PTEN or SMAD4 loss, and activation of KRAS, IDH or NOTCH signalling, thus faithfully recapitulating the human disease at the molecular level. These autochthonous cancer models allow the formation of tumours under the tight control of defined genetic events. While the clear benefit of these models is that tumours arise through different stages of cholangiocarcinogenesis, in some cases including the formation of preneoplastic lesions, GEMMs frequently require complex and time-consuming breeding strategies. A selection of the currently most relevant GEMMs and related models is detailed below.

3.1.1 | Loss of tumour suppressors *Smad4* and *Pten*

The first strictly genetic model of CCA is based on the combined disruption of*Smad4* and *Pten*,[55] two tumour suppressor genes (TSGs) often found mutated in human CCA.[72,73] Liver-specific loss of the TSGs is achieved by crossing mice harboring the conditional *Pten* and *Smad4* alleles to the Albumin Cre (*Alb-Cre*) strain, which expresses a Cre-recombinase under the control of an endogenous albumin promoter.[75] *Alb-Cre* recombines loxP sites in adult hepatocytes but also in hepatic precursor cells that can give rise to both hepatocytes and cholangiocytes. In the *Alb-Cre*;*Smad4*$^{f/f}$;*Pten*$^{f/f}$ model, CCA develops with high penetrance through a sequential, multistep process that involves bile duct hyperplasia, dysplasia, carcinoma in situ and invasive CCA histologically closely resembling the human tumours by 4-7 months of age. At the molecular level, CCAs arising in this model are characterized by increased phosphorylation of mTOR and GSKβ3, consequential of disrupted PTEN/PI3K/AKT signalling, as well as high levels of p-ERK and cyclin D1. Remarkably, the synergistic effect of *Pten* and *Smad4* disruption is experimentally supported by a negative feedback loop between PTEN and SMAD4 in liver cells, suggesting that the absence of one tumour suppressor protein could be compensated by the presence of the other, and highlighting the importance of double pathway activation in CCA development.

3.1.2 | Combined *Kras* activation and *Pten* abrogation

Oncogenic*KRAS* mutations are frequently found in human CCA and associated with poor prognosis.[40,76,77] Ikenoue et al developed a GEMM of iCCA based on the simultaneous *Alb-Cre* driven activation of mutant *Kras* and deletion of *Pten*.[57] Similar to the Smad4/Pten model, *Kras* cooperates with homozygous *Pten* loss and leads to accelerated, albeit stepwise, tumour development. With a median survival of 46 days, mice succumb to non-metastatic CCAs displaying hallmark features of human CCA. Of note, the authors deploy two additional tamoxifen-regulatable GEMMs in order to express Cre recombinase in either adult hepatocytes, *Alb-Cre*$^{ERT2+}$;*Kras*$^{LSL\text{-}G12D/+}$;*Pten*$^{flox/flox}$, or cholangiocytes, *K19Cre*$^{ERT/+}$;*Kras*$^{LSL\text{-}G12D/+}$;*Pten*$^{flox/flox}$) and provide experimental evidence that, in this model, CCA originates from biliary cells. Interestingly, a similar genetic approach has been reported more recently by Lin et al that recapitulates most of the previous findings.[58]

3.1.3 | Concomitant *Kras* activation and *Tp53* deletion

In 2012, a GEMM harboring a*Kras* mutation and *Tp53* deletion, two of the most prevalent genetic events in CCA,[40,76] was described by O'Dell et al.[60] *Alb-Cre*;*Kras*$^{LSL\text{-}G12D/+}$;*Tp53*$^{flox/flox}$ mice developed tumours with complete penetrance as early as 9 weeks of age, with a median survival of 19 weeks. In this model, most tumours (66%) are exclusively CCA, whereas 17% of mice develop

TABLE 3 Genetically engineered mouse models (GEMMS)

Genetically engineered mouse models (GEMM)	Key features	Advantages (A) and disadvantages (D)	Ref.
Liver-specific inactivation of SMAD4 and PTEN (Alb-Cre,Smad4$^{f/f}$, Pten$^{f/f}$)	CCA development through a multistep progression including hyperplasia, dysplasia, carcinoma in situ, and well-established CCA	A: high penetrance (tumours in all mice) D: Long tumour latency (4-5 months), lack of metastasis, Cre activation during embryogenesis	55
Biliary tract activation of KRAS and deletion of PTEN (AhCreER^{T}, Kras$^{V12/+}$, Pten$^{f/f}$)	Multifocal non-invasive papillary neoplasms in the intrahepatic biliary tract (from major interlobular bile ducts to small bile duct radicles in portal tracts)	A: Short latency of CCA, tumour development in adult mice D: Not specific to liver tissue, lack of invasive tumour or metastasis, Cre activation during embryogenesis	56
Liver-specific activation of KRAS and deletion of PTEN (Alb-Cre, Kras$^{LSLG12D/+}$, Pten$^{f/f}$)	CCA from embryonic bipotential cells, invasive tumours with an abundant desmoplasia, primarily showing glandular morphology resembling well-differentiated human CCA.	A: Rapid tumour development (7 weeks of age), high penetrance, only CCA, abundant desmoplastic stroma D: No apparent metastases or invasion to other organs, Cre activation during embryogenesis	57,58
Liver-specific deletion of TP53 (Alfp-Cre;sTp53$^{f/f}$)	Homozygous deletion of p53 leads to tumour formation (advanced HCC/CCA)	A: Tp53 mutation found in human CCA D: long latency (14- to 20-month-old mice), tumours of bilinear origin (mixed HCC/CCA)	59
Liver-specific activation of KRAS and deletion of TP53 (Alb-Cre;Kras$^{LSLG12D/+}$;Tp53$^{f/f}$)	Multistage progression including stroma-rich tumours and premalignant biliary lesions (intraductal papillary biliary neoplasms -IPBN-, and Von Meyenburg complexes -VMC-)	A: Short latency (9-19 weeks post-natal), metastatic lesions D: Wide tumour latency range, CCA in ~ 80% of mice, Cre activation during embryogenesis	60
Cholangiocyte-specific activation of KRAS and deletion of TP53 (Sox9-CreERT2;Kras$^{LSLG12D/+}$, Tp53$^{f/f}$)	CCA tumours accompanied by adjacent extensive ductular reactions and desmoplasia, with areas resembling biliary intraepithelial neoplasia (BIN)	A: Only CCA, recombination in mature cholangiocytes D: 30 weeks average latency	61
Liver-specific activation of KRAS and deletion of TP53 (Kras$^{LSLG12D/+}$;Tp53$^{f/f}$ infected with AAV8-TBG-Cre)	Development of ICC (40%), HCC (40%), mixed HCC/CCA (20%)	A: Recombination events in adult mice, higher CCA frequency in combination with DCC diet (all tumours ICC or mixed HCC/CCA) D: Requires Cre-recombinase administration via adeno-associated virus (AAV), large tumour latency range (12-66 weeks post-AAV infection)	61
Liver-specific activation of KRAS and inactivation of FBXW7 (Alb-Cre;Kras$^{LSL\text{-}G12D/+}$;Fbxw7$^{LSL\text{-}R468C/LSL\text{-}R468C}$)	Dysplastic dust-like structures surrounded by fibrosis in all mice (only bile duct dilation and hyperplasia in some heterozygous Fbxw7$^{LSL\text{-}R468C}$ mice at the age of 8 months)	A: Short latency (2 months of age) D: Homozygous Fbxw7 mutations not occurring in human disease, Cre activation during. embryogenesis	62
Liver-specific activation of KRAS and expression of mutant IDH1 (Alb-Cre;IDH2$^{LSL\text{-}R172}$;Kras$^{LSL\text{-}G12D}$)	Multifocal liver masses of CCA histology in all mice	A: High penetrance (100%), splenic invasion and peritoneal metastases D: Long tumour latency (33-58 weeks), Cre activation during embryogenesis	63
Overexpression of Notch1 intracellular domain (NICD) in livers (Alb-Cre;NotchIC)	Development of transplantable CCA, likely progenitor cell-derived (transplantation of cells from 8 months-old mice in immunodeficient animals gives rise to CCA)	A: Notch expression is characteristic of human disease D: Cre activation during embryogenesis, no obvious cancer development after 8 months in transgenic mice, need of additional transplantation model	64
Liver-specific expression of NICD and suppression of TP53(Alb-Cre;Tp53$^{f/f}$;NotchICD)	Development of CCA abortive glandular pattern (moderate to high pleomorphic nuclei with some atypic mitoses) accompanied by dense fibrous tissue with inflammatory cells	A: High penetrance (100%), development of fibrous and inflammatory microenvironment D: Long tumour latency (>8-9 months), no metastases	65
Liver-specific HSPD1 deletion (Alb-Cre;Hspd1$^{f/f}$)	Cholangiocellular lesions, characterized by irregular glands, loss of polarity, multilayering of cells, and frequent mitosis resembling human BIN	A: Short latency, exclusive CCA formation, possibility of transplanting cholangiocellular lesions, activation of human CC pathways D: Not related to known oncogenic drivers of human disease, no metastases	66

(Continues)

TABLE 3 (Continued)

Genetically engineered mouse models (GEMM)	Key features	Advantages (A) and disadvantages (D)	Ref.
Biliary duct cells-specific activation of KRAS and concomitant deletion of Tgfbr2 and Cdh1 (*Kras*$^{LSL\text{-}G12D}$;*Tgfbr2*$^{flox/flox}$;*Cdh1*$^{flox/flox}$;*Ck19*-Cre^{ERT})	Markedly thickened EHBD wall accompanied by a swollen gallbladder involving invasive periductal infiltrating-type eCCA with lymphatic metastasis	A: Model of eCCA, short latency (4 weeks) B: Concurrent development of lung adenocarcinomas that induce lung failure	48
GEMM-based implantation models			
Bipotent liver progenitor cells (LPCs) from Alb-Cre;Kras$^{LSL\text{-}G12D}$;p53$^{LSLR172H/lox}$ ±FIG-ROS fusion	Xenograted tumours resemble advanced CCA	A: Quick model, orthotopic implantation in the liver, CCA specific, stromal reaction D: technical training to isolate LPC, genetically-engineered mouse strains required	67
Bipotent or cholangiocytic progenitor cells or hepatocytes from Tp53$^{-/-}$ mice	Tumours exhibit a high stromal content and a mixed hepatocellular and cholangiocellular differentiation	A: Quick model D: Not CCA exclusive	59
GEMM-based carcinogenic models			
Administration of TAA (Alb-Cre^{ERT2};R26$^{RlacZ/+}$ and Ck19-Cre^{ERT2};R26$^{RlacZ/+}$)	Macronodular liver cirrhosis containing cells the typical histology of CCA	A: Exclusive formation of CCA with full penetrance D: Long latency (30 weeks)	68
Deletion of TP53 in biliary duct cells and administration of TAA (Ck19-Cre$^{ERT/eYFP}$;Tp53$^{f/f}$)	Mice treatment with TAA to induce oncogenic stress leads to multifocal invasive CCA	A: Exclusive formation of CCA D: 80% penetrance, long latency (>6 months)	69
Deletion of TP53 (Tp53$^{-/-}$) and administration of CCl_4	CCl_4 causes bile duct injury/necrosis, proliferation and fibrosis development	A: Exclusive CCA D: Only half of Trp53$^{-/-}$ mice develop tumours, metastatic lesions sporadically observed	70
Deletion of glutathione-S-transferase (GST) A3 and administration of aflatoxin B1	Macro- and microscopic liver cysts, hepatocellular nodules, cholangiomas, cholangiocarcinomas and oval cell proliferation	A: Model of oval cell driven CCA D: Long latency (12 and 24 weekly AFB1 injections followed by a rest period of 12 and 6 months)	71

combined hepatocellular carcinoma-cholangiocarcinoma (cHCC-CCA) and 17% HCC. This model exhibits varying degrees of CCA differentiation with a prominent stroma and early metastasis, and premalignant lesions adjacent to tumour lesions. While Cre recombinase is already expressed in bipotential hepatic progenitor cells in this GEMM, a recent follow-up study from the same group aimed to identify the cell of origin responsible for CCA formation in response to activation of *Kras* and *Tp53* in the adult liver.[61] First, using an adeno-associated vector to express Cre recombinase in adult hepatocytes under the hepatic-specific thyroid-binding globulin promoter (AAV8-TBG-Cre), the authors show that adult *Kras*$^{LSL\text{-}G12D/+}$;*Tp53*$^{flox/flox}$ hepatocytes are refractory to transformation in the absence of liver injury. However, upon administration of a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, liver tumours recapitulating the full histological spectrum of primary liver cancer developed (CCA: 39%, HCC: 39%, cHCC-CCA: 22%). In line with previous reports,[78] these results suggest that hepatocytes are sensitive to *Ras* and *Tp53*-dependent carcinogenesis and can undergo a phenotypic switch to induce CCA development. In addition, by directing oncogenic signalling towards the ductal compartment (*Sox9-Cre*$^{ERT2+}$;*Kras*$^{LSL\text{-}G12D/+}$;*Tp53*$^{flox/flox}$), the authors demonstrated that adult cholangiocytes are readily predisposed to transformation, with seven of nine mice developing CCA. Collectively, these studies highlight the importance of *Kras* and *Tp53* mutations as well as liver inflammation for CCA development.

3.1.4 | Activating *Idh* mutations

In addition to mutations in the *KRAS* oncogene, activating mutations in *IDH*1 and 2 are found in 20% of human CCA, resulting in the aberrant production of the onco-metabolite 2 HG, which blocks hepatocyte lineage progression. In 2014, the Bardeesy group generated a latent mutant *Idh2* (*Idh2*$^{LSL\text{-}R172K}$) strain.[63] Crossed to *Kras*$^{LSL\text{-}G12D}$ and *Alb-Cre* mice, triple compound mice developed multifocal liver masses with splenic invasion and peritoneal metastases between 33 and 58 weeks (mean 47.3 weeks), histologically resembling CCA. Oval cell expansion and the presence of biliary intraepithelial neoplasia-like lesions adjacent to tumour foci suggested that cholangiocarcinogenesis in this model may involve the formation of these preceding pre-neoplastic lesions before fully malignant CCA appear. At the molecular level, tumours expressed 2HG levels comparable to those in IDH-mutant human IHCC1 cells. Considering the high frequency of IDH mutations and the clinical trajectory of IDH inhibitors, this GEMM highlights the direct preclinical relevance of murine model systems.

3.1.5 | Reactive oxigen species and mitochondrial dysfunction

Considering that CCA frequently arises in the context of chronic liver injury, a more recent GEMM aimed to address the role of mitochondrial dysfunction and reactive oxigen species (ROS) in CCA formation. To mimic hepatic mitochondrial dysfunction, Yuan et al, generated mice with liver specific *Hspd1* deletion (*AlbCre;Hspd1*$^{flox/flox}$).[66] Cholangiocellular lesions resembling human biliary intraepithelial neoplasia are evident as early as 8-weeks post-birth, and, in part, give rise to transplantable tumours with CCA characteristics. Unlike in oncogene-driven models that are highly dependent on the cell-autonomous effects of the respective driver mutations, a main player in the cholangiocellular proliferation and transformation in *AlbCre;Hspd1*$^{flox/flox}$ mice is an altered composition of the microenvironment. As a consequence of ROS accumulation, Tnf-producing Kupffer cells are recruited and induce a JNK/C-Jun dependent cholangiocellular hyperproliferation (63).

3.1.6 | Transplantation models

Implantation of premalignant liver cell populations derived from GEMMs into recipient mice can generate CCA, thus, providing a time-efficient alternative to current GEMMs for functional in vivo studies. A genetically versatile orthotopic allograft mouse model of CCA is based on liver progenitor cells (LPCs) isolated from *Alb-Cre;Kras*$^{LSL-G12D}$;*Tp53*$^{LSL-R172H/lox}$, that are transplanted intrahepatically into recipient mice. Genetic manipulation of LPCs prior to transplantation allows the introduction of additional genetic events such as deletion of tumours suppressor genes (ie, *Pten*) or overexpression of oncogenic fusions (ie, *FIG-ROS*) for their rapid functional characterization in vivo.[67]

3.1.7 | GEMMs and carcinogens

Generation of CCA in mice has also capitalized on the administration of carcinogens to various GEMMs. However, so far there is no carcinogen-induced mouse model with full penetrance. Early studies by Farazi et al reported in 2006 a CCA model dependent on the concomitant deletion of Tp53 and administration of CCL_4.[70] Although an exclusive CCA model, only half of the mice develop tumours. In a similar approach, p53 was deleted from the cholangiocyte compartment, (*Ck19-Cre*$^{ERT/eYFP}$;*Tp53*$^{f/f}$), and mice were treated with thioacetamide (TAA).[69] Experimental animals developed tumours with long latency (>60 weeks) and incomplete penetrance.

3.1.8 | GEMM of pCCA/dCCA

CCA can be classified as intrahepatic, perihilar or distal CCA based on anatomical location and depending on the insertion site of the cystic duct. During embryogenesis, while the extahepatic bile ducts (EHBDs) originated from the embryonic hepatic diverticulum, the intrahepatic bile ducts (IHBDs) arised from the ductal plate within the liver,[79] thus suggesting that the IHBDs and EHBDs may exhibit distinct properties and different carcinogenetic processes. In the context of CCA GEMMs, the previously described models are representative of iCCA, which might limit our understanding of CCA development in the context of specific cells of origin. In an effort to address this issue, Nakagawa et al developed pCCA/dCCA GEMM by generating *Kras*$^{LSL-G12D}$;*Tgfbr2*$^{flox/flox}$;*Cdh1*$^{flox/flox}$;*Ck19*-Cre^{ERT} mice (KTC-*Ck19-Cre*^{ERT}).[48] Histologically, by 4 weeks after tamoxifen administration, 90% of KTC-*Ck19-Cre*^{ERT} mice exhibited a markedly thickened EHBD wall accompanied by a swollen gall bladder, moderately-differentiated adenocarcinoma cells resembling human pCCA/dCCA expanding along the EHBD wall, and infiltration of adenocarcinoma extended to the intrahepatic hilar area including a large IHBDs. A caveat to this model is the simultaneous development of lung adenocarcinomas inducing lung failure and mouse death, which may limit its applicability for survival and therapeutic studies.

3.2 | Transposon-based models

Mouse models of liver carcinogenesis based on the combination of hydrodynamic gene delivery and transposon-mediated stable integration of transgenes in mouse hepatocytes represent an efficient alternative to query the oncogenic or tumour suppressive role of potential liver cancer drivers.[51] Hydrodynamic tail vein injection (HTVI) into 6-8-week-old mice efficiently targets approximately 2-10% of hepatocytes. Thereby, these models mimic the human situation in which "normal" and transformed cells coexist and tumours predominantly develop within adult organisms. As a potential caveat, the preferential cell type targeted by hydrodynamic transfection in these models is the mature hepatocyte, thus limiting studies to the context of oncogenesis triggered in this particular cell population. Table 4 summarizes the transposon-based models described to date, and some of the most representative and/or time-efficient models of CCA disease are discussed below.

3.2.1 | Overexpression of mutant *Ras* oncogenes

The first example of a transposon-based liver cancer model was generated by the exogenous expression of *NRAS* oncogene (G12V) in *Arf*$^{-/-}$ mice.[80] This study provides the proof-of-principle testing on the transposon technology to study liver tumourigenesis. However, despite the advantageous early onset of liver tumours in this model (4-6 weeks), the malignant lesions are both HCC and CCA, thus limiting its application for CCA research.

A later model built upon hydrodynamic injection of *NRAS* and *AKT* oncogenes also induces HCC and CCA.[84] Notably, in the *NRAS/AKT* model deletion of fatty acid synthase (FASN), a lipogenic protein whose expression is increased in multiple tumours, yields tumours that are almost exclusively CCAs by 5 weeks post-injection, implying a different sensitivity to FASN deprivation by CCA and HCC.

3.2.2 | Overexpression of the NOTCH pathway

In 2012, Fan et al investigated the functional role of the NOTCH pathway, a signalling cascade often activated in CCA, by

TABLE 4 Transposon-based models

Transposon-based models	Key features	Advantages (A) and Disadvantages (D)	Ref.
Generation of Novel Mouse Models for Liver Cancer Research	Review on transposon-based models of liver carcinogenesis		51
Overexpression of NRasV12 in Ink4A/Arf$^{-/-}$ mice	Mixed HCC and CCA	A: Quick model (mice moribund by 6 weeks) D: No CCA exclusive, genetically engineered mouse strain required	80
Overexpression of PIK3CA and Yap	CCA spans ~80% of the liver parenchyma. Three tumour types: HCC ~40%), CCA ~10%), and mixed HCC/CCA ~s50%)	A: Relatively short latency (12-13 weeks) D: No CCA exclusive, CCA only rarely forming ductular structures and often lacking desmoplastic stroma, no invasive/metastatic disease	81
Overexpression of NICD1	Development of cystic CCA	A: CCA exclusive, invasion of surrounding liver D: Long latency (5 months)	82
Overexpression of NICD1 and AKT	CCA with signs of malignancy, including necrosis, high mitotic activity and invasion of the surrounding liver parenchyma	A: Quick CCA model (4.5 weeks) D: No stromal reaction, lack of metastases	82
Overexpression of myrAKT and YAPS127A)	Development of CCA	A: Quick model (3 weeks), CCA exclusive D: No metastases	83
Overexpression of myrAKT and NRAS V12D	Mixed HCC/CCA	A: Quick model (3-4 weeks) D: Not CCA exclusive however, additional deletion of fatty acid synthase -FASN- induces almost exclusively CCA)	84,85
Overexpression of NICD in Kras^{LSLG12D} mice	Small ductular tumours with variable amount of desmoplastic stroma. Others with prominent cystic morphology. Invasion and destruction of the surrounding hepatocellular parenchyma.	A: Full penetrance mice develop high tumour burden by 14 weeks post injection) and iCCA exclusive. D: No metastases reported	87
Overexpression of AKT and Jag1	Solid, ductular or cystic tumours observed on the liver surface. Tumours some stromal component	A: Quick model (8 weeks post-injection), CCA exclusive D: Localized, no invasive, no metastases	88
Overexpression of mouse Myc and NrasG12V or mouse Myc and human AKT1 by hydrodynamic tail vein HDTV) or electroporation Epo)	HDTV-mediated transposon delivery induces HCC, whereas delivery of the same vectors via Epo originates ICC with typical tumour stroma	A: Epo favours the development of iCCA D: Implementation of Epo is technically challenging	89
Overexpression of Cas9 and sgRNAs in Kras$^{LSLG12D/+}$;Tp53$^{f/f}$ mice	Development of HCC and CCA	A: Versatile model to perform in vivo loss-of-function screens D: Not exclusive to CCA	90
Overexpression of Cas9 and sgRNAs to knockout *Pten* and *Tp53*	Liver tumours with bile duct differentiation features CK19 positive) 3 months post-injection	A: Quick model to study CCA, versatility of CRISPR/Cas9 strategy allows for further addition of genetic events D: Lack of invasive/metastatic features	91
AKT and YAP overexpression in biliary cells + IL33 injection	Tumour development along the biliary system	A: In contrast with HTVI, biliary cells are targeted and CCA arises in situ from the biliary system D: Challenging surgical approach, incomplete penetrance (72% when combined with IL33 administration)	92

overexpressing the intracellular domain of the NOTCH1 receptor (NICD), either alone or combined with AKT into the mouse liver.[82] NICD induces CCA-like lesions 20 weeks post-injection, whereas similar lesions develop by 3.5 weeks after combined transfection of NICD and AKT. The later model displayed malignant features by 4.5 weeks, including invasive margins, and spanned most of the liver surface. This model represents one of the fastest and reliable models of CCA. In a related transgenic approach, *Alb-Cre* induced overexpression of NICD led to iCCAs in 8 months old animals.[64]

As the NOTCH receptor is activated by its ligand JAG1, the consequences of Jag1 overexpression along with that of activated AKT have also been studied in the context of liver tumourigenesis.[88] Combination of these oncogenic pathways exclusively promotes CCA formation as early as 8 weeks post injection, with multiple large tumours by 11 weeks post injection.

Yes-associated protein (YAP) can activate the Notch Pathway by upregulation of JAG1. Thus, the functional relevance of YAP and AKT overexpression was tested in vivo.[83] YAP/AKT mice form CCA by 3 weeks post-injection and die by 5.5-7.5 weeks. Expectedly, the tumours express active AKT, mTOR and downstream targets of the PI3K-AKTmTOR pathway. Notably, the RAS-ERK pathway is also active in these CCA. Furthermore, tumour cells have high levels of hexokinase 1/2 (HK1/2), pyruvate kinase M1/M2 (PKM1/2) and survivin, suggestive of enhanced glycolysis as well as resistance to apoptosis.

Another model combining overexpression of activated mutant forms of YAP (YapS127A) and PIK3CA (PIK3CAH1047R) induces rapid liver tumour development in mice 12-13 weeks post-injection. However, tumour lesions consist of HCC (40%), CCA (10%) or cHCC-CCA (50%).[81]

Combination of two of the most common genetic events in human CCA, dysregulation of the NOTCH pathway and mutations in*KRAS*, has been studied by the overexpression of a NICD plasmid in $Kras^{LSL\text{-}G12D}$ mice.[87] These mice develop CCA as early as 8 weeks post-injection, with mice requiring euthanasia by 14-16 weeks.

3.2.3 | Transposon-based CRISPR/Cas9 strategies

The advent of CRISPR/Cas9 has revolutionized genome editing and provides an efficient method to disrupt cancer-relevant genes, as well as to create specific mutations by homology-directed repair.[93] The initial proof-of-concept that combined HTVI with CRISPR/Cas9 technology is able to generate intrahepatic tumours was published in 2014. Single guide RNAs targeting *Tp53* and *Pten* gave rise to tumours of biliary differentiation in FVB mice within 3 months after injection.[91] In a similar approach, Weber et al showed that multiplexed mutagenesis using hydrodynamic delivery of 10 guide RNAs into *Alb-Cre;*$Kras^{LSL\text{-}G12D}$ mice can be used in forward genetic screening approaches.[90] 20-30 weeks post HTVI, experimental animals developed multifocal tumours, histologically resembling HCC and CCA.

3.2.4 | In vivo transfection of biliary cells

While HTVI predominantly leads to transfection of hepatocytes, a recent publication by Yamada et al describes a technically challenging approach that allows to exclusively transfect biliary cells in combination with partial bile duct ligation in vivo. Intrabiliary instillation of transposon-constructs encoding for AKT and YAP leads to formation of CCA. Notably, cancer formation was substantially increased from 20% to 72% upon concomitant intraperitoneal injection of the biliary mitogen IL-33.[92]

4 | CELLS OF ORIGIN, CANCER STEM CELLS AND MODELS OF CARCINOGENESIS

Cancer cells of origin (or cancer-initiating cells) are normal cells that have acquired the first cancer-initiating mutation(s).[94] There is growing evidence that distinct cells of origin within an organ can give rise to different subtypes of cancer.[94,95] Tissue-specific stem and progenitor cells are the predominant targets exploited for tumour initiation because of biological properties that predispose them to being targets of transformation.[94]

Different models of carcinogenesis have been proposed to explain the heterogeneity of cancer through the initiation and promotion processes.[94] Stochastic genetic [96] and epigenetic [97] changes are the main determinants of cancer heterogeneity according to the clonal evolution model. Recent observations in primary liver cancers demonstrated that stochastic phenotype switching contributes to intratumour heterogeneity.[98] According to the so-called stem cell model, cancers contain intrinsically different subpopulations of tumourigenic and non-tumourigenic cells organized in a hierarchy where a small population of tumourigenic cells gives rise to phenotypically diverse non-tumourigenic cells.[94] However, responses to extrinsic environmental differences within the tumour and surrounding peritumoural tissue drive metabolic and phenotypic changes. For this reason, cancer cells adjacent to blood vessels are different from cancer cells further from blood vessels.[94] A unifying vision of cancer development considers how cancers may follow the stem cell model yet still be subject to clonal evolution as well as heterogeneity because of environmental differences within tumours.[94]

Recently, defining the molecular profiles of CCA subtypes has been shown to be a dynamic process affecting the molecular heterogeneity of CCAs. For instance, a defined CCA cluster may be initiated by extrinsic (fluke-infection) or intrinsic (IDH1 mutations) carcinogens causing genome-wide epigenetic derangement and subsequent spontaneous changes.[74] Nepal et al confirmed the importance of the hierarchy of molecular events, demonstrating that single nucleotide variants in strong driver genes, namely P53, KRAS and IDH1, determine a defined and clearly distinct imprinting in initiating cells, which leads to further defined and distinct molecular aberrations and associated pathobiological features, including therapeutic responses.[40]

These new insights on the molecular pathobiology of CCA may be seen as the “missing link” to understand the dynamic relationship between the initiation process, which affects the cells of origin, and the promotion and progression steps, which involve CSCs.

4.1 | Cancer stem cells

According to the American Association for Cancer Research Workshop 2006, CSCs are cells within a tumour that: (1) possess the capacity for self-renewal and the generation of heterogenous lineages of cancer cells that comprise the tumour; and (2) are highly tumourigenic, invasive and metastatic, and are responsible for chemo-radio resistance and tumour recurrence. Based on these features, they play a role in self-renewal, plasticity, dormancy, metastasis, and therapeutic resistance of tumours.[94,99]

4.1.1 | Markers of CCA CSCs

Several CSC markers have been reported in human CCAs,[100] including CD133,[101] epithelial cell adhesion molecule (EpCAM),[102] CD44,[103] CD13[104] and CD90.[105] CSCs comprised more than 30%

of the tumour mass in human CCA subtypes.[30] Clinical-pathological studies demonstrated that the expression of CD133,[101] EpCAM,[102] CD44, Sex-determining region Y-box (SRY-box) containing gene 2 (Sox2) [103] and S100A4 epithelial-mesenchymal transition (EMT) marker [106] contributes significantly to the worsening of CCA prognosis. EpCAM and CD133 were expressed by microparticles in a liquid biopsy of CCA patients and showed significant diagnostic and prognostic potential.[107] Moreover, Sox17, a biliopancreatic progenitor transcriptional factor that regulates the differentiation and maintenance of the biliary phenotype, acts as a tumour suppressor in CCA and its restoration may represent a promising new therapeutic strategy.[108] Recently, it has been elucidated that expression of Sox9,[109] and expression of a CSC promoter, inhibitor of differentiation 3 (ID3),[110] are associated, respectively, with the response to chemotherapy and enhanced chemo-resistance in iCCA. These pieces of evidence envisage the possibility to select iCCA patients eligible for efficient chemotherapy based on Sox9 expression, or to use ID3 expression to predict iCCA patient's response to adjuvant chemotherapy.

Unfortunately, all of the described CSC markers in CCA are shared by both CSCs and normal stem cells, thus limiting targeted strategies specific to CSCs in primary liver cancers.[99,100] Interestingly, the investigation of CD44 isoforms, expressed in several CSCs in human CCA tissues, found the CD44 variant 9 (CD44v9) highly expressed in chronic inflammation-induced Opisthorchis viverrini-related CCA, and no CD44v9 staining in the bile duct cells of normal liver tissues.[111]

Moreover, CSCs displayed considerable crosstalk and redundancy in signalling pathways.[99] On top of that, many factors that are needed for the maintenance of CSCs within their niche (cellular components, particularly tumour-associated macrophages, soluble factors, cytokines and growth factors)[42] and that contribute to self-renewal/differentiation, angiogenesis, vasculogenesis, invasion and migration, immune evasion and multiple drug resistance, could be considered as additional potential targets for successful CSC therapeutic strategies.[99,112]

Human CCA cells express EMT markers both in situ and in vitro, and, interestingly, subcutaneous xenografts from highly tumourigenic CD90 + or CD13 + CSCs are dominated by stromal markers.[30] Similarly, CD133 expression in human (non-mucin producing) iCCA indicated poor prognosis of the disease and might be associated with TGF-β related EMT alterations.[113] Highly tumourigenic human CCA CSC subpopulations generate different types of patient-derived xenograft phenotypes depending on the microenvironment.[30] The increased expression of stem and EMT genes in CCAs may imply a process of metastasization possibly determined by tumour cells characterized by an intermediate phenotype, which is largely unknown and represents a research target with important clinical implications.

4.1.2 | Cancer stem cell metabolism

First observed by Otto Warburg, it is well-known that tumour genetic alterations also imply reorganization of tumour metabolism.[114] Indeed, tumour cells produce ATP via glycolysis and accumulate extracellular lactate even under normoxic conditions[115,116] and reduced mitochondrial oxidative phosphorylation (OXPHOS).[115] Although metabolic reprogramming is currently considered an important cancer feature, little is still known regarding CSCs metabolic qualities. Since CSCs are very plastic, several studies proposed a glycolytic associated phenotype of CSCs, whereas other findings suggested a prevalent mitochondrial oxidative metabolism (reviewed in [116]). Recent evidence showed that according to their necessities, CD44 + CCA CSCs modify their redox status contributing to reactive oxygen species (ROS) defense by promotion of glutathione synthesis thus resulting in cell death escape.[117] Moreover, recent evidence demonstrated that liver CD133 + cells are characterized by high glycolytic metabolism and greater extracellular acidification rate, thus indicating that this stem-like subset is more glycolytic compared to CD133- cells. More importantly, stemness characters of liver CD133 + cells are drastically reduced after glycolysis inhibition,[118] and broad transcriptome as well as metabolome analysis revealed a central role of MYC in glycolytic metabolism regulation of liver CD133 + cells.[119]

Beside energetic metabolism there is a growing interest for lipid metabolism as proliferating tumour cells require lipids and cholesterol. Recently, it has been demonstrated that stem-like cells rely on fatty acid oxidation (FAO) for the generation of ATP and NADH.[120] Indeed NAD + concentrations increased in CD133 + cells, and this is directly correlated with SIRT1-dependent enhanced FAO.[119] In liver cancer, genome-wide transcriptional profiling proposed that NANOG could repress OXPHOS and meanwhile activated FAO pathways.[120] Likewise, liver CSCs can be regulated by stearoyl-CoA desaturase 1 (SCD1), a central enzyme involved in saturated fatty acid conversion into monounsaturated fatty acids (MUFAs).[121] Concordantly, SCD1 activation and consequent MUFA production seems to be a probable CSC hallmark.[116]

Notably, peculiar alterations of CSC-iron trafficking can sustain their role in cancer growth. It has been shown that CSCs of different types of tumours are iron-rich in comparison with tumour cells. In particular, ferroportin, the iron exporter protein, is down-modulated in CSCs associated to different tumours,[39,122] and the expression of H ferritin, the iron storage protein, is increased and seems to correlate closely with CSCs features like the capacity to form spheres.[39,123,124] The higher iron content may affect the redox status of CSCs, but information to this regard is still limited. Iron chelation treatment inhibited the stemness, as shown by the decreased expression typical surface markers or the decrease capacity to form spheres, whereas the reverse effect was observed upon iron supplementation.[39,123,124] Accordingly, iron deprivation obtained by overexpressing Fpn in breast cancer cells significantly decreased the expression of specific markers and impaired metastatic capacity.[129] The role of iron is supported by in vivo studies in xenograft mouse tumour models where iron-rich tumour spheres were shown to possess a high tumourigenic potential.[123,125] Notably, dysregulated expression of iron proteins in CSCs has been found to be a negative prognostic factor in human tumours.[39,123,124,130]

All these findings prompt metabolic plasticity as a central force that enables CSCs to modify their replicative capabilities according to specific needs. Furthermore, emerging evidence suggests that CSCs may adopt specific metabolic phenotypes based on their location within the tumour mass.[131]

4.2 | Clinical-pathological studies on the identification of candidate cells of origin in human CCA

A unique feature of CCA is that it recognizes different tissues of origin: namely, the hepatic parenchyma which comprises interlobular bile ducts, and the large IHBDs and EHBDs.[132] Indeed, the EHBDs are composed of ducts which have a wall containing peribiliary glands (PBGs) and are lined by mucin-producing cholangiocytes, while the intrahepatic biliary tree is formed by large bile ducts (segmental, area, septal) which continue the histological organization of the extrahepatic ducts, and by small bile ducts (interlobular and ductules) composed of a single layer of non-mucin producing tall cholangiocytes [2,132,133] (Figure 1). Notably, the hepatic parenchyma and the large intra/extrahepatic bile ducts are furnished by two distinct stem cell niches: the canals of Hering and PBGs, respectively. In determining the origin of CCA, the tissue of origin and its stem cell niche and derived committed lineages should also be taken into consideration [2,132,133] (Figure 1). The human liver parenchyma comprises of epithelial cell types, hepatic stem/progenitor cells, cholangiocyte and hepatocyte lineages, each containing cells at different stages of differentiation and potential targets of cancer initiation [2,132,133] (Figure 1). Evidence regarding the origin of CCA has been obtained by phenotyping candidate tissues/cells of origin with respect to CCA subtypes. Data have demonstrated that cholangiolocarcinoma (CLC) and CK19 + HCC share clinical-pathological features and originate from human hepatic stem/progenitor cells (hHpSCs).[134,135]

Expert researchers have proposed histological classifications based on the anatomy of bile ducts of origin.[136,137] Small bile duct, cholangiolar or mixed-type iCCAs share histomorphological and phenotypical features with cuboidal non-mucin producing cholangiocytes located in or near canals of Hering.[136,137] Additionally, cHCC-CCA shares characteristics of poorly differentiated primitive liver cancers with stem cell traits.[139] More recent studies on transcriptomic signature highlighted a distinct transcriptomic signature of cHCC-CCA.[140,141] In the future, omics of single cells could more precisely define the cells of origin of each CCA subtype.

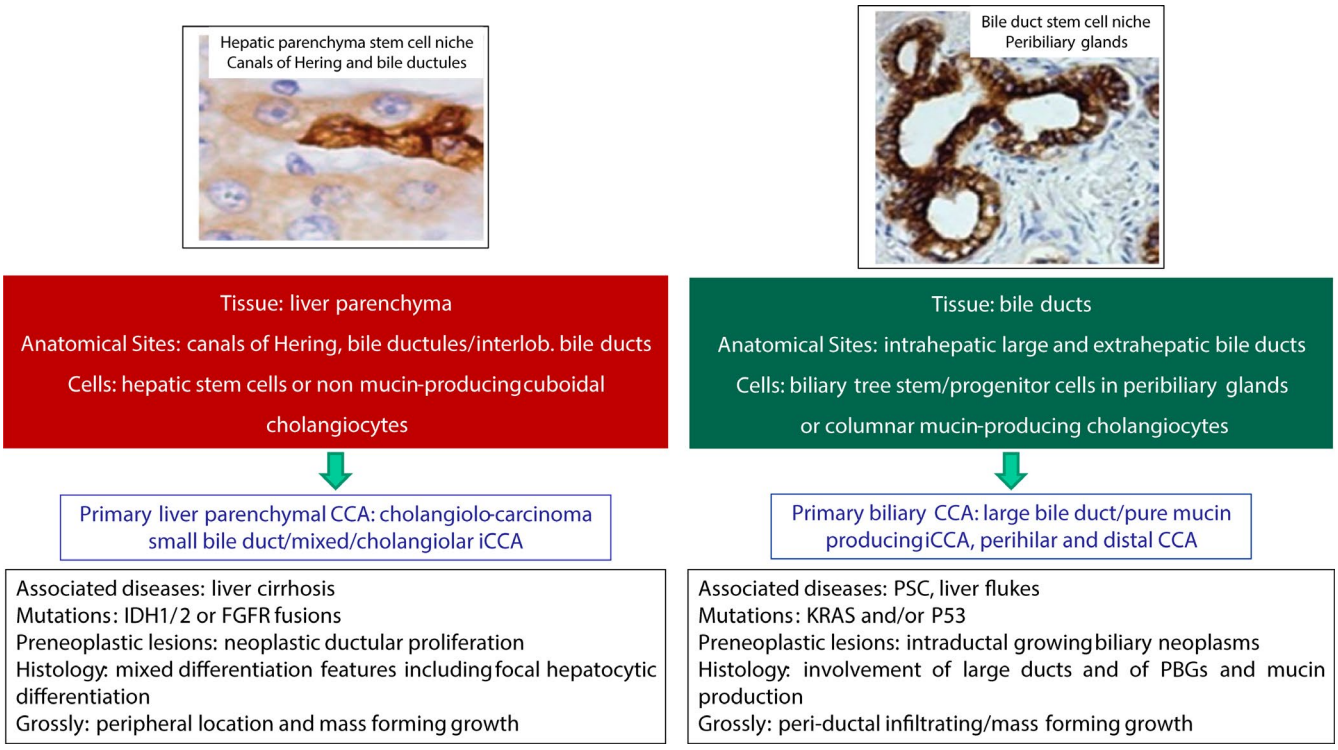

FIGURE 1 Candidate tissues, anatomical sites, cells of origin and related cholangiocarcinoma subtypes. EHBDs and large intrahepatic bile ducts (segmental, area, septal) are composed of ducts that have a wall containing peribiliary glands (PBGs) and are lined by mucin producing cholangiocytes. PBGs represent the stem cell niches of extrahepatic and large intrahepatic bile ducts. Small bile ducts (interlobular and ductules) are composed of a single layer of non-mucin producing tall cholangiocytes. Canals of Hering represent the stem cell niches of the hepatic parenchyma. According to the histomorphological aspects and based on the anatomy of the bile ducts of origin, Nakanuma, Roskams and Liau and collaborators have proposed the reported histology classifications of the CCAs. Thus, multiple tissues, anatomical sites and cells of origin have been recognized for the CCAs and have been associated to different CCA subtypes, presenting specific and separate clinical-pathological and molecular features

In summary, a spectrum of CCA subtypes, including CLC and small bile duct (mixed) iCCA, may originate from the hHpSCs in the canals of Hering or descending biliary lineage cells, such as cuboidal non-mucin producing cholangiocytes in ductules and interlobular bile ducts, while, cHCC-CCA and CK19 + HCC may originate from hHpSCs. It has been proposed that this spectrum accounts for 30% of primary liver cancers.[142]

Indirect evidence regarding the origin of hHpSCs derives from risk factors of iCCA. Chronic liver diseases, especially cirrhosis, are specific risk factors for iCCA as well as HCC.[2,132,133] In light of the recently proposed histological classifications of iCCA, it seems that mixed iCCA, as well as CLC, develop from diseases of the liver parenchyma such as viral hepatitis.[137] Human chronic liver diseases are characterized by a replicative senescence of hepatocytes, whereas hHpSC activation and the extent of ductular reaction correlate with disease activity and stage in chronic liver disease and are associated with primary liver cancer occurrence.[132,143,144] Notably, although liver parenchyma cells are relatively slow cycling when at rest, second only to dormant hematopoietic stem cells as demonstrated in mice,[145] the situation changes completely in liver disease. In humans, the stem cell division rate increases dramatically in the setting of viral cirrhosis and is linearly correlated with the lifetime risk of primary liver cancers.[146] Evidence regarding the activation of HpSCs during chronic hepatic disease and the property of stem cells to elude immune-surveillance bring about a so-called "perfect storm" that culminates in cancer initiation and invasion.[147]

As far as the large intra- and EHBDs, it has been demonstrated that columnar mucin-producing cholangiocytes and PBGs of large bile ducts give rise to large bile duct or pure mucin-producing iCCA and pCCA.[136,137] Moreover, human mucin CCA expresses markers of stem/progenitor cells exclusively located at the bottom of the PBG including LGR5, and the highest density of PBGs in the biliary tree is in the typical sites of CCA origin, such as the hilus, the branching points, and the periampullar region.[30,148] PBGs contain cells implicated in the origin of intraductal papillary neoplasms of the bile duct which are considered a precursor of mucin-CCA.[148]

Chronic bile duct diseases and conditions like PSC and liver flukes are strong risk factors for pCCA,[2] and considering recently proposed histological classifications, it appears that mucin iCCAs as well as pCCAs develop in the context of large bile duct diseases such as PSC.[136-138] Human PBG biliary progenitor cells are able to respond to bile duct epithelial loss by proliferating, differentiating, and maturing in order to restore the epithelial integrity.[150] PBGs are activated and undergo hyperplasia and mucinous metaplasia in pathologies at risk for mucinous CCA development, including PSC.[151] In diabetes, biliary tree stem/progenitor cell (BTSC) activation in humans and mice has also been observed.[152] PSC represents a human model of biliary carcinogenesis.[33] PBG cell proliferation, mucinous metaplasia and dysplasia to cancer progression take place within bile ducts and along the biliary tree in PSC, mimicking the cancerization field ("field defect") described in ulcerative colitis.[33] CCA arising from chronic damage of the large intrahepatic and EHBDs, like liver flukes, PSC and different types of cholangitis, may also follow this pattern of cancerization.

4.3 | Mouse models for tracing the cellular origin of CCA

Lineage tracing is increasingly being used to probe the origin of different cell types that exist within cancers,[153] including CCAs.[141,154] Controversies exist regarding the cellular origins of iCCA in lineage tracing studies in experimental carcinogenetic models. There is evidence in favour of a hepatic progenitor cell, cholangiocyte or hepatocyte origin. For instance, Dill et al described a HpSC origin because of aberrant activation of Notch2 signalling in AlbCre/N2ICD (Notch 2 intracellular domain) plus diethylnitrosamine (DEN).[155] Villanueva et al reached a similar conclusion following the aberrant expression of the active form of Notch1 in AFP-NICD mice.[142] Evidence contradicting a HpSC origin has been provided by Shin et al by tracing the thyroxine-binding globulin in mice subjected to diethylnitrosamine (DEN) as well as multiple injections of CCl4 or TCPOBOP.[156] Guest et al used a cholangiocyte-lineage tracing system (CK19-lineage tracing) to target p53 loss in biliary epithelia. In the context of chronic inflammation and p53 loss, which is common in human disease, biliary epithelia are a target of transformation and an origin of iCCA.[157]

Hepatocytes have also been investigated as candidate cells of origin of CCA in lineage-tracing studies tracing albumin[68] or transthyretin positive parenchyma cells in NICD/AKT mice.[82] More recently Wang et al, through the adoption of an established murine hepatocyte-derived iCCA model by hydrodynamic injection of activated forms of AKT (myr-AKT) and Yap (YapS127A) proto-oncogenes, found that AKT/Yap-induced iCCA formation is hepatocyte derived and this process is strictly dependent on the canonical Notch signalling pathway in vitro.[158] Finally, in studying the effect of E-cadherin deletion in addition to Kras activation and TGFβR2 deletion on the biliary tree (KTC-K19CreERT), it was revealed that biliary epithelial injury-induced regenerative response mediated by IL-33 accelerates development of pCCA from peribiliary glands.[48] Experimental models of liver disease and associated cholangiocarcinogenesis are affected by a translational issue that may impact the results and interpretation of lineage tracing studies conducted on these models. Indeed, the murine models of liver injury do not cause a significant decrease in hepatocyte proliferation.[132] In a novel mouse model, in which apoptosis, necrosis and senescence are induced in nearly all hepatocytes, HpSC activation was crucial for survival and complete functional liver reconstitution, with the emergence of cholangiocyte-derived hepatocytes.[159,160]

Whilst the lineage tracing technique is useful for tracking cells in vivo and dissecting the roles of different cellular subsets in development, homeostasis and oncogenesis, there are important caveats associated with lineage tracing strategies.[153] For example, a pathophysiological issue may arise with respect to the origin of primary liver cancers when the gene used for cell tracing can be expressed in multiple cells and lacks specificity.[161] Also, the experimental damage can induce specific hepatocyte alterations and mutagenesis, such as in the thioacetamide-induced mouse model (hepatotoxin was used in the study of HCC.[162] Technical issues, such as artifacts induced by tamoxifene administration should also be taken into account.

Indeed, it was revealed that tamoxifen induced ectopic stem cell marker expression.[163,164] Notably, cholangiocytes and CCA are oestrogen sensitive,[165] and tamoxifen administration could affect stem cell activation and determine a relatively hyper activation and the expansion of other cell types involved in hepatic regeneration. Other factors that may influence lineage tracing results include the scoring of clones, the timing of induction and the marked variability in labelling efficiency.[153] Thus, experts of the lineage tracing technique have suggested viewing extensive areas of tissue and taking into consideration the intricacies of the methodology for lineage tracing studies on normal tissues and on potential cancer cells of origin.[153]

In conclusion, a definitive determination of the origin of iCCA in experimental models cannot be reached based on current evidence. Indeed, it appears that experimental models of liver damage do not reproduce the exhaustive proliferative potential of hepatocytes typical of human chronic hepatic disease, and that lineage tracing studies must be conducted and interpreted cautiously.

4.4 | Interaction between cells of origin, molecular alterations and underlying pathologies in the origin of different CCA subsets

Intertumoural heterogeneity is the result of a dynamic interaction of intrinsic and extrinsic mechanisms, such as, the genetic/epigenetic mutational profile of cells, the nature of the cell of origin and the tissue microenvironment.[94] The network of liver and BTSC niches should be considered a framework for understanding liver and biliary regeneration after extensive or chronic injury and for the study of related diseases.[132] Chronic liver diseases affecting interlobular bile ducts or large intrahepatic bile ducts could activate HpSCs in the canals of Hering or BTSCs in PBGs respectively.[144,166] Furthermore, these stem/progenitor cell niches may contain the cells of origin of CCA subtypes.[144] Etiologies of chronic hepatic parenchymal diseases, such as alcoholic, viral and metabolic (NAFLD/NASH) injuries, trigger hHpSC activation while PSC triggers PBG activation.[144] Interestingly, among cholangiopathies a clear distinction has been described. Indeed, primary biliary cholangitis massively activates hepatic progenitor cells and only minimally activates PBGs, while the opposite has been described in primary sclerosing cholangitis.[166] Since somatic mutagenesis and epigenome features are cell/lineage specific [95,167] and are driven by the inflammatory pathological milieu characterizing the CCA risk factors,[74] the multiple niches of origin plus the specific related genomic alterations may explain the intertumoural heterogeneity observed at any level of CCA, including molecular clustering (Figure 2).

A comprehensive integrative molecular analysis of 10,000 specimens from 33 types of cancer highlighted the influence of cell type in DNA-methylation-based clustering.[95] This observation confirms the molecular similarities between histologically or anatomically related cancer types. As far as CCAs are concerned clinical-pathological or etiological classes clearly influence molecular clustering.[74] The spectrum ranged from IDH mutations typically observed in iCCA to KRAS mutations observed in pCCA, including PSC-associated

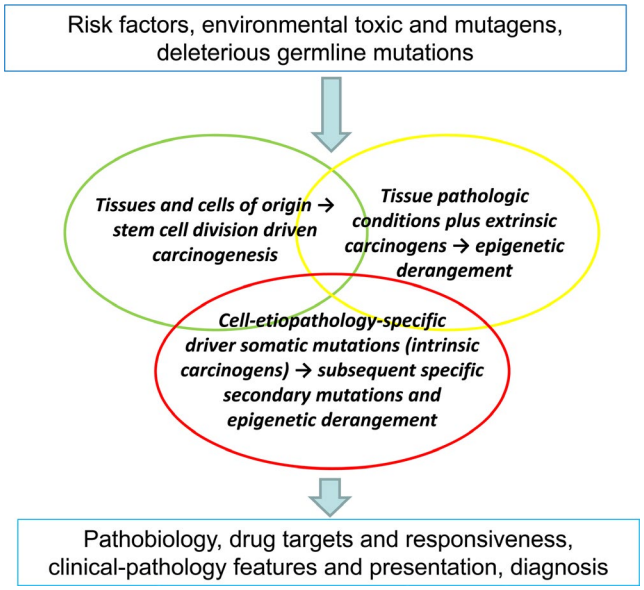

FIGURE 2 A proposed model of cholangiocarcinoma origin and heterogeneity. The interplay of the tissue/cell of origin, the pathological condition, and the cell-etiopathology-specific somatic mutations and epigenetic modifications may dictate the origin and the pathobiology of cholangiocarcinoma (CCA). Heterogeneity of CCA may be because of the interplay of the distinct tissues and cells of origin, the underlying diseases, and the associated molecular clustering based on driver mutations which shape the pathobiological features of the different CCA subtypes

CCA.[40,73,74] Thus, the intertumoural heterogeneity of CCA may be because of the interplay of the distinct tissues of origin, the underlying diseases, and the associated molecular clustering based on driver mutations which shape the pathobiological features of the different CCA subtypes.[40] Emphasizing this complex pathogenesis of CCAs would have implications on preventive strategies or early diagnosis in patients with clinical or subclinical underlying hepatic or biliary diseases, and may inform a rational approach to the personalized medicine of CCA subtypes.

5 | CONCLUSIONS

Numerous alterations associated with and/or responsible for CCA development and progression have been identified in the recent years, mainly because of the establishment and application of high-throughput methodologies to this deadly disease. These approaches have led to the generation of an extremely large body of data that remain to be properly interpreted and, eventually, applied to the clinical practice. For this purpose, several in vitro and in vivo models recapitulating many of the alterations detected in human CCA specimens have been developed. These models, each of them possessing significant advantages and drawbacks, provided important information on the role and impact of genes believed to either favour or suppress CCA cell growth. Nonetheless, many crucial questions remain unanswered. First of all, the relevance of the

experimental data for the human disease requires to be validated in further studies. In addition, critical issues have been either marginally addressed so far (inter- and intra-tumour heterogeneity, role of the tumour microenvironment, mechanisms of resistance to therapy, pathways, cellular crosstalk, tumour metabolism, metastases, etc) or remain highly debated (cells of origin of CCA, importance of cancer stem cells in tumour development and maintenance). Furthermore, the most recent results obtained by using large-scale approaches clearly showed that CCA is a heterogeneous disease, both at the molecular and clinical level. This implies that the generation of an ideal model recapitulating the whole spectrum of the human disease is unfeasible. On the other hand, this observation indicates that existing and future experimental models should be devoted to address more specific, precise issues on CCA. In particular, the generation of ad hoc models mimicking the biologic and molecular features of subsets of human CCA would be a powerful tool both to better understand its pathogenesis and for the development of novel, personalized and effective treatments against this aggressive tumour.

With the recent creation of the European Network for the Study of Cholangiocarcinoma (ENSCCA: www.enscca.org / www.cholangiocarcinoma.eu), a pan-European and multidisciplinary collaborative group, an ideal platform at basic and clinical level has been developed both for further investigation of the experimental models as well as for their prompt validation and translational application in patients.

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CONFLICT OF INTEREST

The authors do not have any disclosures to report.

ORCID

Monique M. A. Verstegen https://orcid.org/0000-0001-9908-6673
Pietro Invernizzi https://orcid.org/0000-0003-3262-1998
Diego F. Calvisi https://orcid.org/0000-0002-6038-8567
Vincenzo Cardinale https://orcid.org/0000-0003-0234-3341

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