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REVIEW ARTICLE

Diverse functions of SOX9 in liver development and homeostasis and hepatobiliary diseases

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Abstract The liver is the central organ for digestion and detoxification and has unique metabolic and regenerative capacities. The hepatobiliary system originates from the foregut endoderm, in which cells undergo multiple events of cell proliferation, migration, and differentiation to form the liver parenchyma and ductal system under the hierarchical regulation of transcription factors. Studies on liver development and diseases have revealed that SRY-related high-mobility group box 9 (SOX9) plays an important role in liver embryogenesis and the progression of hepatobiliary diseases. SOX9 is not only a master regulator of cell fate determination and tissue morphogenesis, but also regulates various biological features of cancer, including cancer stemness, invasion, and drug resistance, making SOX9 a potential biomarker for tumor prognosis and progression. This review systematically summarizes the latest findings of SOX9 in hepatobiliary development, homeostasis, and disease. We also highlight the value of SOX9 as a novel biomarker and potential target for the clinical treatment of major liver diseases.

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Introduction

The liver is a crucial hub for multiple physiological processes such as lipid homeostasis, nutrient metabolism, immunoregulation, and drug detoxification. It is also the largest gland in the body with endocrine properties for the secretion of angiotensinogen and thrombopoietin and exocrine properties for the formation of bile. Importantly, glucose can be stored as glycogen and synthesized via the gluconeogenic pathway in the liver, which is an effective strategy for coping with fasting. In addition, the liver is responsible for amino acid metabolism and urea metabolism for the disposal of nitrogenous waste from protein degradation.[1,2] Since the liver is a crucial regulator of normal physiological processes, liver failures and/or diseases, such as viral infection, drug-induced injuries, cirrhosis, liver fibrosis, and cancers, result in high rates of morbidity and mortality.[3,4] The high health burden from liver diseases has reminded people to improve their cognition of the mechanisms controlling liver development, homeostasis, and disease progression.[5–7]

Transcription factors of the sex-determining region Y (SRY)-related high-mobility group (HMG) box (SOX) family have been shown to mediate liver development and liver disease progression.[8,9] More than 20 SOX transcription factors in mammals can be classified into eight subgroups based on their sequences and functions.[10] SOX9, SOX8, and SOX10 belong to subgroup *E*, in which proteins share high sequence similarity and regulate cell differentiation and fate determination. SOX9 can regulate various cellular processes, including cell proliferation,[11] metastasis,[12] maintenance of stemness,[13] and chemoresistance.[14] Importantly, multiple studies have identified the role of SOX9 in various cancers, including colon cancer,[15] liver cancer,[16] prostate cancer,[17] bladder cancer,[18] melanoma,[19] and cervical cancer.[20]

SOX9 is crucial for regulating hepatobiliary development and cell fate determination during embryogenesis and liver homeostasis in adulthood. In the liver, alterations in SOX9 protein activity and *Sox9* gene transcription may lead to malignant hepatobiliary diseases. A deeper understanding of SOX9 from the perspective of liver development and pathology could benefit the development of novel therapeutic interventions and personalized medicine. This review focuses on the latest advances in SOX9 function in liver physiological or pathological status, as well as the molecular regulation of SOX9 expression during liver development.

SOX protein family, groups, and domain structures

The sex-determining region Y (SRY)-related high-mobility group (HMG) box (SOX) family consists of more than 20 members in vertebrates, which can be classified into eight subgroups (SoxA–H) based on their sequences and functions (Fig. 1A). This group of genes originated from a series of evolutionary processes and was first discovered in 1990.[21] Since then, multiple studies have demonstrated their dynamic and essential functions during embryonic development and disease progression. The molecular functions and novel insights of SOX transcription factors were comprehensively reviewed in two recent studies.[22,23]

Generally, SOX proteins in the same subgroup have similar biochemical properties and may perform overlapping functions. For example, SOX5 and SOX6 proteins of the SoxD subgroup display cooperative and overlapping functions during cartilage development.[24] On the other hand, SOX proteins from different subgroups usually show distinct biological functions despite their recognition of the same DNA consensus motif.[25] SOX proteins can recognize different DNA binding sites and selectively regulate gene expression. Possible factors leading to this difference include flanking sequences that affect binding affinity, the post-translational modification of SOX factors, SOX proteins dimerization, and interactions between SOX proteins and other transcription factors due to their adjacent binding sites.[26] All these events contribute to the diverse functions of SOX proteins.

SRY was the first SOX protein to be discovered and is the only member of the SoxA group. It plays an important role in sex determination and the HMG box of this SOX protein is highly conserved between species. The SoxB group can be classified into SoxB1 and SoxB2 subgroups. SoxB1 factors, including SOX1, SOX2, and SOX3, which contain transcriptional activators, share a high degree of sequence similarity and have almost identical biological activities.[27] Unlike SoxB1 factors, SOX14 and SOX21 involved in the SoxB2 group have a transrepression domain in the C-terminal region instead of a transactivation domain.

The domain structure of factors in SoxC, SoxE, and SoxF groups are similar to SoxB1 proteins, with a total length of 300–500 amino acids containing HMG domains located close to the N terminus and an activation domain in the C-terminal region. The SoxC group is comprised of SOX4, SOX11, and SOX12 factors and has been reported to be associated with the central nervous system (CNS) development including neuronal subtype specification and dendritogenesis.[28] SOX8, SOX9, and SOX10 belong to the SoxE group. These factors contain a self-dimerization domain located proximally to the HMG box, which is required for the chondrogenesis.[29] SoxF proteins including SOX7, SOX17, and SOX18, are found to participate in cardiogenesis and lymphangiogenesis.[30]

SoxD factors, which consist of the Sox5, Sox6, and Sox13, have a long N-terminal sequence containing a coiled-coil domain which allows dimerization with other SoxD proteins.[31] SoxG (SOX15) and SoxH (SOX30) proteins are structurally related to SoxB1 and SoxD proteins, respectively. Recent studies have shown the antitumor role of SOX15 in glioma and prostate cancer.[32,33] As the only identified member of the SoxH group, SOX30 has no obvious homology outside the HMG box to other SOX groups.[34] Studies have identified this factor as a prognostic biomarker for ovarian cancer[35] and a potential tumor suppressor in lung cancer.[36]

SOX9 structure and functions

SOX9 belongs to the SOXE subgroup, and its coding gene is located in a 3-Mb region on chromosome 17.[37] SOX9 mainly consists of three domains: an HMG domain, a self-dimerization domain (DIM) located on the N-terminal side, and a

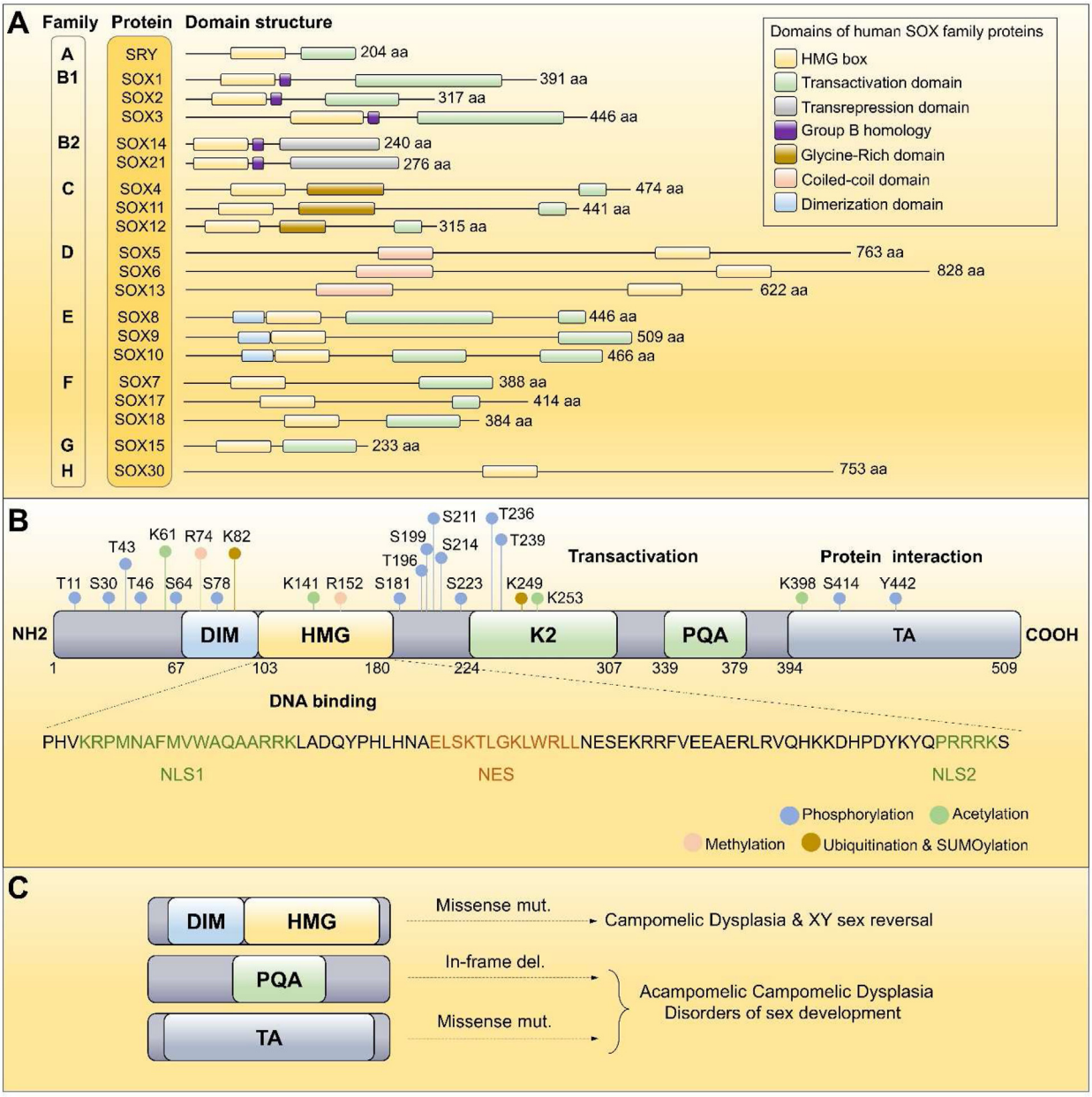

Figure 1 Schematic diagram of domain structures of human SOX transcriptional factors. **(A)** Based on the degree of sequence identity of the HMG motif, the human SOX family of transcription factors has been subdivided into eight groups (SoxA through SoxH). The HMG motif is an evolutionarily conserved DNA-binding motif and is also a representative signature of the SOX family. **(B)** SOX9 protein domain organization and post-translational regulation. SOX9 protein has five different domains: the dimerization domain (DIM) is located at N-terminus, followed by the high-mobility group (HMG) domain, two separate transactivation domains (K2 and PQA) located in central position, and the TA domain is located at C-terminus. The HMG domain consisting of one nuclear export signal (NES) sequences and two nuclear localization signal sequences. Post-translational modifications including phosphorylation (blue), acetylation (green), methylation (pink), and ubiquitination/sumoylation (brown) are highlighted. **(C)** Mutations located at different position of *Sox9* can cause severe diseases.

transactivation domain located on the C-terminal side (Fig. 1B). The DIM is essential for dimerization with the HMG domain of other SOX members, whereas the C-terminal domain (also called TA, transactivation domain at the C-terminus) interacts with transcription machinery or coactivators, such as CBP and MED25.[38–40] Mutations located at different positions of Sox9 can cause severe diseases (Fig. 1C). Importantly, the DNA-binding HMG domain consists of one nuclear export signal (NES) sequence and two nuclear localization signal (NLS) sequences, which guarantee a high-rate translocation of SOX9 between the cytoplasm and nuclear.[41]

SOX9 is widely expressed and is essential for cell lineage determination during embryonic development and

adulthood. The role of SOX9 in development was first reported in campomelic dysplasia (CMPD), a severe skeletal malformation syndrome in which heterozygous mutations of the gene also cause XY sex reversal and skeletal dysplasia.[42,43] SOX9 has been reported to play a pivotal role in the development of organs such as cartilage,[44,45] liver,[8,46,47] breast,[48–50] lung,[51–53] testis,[54,55] brain,[56,57] eye,[58–60] stomach,[61,62] pancreas,[63,64] kidney,[65–67] heart,[68,69] prostate,[70,71] neural system,[72,73] and skin[74,75] (Table 1). Notably, *Sox9* homozygous mutant mice died on E11.5[46]. SOX9 is vital for the development of organs from the endoderm (*e.g.*, liver, lung, pancreas), mesoderm (*e.g.*, heart, male gonad, bones), and ectoderm (*e.g.*, skin, eye, hair) at the embryonic stage (Fig. 2).

SOX9 is expressed in multiple adult cell types such as intestinal epithelial cells, neural crest cells, and pneumonocytes. In the developmental stage of the neural crest, SOX9 can regulate the differentiation direction of neurons and glial cells by inhibiting the expression of genes such as Pax6, Pax7, and Nkx6.[76] In human outer root sheath cells, high *Sox9* expression can maintain the number of hair follicle stem cells. For the stem cell population, SOX9 can not only maintain the high activity of enhancers but also relieve the silencing state of some genes.[37] Moreover, SOX9 participates in various important processes, including sex determination, the cardiovascular system, and chondrogenesis.[55,77,78]

Molecular mechanisms of SOX9 regulation

SOX9-partner complexes

SOX9 generally acts as both an activator and repressor in transcription, which relies on partner proteins, target genes, and the subsequent recruitment of either coactivators or repressors.[37,79] Partner proteins can be factors from another protein family: heterologous or homologous SOX proteins. Target genes usually have binding sites for partner proteins, and SOX-partner complexes are first formed before binding to the target DNA sites. For example, the dimerization of SOX9/SOX9 is essential for the transactivation of *Col11a2* and *Col9a2*, and the missense mutation A76E in this dimerization disrupts the transactivation activity of SOX9 during chondrogenesis.[29,80] SOX5/SOX6 dimers consolidate the combination of SOX9 and DNA sites by binding to sites on enhancers close to SOX9, thereby improving SOX9 activity.[24] Importantly, the

Table 1 The role of SOX9 in development and disease.

Tissue	Relevance to SOX9	Reference
Cartilage	Chondrogenesis	44,45
	Regulates cartilage ECM components	
	Regulates β-catenin degradation	
Liver	Biliary development	8,46,47
	Activation of HSCs in liver fibrosis	
	Liver injury and liver cancer progression	
Breast	Regulates invasion and metastasis of breast cancer cells	48–50
	Drives breast cancer endocrine resistance	
Lung	key factor for tracheal formation; Loss of SOX9 result in tracheal defects	51–53
	Drives EMT in non-small-cell lung cancer	
	SOX9 dependent FOXA1 expression promotes tumorigenesis in lung carcinoma	
Testis	Mediates testis differentiation and male development	54,55
	Promotes Pgd2 synthesis	
Brain	Ischemic Brain Injury	56,57
	Astrocyte expression specifically (astrocyte-specific nuclear marker)	
Eye	Regulates lacrimal gland branching and differentiation	58–60
	Retinal differentiation	
Stomach	Specifies pyloric sphincter epithelium	61,62
	Gastric cancer progression	
Pancreas	Endocrine specification	63,64
	Maintains progenitor cell population	
Kidney	epithelial branching of ureter	65–67
	Activation of SOX9+ cells contribute to kidney repair after partial nephrectomy	
	Kidney fibrosis progression	
Heart	Vascular calcification	68,69
	heart valve formation	
Prostate	Prostate development	70,71
	Regulates invasion and metastasis of prostate cancer cells	
Neural	Key factor for neural stem cells formation and maintenance	72,73
	Phosphorylation of SOX9 is required for neural crest delamination	
Skin	Maintenance and differentiation of hair follicle stem cells	74,75
	Decreased proliferation in melanoma xenografts *in vivo*	

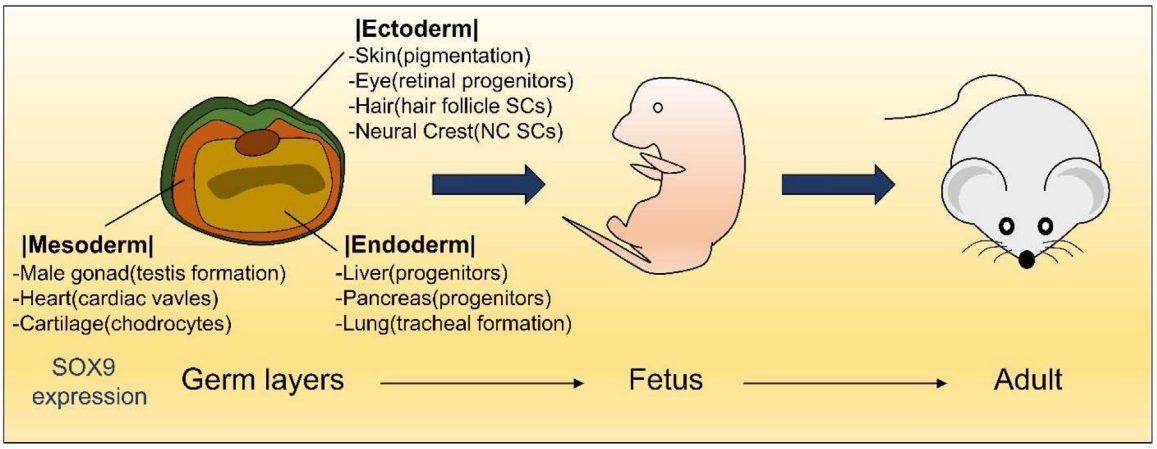

Figure 2 SOX9 is widely expressed and essential for cell lineage determination during embryonic development and through to adulthood. SOX9 is expressed throughout development, initially in pluripotent progenitors and subsequently maintained in fetal and adult progenitors.

SOX9 and SOX5/SOX6 trio (also known as the SOX trio) are required for the activation of *Col2a1*, a key gene that controls chondrogenic differentiation and extracellular matrix (ECM) deposition.[81–83] The transcriptional coactivator CBP/p300 also enhances *Col2a1* promoter activity by binding to the C-terminal domain of SOX9.[84]

SOX9 is also an important testis-determining gene involved in gonadal development in mice. SRY forms a complex with SF1, which binds to the testis-specific enhancer of *Sox9* and regulates the expression of SOX9.[85] In addition, up-regulated SOX9 facilitates the formation of a feedback loop, in which SOX9 can replace SRY to activate its expression by binding to SF1. The self-perpetuating pathway of SOX9 expression appears to positively regulate its transcription.

Transcriptional regulation of SOX9 expression

Generally, the expression of SOX9 is regulated by multiple transcription factors, and the subsequent effects usually influence cell fate and functions. Transcription factor EB (TFEB) is critical for controlling cell fate and proliferation in the liver during tissue repair and embryogenesis. As a downstream target of TFEB, SOX9 is involved in the regulation of cell fate decisions. The TFEB-SOX9 axis drives the differentiation status of liver progenitor cells (LPCs) into progenitor/cholangiocyte lineage during liver development and regeneration.[86] Additionally, the NF-κB subunit p65 positively regulates SOX9 expression by directly binding to the *Sox9* promoter, which may influence the subsequent invasiveness of pancreatic cancer stem cells (CSCs).[87] Studies have shown that the *Sox9* promoter contains several key cis-acting elements, which can be targeted by the NF-κB member RELA,[88] HIF-1α,[89] and the Notch signaling mediator RBPJ.[90] These key factors play positive or negative regulatory roles in *Sox9* gene expression.[91]

DNA methylation plays an essential role in embryonic development and cancer progression. Histone lysine demethylase 4D (KDM4D or JMJD2D) is highly expressed in liver CSCs and promotes the self-renewal of hepatic CSCs by enhancing the expression of SOX9. Specifically, JMJD2D enhances the transcription of *Sox9* by reducing H3K9me3 levels on the *Sox9* promoter via interaction with the Notch1 intracellular domain.[92] Studies have shown that the methylation status of the *Sox9* promoter region is aberrant in several tumor types.[93,94]

Post-transcriptional regulation of SOX9 expression: MicroRNAs, LncRNAs, and circRNAs

MicroRNAs (miRNAs) are endogenous, short non-coding, single-stranded RNAs that induce either translation blockade or mRNA degradation by binding to the complementary 3′-untranslated region (3′-UTR) of target mRNAs.[95] Sequence analysis revealed that the 3′-UTR of human *Sox9* mRNA contained distinct sites that can be recognized by six different miRNAs: miR-101, miR-145, miR-1/205, miR-300, miR-384—5p, and miR-590/590—3p.[91] In hepatocellular carcinoma (HCC), miR-101 was the first miRNA found to reduce HCC cell proliferation and tumorigenicity through directly binding to SOX9.[96] Similarly, several inhibitory miRNAs have been found to mediate cancer properties by targeting SOX9.[97–102] In contrast, miR-126 positively regulates the expression of SOX9 by suppressing the translation of homeobox b6 (Hoxb6), thereby regulating the fate of $SOX9^+$ liver progenitor cells (LPCs) during liver regeneration.[103] These findings suggest that miRNAs are potent regulators of SOX9 expression, indicating their possible roles in improving the treatment of liver diseases (Fig. 3).

LncRNAs are non-coding RNA transcripts longer than 200 nucleotides.[104,105] LncRNAs regulate multiple biological processes, such as proliferation, differentiation, apoptosis, invasion, and tumorigenesis, by targeting miRNAs and genes.[106,107] Several studies have shown that lncRNAs mediate the regulation of SOX9 expression in liver disease. LncRNA NEAT1 inhibits the expression of miR-139—5p, which regulates liver fibrosis by targeting the β-catenin/SOX9/TGF-β1 pathway.[108] The lncRNA DUXAP9 positively regulates HCC cell stemness by directly binding to the 3′-UTR of SOX9 and increasing the expression of SOX9.[109] In

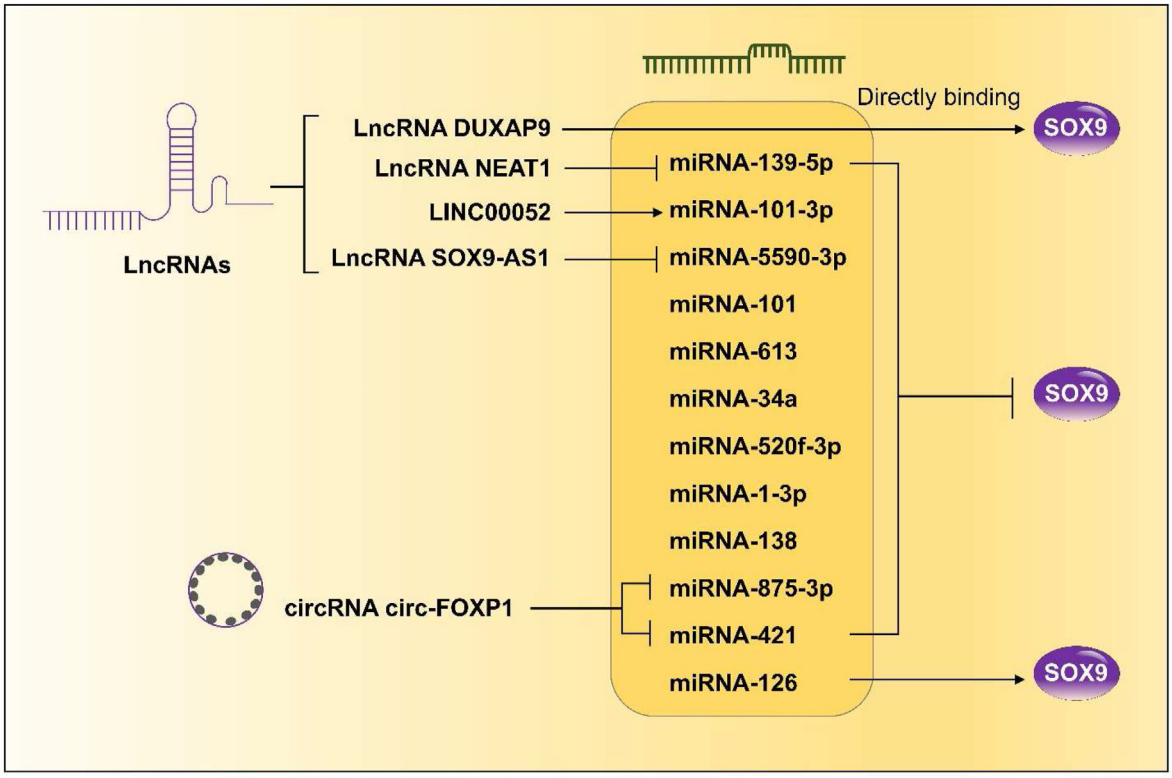

Figure 3 Post-transcriptional regulation of SOX9 expression in the liver. A schematic representation of miRNA/SOX9 axis regulated by lncRNAs and circRNAs. LncRNAs/circRNAs displays crucial roles during liver development and disease progression as potential upstream regulators of miRNA/SOX9 axis.

addition, the lncRNA SOX9-AS1 can down-regulate miR5590—3p through sponging, and the SOX9-AS1/miR-5590—3p/SOX9 positive feedback loop regulates HCC progression through the Wnt/β-catenin pathway.[110] In contrast, LINC00052 positively affects the expression of miR-101—3p, which subsequently decreases the expression of SOX9, resulting in a reduction in the proliferation of cancer cells.[111] The circular RNAs (a special type of endogenous non-coding RNAs, circRNAs) also play a role in the miRNA/SOX9 axis in cancer cells. SOX9 up-regulates the expression of circ-FOXP1 in HCC cells, and circ-FOXP1 further affects the expression of SOX9 through a positive feedback loop, which is mediated by the reduced expression of miR-875—3p and miR-421 via sponging.[112]

Post-translational regulation of SOX9: phosphorylation, acetylation, methylation, and ubiquitination

Post-translational modifications, such as phosphorylation, methylation, acetylation, and ubiquitination, play essential roles in mediating the function of SOX9. SOX9 undergoes post-translational modifications at different amino acid sequences (Fig. 1B). First, SOX9 function is modulated by phosphorylation during development. The phosphorylation of two serine residues (S64 and S181) is regulated by cyclic AMP-dependent protein kinase A (PKA). Phosphorylation targeting S64 and S181 has been reported to improve SOX9 activity and transactivation reporter genes *in vitro*.[113,114] Second, SOX9 protein usually needs to be transported from the cytoplasm to the nucleus as a transcription factor. The acetylation of SOX9 protein does not affect its stability but prevents it from entering the nucleus.[115] Histone deacetylase SIRT1 (silent mating type information regulation homolog1) has been found to be one of the main upstream factors targeting SOX9 deacetylation. SIRT1-induced deacetylation facilitates SOX9 nuclear entry by increasing the affinity of SOX9 protein and transport receptors,[116] leading to high expression of chondrocyte extracellular matrix proteoglycans and type II collagen.[117] Third, coactivator-associated arginine methyltransferase-1 (CARM1, also known as PRMT-4) methylates arginine residues at the N-terminus of the SOX9 protein, which prevents the interaction of SOX9 with β-catenin.[118] Finally, E3 ubiquitin ligase E6-AP affects the activity and stability of SOX9 protein through ubiquitination, which mediates the dynamic turnover of SOX9 during development.[119]

Additionally, the expression of SOX9 is also directly or indirectly mediated by several signaling pathways, such as Wnt/β-catenin,[44,72,120—122] Notch,[123—127] Hedgehog,[128,129] FGF,[58,130—132] TGF-β,[133,134] and BMP[135—137] (Table 2).

SOX9 in liver development: participate in biliary morphogenesis and differentiation

Liver development is a highly complex and dynamic process that mainly includes four stages: liver endoderm specification, liver bud growth, hepatoblast differentiation, and

Table 2 Signaling pathways regulating SOX9 during development and in human diseases.

Key factors	Relevance to SOX9	Reference
Wnt/β-catenin	Wnt5 upregulates SOX9 during chondrogenesis and inhibits it during chondrocyte maturation SOX9 inhibits the transcriptional activity of β-catenin Paneth cell differentiation Stemness properties of Colorectal cancer Phosphorylates SOX9 for neural crest delamination	44,72,120–122
Notch	Development of intrahepatic bile ducts Inhibits SOX9 expression *in vivo* and *in vitro* Upregulates *Hes1* and *Hey1*, which compete for the SOX9 binding of the *Col2a1* enhancer to prevent SOX9-mediated activation Notch signaling controls chondrocyte hypertrophy via indirect regulation of SOX9 Astrogliogenesis and stem cell maintenance	123–127
Hh	Sonic hedgehog upregulates SOX9 to mediate both formation and patterning of tracheal cartilage Upregulates SOX9 to modulate OPN in liver fibrosis	128,129
FGF	Activates SOX9-SOX10 pathway for the formation and branching morphogenesis of mouse ocular glands Controls the specification of hair placode-derived SOX9 positive progenitors to Merkel cells The SOX9/Fgf feed-forward loop maintains pancreatic organ identity	58,130–132
TGF-β	Upregulates SOX9 and *Smad3* Activates SOX9 *in vitro* to mediate chondrogenic commitment Atrial fibrosis TGF-β/SOX9 axis-inducible COL10A1 promotes invasion and metastasis of gastric cancer cells	133,134
BMP	Regulates SOX9 expression in endoderm differentiation along with Activin and FGF pathway BMP2 induces chromatin remodeling, and modifies the Sox9 promoter BMP4 upregulates SOX9 in semilunar valve cells	135–137

liver morphogenesis. SOX9 is the earliest sign of biliary differentiation, which is first expressed in endodermal cells lining the liver diverticulum at E10.5, and its expression becomes undetectable when hepatoblasts invade the septum transversum. Intriguingly, SOX9 reappears in hepatoblasts at E11.5, and these SOX9$^+$ cells align around the portal vein to form a monolayer of ductal plates in response to signals from the portal mesenchyme at E15.5.[46] The primary ductal plate undergoes a remodeling process that leads to bile duct formation. Specifically, the primary ductal structures have an asymmetrical distribution composed of SOX9$^+$ cholangiocytes on the portal side and SOX9$^-$ undifferentiated hepatoblasts on the parenchymal side. This asymmetric cellular arrangement is temporary, as hepatoblasts on the parenchymal side differentiate into cholangiocytes, thus producing mature ducts entirely lined with cholangiocytes[46] (Fig. 4A). In contrast, intrahepatic bile ducts in zebrafish are formed by the direct joining of cellular processes among individual biliary cells, and only SOX9b (an ortholog of mammalian SOX9) is expressed in biliary cells.[138–140] Studies using mice with liver-specific inactivation of SOX9 have revealed that SOX9 is dispensable for biliary differentiation, although it is the earliest marker of intrahepatic bile duct formation.[46] Notably, SOX9 is also expressed in the extrahepatic biliary tract in mouse embryos at E13.5, and mice with SOX9 homozygous mutant die at E11.5.[46,141]

As previously reported, the TGF-β signaling pathway is pivotal for intrahepatic bile duct development. The number of bile ducts and CK19$^+$ cholangiocytes was reduced along with decreased expression of the Notch ligand Jagged1 in the portal vein mesenchyme when TGFβ signaling was blocked at E10.5. As a downstream factor of the Notch signaling pathway, SOX9 is also down-regulated after blocking the TGF-β signaling pathway, indicating that the Jagged1-Notch-SOX9 axis regulated by TGF-β signaling may be crucial in controlling the bile duct development.[127] Notch ligand jagged1 produced by the portal vein mesenchyme mediates SOX9 expression in biliary cells, and mutations in jagged1 and Notch2 cause Alagille syndrome.[142] Notably, the combined absence/deletion of SOX9 and SOX4 suppresses biliary morphogenesis, suggesting that SOX factors synergistically integrate into the biliary gene network to regulate biliary development[143] (Fig. 4B).

SOX9 in liver homeostasis and regeneration: crucial role in cell fate determination and transdifferentiation

In the adult liver, SOX9 is continuously expressed and is important for maintaining homeostasis.[144] SOX9$^+$ cells located in the Hering canals and glands of intrahepatic bile ducts are considered hepatic progenitor cells (HPCs) expressing stem

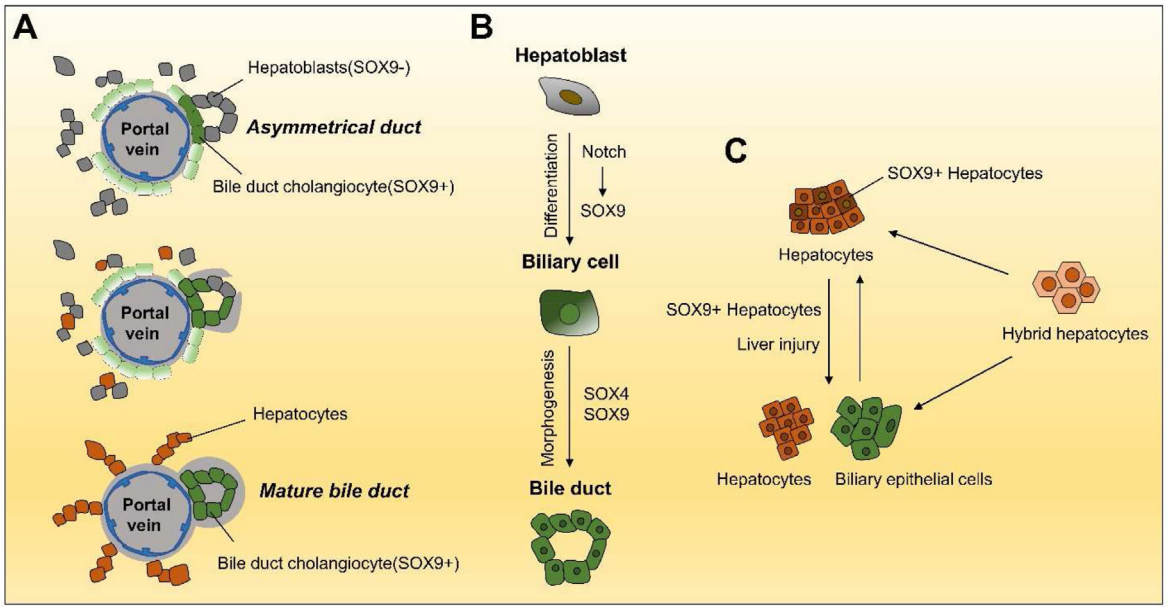

Figure 4 SOX9 in hepatobiliary development and injury. (**A** and **B**) Development of the bile ducts. During the progression from asymmetrical to symmetrical ducts, SOX9^{-} hepatoblasts differentiate into SOX9^{+} biliary cells. SOX9 and SOX4 cooperate to regulate differentiation of the biliary cells and morphogenesis of bile ducts. (**C**) Consistent with the hybrid hepatocytes during liver homeostasis, SOX9^{+} hepatocytes can differentiate into HNF4a^{+} hepatocytes and SOX9^{+} biliary epithelial cells after liver injuries.

cell markers.[145,146] Adult hepatocytes, biliary cells, and pancreatic acinar cells are thought to be supplied by SOX9^{+} progenitors based on a study using SOX9$^{IRES\text{-}CreERT2}$ lineage tracing.[141] However, several studies have reported that there was no detection of colonization of SOX9^{+} cells in the liver using the SOX9^{CreERT2} mouse line.[144,147,148] These discrepant results may be attributed to the differences in the SOX9^{CreERT2} strains used. Moreover, a high dosage of tamoxifen may result in ectopic expression of SOX9 in hepatocytes.[147,148] In this case, lineage tracing would not be able to distinguish between the progeny of SOX9^{+} cells and hepatocytes expressing tamoxifen-induced SOX9.

SOX9 has been considered as a progenitor/stem cell marker for cell fate determination.[149,150] During liver injury, SOX9^{+} hepatocytes are converted to biliary cells.[151,152] In contrast to SOX9^{-} hepatocytes, the contribution of SOX9^{+} hepatocytes to biliary cells is more efficient after DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine) or bile duct ligation (BDL)-induced liver injuries.[151] Importantly, a previous study indicated that a single SOX9^{+} hepatocyte could differentiate into both biliary cells and hepatocytes after injury using a dual recombinase-based lineage-tracing strategy[152] (Fig. 4C). Another study found that overexpression of TFEB in hepatocytes does not affect hepatocyte proliferation and identity in homeostasis conditions, while it induces differentiation of hepatocytes into ductular reaction (DR)-associated cholangiocytes after injury by regulating the expression of SOX9.[86] In addition, a recent study has shown that metabolic nuclear receptors, farnesoid *X* receptor (FXR) and PPARα, play critical roles in regulating the dynamic fate of Sox9^{+} hepatocytes during physiological (maintenance) and pathological (repair) regeneration.[153] Signaling pathways that regulate the conversion of hepatocytes to biliary cells include Hippo-Yap,[154] Notch,[155] and TGF-β.[156]

Furthermore, facultative HPCs/stem cells may be activated to replenish lost hepatocytes under severe injury conditions.[157] Transplanted SOX9^{+} HPCs contributed to liver regeneration in an *Mdm2* mutant mouse model of hepatocytes undergoing apoptosis, necrosis, and senescence.[158] In zebrafish, inhibition of HDAC1 activity by MS-275 treatment enhances SOX9b expression in LPCs and impairs LPC-to-hepatocyte differentiation.[159] In contrast, inhibition of Notch signaling could promote LPC-to-hepatocyte differentiation by repressing SOX9b expression.[160] Moreover, the EGFR-ERK-SOX9 axis also suppresses LPC-to-hepatocyte differentiation during liver regeneration, suggesting that SOX9 inhibitors are therapeutic drugs for patients with liver disease.[161]

SOX9 in liver diseases: a potential target for clinical treatment of major hepatobiliary diseases

SOX9 in acute liver diseases

SOX9 in two-thirds partial hepatectomy

Multiple studies on the dynamics of hepatocyte proliferation have focused on the periportal region since hepatocytes located here are the first and the most vigorous responders after two-thirds partial hepatectomy.[162,163] A subpopulation of periportal HNF4α-positive hepatocytes expressing SOX9 and the ability of such cells to clonally expand after a liver injury has been noted. A study termed these cells hybrid hepatocytes, indicating that these hepatocytes expanded *in situ* after tetracarbon chloride (CCL_4) liver injury and migrated toward the pericentral

regions. A major problem is whether these hepatocytes are committed to only the hepatocyte lineage or cholangiocyte lineage, whereas some may be able to generate both cell types. The SOX9-CreER mice with a single reporter used for tracing $SOX9^+$ $HNF4\alpha^+$ is not precise because the system will label both bile duct epithelial cells (BECs) and $SOX9^+$ hepatocytes. To overcome this shortcoming, another recombinase Dre-rox was recently introduced to delineate the lineage potential of $SOX9^+$ hepatocytes. This study provides direct evidence that $SOX9^+$ hepatocytes can produce both hepatocytes and ductal cells for liver regeneration.[152]

SOX9 in drug/chemical-related liver injury

Drug/chemical-related liver injuries caused by CCl_4, DDC, and thioacetamide (TAA) lead to hepatocyte damage in different zones. CCl_4 and TAA can cause acute injuries in zone 3 hepatocytes, whereas DDC or bile duct ligation (BDL) is regarded as a zone 1 injury model.[6,164,165] $SOX9^+$ hepatocytes behave differently in response to different liver injuries. Multiple injections of CCl_4 increased the number of $SOX9^+$ hepatocytes, whereas a DDC diet or BDL decreased the percentage of $SOX9^+$ hepatocytes.[164] Cell fate conversion is observed in multiple types of diseased/injured mammalian tissues and organs. Hepatocytes and cholangiocytes in the liver exhibit significant plasticity under injurious conditions. Hepatocytes can transdifferentiate into $SOX9^+$ BECs upon DDC treatment using cre-based lineage tracing.[151] Meanwhile, in a TAA-induced intrahepatic cholangiocarcinoma (iCCA) mouse model, hepatocyte-derived biliary cells give rise to $SOX9^+$ cholangiocarcinoma.[165]

SOX9 in liver ischemia-reperfusion

Liver ischemia-reperfusion injury (IR) is a clinical phenomenon in which liver injury and tissue damage are more serious than ischemic conditions when the liver tissue is reperfused with blood flow after ischemia.[166] In the initial stage of IR, a large number of reactive oxygen species (ROS) are produced. Thus, overloaded ROS can induce cell apoptosis, form damage-associated molecular patterns (DAMPs), and further attract macrophages and neutrophils, activating the inflammatory reaction.[167] SOX9 promotes the generation of extracellular matrix to enhance hepatic injury.[168] It has been reported that SOX9 is overexpressed in the liver of IR mice. Suppression of SOX9 leads to lower liver IR injury by reducing apoptosis and inflammation.[169] Blocking TGFβ1 expression diminished apoptosis and inflammation induced by SOX9 overexpression in the liver following IR injury. Therefore, SOX9 regulates inflammation and apoptosis via TGFβ1 expression, and reducing SOX9 expression may be a potential efficient target for developing therapeutic strategies against liver IR injury.[169]

SOX9 in chronic liver inflammation

Liver fibrosis

Liver fibrosis is characterized by excessive synthesis and deposition of ECM, resulting in pathological scarring and tissue dysfunction. In response to injury, hepatic stellate cells (HSCs) are activated and transdifferentiated into myofibroblasts, which secrete tissue-damaging ECM, the major component of which is collagen. Excessive accumulation of ECM changes the liver structure and leads to fibrosis. If this process cannot be effectively controlled, it may lead to the occurrence of liver cancer.[170,171] Thus, delaying, restraining, or reversing the process of liver fibrosis is of great significance in patients with cirrhosis.

Studies have shown that SOX9 plays an important role in regulating multiple components of fibrotic ECM in liver diseases.[47,172] SOX9 transcriptionally activates multiple cartilage-specific ECM genes, such as collagens type-2,9, matrilin-1, and cartilage oligomeric protein during bone development and chondrogenesis.[44,173] HSCs also express SOX9 in response to pro-fibrotic signaling factors. A previous study demonstrated that SOX9 is present in activated HSCs and produces profibrotic collagen COL1 under the regulation of TGF-β signaling.[47] Mice lacking SOX9 have reduced scarring and inflammation in a fibrosis model, and the extent of SOX9 in biopsies of patients with liver disease correlates with fibrosis severity. Importantly, the abundance of SOX9 in biopsy samples for the detection of disease severity and the prediction of disease progression outperformed other fibrosis risk factors.[174] The transcriptomic analysis combined with previous studies has profiled several downstream targets of SOX9 in patient serum samples, including vimentin, osteopontin, osteonectin, osteoactivin, and fibronectin.[175] Further analysis revealed that these targets were also localized to fibrotic regions in biopsies from patients with severe fibrosis, commensurate with increased SOX9 levels.

LncRNAs play a crucial role in liver diseases. LncRNA H19 is highly expressed in the embryonic liver but is remarkably reduced after birth.[176] Hepatic H19 expression level was correlated with the severity of liver fibrosis in mouse liver injury models and human primary biliary cholangitis (PBC) patients.[177,178] SOX9 transcriptionally induces H19 by directly binding to its promoter region during liver fibrosis development. *In vivo*, the ectopic expression of H19 abolished the inhibitory effects of SOX9 depletion on liver fibrosis.[179] Another study verified that lncRNA NEAT1 could target and suppress the expression of miR-139—5p directly to promote HSCs activation and increase β-catenin expression, which subsequently binds to SOX9 and positively regulates TGFβ, thus promoting liver fibrogenesis. The NEAT1/miR-139—5p axis promotes HSC activation and modulates β-catenin/SOX9/TGF-β1 signaling, providing potential targets for the early diagnosis and treatment of liver fibrosis.[108]

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease with a prevalence of 25%.[180] NAFLD ranges in severity from hepatic steatosis to a more serious condition, with inflammation, hepatocyte damage, and pericellular fibrosis (non-alcoholic steatohepatitis, NASH).[181] The common pathological drivers of NAFLD are the accumulation of toxic lipid species, which induces hepatocellular stress and injury, leading to fibrogenesis and hepatocellular carcinoma.[182] Bioinformatic analysis suggests that SOX9 is a potential biomarker for fibrosis progression in NASH.[183] Studies have shown that Notch activity in the liver is increased in obese rodents and NASH.[184,185]

Notch signaling induces the expression of SOX9 and subsequently increases the transcriptional activity of the downstream target osteopontin, which regulates the activation of HSCs and leads to liver fibrosis.[186]

SOX9 in HBV-related diseases and hepatic cystogenesis

HBV infection causes serious liver diseases, which have become a global public health problem. A previous study has demonstrated a distinct negative feedback mechanism underlying the regulation of SOX9 expression and HBV replication. HBV induces SOX9 expression in human hepatoma cells by activating the SOX9 promoter. In contrast, SOX9 decreases HBV replication by binding to HBV EnhII/Cp via the HMG domain.[187] This study identified SOX9 as a novel therapeutic agent for the treatment of HBV-associated diseases. In addition, liver-specific deletion of SOX9 causes hepatic cystogenesis in mice through transcriptional down-regulation of Sec63, which plays a crucial role in hepatic cystogenesis. Hepatic cysts were observed in six-month-old liver-specific Sox9 knockout mice, and the number and size of cysts increased with age.[188]

SOX9 in liver cancer

Liver cancer is the sixth most common cancer worldwide, and primary liver cancer is a heterogeneous group of malignancies, including the two common subtypes of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA).[189] Although continuous progress has been made in clinical detection and treatment strategies, the molecular mechanisms underlying hepatocellular carcinogenesis and its progression have not been fully elucidated. Hence, the identification of key mediators of liver cancer development and progression will contribute to the understanding of liver cancer pathogenesis and improve patient prognosis.

SOX9 is reportedly regulated by Yap/Taz in the liver.[154] In mouse livers, the activation of YAP or inactivation of Hippo signaling promotes HCC and iCCA tumor formation, with an apparent expansion of $SOX9^+$ cells.[154,190,191] A recent study investigated the specific function of SOX9 in YAP-induced hepatocyte cell fate plasticity during HCC progression in mice using lineage tracing and genetic models.[8] YAP activation in hepatocytes leads to a transition from mature hepatocytes to liver progenitor cells, and SOX9 is required for the second step of mouse hepatocarcinogenesis. Importantly, the intrinsic difference in SOX9 expression between HCC and hepatocyte-derived iCCA tumors determines the tumor types in both mouse and human liver tumors with high YAP activity.[8]

Liver cancer can be induced by liver CSCs.[157,192] SOX9 is highly expressed in liver CSCs, and high expression of SOX9 correlates with poor prognosis in liver cancer patients. SOX9 is regarded as a CSC marker.[16] In addition, Sox9 is required for the maintenance of self-renewal and tumorigenicity of liver CSCs. In contrast to differentiated cancer cells or liver CSCs with inhibited Notch signaling, spheroid-cultured liver CSCs exhibit reduced asymmetrical cell division. In liver CSCs, SOX9 is responsible for the asymmetrical-to-symmetrical cell division switch. Meanwhile, SOX9 also negatively regulates Numb expression and maintains Notch activity in liver CSCs.[16]

Previous studies have demonstrated that activation of AKT signaling could be considered a hallmark of the acquisition of CSCs traits.[193–195] CD73 promotes the progression and metastasis of liver cancer by activating AKT signaling.[196] SOX9 is an important downstream regulator of CD73 in liver cancer. Interestingly, CD73 up-regulated SOX9 expression through two distinct mechanisms: increasing SOX9 transcription by c-Myc and stabilizing SOX9 protein by inhibiting GSK3β activity, resulting in an enhancement effect that further facilitates stemness characteristics. Moreover, the combined analysis of CD73 and SOX9 has the potential to achieve a more accurate prognosis.[197] *Pten* (phosphatase and tensin homolog deleted on chromosome 10) is one of the most deleted tumor suppressor genes in human cancers, which negatively regulates the PI3K/AKT pathway.[198,199] Deletion of *Pten* in albumin-expressing liver cells results in steatosis and tumor formation.[200,201] $SOX9^+$ liver cells with *Pten* deletion have the potential to function as liver tumor-initiating cells and induce the formation of mixed-lineage tumors.[202] Chronic steatosis induces the proliferation of $SOX9^+$ liver cells with *Pten* deletion and promotes liver cancer progression. Specifically, Wnt/β-catenin serves as a crucial signal for tumor formation in $SOX9^+$ liver cells, and this effect is mainly mediated by Frizzled-7, the key receptor of the Wnt pathway regulated by SOX9.[203] Recent advances in epigenomics have demonstrated that epigenetic regulation, including DNA methylation and histone modification, participates in CSC self-renewal maintenance.[204,205] Histone lysine demethylase 4D (JMJD2D) is highly expressed in liver and colon tumors.[206–208] A recent study has shown that the down-regulation of JMJD2D inhibits the self-renewal of liver CSCs *in vitro* and *in vivo*. Mechanistically, JMJD2D reduced H3K9me3 levels on the promoters of EpCAM and SOX9 to enhance their transcription via interactions with the β-catenin/TCF4 and Notch1 intracellular domains, respectively. This finding indicates that the JMJD2D-SOX9/EpCAM axis may be a potential therapeutic target for liver CSCs.[92] In addition, long non-coding RNA, small nucleolar RNA host gene 10 (SNHG10), and its homolog SCARNA13 participate in a positive feedback loop for hepatocarcinogenesis, in which SCARNA13 up-regulates the expression of SOX9 to mediate cell proliferation and migration.[209]

One of the most remarkable characteristics of CSCs is their resistance to chemotherapies. Sorafenib is a multi-kinase blocker used to treat aggressive HCC. A study focusing on the mechanism of sorafenib-related resistance found that SOX9 induces sorafenib resistance by regulating ATP-binding cassette subfamily *G* member 2 (ABCG2) expression, suggesting SOX9 as a prognostic index for sorafenib treatment.[210] The complex interplay between hepatoma cells and the surrounding environment contributes to the metastasis and recurrence of liver cancer. $SOX9^+$ hepatoma cells activate HSCs in a paracrine manner by inducing INHBB expression and activin B secretion. In turn, HSC activation and liver fibrosis in the surrounding tissue strengthen the growth and metastasis of liver cancer.[211]

Notably, studies have suggested that there is a gender discrepancy in liver cancer occurrence.[212–214] SOX9 is

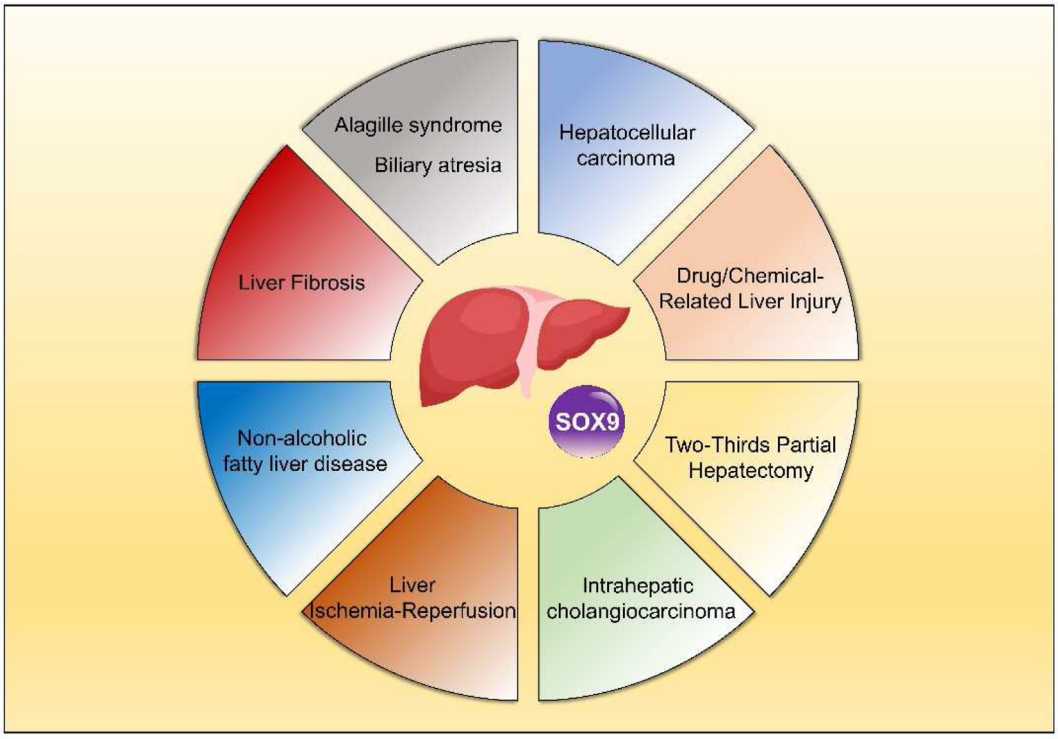

Figure 5 SOX9 in liver diseases. SOX9 is associated with different hepatobiliary diseases and as a potential target for the clinical treatment of major liver diseases.

thought to be a target gene of SRY.[85] A study demonstrated that activation of SRY and its downstream SOX9 and PDGFRα pathways are commonly involved in male hepatocarcinogenesis, which provides new insights into gender disparity and sex-specific therapeutic strategies for liver cancer.[215] However, another study found that neither expression intensity nor the spatial position of SRY was correlated with gender disparity in liver cancer.[216] The mechanism of SRY and SOX9 as transcription factors that participate in liver cancer progression warrants further in-depth investigation.

Biliary tract carcinomas include cholangiocarcinoma (CCA) and gallbladder carcinoma (GBC). Generally, CCA arising from the biliary epithelium can be defined as intrahepatic CCA (iCCA), and extrahepatic CCA comprises the perihilar CCA (pCCA) and distal CCA (dCCA). Clinically, iCCA is highly invasive and often accompanied by lymph nodes and distant metastases, and its incidence has significantly increased worldwide. Decreased SOX9 expression relates to the early stage of iCCA carcinogenesis, whereas increased SOX9 expression in iCCA is correlated with tumor proliferation and invasion.[217] Notably, SOX9 has also been implicated in iCCA chemoresistance and holds promise as a biomarker for selecting iCCA patients to receive effective chemotherapy.[14,218] In a study of 59 iCCA patients, the survival of iCCA patients with high SOX9 expression was significantly shorter than that of patients with low SOX9.[14] Interference with siRNA targeting SOX9 significantly reduced the expression of genes related to drug metabolism and multidrug resistance. Moreover, the phosphorylation of checkpoint kinase 1 (CHK1) induced by gemcitabine was inhibited after *Sox9* knockout, significantly increasing cell apoptosis in iCCA cells. Thus, SOX9 confers chemoresistance to iCCA through the activation of CHK1, indicating that the sensitivity of gemcitabine could be enhanced by targeting SOX9 in iCCA patients.[14]

SOX9 in other biliary diseases

Biliary atresia (BA) is the most common cause of neonatal cholestasis and a major indication for liver transplantation in pediatric patients. One of the characteristics of BA is rapidly progressive fibrosis, which can lead to cirrhosis at an early age.[219] Targeting SOX9 has been proposed as a therapeutic strategy for inhibiting fibrosis progression in BA.[220,221] The ectopic appearance of SOX9-positive peribiliary glands has been detected in the gallbladders of SOX17$^{+/-}$ BA neonate mice.[222] Alagille syndrome (ALGS) is a multisystem developmental disorder caused by dysregulated Notch signaling characterized by severe bile duct paucity leading to cholestasis.[223,224] A liver-specific increase in SOX9 expression is a potential therapeutic approach for ALGS.[225]

Other SOX proteins in hepatobiliary development, homeostasis, and diseases

SOX17 is expressed in the foregut endoderm early in development and acts as an important regulator of endoderm formation across vertebrate species.[226] At the late stage of endoderm development, SOX17 mediates segregation of the liver, biliary system, and ventral pancreas.[226]

Tissue-specific knockout of *Sox17* leads to improper localization of the endoderm, which results in the complete loss of the gallbladder and cystic duct, suggesting the crucial role of SOX17 in the specification of extrahepatic biliary system.[227] As a member of the SoxD group, SOX13 has been identified as an autoantigen in primary biliary cirrhosis and an oncogene in HCC.[228,229] SOX30 was reported to inhibit HCC progression through binding with TP53,[230] while SOX2 has been found to regulate non-alcoholic fatty liver disease development and HCC progression.[231,232]

At present, a substantial number of discoveries have documented the significance of SOX family in the initiation and progression of HCC, and regard them as potential therapeutic targets for HCC.[9] It is worth noting that members of the SOX family may act as HCC oncogenes, suppressor genes, or both, depending on the cellular environment, and can be stimulated or incapacitated through diverse genetic and epigenetic mechanisms.

Conclusion and future perspectives

Driven by significant technical advances, research focusing on liver development has made significant progress over the past two decades, which has identified a series of transcription factors, signaling molecules, and cellular events. Members of the SOX family of transcription factors are expressed in the liver throughout development. Among those, SOX9 is essential for the differentiation and morphogenesis of intrahepatic biliary cells. Throughout the process of bile duct formation, SOX9 can be detected at the portal site of the developing duct, continues to be expressed in the liver beyond the embryonic stage, and plays a critical role in organ homeostasis. Importantly, liver-specific SOX9 inactivation resulted in delayed bile duct differentiation. SOX9 was also observed to regulate the expression of genes involved in liver development, including genes in the transforming growth factor-β and Notch pathways.

Liver diseases, such as hepatic steatosis, liver inflammation, liver fibrosis, and liver cancer, cause security problems and have evolved into global safety issues. Recent studies have suggested that SOX9 is a key player in various liver diseases (Fig. 5). The identification, verification, and application of biomarkers are complex. Immunostaining of SOX9 in pathological sections is helpful in diagnosing patients. For example, SOX9 staining, along with its targets *COL1* and *COL3* can be included in liver biopsy analysis as a staging strategy for liver fibrosis.[233]

Notably, several questions remain unanswered. SOX9 often works in a dose-dependent manner, and studies on new post-translational modifications that may mediate the dosage of SOX9 expression during liver development and the progression of diseases will be exciting. A systematic study on the expression and function of SOX9 in the liver under different physiological conditions remains to be reported, as well as the dynamic changes in SOX9 expression in the liver from development to maturity, and then to aging (disease occurrence). Although the role of SOX9 in intrahepatic bile duct development and iCCA progression has been reported, the expression and function of SOX9 in the extrahepatic bile duct and gallbladder remain unclear. SOX9 is considered a marker of stem cells. Introducing SOX9 may enhance the function and maturity of stem cell-derived cells; however, this requires further validation. In summary, SOX9 is a crucial factor in the developmental biology of the liver and an appealing molecular target for the treatment of liver disease. The incorporation of SOX9 into clinical treatment requires further studies on its upstream regulators and downstream molecular and cellular events.

Author contributions

T.Y.S. and T.Y.J. drafted the manuscript and drew the figures with the help of X.W.C., Y.F.P., and X.F.F. T.Y.S., H.Y.W., and L.W.D. discussed and revised the manuscript. H.Y.W. and L.W.D. designed the study. All authors read, revised, and approved the final manuscript.

Conflict of interests

The authors declare no competing interests.

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