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Estrogen acts via estrogen receptor 2b to regulate hepatobiliary fate during vertebrate development

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Abstract

During liver development bipotent progenitor cells differentiate into hepatocytes and biliary epithelial cells (BECs) to ensure a functional liver required to maintain organismal homeostasis. The developmental cues controlling the differentiation of committed progenitors into these cell types, however, are incompletely understood. Here, we discover an essential role for estrogenic regulation in vertebrate liver development to affect hepatobiliary fate decisions. Exposure of zebrafish embryos to 17 β -estradiol (E2) during liver development significantly decreased hepatocyte-specific gene expression, liver size, and hepatocyte number. In contrast, pharmacological blockade of estrogen synthesis or nuclear estrogen receptor signaling enhanced liver size and hepatocyte marker expression. Transgenic reporter fish demonstrated nuclear

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S.C., M.K.G., T.E.N. and W.G. conceived, designed, analyzed the experiments and wrote the manuscript. S.C. generated the *esr2b* TALEN mutant and performed all zebrafish experiments. A.S. performed confocal and time-lapse imaging analysis. K.L. performed BMP studies. S.C. and C.C.L. performed cell culture and immunofluorescent analysis. S.C., M.K.G., and C.C.C. performed chemical exposures and MO experiments. K.L. and N.B. performed environmental estrogen experiments. K.J.C. gave advice on estrogen concentrations. D.A.G. provided ERE reporter fish. A.P. and K.D.T. provided insight into murine liver development. All authors reviewed and edited the manuscript.

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estrogen receptor activity in the developing liver. Chemical inhibition and morpholino knockdown of *nuclear estrogen receptor 2b* (*esr2b*) increased hepatocyte gene expression and blocked the effects of E2 exposure. *esr2b*^{-/-} mutant zebrafish exhibited significantly increased expression of hepatocyte markers with no impact on liver progenitors, other endodermal lineages or vasculature. Significantly, E2-stimulated ESR2b activity promoted biliary epithelial differentiation at the expense of hepatocyte fate, while loss of *esr2b* impaired biliary lineage commitment. Chemical and genetic epistasis studies identified bone morphogenetic protein (BMP) signaling as a mediator of the estrogen effects. The divergent impact of estrogen on hepatobiliary fate was confirmed in a human hepatoblast cell line, indicating the relevance of this pathway for human liver development.

Conclusion: Our studies identify E2, *esr2b* and downstream BMP activity as important regulators of hepatobiliary fate decisions during vertebrate liver development. These results have significant clinical implications for liver development in infants exposed to abnormal estrogen levels or estrogenic compounds during pregnancy.

Introduction

The liver is critical in maintaining metabolic homeostasis of the organism, regulating lipid and carbohydrate metabolism and detoxifying both endogenous and exogenous waste products throughout life. To ensure adequate function at birth, bipotential liver progenitors, hepatoblasts, differentiate into hepatocytes to perform its metabolic tasks, and into biliary epithelial cells (BEC) to transport bile. While many key factors have been identified that regulate particular aspects of liver specification and differentiation, the signaling networks that function to ensure the balanced and timely maturation of hepatoblasts into hepatocyte and biliary lineages are largely unknown.

During pregnancy, temporal and spatial expression of steroid hormones are crucial for fetal development, growth and organogenesis (1,2). Estrogen, in particular, is present throughout all stages of gestation and regulates many intrauterine processes (3). Furthermore, estrogen is necessary for proper embryonic development, as early inhibition of estrogen synthesis leads to defects in embryogenesis (4). Interestingly, excessive exposure to estrogenic compounds, such as diethylstilbestrol (DES), during pregnancy has been shown to alter fetal organogenesis and is associated with later development of cancer (5,6). While these studies indicate the potential effects of estrogenic exposure in the liver later in life, the direct impact of estrogen signaling on liver differentiation and hepatobiliary fate decisions have not been characterized.

17 β -estradiol (E2) is the most prevalent and active form of estrogen in vertebrates. Classical E2 signaling involves binding of E2 to the nuclear hormone receptors estrogen receptor alpha (ESR1 or ER α) and estrogen receptor beta (ESR2 or ER β) in the cytoplasm, which then dimerize, translocate to the nucleus, and activate transcription of target genes via estrogen response elements (ERE). While both ESR1 and ESR2 contain highly conserved DNA-binding and ligand-binding domains and interact with the same co-regulators, they possess an overlapping, yet unique, repertoire of target genes (7,8). Importantly, ESR1 and ESR2 exhibit different tissue distributions, biological functions and pathological phenotypes (9–11). During embryonic development, ESR1 and ESR2 are differentially expressed: in the

human mid-gestational fetus (16–23 weeks), ESR1 is abundant in the uterus, while ESR2 is expressed in ovary, testes, adrenal gland, spleen, and liver. ESR2 expression in the embryonic liver is of particular interest (12,13), as it directly contrasts with the adult liver, which predominantly expresses ESR1 (14). These differences in estrogen receptor expression suggest the possibility for a physiological role of ESR2-specific regulation during liver development. While we have recently reported a role for E2 signaling through a G protein-coupled receptor on adult liver growth and cancer formation (15), the role of ESR2 during liver development has not been defined.

Here, we identify an essential novel function for E2 acting through *Esr2b* to control hepatobiliary differentiation during zebrafish liver development. Enhanced estrogen activity results in decreased liver size and hepatocyte number in a BMP-dependent fashion, while chemical inhibition of E2 synthesis or blockade of receptor signaling leads to expansion of differentiated hepatocytes. Transgenic reporter fish demonstrate activation of estrogen response elements in the liver upon E2 exposure. Chemical inhibition of *Esr2*, and knockdown or genetic loss of *esr2b* increased liver size and protected the liver from the inhibitory effects of estrogen exposure. E2/*Esr2b* regulated the timing and scale of hepatoblast differentiation towards hepatocytes, but had no effect on hepatoblast production or viability, other endodermal organs, or vasculature. *Esr2b* activity determined hepatobiliary fate decisions, as loss of *esr2b* enhanced hepatocyte commitment at the expense of biliary differentiation. These findings were confirmed *in vitro* in human induced hepatoblast culture. Together, these data elucidate a previously uncharacterized function of estrogen in a non-reproductive tissue as a key differentiation determinant during embryonic liver development.

Experimental Procedures

Zebrafish husbandry

Male and female WT Tu zebrafish, *Tg(-2.8fabp10a:eGFP)^{as3}* (4,16), abbreviated *fabp10a:GFP*, *Tg(fabp10a:DsRed)^{gz15}* (17) *Tg(Tp1b:glob:eGFP)^{um14}* abbreviated *tp1:GFP* (18), *Tg(flk:mCherry)^{is5}* (19), *Tg(5xERE:GFP)^{c262}* (4,20), and *Tg(hsp70l:dnXla.Bmpr1a-GFP)* (21) were used.

Chemical exposures

Zebrafish embryos were exposed to chemicals for 48 hours from 24–72 hours post fertilization (hpf) and analyzed at 72hpf (unless otherwise specified). Chemicals used are listed in Supporting Table 1.

Morpholino injection

ATG morpholino oligonucleotides (MO) designed against *esr1*, *esr2a*, *esr2b* (Gene Tools) were previously validated (22). MOs were injected into WT Tu embryos at the one-cell stage. *esr1* MO: 5'-AGGAAGGTTCTCCAGGGCTTCTCT-3' (10 μ M), *esr2a* MO: 5'-ACATGGTGAAGGCGGATGAGTTCAG-3' (10 μ M), *esr2b* MO: 5'-AGCTCATGCTGGAGAACAAGAGA-3' (10 μ M).

Generation of *esr2b*^{-/-} mutant

TALEs targeting endogenous *esr2b* were generated according to published protocols (4,23) and obtained from The Broad Institute Genetic Perturbation Platform. mRNAs of TALEN pairs were synthesized by mMESSAGE mMACHINE kit (Ambion) and injected into 1-cell stage WT embryos. Somatic mutation rate was determined from pooled-larvae by sequencing. Primers used for *esr2b* PCR: Forward 5'-GCCAGGGTCTCTCTTGTGTT-3', Reverse 5'-TGACAGCTGCCACCTAAAGA-3'. F₁ with mutations at TALEN-targeted site were raised and out-crossed for at least 4 generations to avoid possible TALEN-induced off-target effects.

Whole mount *in situ* hybridization

Embryos were fixed with 4% paraformaldehyde and performed WISH according to standard protocols (24,25) for the following markers: hepatocytes - *fabp10a* and *transferrin*, endoderm - *foxa3*, hepatoblasts - *prox1*, biliary tree - *sox9*, endocrine pancreas - *insulin*, exocrine pancreas - *trypsin*.

Cell culture and Immunofluorescence

iCell hepatoblasts (Cellular Dynamics International, HBC-100-020-001-PT) were maintained according to manufacturer's protocol and exposed to DMSO, E2 (1μM, 2μM), or PHTPP (100μM, 250μM) for 48 hrs. Cells were grown in media for additional 3 days and then fixed and stained with antibodies (CK7, albumin) and nuclear staining. The antibodies used are listed in Supporting Table 2.

Expression and fluorescent image analysis

Embryos processed for *in situ* hybridization, as well as transgenic embryos were imaged on a Zeiss Discovery V8 stereoscope, and Zeiss LSM800 and Nikon/Yokagawa W1 confocal microscopes, respectively. Quantification of gene expression areas and organ volumes was performed using ImageJ and Imaris 9. Analyses shown in each figure represent one of 3 technical replicates and 3 biological replicates (3 different clutches of embryos).

Results

Estrogenic regulation is required for normal liver development

We previously performed a chemical genetic screen in zebrafish embryos to identify novel regulators of liver development (4,26) and uncovered several estrogen-related compounds that affected hepatogenesis (Supporting Fig. S1A). Exposure to physiological estrogens E2 and estriol, synthetic estrogens 17α-ethinylestradiol (EE2) and diethylstilbestrol, and the phytoestrogen quercetin from 24–72hpf each decreased liver-specific gene expression, as assessed by whole mount *in situ* hybridization (WISH) for the hepatocyte-specific gene *liver fatty acid binding protein 10a* (*fabp10a*) at 72hpf (Supporting Fig. S1A–C). In contrast, treatment with the aromatase inhibitor chrysin and the estrogen receptor antagonist tamoxifen increased the area of *fabp10a* expression (Supporting Fig. S1A). Exposure to the environmental estrogenic compound bisphenol A (BPA) decreased *fabp10a* expression. Any BPA possibly contained in the plastic Petri dishes, however, is not sufficient to affect

hepatogenesis (Supporting Fig. S1D–G). In addition, exposure to steroid hormones progesterone and testosterone had no impact on liver formation (Supporting Fig. S2A), indicating the specificity of estrogen-associated signaling or transcriptional activity for this process.

To corroborate these findings and further investigate the impact of E2 on liver development, zebrafish embryos were exposed to physiological levels of E2 (22) from 24–72hpf, a time period during which hepatic specification, progenitor differentiation, and hepatocyte maturation successively take place. Alterations in liver formation were assessed by WISH for *fabp10a* at 72hpf, with quantification of liver area by ImageJ. Exogenous E2 exposure resulted in a dramatic reduction in liver size compared to DMSO-exposed controls (Fig. 1A,B; $p < 0.01$). Quantitative analysis of hepatocyte number using fluorescence activated cell sorting (FACS) (Supporting Fig. S2B; $p < 0.001$) and liver volume by confocal microscopy (Fig. 1C,D; $p < 0.0001$) in Tg(*fabp10a*:GFP) reporter embryos at 72hpf confirmed a decrease in total hepatocyte number following E2 exposure. To determine whether the observed decrease in liver area was specific to endogenous estrogen activity, zebrafish were exposed to Anastrozole (ANAS), an inhibitor of the estrogen synthesis enzyme aromatase. ANAS exposure resulted in enlarged livers (Fig. 1A,B; $p < 0.01$, Fig. 1C,D; $p < 0.0001$), indicative of a specific role for endogenous estrogenic modulation in liver development. Taken together, these data indicate that optimal levels of both endogenous and exogenous E2 signaling are important for normal liver formation.

The effect of E2 on liver development is mediated by *esr2b*

Analysis of previously published microarray expression profiling of endodermal progenitors (27) indicates the presence and dynamic expression of three ESR genes, *esr1*, *esr2a* and *esr2b*, during liver development (Supporting Fig. S2C). To identify the specific estrogen receptor mediating the effects of E2 on early liver development, a chemical inhibitor approach was employed: embryos were exposed to E2 concomitantly with the specific ESR1 antagonist MPP or ESR2 antagonist PHTPP (24–72hpf). Embryos exposed to PHTPP alone had increased *fabp10a* expression at 72 hpf ($p < 0.0001$), while MPP-exposed embryos were unchanged (Fig. 2A,B). Furthermore, PHTPP, but not MPP, blocked the effect of E2 on liver size, indicating that E2 regulates liver development via ESR2. The results of these chemical modulations were confirmed by morpholino (MO)-mediated knockdown of individual zebrafish estrogen receptors: *esr2b* morphants exhibited a significant increase in liver size compared to *esr1* and *esr2a* morphants and uninjected controls, as measured by *fabp10a* expression area (Fig. 2C,D; $p < 0.0001$, Supporting Fig. S2D; $p < 0.01$). Furthermore, while E2 exposure in *esr1* and *esr2a* morphants still reduces liver size, the negative impact of E2 exposure was significantly blunted in *esr2b* morphants (Fig. 2C,D, Supporting Fig. S2D), further indicating that E2 specifically acts through *esr2b* to regulate liver development.

These chemical and genetic inhibition studies prompted further examination of the developmental role of *esr2b* by generating *esr2b* knockout zebrafish using transcription activator-like effector nucleases (TALENs) (4,28). TALEN-generated *esr2b* mutants contain a 5-base pair deletion in the first exon, predicted to cause a premature stop codon (Supporting Fig. S2E). Indeed, compared to wild-type (WT) siblings, *esr2b* expression was

not detected in *esr2b*^{-/-} homozygous mutant embryos, indicative of complete loss of *esr2b* (Fig. S2F). Both *esr2b*^{+/-} and *esr2b*^{-/-} embryos exhibited no gross developmental abnormalities compared to their WT siblings (*esr2b*^{+/+}), survived to adulthood and were fertile as described (4,29). While exhibiting normal liver histology (Supporting Fig. S2G), *esr2b*^{-/-} embryos, exhibited a ~40% increase in *fabp10a* expression area compared to WT at 72hpf (Fig. 2E,F); further, *esr2b*^{-/-} embryos completely lacked a response to E2 in the liver, confirming *esr2b* as the mediator of estrogenic regulation of liver development. Importantly, the negative impact on hepatocyte growth was maintained into early adulthood, leading to significantly decreased liver:body weight ratios in animals at 6 weeks that were exposed to E2 during development, while *esr2b* mutants exhibited increased liver:body weight ratios (Fig. 2G).

E2/*esr2b* signaling functions during a specific temporal window of liver development

To precisely delineate the developmental period influenced by estrogenic activity, WT embryos were exposed to DMSO and E2 at select intervals designed to target hepatic progenitor specification (18–24hpf), hepatoblast budding (24–42hpf), and hepatocyte differentiation (42–72hpf; Fig. 3A). Expression of the endodermal progenitor marker *foxa3* and hepatoblast marker *hhex* were evaluated at 48hpf, while the hepatocyte marker *fabp10a* was assessed at 72hpf. E2 exposure from 18–24hpf and 24–42hpf had no or minimal effects on both *foxa3* or *hhex* expression (Fig. 3B–F); additionally, no significant effects were observed on hepatocytes during the earliest exposure window, with modest increases in the number of embryos with reduced *fabp10a* expression after E2 exposure from 24–42hpf (Fig. 3E). In contrast, later developmental exposure to E2 from 42–72hpf, during the window of hepatocyte differentiation, consistently caused the most profound decrease in *fabp10a* expression (Fig. 3E,F; *p*<0.01). Importantly, E2 exposure did not alter endodermally derived exocrine or endocrine pancreatic cell populations, as assessed by WISH for *trypsin* and *insulin* at 72hpf (Supporting Fig. S3A). Together, these data indicate that E2 signaling specifically impacts the window of hepatoblast differentiation toward mature hepatocytes during liver development.

To confirm that the impact on hepatocyte differentiation was mediated by *Esr2*, WT and mutant embryos were exposed to E2 from 24–72hpf, and the relative areas of hepatoblast (*prox1*) and hepatocyte (*transferrin*, *fabp10a*) expression were quantified by WISH and ImageJ analysis at 72hpf. E2 exposure significantly reduced levels of the hepatocyte marker *transferrin* similarly to *fabp10a* (Supporting Fig. S3B,C) in WT embryos; in contrast, *prox1* expression was unchanged (Figure. 3G,H). While *esr2b*^{-/-} mutants exhibited increased expression of hepatocyte markers *transferrin* and *fabp10*, there was no significant impact on *prox1*, confirming that hepatoblast formation is not impacted by E2 signaling (Fig. 3G,H, Supporting Fig. S3B,C). These observations indicate that E2/*esr2b* signaling has a selective and important role in hepatoblast differentiation towards hepatocytes.

We previously demonstrated that E2 is present in the embryo during the window of liver development and significantly increased within the physiological range upon exposure to exogenous E2 (22). To examine whether *Esr* signaling is active in the region of the developing liver and increases in response to E2 treatment during hepatocyte differentiation,

we utilized transgenic reporter zebrafish with estrogen response element (ERE)-driven GFP expression, Tg(*5xERE:GFP*) (4,20), crossed into a hepatocyte-specific reporter background Tg(*fabp10a:DsRed*). Baseline levels of ERE activation were observed in the livers of control embryos (Fig. 3I,J). E2 exposure from 24–72hpf increased ERE activity in the hepatoblast region at 48hpf as well as in hepatocytes at 72hpf, and concomitantly reduced hepatocyte-specific fluorescence (Fig. 3I,J). Together, these data demonstrate that precise E2/*esr2b* signaling is necessary for proper hepatocyte differentiation during liver development.

E2/*esr2b* signaling controls hepatobiliary fate decisions

By 72hpf, the embryonic liver is comprised of a mixture of bipotent hepatic progenitors, endothelial cells, and differentiating cells of the hepatocyte and biliary lineage. Given that modulation of E2/*esr2b* activity altered hepatocyte development, we sought to assess whether other hepatic cell types were also affected. We previously showed that early (12–24hpf) vascular development and specification can be influenced by estrogenic activity (22). To determine whether alterations in endothelial population contributed to the effects of E2 on liver development, endothelial-specific reporter fish Tg(*flk1:mCherry*) were crossed into the Tg(*fabp10a:GFP*) liver reporter background and exposed to E2 from 24–72hpf. Whereas hepatocyte-specific GFP expression was reduced, no alterations were observed in gross vascular structure by fluorescence imaging (Supporting Fig. S4A–C). Similarly, *cloche* mutants, which lack all vascular endothelium (30), demonstrated a decrease in hepatocyte-specific gene expression in response to E2 exposure compared to WT embryos (Supporting Fig. S5A,B). Consistent with prior reports indicating that endothelial cells are required for liver growth, but not specification (31,32), these findings demonstrate that the impact of E2/*esr2b* signaling on hepatic differentiation is independent of vasculature.

During embryonic development, bipotential hepatoblasts are specified from common endodermal precursors, which subsequently differentiate to become hepatocytes or BECs, also known as cholangiocytes. Given the strong impact of E2/*esr2b* on hepatocytes, we examined the contemporaneous effect on BEC differentiation within the same organism. Bigenic Tg(*fabp10a:GFP; tp1blob:mCherry*) embryos were examined, marking hepatocytes and BECs. E2 exposure from 24–72hpf decreased liver size (Fig. 4A,B,E; $p < 0.05$) and enhanced BEC formation at 72hpf (Fig. 4A,C–E; $p < 0.0001$). Consistently, *esr2b* knockdown dramatically reduced area and complexity of notch+ BECs (Fig. 4F,H–J; $p < 0.0001$) despite a significant increase in overall *fabp10a+* liver size (Figure. 4F,G,J; $p < 0.05$). Similar impact on hepatobiliary differentiation was seen using *sox9b* as a biliary marker versus *fabp10a*-based hepatocyte differentiation after E2 and ESR antagonist Fulvestrant exposure (Supporting Fig. S5C,D) and in *esr2b* morphants assessing BEC-associated Annexin 4A via 2F11 epitope expression (33) (Supporting Fig. S5E,F; $p < 0.0001$). Time-lapse imaging further detailed the dynamic changes in enhancing BEC growth upon E2 treatment (Fig. 4K,L, Supporting Fig. S6A, Supporting Movies 1,2). Together, these findings demonstrate that E2 controls hepatocyte-versus-biliary fate decisions during development via *Esr2b*.

E2/ESR2 signaling affects hepatobiliary differentiation in human hepatoblast

To directly test the impact of E2/*Esr2b* signaling on hepatoblast differentiation and to determine the conservation of the roles of E2 signaling in hepatobiliary development across

species, we utilized bipotent hepatoblasts derived from human induced pluripotent stem (iPS) cells. These progenitor cells are capable of differentiating into hepatocytes and BEC/ cholangiocytes as assayed by Albumin and cytokeratin 7 (CK7) expression (34), respectively (Fig. 5A). Upon E2 exposure for 48 hours, CK7+ BECs increased, while Albumin+ hepatocytes significantly decreased (Fig. 5B,C,D; $p < 0.0001$). In contrast, the ESR2 antagonist PHTPP decreased CK7+ BECs, while enhancing Albumin+ hepatocytes (Fig. 5B,C,D; $p < 0.0001$). These changes in hepatocyte and BEC populations upon E2 and PHTPP exposures occurred in a dose-dependent manner suggesting the specific impact of E2/ESR2 modulation on hepatocyte and BEC differentiation. Importantly, cell number by nuclear staining was not changed compared to DMSO-treated controls (Fig. 5B,E), demonstrating that the impact of E2 and PHTPP are specific to hepatoblast differentiation rather than overall cellular proliferation. These results reveal that E2/ESR2 regulation of hepatobiliary fate decisions of hepatoblasts is conserved across vertebrate species.

E2 signals through BMP pathway to impact hepatobiliary development

To delineate the downstream signaling pathways mediating E2 effects on hepatobiliary differentiation, we utilized a targeted approach: Bone morphogenetic proteins (BMPs) signaling has been shown to be activated downstream of ESR in osteoblasts and mesenchymal stem cells (35,36). We exposed zebrafish to selective BMP inhibitors, Dorsomorphin and K02288, to determine the involvement of BMP signaling in E2-mediated impact on hepatobiliary differentiation. Inhibition of BMP during hepatobiliary differentiation had minimal impact on liver and BEC formation compared to DMSO-treated embryos (Fig. 6A–C,E–F). Co-exposure of E2 with Dorsomorphin or K02288 from 42–72hpf, however, significantly blocked the E2-mediated reduction in liver size (Fig. 6A–C; $p < 0.001$, 6E–F; $p < 0.0001$, Supporting Fig. S7A; $p < 0.01$) and normalized the E2-mediated increase in BECs (Fig. 6B,C; $p < 0.001$). Western blot for p-Smad confirmed specific activation of BMP signaling in E2-exposed embryos (Supporting Fig. S7B). Increased p-Smad levels were normalized when E2 was co-exposed with Dorsomorphin. To confirm these results, we employed Tg(*hsp70l:dnBmpr-GFP*) (*dnBmpr*) zebrafish that express a dominant-negative form of the BMP receptor upon heat-shock activation (21). *dnBmpr* was induced at 28hpf, prior to the onset of hepatoblast differentiation, and subsequently at 44hpf and 56hpf to achieve sustained expression throughout liver differentiation. Heat-shocked embryos displayed normal liver formation at 72hpf and significantly mitigated the E2 impact on liver size (Fig. 6G,H; $p < 0.0001$). These data indicate that E2 specifically activates BMP signaling pathway to promote BEC differentiation at the expense of hepatocytes.

Discussion

In this study, we employed a chemical genetic screening approach to discover a novel role for estrogen during liver development. Optimal levels of E2 and *Esr2b* activity are essential for normal liver development, specifically during the time window of hepatobiliary differentiation. Importantly, elevated E2 activity results in enhanced BMP signaling to impair hepatocyte differentiation and enhance biliary lineage development, while inhibition of E2 or loss of *esr2b* directs hepatoblast differentiation towards hepatocytes at the expense of biliary lineage.

Estrogen regulates hepatobiliary fate decisions

In the liver, hepatocytes and BECs arise from the same hepatic progenitor pool (37). While several pathways have been described that are important for the differentiation of either hepatocytes or BECs, only very few signals have been found to affect hepatobiliary fate decisions in embryonic hepatoblasts. The transcription factor *Sall4* promoted biliary over hepatocyte fate in isolated murine hepatoblasts by enhancing BEC proliferation, while suppressing hepatic differentiation (4,38). Conversely, loss of *Tbx3* in murine embryos resulted in impaired hepatocyte differentiation with increased BEC differentiation (4,39). These studies, highlighting transcription factors acting in opposite directions, together with our own findings, indicate the critical need for a precisely balanced and tightly regulated hepatobiliary differentiation process. Furthermore, prior studies suggest the relevance of E2 signaling for BEC proliferation: adult vertebrate animal studies have shown differential expressions of *Esr* subtypes in distinct adult liver cell populations with *Esr2* being absent in hepatocytes, but highly expressed in BECs (14). Functionally, *Esr2* has been shown to promote proliferation of biliary cells, and its high expression levels have been implicated in biliary cirrhosis and cholangiocarcinoma (4,40). BMPs are part of the Transforming Growth Factor- β superfamily of proteins and play crucial roles during embryogenesis, including gastrulation, hepatic specification (41), and liver regeneration (42). Shin et al. describe a required role for BMP signaling and *alk8* activity during hepatoblast formation (41) and in biliary-driven liver regeneration (42). While our data demonstrate BMP activation in response to E2 exposure, there was no effect of E2 signaling on hepatoblasts, demonstrating the importance of precise timing of developmental signals for cell-specific effects, and further demonstrate the complexity of BMP signaling in various contexts.

Estrogen signaling modulates hepatocyte differentiation in vitro

Currently, the only clinically viable approach to acute and acute-on-chronic liver failure is liver transplantation. While theoretically attractive (43), fully functioning human hepatocytes cannot be generated *in vitro* at scale for both therapeutic and investigational purposes. While several protocols have been generated to produce hepatocyte-like cells from various progenitor populations, these cells do not capture the complexity of metabolic and synthetic functions of mature hepatocytes (44–46). Our previous work has highlighted the impact of chemical screens to improve *in vitro* differentiation and proliferation of hepatocytes (47). Our current data demonstrate increased hepatocyte differentiation from human bipotent hepatoblasts, generated from iPSCs, with chemical blockade of *ESR2*, arguing further for the importance of developmental signals for *in vitro* differentiation of functional hepatocytes.

Embryonic estrogen signaling and adult diseases

While alteration of E2 signaling during gestation can directly affect embryonic organogenesis, it may also lead to developmental reprogramming, resulting in long-lasting impact on adult homeostasis. Multiple studies have shown that maternal exposure to high levels of environmental estrogenic compounds led to fetal epigenetic reprogramming that predisposed these offspring to diseases in adulthood. Prior studies have noted the plasticity of the ER regulatory region epigenomes as they could be altered upon prenatal exposure to

estrogenic compounds such as BPA (48). Interestingly, our results revealed the long-term impact of E2 exposure during liver development in adult liver size. Given that epigenetic modifications take place throughout organ differentiation and that epigenetic reprogramming during organogenesis can affect disease susceptibility in adulthood, it is tempting to speculate that the observed effect of E2 signaling on hepatocyte differentiation could imprint a long-term influence on adult liver functions and homeostasis.

In summary, our work reveals a novel and unexpected role for estrogen in regulating hepatobiliary fate during liver development. Our findings are of immediate relevance for human physiology, and in particular for improving protocols for *in vitro* differentiation of hepatoblasts for research and clinical purposes and for anticipating the increasing impact of environmental exposure to estrogenic pollutants. Further studies will be needed to prospectively determine the long-term impact of embryonic estrogenic exposure on liver function, disease, and regenerative capacity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

ANAS	anastrozole
BEC	biliary epithelial cell
BMP	Bone morphogenetic protein
BPA	bisphenol A
DES	diethylstilbestrol
dlc	delta C
DMSO	dimethylsulfoxide
E2	17 β estradiol
EE2	ethinyl estradiol
ESR	estrogen receptor
ERE	estrogen response element

fabp10a	fatty acid binding protein 10a
FACS	fluorescence activated cell sorting
flk	fetal liver kinase
foxa3	forkhead box A3
FUL	fulvestrant
hhex	hematopoietically-expressed homeobox
iPSC	induced pluripotent stem cell
MO	morpholino
prox1	prospero homeobox 1
SALL4	Spalt Like Transcription Factor 4
Sox9	SRY Box9
TALEN	transcription activator-like effector nuclease
Tbx3	T-Box3
tfa	transferrin A
WISH	whole mount in situ hybridization

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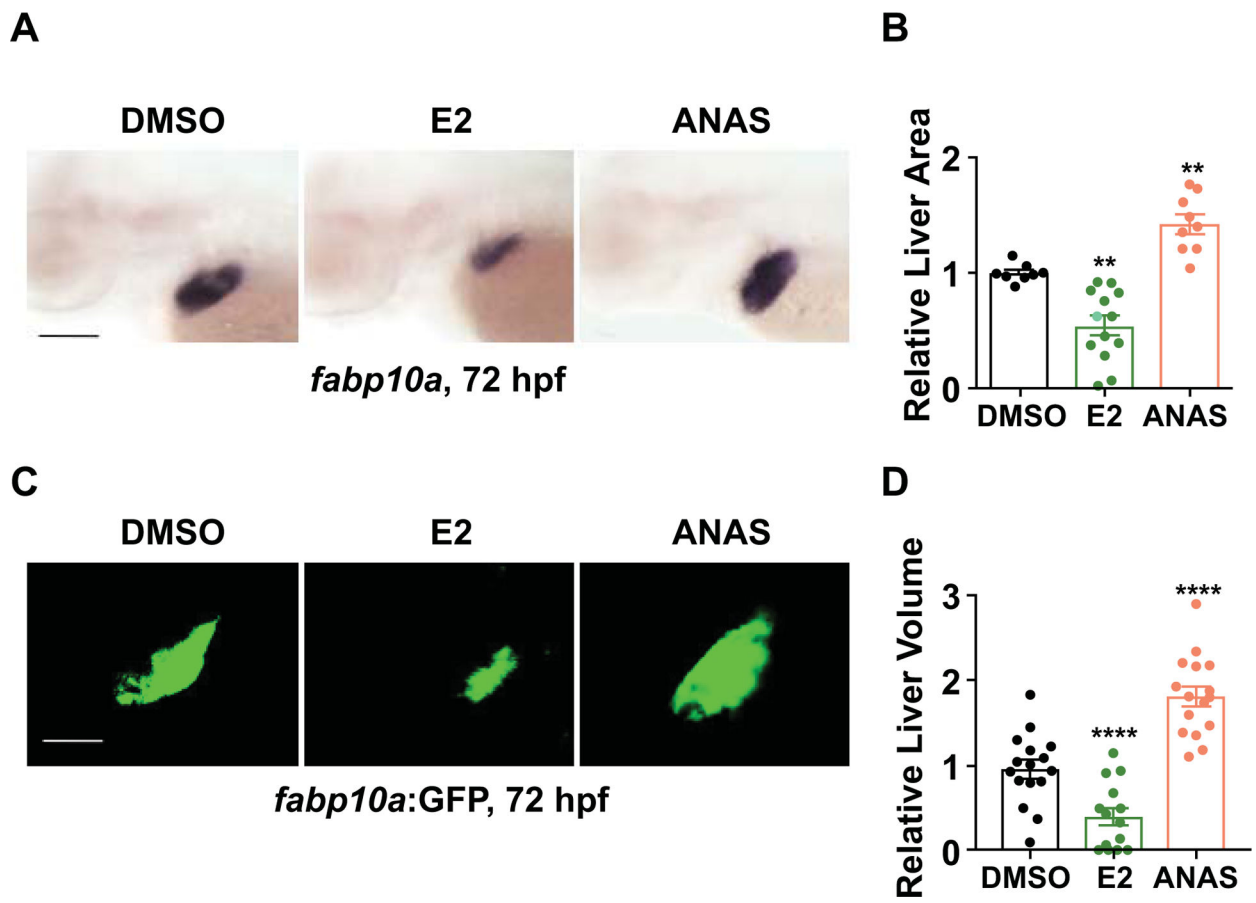


Figure 1. E2 signaling is required for normal embryonic liver formation

(A) Representative images of zebrafish embryos exposed to DMSO, E2 (10 μ M), or ANAS (10 μ M) from 24–72hpf. Liver size assessed by whole mount *in situ* hybridization (WISH) for *liver fatty acid binding protein10a* (*fabp10a*) at 72hpf. (B) Liver marker *fabp10a* expression area quantified by ImageJ analysis. $n = 8$, $**p < 0.01$, one-way ANOVA. (C) Representative images of Tg(*fabp10a:GFP*) embryos exposed to DMSO, E2 (10 μ M), or ANAS (10 μ M) from 24–72hpf. (D) Quantification of liver volume in Tg(*fabp10a:GFP*) embryos exposed to DMSO, E2, or ANAS from 24–72hpf by confocal microscopy analysis at 72 hpf. $****p < 0.0001$, $n = 10$, one-way ANOVA. All values represent mean \pm SEM, all scale bars, 200 μ m.

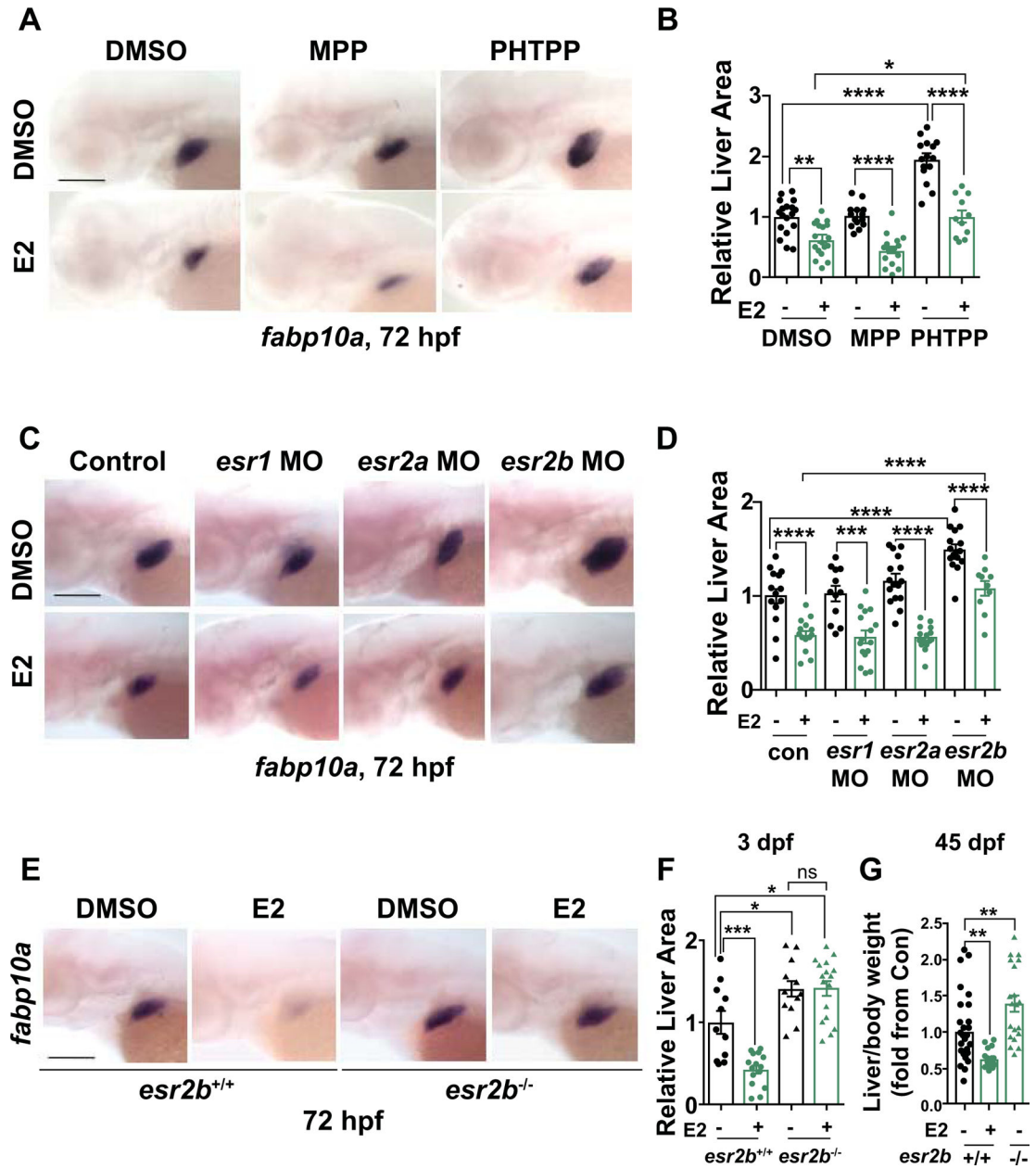


Figure 2. Estrogen receptor 2b mediates impact of E2 on embryonic liver development
 (A) Representative images of WT embryos exposed to DMSO, ESR1 antagonist (MPP), and ESR2 antagonist (PHTPP) alone or together with E2 from 24–72hpf at 72hpf. (B) Quantification of liver size by ImageJ analysis. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, $n = 11$, one-way ANOVA. (C) Representative images of *fabp10a* expression at 72hpf in *esr1*, *esr2a*, or *esr2b* morphants exposed to DMSO or E2 from 24–72hpf. (D) Quantification of *fabp10a* liver area by ImageJ analysis. *** $p < 0.001$, **** $p < 0.0001$, $n = 10$, one-way ANOVA. (E) Representative images of WISH for *fabp10a* at 72hpf of *esr2b*^{-/-} mutants and WT siblings upon exposure to DMSO or E2 from 24–72hpf. (F) Quantification of liver size at 72hpf. ns=not significant, * $p < 0.05$, *** $p < 0.001$, one-way ANOVA. (G) Liver/body weight of 45

dpf *esr2b*^{+/+} and *esr2b*^{-/-} fish exposed to E2 from 24–72hpf, $n = 11$, ** $p < 0.01$, one-way ANOVA. All values represent mean \pm SEM, all scale bars = 200 μm .

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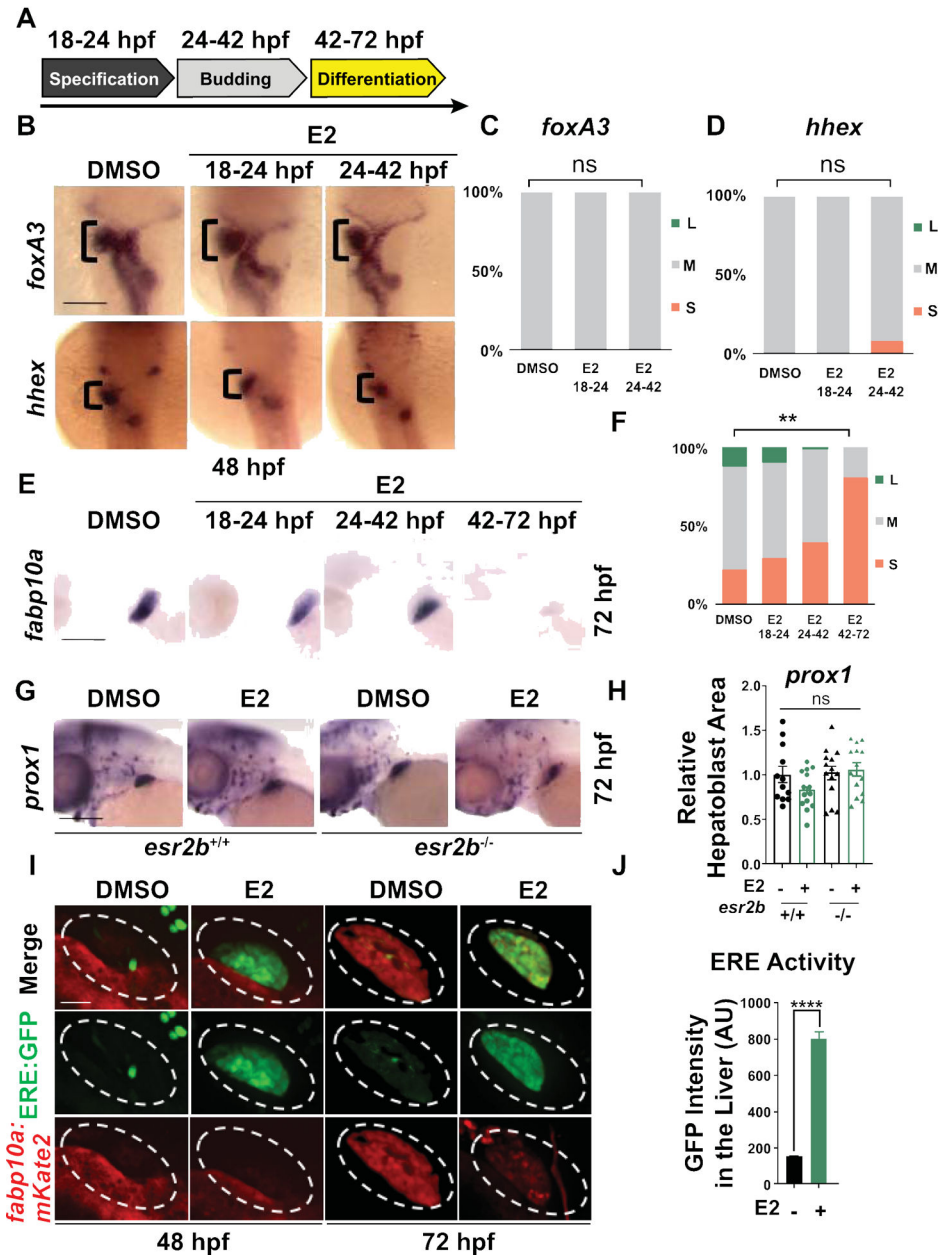


Figure 3. E2 inhibits hepatocyte differentiation

(A) Scheme illustrating E2 exposure time-windows: 18–24hpf targeting hepatic specification, 24–42hpf targeting hepatoblast budding, 42–72hpf targeting hepatoblast differentiation and maturation. (B) Endoderm (*foxa3*) and hepatoblast (*hhx*) marker expressions as assayed by WISH at 48hpf remained mostly unaffected by E2 exposures from 18–24hpf or 24–72hpf. (C–D) Distribution graph of liver size showing % of embryos with large (L, blue), medium (M, gray) or small (S, red) liver. $n = 20$, ns=not significant, two-tailed Student’s *t*-test. (E) E2 exposure from 42–72hpf exposure time-window has the most significant impact on *fabp10a* hepatocyte marker expression. (F) Liver size distribution of embryos exposed to E2 as assessed by WISH for *fabp10a* at 72hpf as % of embryos with large (L, blue), medium (M, gray) or small (S, red) livers. $n = 51$, ** $p < 0.01$, two-tailed

Student's *t*-test. (G) Expressions of hepatic progenitors (*prox1*) of *esr2b*^{+/+} and *esr2b*^{-/-} embryos exposed to DMSO or E2 from 24–72hpf at 72hpf. (H) Quantification of *prox1* expression at 72hpf by ImageJ. *n* = 11, ns=not significant, two-way ANOVA. (I) Co-localization of nuclear estrogen receptor activity in hepatocytes in bigenic zebrafish Tg(*fabp10a:DsRed*; *5xERE:GFP*) exposed to DMSO or E2 from 24–72hpf at 48 and 72hpf. Scale bar =70μm (J) Quantification of GFP intensity in the liver area (AU), *****p*<0.0001, *n* = 10, one-way ANOVA. Scale bars =200μm. All values represent mean ± SEM.

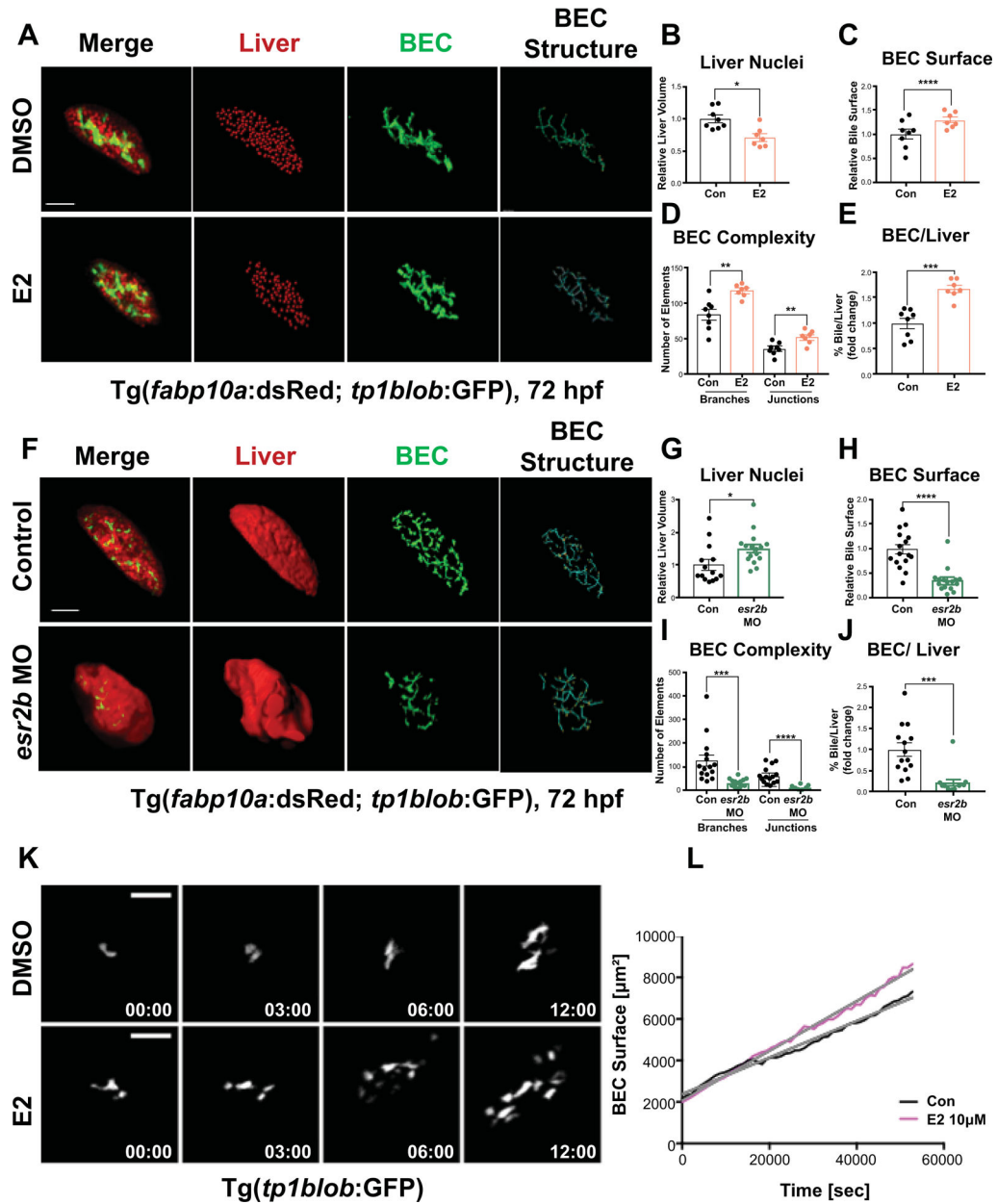


Figure 4. E2/esr2b activity controls hepatobiliary fate decisions

(A) Representative images of bigenic *Tg(fabp10a:dsRed; tp1blob:GFP)* embryos at 72hpf exposed to DMSO or E2 from 24–72hpf. Quantification of number of liver nuclei (B), Biliary surface area (C), Biliary tree complexity (D), and biliary surface area/number of liver nuclei ratio for individual embryos (E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA. (F) Representative images of *esr2b* MO injected or non-injected control bigenic *Tg(fabp10a:dsRed; tp1blob:GFP)* embryos at 72 hpf. Quantification of number of liver nuclei (G), Biliary surface area (H), Biliary tree complexity (I), and biliary surface area/number of liver nuclei ratio for individual embryos (J). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA. (K) Time-lapse images showing dynamic growth of BEC in *Tg(tp1blob:GFP)* embryos for 16 hours from 55–72 hpf. These embryos were exposed to

DMSO or E2 starting at 30 hpf. (L) Quantification of BEC surface areas (μm^2) of Tg(tp1blob:GFP) embryos exposed to DMSO or E2 from 30 hpf. All images were quantified by Imaris 9 software, all values represent mean \pm SEM, scale bars = 70 μm .

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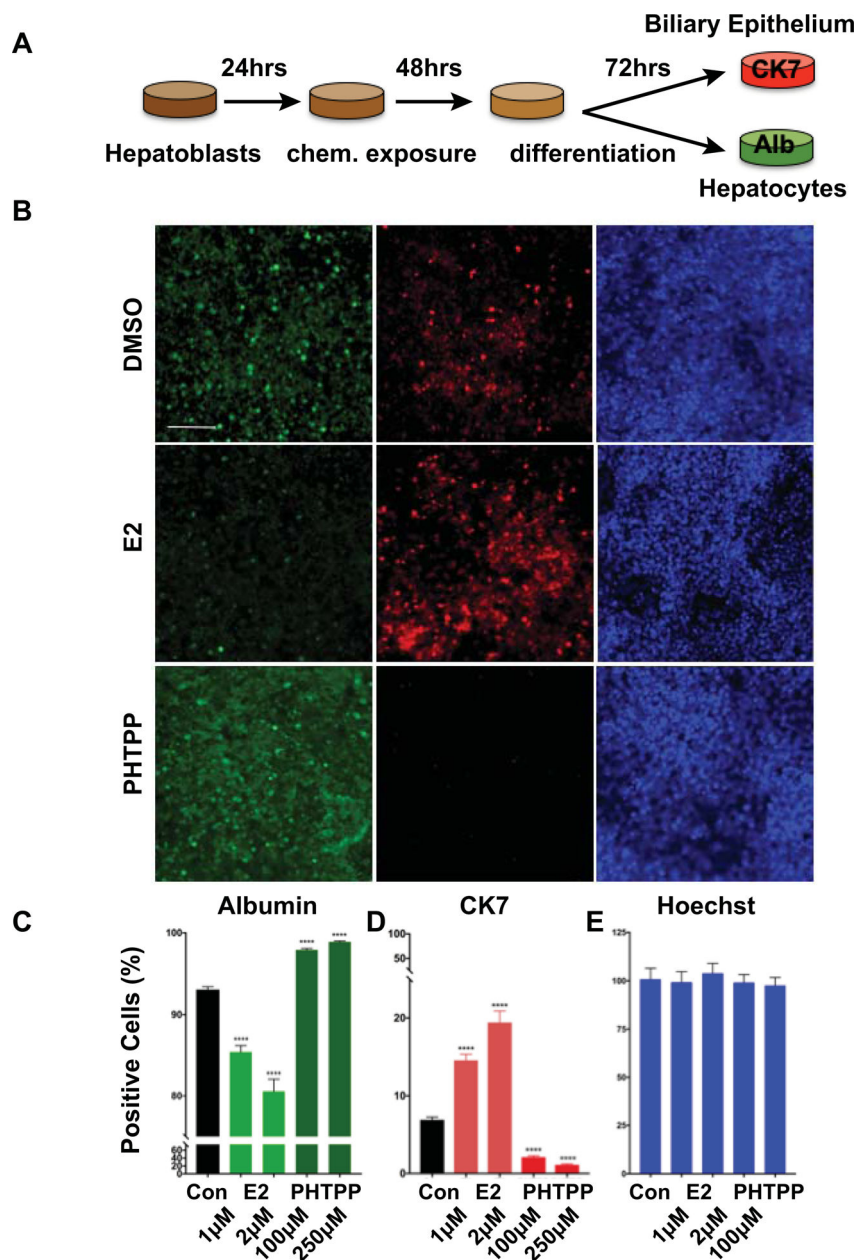


Figure 5. E2/ESR2 signaling affects hepatobiliary differentiation in human hepatoblasts
 (A) Schematic illustration of the human hepatoblast differentiation experiment. Human hepatoblasts were cultured for 24 hours prior to exposure to E2 (1µM, 2 µM) or PHTPP (100nM, 250nM) for 48 hours. The culture was allowed to grow for another 72 hours before immunofluorescent analysis labeling CK7 (BEC) and Albumin (hepatocyte). (B) Representative images of cells after immunofluorescence marking hepatocytes (Albumin), BEC (CK7), and DNA (Hoechst). Quantification of Albumin+ (C), CK7+ (D), and Hoechst + (E) cells in E2 or PHTPP treated hepatoblasts relative to DMSO treated controls. **** $p < 0.0001$, one-way ANOVA. All values represent mean \pm SEM, scale bar = 100 µm.

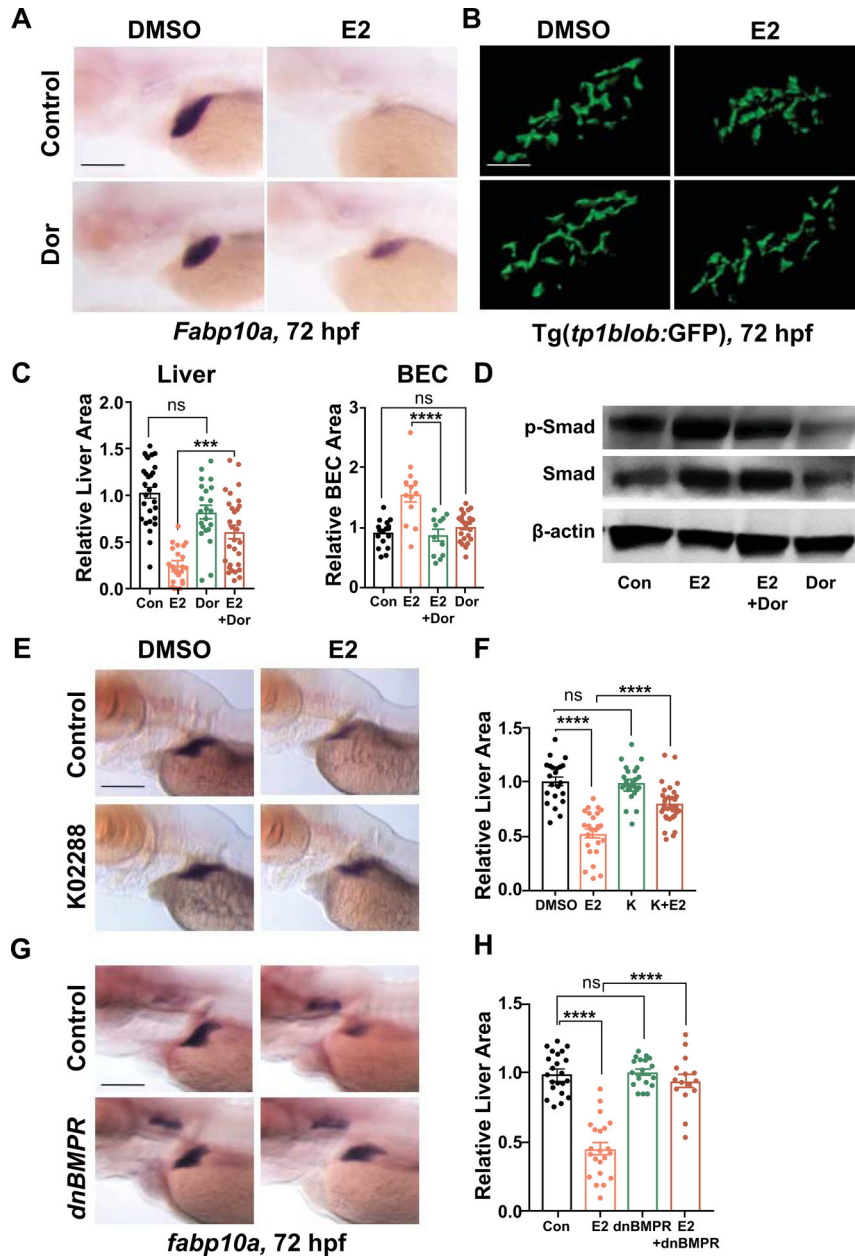


Figure 6. E2 signals through BMP pathway to impact hepatobiliary development
 (A) Representative images of zebrafish embryos exposed to DMSO, E2 (10 μM), Dorsomorphin (10μM), or E2+Dorsomorphin from 24–72hpf at 72hpf. Liver size assessed by WISH for *fabp10a* at 72 hpf. (B) Confocal images of BEC surface area of Tg(tp1blob:GFP) embryos exposed to DMSO, E2 (10μM), Dorsomorphin (10μM), or E2+Dorsomorphin from 24–72hpf at 72hpf. Scale bar = 40μm. (C) Liver area quantified by ImageJ analysis and BEC surface area quantified by Imaris 9 imaging software. *n* = 12, ns=not significant, ****p*<0.001, *****p*<0.0001, one-way ANOVA. (D) Immunoblot of p-Smad, total Smad, and β-actin of embryos treated with DMSO, E2 (10μM), Dorsomorphin (10μM), or E2+Dorsomorphin from 24–72 hpf at 72 hpf. (E) Representative images of embryos exposed to DMSO, E2 (10μM), K02288 (5μM), or E2+K02288 from 24–72hpf at 72hpf. (F)

Quantification of liver area at 72hpf. ns=not significant, **** $p<0.0001$, $n = 22$, one-way ANOVA. **(G)** Representative images of dnBMPR embryos exposed to DMSO or E2 (10 μ M), from 24–72hpf at 72hpf. **(H)** Quantification of liver area at 72hpf. ns=not significant, **** $p<0.0001$, $n = 15$, one-way ANOVA. All values represent mean \pm SEM, scale bars = 200 μ m.