Roles of Estrogen Signaling in Vertebrate Liver

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th>Citation</th>
<th>Chaturantabut, Saireudee. 2017. Roles of Estrogen Signaling in Vertebrate Liver. Doctoral dissertation, Harvard University, Graduate School of Arts &amp; Sciences.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:41142053">http://nrs.harvard.edu/urn-3:HUL.InstRepos:41142053</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
Roles of estrogen signaling in vertebrate liver

A dissertation presented

by

Saireudee Chaturantabut

to

The Department of Molecular and Cellular Biology

in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of

Biochemistry

Harvard University
Cambridge, Massachusetts

February 2017
Abstract

Estrogen (17β-estradiol; E2) signaling has been implicated in many biological processes, particularly in reproductive biology, organ development and carcinogenesis. E2 plays crucial roles in adult reproductive organ growth and size determination; however, the impact of E2 on non-reproductive organs such as liver is not well understood. In this dissertation work, I have investigated the roles of E2 signaling throughout embryonic liver development and adult liver homeostasis. I demonstrate in Chapter 2 that early activation of E2 signaling during hepatic formation in the zebrafish embryo from 24 hour post fertilization (hpf) progressively decreased liver size, with the most severe impact on hepatic differentiation. These E2 effects are mediated by estrogen receptor 2b (esr2b) as specifically generated esr2b knockout embryos blocked estrogenic effects and had increased liver size. Importantly, while pharmacological blockade of estrogen receptors increased liver size, it negatively impacted cholangiocyte formation, suggesting the potential role of E2 signaling in determining hepatic lineages: promoting biliary tree while suppressing hepatocyte formation.

I describe further in Chapter 3 that activation of E2 signaling after hepatic differentiation increased liver size. This increase in liver size induced by E2 exposure was blocked in our generated G protein-coupled estrogen receptor 1 (gper1) knockout embryos, demonstrating that E2 signals via gper1 to drive liver growth. Intriguingly,
impacts of E2 on liver size were observed after a brief 5 hour E2 exposure, suggesting that E2 activates downstream rapid signaling. Epistasis analysis revealed that E2 signals via \textit{gper1} to activate Akt/mTOR signaling to promote liver growth. Indeed, hypomorphic \textit{mtor} mutant embryos blocked estrogenic effects on liver size. E2/GPER1 signaling further promoted liver regeneration in a hepatocyte-targeted ablation model. Importantly, E2 accelerated, whereas loss of GPER1 in \textit{gper1} knockout or in GPER1 inhibitor-treated adults significantly reduced, liver cancer incidence and progression, specifically in males. Finally, E2 increased proliferation of primary human hepatocytes and \textit{GPER1} expression increased in human liver tissues with cirrhosis and hepatocellular carcinomas, suggesting its positive role during liver carcinogenesis. Taken together, I have provided substantially novel mechanistic insight into the roles of E2 signaling during normal liver organogenesis, regeneration as well as liver cancer.
Table of contents

Acknowledgements ......................................................................................................................... vi

Chapter 1: Introduction
1.1 LIVER GROWTH DURING DEVELOPMENT AND DISEASE ............................................. 2
1.2 LIVER DEVELOPMENT IN MAMMALS AND ZEBRAFISH ........................................... 3
   1.2.1 LIVER DEVELOPMENT IN MAMMALS .................................................................. 3
   1.2.2 LIVER DEVELOPMENT IN ZEBRAFISH ............................................................... 6
1.3 MODELING LIVER RESEARCH IN ZEBRAFISH ............................................................. 9
   1.3.1 ZEBRAFISH AS A MODEL FOR LIVER DEVELOPMENT ..................................... 9
   1.3.2 ZEBRAFISH AS A MODEL FOR LIVER REGENERATION ................................... 10
   1.3.3 ZEBRAFISH AS A MODEL FOR LIVER CANCER ................................................. 12
1.4 ESTROGEN SIGNALING ................................................................................................. 13
   1.4.1 ESTROGEN SIGNALING VIA ESTROGEN RECEPTORS ................................... 13
   1.4.2 ESTROGEN SIGNALING IN LIVER BIOLOGY ..................................................... 16
   1.4.3 SEXUAL DIMORPHISM OF THE LIVER .............................................................. 17
1.5 REFERENCES .................................................................................................................... 20

Chapter 2: Estrogenic regulation via Estrogen receptor 2b controls differentiation of hepatic progenitors during vertebrate embryogenesis
2.1 ABSTRACT ......................................................................................................................... 30
2.2 INTRODUCTION ............................................................................................................... 31
2.3 RESULTS .......................................................................................................................... 33
2.4 DISCUSSION ..................................................................................................................... 50
2.5 MATERIALS AND METHODS .......................................................................................... 54
2.6 REFERENCES .................................................................................................................... 57

Chapter 3: The hepatic estrogen sensor GPER1 activates PI3K/mTOR to promote gender dimorphism in liver growth and cancer
3.1 ABSTRACT ........................................................................................................................ 63
3.2 INTRODUCTION ............................................................................................................... 64
3.3 RESULTS .......................................................................................................................... 66
3.4 DISCUSSION ..................................................................................................................... 84
3.5 MATERIALS AND METHODS .......................................................................................... 88
3.6 REFERENCES .................................................................................................................... 94

Chapter 4: Conclusion, discussion, and future direction
4.1 CONCLUSION .................................................................................................................... 101
4.2 DISCUSSION AND FUTURE DIRECTION ..................................................................... 104
4.3 REFERENCES .................................................................................................................... 113

Appendices
APPENDIX 1: SUPPLEMENTAL MATERIALS FOR CHAPTER 2 ............................................ 119
APPENDIX 2: SUPPLEMENTAL MATERIALS FOR CHAPTER 3 ............................................. 125
Acknowledgements

I am deeply thankful how far I have come and to those who have enabled me to get here. I would like to start by thanking my past mentors during my undergraduate years at Columbia University. Thank you Prof. Koji Nakanishi and Gorge Ellestad for adopting me into your lab and sparking within me the curiosity for research during my freshmen year. Thank you for teaching me and for trusting me to be a part of your organic chemistry project. Thank you Dr. Yinghui Mao for welcoming me and helping me in so many ways as I transitioned into biology research. Thank you for all the wise advice, support, and career guidance you have given me over the years. Thank you Sana Ahmad for inspiring me with your hard work and your love for science and for being a wonderful friend and mentor in Dr. Mao’s lab. You will be an amazing physician scientist!

I also have a tremendous amount of gratitude to my advisor, Wolfram. Though it has not always been easy, these past years have been a truly wonderful journey. Your passion and love for science have been inspirational and contagious. Thank you for believing in me through the ups and downs and for giving me the courage to pursue my passions. Thank you for being available and for going above and beyond to provide me with all the resources I need to do the best science possible. Your scientific wisdom, creativity, and exceptional writing skills are remarkable, and I am fortunate to get to learn from you. Your determination, humility, and positivity through challenges are truly inspirational. Thank you also for your genuinely caring about every aspect of my life: scientifically, professionally, and personally. It has been a great privilege to be a part of Goessling lab, thank you.
Thank you the Goessling lab members for making this accomplishment possible. Thank you past and present members: Allison Tsomides, Katie Kwang, Julia Wucherpfennig, Kristen Alexa, Olivia Weeks, Sahar Nissim, Maija Garnaas, Chad Walesky, Paul Wrighton, Emily Kurdzo, Hongchao Zhou, AJ Kim, John Hedgepeth, Steph Schatzman-Bone, Diane Saunders, Rachel Lucier, Alexandra Nano, and Nadine Budrow. Your knowledge, skills, enthusiasm, and expertise throughout these years have greatly shaped me as a student, teacher, and researcher. Your friendships and support mean so much to me and have made my time in lab truly enjoyable. Thank you Trista North for being a wonderful mentor. I have learned a lot from your scientific insights and I am encouraged by your excitement for science. Thank you Leah Liu for all your helpful advice, especially during my first few years of trying to get the project going. Your hard work, persistence, and fun attitude have been a great encouragement. Thank you Andy Cox, a very talented postdoc that I was fortunate to work with and to learn from. Your critical thinking, rigorous scientific approach, and dedication to pursuing your passion is inspiring and have kept me motivated. I am thankful for your genuine care and friendship. Australia is lucky to have you!

The completion of this dissertation would not have been possible without the generous help, expertise, insights, and commitments from all my committee members. Thank you Alex Schier for always sharing valuable insights during the committee meetings. Thank you for truly caring about my project and for challenging me to push the boundaries, to be meticulous, and to carry out the best experiments possible. Thank you Craig Hunter for always making time for me and for investing your thoughts and energy into my project. Thank you also for allowing me to rotate in your lab and for
teaching me so much about genetics. Thank you for your encouragements and precious advice on science and life throughout the years. Thank you David Cohen for putting a great deal of time and effort into my project. Thank you for always encouraging me not to lose sight of the big picture of how our findings in fish can impact humans and for providing me with valuable advice. Thank you also for introducing me to Mariana Acuna, and I am grateful for all her help with our project.

My classmates at the MCO program, you are wonderful. Being surrounded by such a talented, knowledgeable, and fun group of people has been truly amazing. I have been fortunate to be part of a group that help and support each other during difficult times, especially during our first year classes and qualifying exams. To Sara Leiman and Alicia DeFrancesco, I am thankful for your friendships and support. I have learned so much from you. I am grateful to the MCO program for the opportunities to have been a teaching fellow for undergraduate classes. Thank you Jill Penn for being a wonderful preceptor. Your love for teaching and your genuine care for the TFs and students are inspirational.

Thank you to my church family at Park Street Church. Thank you Debra Zhang, Ivy Chang, and Tiara Lin for your amazing friendship and invaluable support. Thank you for always be there to encourage me, give me precious advice, and to talk with me over countless boba. Thank you Ray and Janice Kim for your example in living to serve others sacrificially, for deeply caring about me, and for your endless support over the years. Thank you Vinca Chow. I could not have asked for a better partner with whom to lead Sunday Bible studies. Your love and commitment to serve patients, students, the homeless, and everyone around you, as well as your great time management skills, are
truly inspiring! To my fellow graduate students at Harvard graduate fellowship (GCF), Teoh Zhi Ern, Bronwyn Isaacs and Janet Zong, thanks for bringing in such a great knowledge and wisdom from each of your field of expertise. I learned a lot from you and I am so thankful for your support. Thank you Sze-Yi Lau, Rapeechai Navawongse (Pom), Jessica Liu, and Steven Chao for helping me transition to Boston, for taking care of me, and for the delicious meals and board game nights. Thank you Tee Ariyachet for being a wonderful and fun roommate, for taking care of me, cooking me Thai food, and for introducing me to Wolfram! Thank you Pan-Yu Chen for being awesome. Going through graduate school together, I am thankful for your encouragements and for our endless talk and laughs sharing our lives together after long day at labs.

Jonathan, my lovely husband, I could not have asked for a better friend to walk with through all the ups and downs of life. You are so sweet, thoughtful, and fun. Being with you makes everyday full of life and meaning. Thank you for your love, sacrifices, and support. Thank you for keeping our home functioning during my busy times. You know my strengths and weaknesses, and you bring out the best in me. Thank you for believing in me and my work and for giving me the courage to pursue my passions. Thank you for being so positive and for helping me see the best in every situation. Thank you for your love and dedication in serving your patients. You inspire me to pursue excellence in all I do. I love you.

Thank you Ja and Fon, my wonderful sisters, for your love, generosity, support, and wisdom. Thank you for supporting me through all the ups and downs. Your wise advice have helped me through so many major decisions. Thank you for your
unconditional love and for investing in me with time, energy, and much wisdom. Thank you Joseph, my awesome brother-in-law, for all your support and encouragements. Your kindness and sense of humor have brought us joy and laughter and have brightened so many of our days.

No words can describe my gratitude towards my family. Thank you mom and dad for modeling to me what unconditional love is like. Thank you for always being there for me. Thank you for instilling in me a passion for learning, for creating wonderful educational opportunities for me from the time I was young, for prioritizing your children above your work, and for always offering a listening and supportive ear. Thank you for your precious wisdom that brought me through critical moments and overcome countless obstacles throughout the years. Thank you for your passions and dedication to your work have inspired me to pursue my passions and to work hard. Thank you for teaching me not to take the skills and talents I have for granted, but to cultivate them and to use them for others. I love you, am so proud of you, and am so grateful to be your daughter. I would also like to thank my wonderful parents-in-law, Seree and Sunee, for your support, encouragements, advice, and for always loving me as your daughter. Thank you for your example in dedicating your lives to serving others. Your kindness and generosity is truly inspirational. Thank you Michelle and Lindle Barkley for adopting me into your family starting from my high school exchange program. Your generosity, support, and your presence at our wedding meant so much to us! Love y’all!

To all of you, I am deeply thankful. Each and everyone of you had made my journey an incredible one and for those who are far away, I am looking forward to the day that our paths will cross again.

x
CHAPTER ONE

Introduction: Estrogen signaling and vertebrate liver
1.1 LIVER GROWTH IN DEVELOPMENT AND DISEASE

Cellular and molecular basis for liver growth is crucial not only during development and maturation of hepatocytes, but also during liver homeostasis and pathogenesis. Liver growth involves the process of increasing hepatic cell number and organ size through developmental stages. Early liver organogenesis is broadly characterized as endoderm patterning, hepatic progenitor specification, and hepatic differentiation and growth. Accelerated hepatic growth predominantly takes place during these two early stages, liver budding and liver outgrowth. Towards the end of hepatic specification, hepatic progenitors undergo rapid growth and proliferation to form the liver bud, just prior to becoming the major site of fetal hematopoiesis in mammals. A variety of growth factors are involved during this developmental phase, including Wnt, TGFβ, hepatic growth factor (HGF) and fibroblast growth factor (FGF) signaling and its downstream MAP kinase and PI3 kinase signals (Micsenyi et al., 2004; Calmont et al., 2006). Upon hepatic differentiation and maturation, liver growth and size continues to be tightly regulated by pathways such as Hippo and mTOR signaling (Tumaneng et al., 2012). This closely knitted network of signaling cascades controls normal hepatic growth throughout liver development and adult liver homeostasis.

Due to its central roles in detoxification and metabolism, the liver has a unique capacity to fully regenerate upon injury (Michalopoulos and DeFrances, 1997). Notably, signaling pathways necessary for the maintenance and growth of hepatic progenitors and hepatocytes are often important during hepatic repair. Indeed, these pro-growth developmental signals mentioned, such as HGF and TGFβ, are induced during the regenerative process (Michalopoulos, 2007). Nevertheless, chronic injury such as
chronic viral hepatitis or long-term use of alcohol, often impair hepatic regenerative abilities and lead to chronic liver diseases, including fibrosis, cirrhosis and liver cancer. The underlying process leading to cancer formation is an unregulated proliferation of cells. Indeed, signaling pathways shown to be essential during developmental liver growth have been implicated in liver cancer, including Wnt, PI3 kinase, Hippo, and mTOR signaling (Whittaker et al., 2010). Our knowledge of molecular pathways governing liver growth and proliferation during hepatogenesis can therefore be translated to an improved understanding of mechanistic details involved in hepatocarcinogenesis, and the identification of new molecular targets for liver cancer therapies.

1.2 LIVER DEVELOPMENT IN MAMMALS AND ZEBRAFISH

1.2.1 Liver development in mammals

In mammals, over 70% of the mature liver is composed of hepatocytes, which are derived from the endodermal germ layer. The mammalian endoderm emerges from the anterior end of primitive streak during gastrulation at embryonic day (E) 7.5 in mouse and at ~21 days of gestation in humans, and later undergoes patterning into foregut, midgut, and hindgut as determined by overlapping Wnt, fibroblast growth factor (FGF), and bone morphogenetic protein (BMP) signaling gradients. Upon induction by FGF and BMP signals, the foregut gives rise to hepatic progenitors or hepatoblasts. Specifically, FGF-mitogen activated protein kinases (MAPK) pathway drives hepatic induction, while the FGF-phosphoinositide 3 kinase (PI3K) pathway promotes hepatic growth (Calmont et al., 2006). Wnt/β-catenin signaling is required for hepatoblast expansion, as its inhibition leads to increased apoptosis (Suksaweang et al., 2004) and
levels of β-catenin correlate with hepatoblast proliferation (Micsenyi et al., 2004). By E9.5 in mouse or ~32 days of gestation in humans, hepatoblasts migrate into the septum transversum mesenchyme (STM) to form a nascent liver bud and express liver genes, such as Albumin and hepatic nuclear factor 4 alpha (HNF4α) (Suzuki et al., 2000). During this period, the liver bud also becomes the major site of fetal hematopoiesis. From E13.5-18.5 in mouse or ~56-210 days of gestation in humans, hepatoblast differentiation takes place: hepatoblasts in contact with the portal vein at the ductal plate become cholangiocytes, while those located away from the portal vein differentiate into hepatocytes (Zorn and Wells, 2009) (Figure 1.1; Figure 1.2). Subsequently, hepatocytes continue to mature and compartmentalize based on their localization by expressing different enzymes and transporters forming the classic zonal function of the liver (Jungermann and Kietzmann, 1996).
**Figure 1.1 Hepatic lineages.** During embryonic liver development, uncommitted endoderm is patterned via graded Wnt, FGF, BMP signals to become foregut midgut and hind gut. Foregut is further specified to become hepatic progenitor (hepatoblast). Hepatoblasts subsequently differentiate into hepatocyte and biliary epithelium, which continue to grow and mature. Hepatic lineages are highlighted in red (Zorn, 2008).

**Figure 1.2 Mouse liver development.** Timeline showing various stages of liver development in mouse embryos. Gastrulation takes place during embryonic day (e) 6.5-e7.5 when the endoderm is formed. Endoderm patterning occurs during e7-e8.5 giving rise to foregut (fg), midgut (mg) and hindgut (hg). Liver bud (lb) is formed by e10 from liver diverticulum (ld). At e15, hepatoblasts differentiate into hepatocytes and biliary cells, which undergo growth and maturation. Endoderm is highlighted in yellow, liver in red, and gall bladder in green (Zorn, 2008).
1.2.2 Liver development in zebrafish

The process of hepatogenesis is conserved among vertebrates, including zebrafish (*Danio rerio*). Similar to mammals, the zebrafish liver arises from anterior endodermal progenitor cells, which are first identified at 6 hours post fertilization (hpf) or at shield stage. The endodermal transcription factor *sox32* activates *sox17* to regulate expression of genes required for endoderm precursors, identified by expression of forkhead box transcription factors (*foxa1*-3) (Field et al., 2003; Shin et al., 2007). Endoderm precursors are specified to become hepatic progenitors at 18-24 hpf and can be identified by expression of the transcription factor *hematopoietically expressed homeobox* (*hhex*) and *prospero homeobox 1* (*prox1*) (Shin et al., 2007). Hepatic nuclear factors, such as hepatic nuclear factor 1β (*hnf1b*), are also expressed and essential for hepatic specification (Cheng et al., 2006; Lokmane et al., 2008). From 24-28 hpf, the endoderm thickens to form a solid bar of midline cells called intestinal rod (Field et al., 2003), and by 28 hpf, the more posterior domain of the rod contributes to the pancreas, while the anterior domain to the liver forming a liver bud. By 48 hpf, hepatic progenitors aggregate to form gut tube and by 72 hpf, hepatoblasts differentiate to become hepatocytes, identified by expression of *liver fatty acid binding protein 10a* (*fabp10a*) (Her et al., 2003). From 72 hpf, the liver continues to grow and is fully developed and functional by 120 hpf.

Signaling pathways regulating zebrafish liver formation are also conserved in mammals (Chu and Sadler, 2009). Key signals during hepatogenesis include Nodal, FGF, BMP, and Wnt signaling pathways. In zebrafish, an extra-embryonic yolk syncytial layer signal activates Nodal signaling to specify mesoderm and endoderm progenitors.
(Shen, 2007). By the end of gastrulation, endoderm is patterned along the anterior-posterior axis to become foregut and hindgut progenitors. The precise regulation of Wnt signaling levels, such as that of zebrafish Wnt11, is required for proper endoderm patterning (Stuckenholz et al., 2013). Foregut epithelium is subsequently specified to become hepatic progenitors, and this process is driven by FGF as well as BMP signaling pathways (Shin et al., 2007; Naye et al., 2012). Initially, canonical Wnt/β-catenin signaling must be repressed to allow differentiation of foregut into hepatic progenitors. Conversely, Wnt/β-catenin is required later for hepatocyte differentiation, proliferation and growth (Ober et al., 2006; Goessling et al., 2008). For instance, loss of Wnt2 and Wnt2bb results in liver agenesis (Poulain and Ober, 2011) while overexpression of Wnt8a leads to direct conversion of pancreatic progenitors to hepatoblasts (So et al., 2013) in zebrafish embryos (Figure 1.3).
**Figure 1.3 Gut formation during zebrafish development.** Purple represents endodermal-derived structures. During zebrafish embryonic development, the liver is derived from the endoderm germ layer. At 48 and 72 hours post fertilization (hpf), the liver bud and pancreatic buds (yellow) are visible as outgrowths from the gut tube. Endoderm precursors are specified to become hepatic progenitors at 18-24 hpf and can be identified by *hhex* and *prox1* expression. From 24-48 hpf, hepatic progenitors aggregate to form a gut tube. From 48-72 hpf, hepatic progenitors differentiate to become hepatocytes identified by expression of liver fatty acid binding protein (*fabp10a*) (green). From 72 hpf, the liver continues to grow and is fully developed and functional by 120 hpf (drawing adapted from Diane Saunders).

The liver is composed of not only hepatocytes but also cholangiocytes or biliary epithelial cells. Hepatoblasts differentiate into cholangiocytes at ~56 days of gestation in humans, ~E13.5 in mouse, and ~24 hpf in zebrafish. In humans, bipotential hepatoblasts express alpha-fetoprotein (AFP), while cholangiocytes are specifically marked by high levels of cytokeratin-19 (CK19) (Haruna et al., 1996). In zebrafish, biliary cells mature and become functional at 120 hpf; marked by expression of *cytokeratin-18* (*krt18*) (Lorent et al., 2004). Notch and Wnt signaling pathways are shown to be essential to biliary cell differentiation, development, and survival (Lorent et al., 2004; Zong et al., 2009; Tan et al., 2008). Besides endodermal-derived hepatocytes
and biliary cells, the mature liver also contains other cell types, including endothelial cells, Kupffer cells (liver macrophages), and hepatic stellate cells, which are of mesodermal origin. As a result, having a fully functional liver does not depend only on endodermal factors, but also the signals produced by the neighboring mesoderm. Finally, given the diverse cell types in the liver and their complex regulatory networks, investigating the interplay between these signaling pathways in regulating liver development will enhance our understanding of the liver biology as well as liver pathogenesis.

1.3 MODELING LIVER RESEARCH IN ZEBRAFISH

1.3.1 Zebrafish as a model for liver development

The liver is an essential organ that plays a vital role in lipid, glucose and toxin metabolism. Understanding the mechanisms underlying liver development and pathogenesis is thus crucial for the prevention and treatment of liver diseases. Human liver development and diseases have been successfully modeled both *in vitro* in cell culture systems and *in vivo* using animal models, including zebrafish (Goessling and Sadler, 2015). Major advantages of using zebrafish as a liver development model include the fact that zebrafish embryos develop externally and rapidly, having a functional mature liver by 5 dpf. Zebrafish embryos are also transparent during the first week of life, enabling *in vivo* imaging of liver developmental processes. Specific organs and tissue types can also be visualized using zebrafish transgenic lines expressing fluorescent proteins under the promoters expressed in hepatocytes, *fabp10a*; Tg(-2.8fabp10:EGFP) (Her et al., 2003), biliary epithelial cells, *krt18*; Tg(*krt18:EGFP*) (Wilkins et al., 2014), or hepatic stellate cells, *hand2*; Tg(*hand2:EGFP*) (Yin et al.,
They are particularly suited for studying liver organogenesis since, unlike murine liver development, the zebrafish liver is not a site for hematopoiesis, enabling the study of mutations that affect the liver without causing anemia or embryonic lethality (Tao and Peng, 2009). In addition, the zebrafish genome sequencing indicates that approximately 70% of human genome has zebrafish orthologues (Howe et al., 2013). Recent advancement in genome editing technologies such as transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 further allows genetic modifications and identifications of novel gene functions in zebrafish (Bedell et al., 2012; Li et al., 2016). Finally, zebrafish produce large numbers of offspring, up to 200 per week, allowing large chemical and genetic screens to identify crucial regulators of liver development and disease (Driever et al., 1996).

1.3.2 Zebrafish as a model for liver regeneration

Similar to mammals, the zebrafish liver has striking regenerative capacities and has been widely used as liver injury and regeneration model. One of the most established models for liver regeneration is partial hepatectomy (Michalopoulos, 2013). Using this procedure, the study using zebrafish as a liver regeneration model was carried out (Goessling et al., 2008). In murine two-thirds hepatectomy studies, three out of five liver lobes are removed and the two remaining lobes proliferate until the liver regain its original size. In contrast to the compensatory growth of the remaining liver in mammals, zebrafish exhibit a different pattern after removal of one of three liver lobes: after surgical resection of the ventral lobe, this lobe regrows to recover its original structure. *In vivo* ultrasonographic analysis further reveals similar kinetics and 1-week
recovery time of the zebrafish liver repair process compared to that of mice (Goessling et al., 2007). With this approach, studies from our laboratory and others have uncovered signaling pathways involved in liver regeneration. For instance, the interaction between prostaglandin E2 and Wnt pathways during liver regeneration was revealed (Nissim et al., 2014), and the inhibition of nitric oxide signaling repaired zebrafish liver regeneration, suggesting its necessary role during this process (Cox et al., 2014). Wnt, BMP and FGF signaling pathways were further shown to be crucial for hepatic repair (Goessling et al., 2009; Kan et al., 2009).

Since the first use of zebrafish as a liver regeneration model in 1999 (Burkhardt-Holm et al., 1999), many models of liver regeneration have been successfully developed, including the more recent use of nitroreductase (NTR)-metronidazole (Mtz) for spatial and temporal-controlled ablation of hepatocytes (Curado et al., 2007; Curado et al., 2008). These studies are based on the principle in which a nontoxic prodrug, Mtz, is converted to a DNA cross-linking agent by NTR activity causing cell death (Edwards, 1993). Mtz has much reduced off-target effects compared to the previous commonly used NTR-substrate CB1954 (Bridgewater et al., 1997). To achieve tissue-specific ablation, transgenic animals expressing NTR under organ-specific promoters such as Tg(fabp10a:CFP-NTR) for liver-specific ablation and Tg(insulin:CFP-NTR) for pancreatic β-cell ablation were generated and shown to efficiently and rapidly ablate target tissues. Liver regeneration then takes place upon removal of Mtz, and the ablated hepatocytes are quickly replaced by newly proliferating cells (Curado et al., 2007). Together, these zebrafish liver regeneration models contribute to and continue to enhance our mechanistic understanding of liver regeneration.
1.3.3 Zebrafish as a model for liver cancer

Over the past decade, zebrafish have also been increasingly used as a vertebrate model for liver cancer studies. While fish spontaneously develop cancers, the extent of tumorigenesis can be difficult to measure in nature (Stern and Zon, 2003). Carcinogen-induced zebrafish tumor models have been developed to accelerate cancer formation with specific mutagens causing different types of tumors. For instance, zebrafish treated with N-methyl-N’-nitro-M-nitrosoguanidine (MNNG) are prone to hepatic neoplasms and mesenchymal neoplasms, such as rhabdomyosarcoma and chondroma formation (Spitsbergen et al., 2000). Zebrafish exposed to 7,12-imethylbenz(a)anthracene (DMBA) develop liver, gill, and blood vessels tumors (Spitsbergen et al., 2000). These zebrafish develop a wide range of liver tumors, including hepatic adenomas, hepatocellular carcinoma (HCC), and cholangiocarcinomas. Importantly, zebrafish liver tumor histology as well as their gene expression profiles resemble that of human liver cancer (Goessling and Sadler, 2015; Lam et al., 2006), suggesting a conservation of mechanisms underlying liver carcinogenesis. Moreover, oncogenes and tumor suppressors identified in mice and humans, including p53, β-catenin, myc, ras, have zebrafish orthologues (Langheinrich et al., 2002; Schreiber-Agus et al., 1993). Zebrafish oncogene-induced liver tumor models have also been developed and shown to induce hepatic neoplasms. For example, hepatocyte-specific activation of kras induced tumors that progressed to HCC within 4 weeks (Nguyen et al., 2012). Constitutively active β-catenin in the hepatocytes in Tg(fabp10a:pt-β-cat) zebrafish also led to HCC development in 78% of transgenic animals at 6 months post fertilization (Evason et al.,
This transgenic modeling of liver cancer further provides a chemical screening platform for discovery of new anti-liver cancer drugs.

1.4 ESTROGEN SIGNALING PATHWAY

1.4.1 Estrogen signaling via estrogen receptors

Estrogens are naturally occurring molecules that regulate cellular proliferation, differentiation and homeostasis. While there are three major forms of estrogens, estrone (E1), estradiol (E2), and estriol (E3), estradiol (E2) is the most dominant and active form of estrogens. While estrogens are predominantly found in females, males also contain estrogens but at a far lower extent. The principal source of E2 is the ovary, however, E2 can also be produced from extragonadal tissues, including adipose tissue, bones, vascular endothelium, smooth muscle cells in the heart, as well as multiple sites of the brain, and can act as a paracrine or intracrine factor (Simpson, 2003). E2 signals directly through three major estrogen receptors, estrogen receptor 1 (ESR1 or ERα), estrogen receptor 2 (ESR2 or ERβ), and the G protein-coupled estrogen receptor 1 (GPER1). ERα and ERβ belong to the nuclear receptor family of transcription factors and contain evolutionarily highly conserved DNA binding (DBD) domains, ligand binding domain (LBD), and the most variable N-terminal domains. Two activation function (AF) domains, activation function 1 (AF1), which is constitutively active and activation function 2 (AF2), which is ligand-dependent regulate the receptor transcriptional activity (Nilsson et al., 2001) (Figure 1.4). Both ERα and ERβ bind to the same DNA response elements and have similar affinity towards E2 with $K_i$ values of 0.12 and 0.13nM for ERα and ERβ, respectively. Nevertheless, ERα and ERβ are products of different genes located on separate chromosomes (Enmark et al., 1997).
Classical or ligand-dependent E2 signaling via ERα and ERβ involves binding of E2 to the receptor localized in the cytoplasm. This process leads to conformational changes, dimerization of the receptors, and translocation of these receptors to the nucleus. The E2-bound estrogen receptor complex then binds to estrogen response elements (EREs) near their target gene promoters. This binding triggers recruitment of coregulators as well as RNA polymerase II transcription machinery to promote target gene transcription. Interestingly, besides the direct binding of E2-ER complex on their target genes, over 35% of human primary E2-responsive genes are regulated via indirect binding of ERs to mediatory transcription factors, a process referred to as transcriptional crosstalk (O’Lone et al., 2004; Göttlicher et al., 1998). Examples of proteins mediating this indirect association between target DNA and E2-ER include stimulating protein-1 (Sp-1) and c-rel subunit of nuclear factor-κB (NF-κB). Genes shown to be regulated by this mechanism are the low-density lipoprotein (LDL) receptor, cyclin D1, and the cytokine interleukin-6 (IL-6) (Li et al., 2001; Castro-Rivera et al., 2001; Kalaitzidis and Gilmore, 2005). Furthermore, ERs can control transcription of genes via the activator protein-1(AP-1) complexes such as regulation of collagenase, insulin-like growth factor (IGF)-1 receptor and cyclin D1 expression (Marino et al., 2002). While having similar DNA-binding domains, the impact of E2 on its target gene regulation are unique for ERα and ERβ. Expression of cyclin D1, for example, is enhanced through E2-ERα-mediated induction, but inhibited in the presence of E2-bound ERβ (Liu et al., 2002). In breast cancer cells, ERβ has been shown to repress ~70% of ERα-induced target genes (Williams et al., 2008), and ERα also displays
different DNA binding regions from ERβ (Zhao et al., 2010), revealing further a complex interaction between these receptors.

**Figure 1.4 Structure of estrogen receptors.** Schematic representing six domains of estrogen receptor alpha ERα and estrogen receptor beta ERβ. Functions of each of the domain presented are listed in the table underneath. The percent homology between different domains in ERα and ERβ is indicated below each domain (Adapted from Karimian et al., 2011).

GPER1 is a seven transmembrane G protein-coupled receptor. GPER1 has been shown to signal via a non-genomic transduction mechanism in contrast to genomic signaling mediated by ERα and ERβ. Several studies have shown that GPER1 activates rapid signaling pathways, including stimulation of phosphatidylinositol 3-kinase (PI3K)/Akt via epidermal growth factor (EGF) receptor transactivation in breast cancer.
cell lines (Revankar et al., 2005; Zekas and Prossnitz, 2015). GPER1 is also reported to activate EGFR/MAPK/Erk in endometrial cancer cells and thyroid cancer cells (Vivacqua et al., 2006). GPER1 can activate cAMP/PKA and the Ca\(^{2+}\)/Calmodulin (CaM) network in smooth muscle cells (Yu et al., 2014) and vascular endothelial cells (Tran et al., 2016) respectively. Notably, GPER1 can initiate different downstream signaling cascades in different cell types, indicating that GPER1 signaling pathways are tissue-specific. Additionally, while GPER1 has been reported to promote migration and metastasis of breast and ovarian cancer cells (Chen et al., 2014; Yan et al., 2013), GPER1 is shown to inhibit prostate cancer cell growth (Chan et al., 2010), highlighting further that not only the downstream signaling, but also the impact of GPER1 are specific to target organs.

1.4.2 Estrogen signaling in liver biology

As previously mentioned, E2 is the most abundant and most biological active form of a sex steroid hormone and suspected to play a significant role in gender differences of the liver. While the roles of E2 in the development and the disease of reproductive organs in both males and females are extensively investigated (Barros and Gustafsson, 2011), studies on the effect of E2 in non-reproductive organ such as the liver are less known. Indeed, the liver is deemed to be a target tissue for estrogen signaling due to the presence of hepatic nuclear estrogen receptors. In humans, the presence of ER\(\alpha\) and ER\(\beta\) in embryonic liver during the perinatal period (Lax et al., 1983) as well as ER\(\alpha\) and GPER1 in adult livers (Alvaro et al., 2000) suggests that direct E2 signaling in hepatocytes may be relevant for both embryonic liver development and adult liver homeostasis. In rodents, hepatic ER\(\alpha\) is predominantly expressed and
has been shown to play a significant role in regulating hepatic lipid signaling and metabolism (Pedram et al., 2013), whereas expression of hepatic ERβ is minimal and its role is unclear. The role of hepatic GPER1 is far less understood due to its more recent discovery and its extremely low expression in mouse and rat hepatocytes (O'Dowd et al., 1998). Nevertheless, GPER1 was shown to have a gender-specific role in mouse liver. For instance, GPER1 is crucial for normal lipid metabolism of the male liver (Sharma et al., 2013) and is required for optimal body and liver weight of female livers (Mårtensson et al., 2009; Otto et al., 2009). Given that human livers highly express GPER1, it may play a significant role in the maintenance of adult male and female livers.

1.4.3 Sexual dimorphism of the liver

The liver is a sexual dimorphic organ in regards to size, content, toxin clearance ability, and the metabolism of nutrients, drugs and hormones. The major signaling pathway determining the gender-specific properties of the liver is growth hormone signaling. Pulsed release of growth hormone results in activation of the tyrosine phosphorylation cascade and transcription of male-specific genes in the male liver. Conversely, low and continuous release of growth hormone maintains the default transcription of female-specific genes in the female liver (Waxman, 2000; Clodfelter et al., 2006; Zhang et al., 2011). Steroid hormones have been shown to be key regulators of growth hormone secretion patterns via activation of the anterior pituitary that results in gender differences of the liver. While steroid hormones can contribute to liver sexual dimorphism indirectly via modulating pituitary release of growth hormone, they can also directly act on the liver by binding to their receptors as evidenced by the presence of
steroid receptors in hepatocytes, including estrogen receptors as mentioned previously. Indeed, sexual dimorphism of the liver has not only been observed in murine and human studies, multiple reports using fish have shown gender differences in hepatic gene expression (Qiao et al., 2016; Zheng et al., 2013), suggesting the importance of gender-specific hepatic gene expression in male and female liver homeostasis.

The liver responses towards steroid hormone exposure are also gender biased, depending upon the level of hormones, expression or post-translational modification of the receptors and receptor functions specific to male and female livers (Rando and Wahli, 2011). Multiple studies have further shown that this direct interaction between liver and steroid hormones is crucial to gender differences in the liver diseases, such as non-alcoholic fatty liver disease, liver cirrhosis or liver carcinogenesis (Molodecky et al., 2011; Sato et al., 2001; Clocchiatti et al., 2016). Besides cancers of reproductive organs such as breast cancer, E2 signaling has been implicated in the sexual dimorphism of cancers in non-reproductive organs, including colon cancer, squamous cell carcinoma, and liver cancer incidence (Clocchiatti et al., 2016). For instance, E2 signaling via ERβ is believed to protect women against colon cancer, as males exhibited higher incidence, and low ERβ expression was associated with colorectal cancer patients (Passarelli et al., 2013). Specifically, it has long been established that liver cancer is a male-predominant disease, leading to various attempts to elucidate the role of E2 in liver carcinogenesis. Studies have shown impacts of androgen signaling in promoting liver cancer, while E2, acting through ERα and FoxA1/2, prevented it (Li et al., 2012). Others have reported effects of E2 in suppressing inflammatory response directly or indirectly via secretion of growth hormone from pituitary glands (Naugler et
al., 2007; Mueller et al., 2011). Nevertheless, most of these studies were carried out in murine models and given the species differences in E2 signaling components, the complete roles of E2 pathway in liver carcinogenesis require further investigations.
1.5 REFERENCES


CHAPTER TWO

Estrogenic regulation via Estrogen receptor 2b controls differentiation of hepatic progenitors during vertebrate embryogenesis


This chapter contains a prepared manuscript draft. It has been modified to fit the style of this dissertation. Supplemental data can be found in Appendices.

Author contributions: S.C. generated and characterized esr2b TALEN mutants, performed vasculature and biliary tree analysis, and carried out all zebrafish experiments. T.E.N. and W.G. conducted original chemical screen. S.C., M.K.G., and C.C.C. confirmed the screen, analyzed hepatoblast expression and performed MO experiments. C.A.G and A.M. generated and analyzed ChIP-Seq data on human ESC, endoderm, and hepatocytes. D.A.G. provided ERE reporter zebrafish. K.D.T. performed mouse explant culture experiments. S.C., M.K.G., T.E.N., and W.G. wrote the manuscript. K.J.C. and T.E.N. provided overall input.
2.1 ABSTRACT

Estrogen is an important regulator of reproductive organ development and gender differentiation, but much less is known about its role for the development of other organs. The liver originates from endoderm-derived progenitor cells through a temporally and spatially orchestrated process of hepatic differentiation. The developmental cues controlling the differentiation of hepatocytes from committed hepatic progenitor cells, however, are incompletely understood. Here, we define an important role for estrogenic regulation in vertebrate liver development: in zebrafish embryos, exposure to 17\(\beta\)-estradiol (E2) during hepatocyte development decreases hepatocyte number and hepatocyte-specific gene expression, while pharmacological blockade of aromatase-mediated estrogen synthesis or nuclear hormone receptor signaling enhances expression of differentiated liver transcripts. These effects are similarly elicited by exposure to xenoestrogenic compounds. Chemical inhibition and morpholino knockdown reveal that E2 effects are mediated via the nuclear estrogen receptor 2b (esr2b). Surprisingly, esr2b activation promotes biliary differentiation at the expense of hepatocyte differentiation. In contrast, esr2b mutant zebrafish exhibit increased hepatocyte marker expression and diminished biliary differentiation without an effect on temporal regulation of liver differentiation. Importantly, the role of estrogen in early liver formation is conserved, as E2 exposure disrupts murine liver development. Our studies identify estrogen and esr2b activity as important regulators of hepatobiliary differentiation that directly target hepatic progenitor fate decisions during embryonic liver formation. This investigation has important clinical implications for infants exposed to abnormal estrogen levels or estrogenic compounds during pregnancy.
2.2 INTRODUCTION

The liver is the central metabolic organ, regulating lipid and carbohydrate homeostasis and detoxifying both endogenous and exogenous waste products throughout life. It is therefore essential that the vertebrate liver is adequately differentiated at birth, concomitant with the initiation of food intake. While many key factors have been identified, which regulate particular aspects of liver specification and differentiation, the signaling network(s) that function to ensure sufficient and timely maturation of multi-potent progenitors to differentiated hepatic components are not widely understood.

During pregnancy, temporal and spatial expression of steroid hormones are crucial for fetal development, growth and organogenesis (Mesiano and Jaffe, 1997; Thompson et al., 2002). Estrogen, in particular, is present throughout all stages of gestation and contributes to the regulation of many intrauterine processes (Kaludjerovic and Ward, 2012). Furthermore, estrogen is similarly necessary for proper embryonic development, as early inhibition of estrogen synthesis leads to defects in embryogenesis (Wu and Doong, 1984). Interestingly, excessive exposure to estrogentic compounds, such as diethylstilbestrol (DES), during pregnancy has been shown to alter fetal organogenesis and is associated with the later development of carcinoma (Bibbo et al., 1975; Ostrander et al., 1985). Specifically, estrogen signaling modulation upon exposure to xenoestrogens, such as bisphenol A (BPA), during embryogenesis was shown to correlate with liver damage and liver cancer in adult rodents (Xia et al., 2014; Weinhouse et al., 2014). While these studies indicate the potential developmental effects of estrogenic exposure in the liver later in life, the direct impact of estrogen
signaling on hepatogenesis or the mechanism(s) leading to subsequent dysregulation have not been characterized.

17β-estradiol (E2) is the most prevalent and active form of estrogen. Classical E2 signaling involves two nuclear estrogen receptors: estrogen receptor alpha (ESR1, formerly ERα) and estrogen receptor beta (ESR2, formerly ERβ). Upon binding to E2, cytoplasmic estrogen receptors dimerize, translocate into the nucleus, and activate transcription of their target genes via the estrogen response element (ERE) (Heldring et al., 2007). While both ESR1 and ESR2 contain highly conserved DNA-binding and ligand-binding domains and interact with the same pool of coregulators, studies have shown they possess overlapping yet unique repertoire of target genes (Liu et al., 2008; Hall et al., 2001). Likewise, ESR1 and ESR2 also exhibit different tissue distributions, biological functions and pathological phenotypes (Dupont et al., 2000; Couse and Korach, 1999; Liu et al., 2002). Differential expression of ESR1 and ESR2 is also apparent during development: in the human midgestational fetus (16-23 weeks), ESR1 is abundant in the uterus, while ESR2 is expressed in the fetal ovary, testes, adrenal gland, spleen, and liver. The expression of ESR2 in the embryonic liver is of particular interest (Brandenberger et al., 1997; Takeyama et al., 2001), as it is in direct contrast that of the adult, which shows predominant ESR1 expression (Alvaro et al., 2000), as it suggests there may be a physiological role of ESR2 during early liver development.

Here, we identify an essential role for E2 regulation, acting through esr2b, in the control of embryonic liver development. Enhanced E2 activity results in decreased hepatocyte differentiation, while chemical inhibition of E2 synthesis or signaling leads to expansion of the differentiated hepatocyte pool. Analysis of esr2b knockout zebrafish
confirmed the role of estrogen as a central regulatory of hepatocyte maturation. Notably, esr2b expression is downregulated during normal hepatoblast differentiation; selective modulation of E2 activity during select developmental time windows revealed that E2/esr2b specifically functions to regulate the timing and scale of hepatoblast proliferation and differentiation. Furthermore, E2/esr2b regulates hepatobiliary fate decisions, without impacting endothelial cells, endocrine, or exocrine pancreas populations. Finally, this role of estrogenic regulation in hepatoblast biology is evolutionarily conserved in murine liver development. Our data elucidate a previously uncharacterized function of estrogen as a key differentiation factor of a non-reproductive tissue type.

2.3 RESULTS

**Estrogenic regulation is required for normal liver development**

We previously performed a chemical genetic screen to identify novel regulators of liver development (Garnaas et al., 2012) and uncovered several estrogen-related compounds that affect hepatogenesis (Figure S2.1A). Exposure to physiological estrogens 17β-estradiol (E2) and estriol, synthetic estrogens 17α-ethynylestradiol (EE) and diethylstilbestrol, and the phytoestrogen quercetin from 24-72 hours post fertilization (hpf) 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 72 hours post-fertilization (hpf). In contrast, treatment with the aromatase inhibitor chrysin and estrogen receptor antagonist tamoxifen increased the region of fabp10a expression (Figure S2.1B), suggesting estrogen associated signaling or transcriptional activity may regulate liver formation.
To corroborate these findings and further investigate the impact of E2 on liver development, zebrafish embryos were exposed to physiological levels of E2 from 24 to 72 hpf, a time period during which zebrafish hepatic specification, progenitor differentiation, and hepatocyte maturation each take place. Alterations in liver formation were assessed by WISH for fabp10a at 72 hpf, with quantification of liver area using ImageJ. Exogenous E2 exposure resulted in a dramatic reduction in liver size compared to DMSO-exposed controls (Figure 2.1A,B; p<0.01). 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. In contrast to the reduced liver area seen in E2-treated embryos, ANAS exposure resulted in enlarged livers (Figure 2.1A,B; p<0.001), indicative of a specific role for estrogenic modulation in liver development. Quantitative analysis of hepatocyte number using fluorescence activated cell sorting (FACS) in Tg(fabp10a:GFP) reporter embryos at 72 hpf further confirmed a decrease in total hepatocyte number following E2 exposure (Figure 2.1C). To assess whether the effect of E2 was specific to fabp10a expression, an alternative hepatocyte marker, transferrin, was assessed, yielding a similarly reduced liver (Figure S2.2A). As non-amniotes with highly permeable skin, zebrafish embryos are known to be susceptible to hormones and hormone-mimicking compounds present in the environment. (Schug et al., 2013). Importantly, xenoestrogens such as bisphenol A (BPA) had a similarly inhibitory effect on liver formation, indicating the immediate relevance of our findings for environmental toxicology (Figure S2.2B). These data indicate that exogenous estrogen and xenoestrogenic compounds inhibit liver formation.
Evaluation of estrogen content using a previously established enzyme immunoassay (EIA) (Carroll et al., 2014), indicated that endogenous E2 levels were significantly increased upon exposure to exogenous E2 but remained within the physiological range for embryonic development, suggesting that the observed liver phenotypes likely reflect the role of intrinsic E2 signaling during normal embryonic liver development.

As part of the nuclear hormone receptor family, classic estrogen-associated genomic regulation involves estrogen receptor dimerization after E2 binding, translocation of receptor dimers into the nucleus and binding to estrogen response elements (ERE) in target gene promoters (Monteiro et al., 2014). To examine whether E2 signaling exerts hepatic transcriptional regulation, we utilized transgenic reporter zebrafish with ERE-driven GFP expression, Tg(5xERE:GFP) (Gorelick and Halpern, 2011), crossed into a hepatocyte-specific reporter background Tg(fabp10a:DsRed) to visualize E2-mediated genomic activation within the developing liver. E2 exposure (24 to 72 hpf) increased ERE activity in hepatocytes and concomitantly reduced liver-specific fluorescence, as indicated by a relative increase in GFP signal intensity, and a decrease in DsRed fluorescent area compared to controls (Figure 2.1D). To further confirm that estrogen acts through nuclear hormone receptor signaling, embryos were exposed to the direct receptor blocker fulvestrant (Ful), which led increase in liver-specific gene expression in fluorescent reporter fish and by WISH for transferrin at 72 hpf (Figure S2.2B). Taken together, these data reveal the requirement of optimal E2 signaling for normal liver development; E2 signaling overactivation results in transcriptional activation of E2 target genes in hepatocytes, which leads to impaired
liver development and decreased liver size while decreased E2 signaling led to enhanced liver growth.

**Figure 2.1** Optimal E2 signaling is required for normal embryonic liver formation
(A) Representative images of zebrafish embryos exposed to DMSO, E2 (10 μM), or ANAS (10 μM) from 24-72 hpf and assessed liver size by in situ hybridization (ISH) for liver fatty acid binding protein 10a (fabp10a) at 72 hpf. (B) Areas of fabp10a expression were quantified using ImageJ. *n* as indicated, **p<0.01, ****p<0.0001, two-tailed Student’s *t*-test. (C) Number of hepatocytes were quantified in embryos exposed to DMSO or E2 from 24-72 hpf using fluorescence-activated cell sorting (FACS) for GFP+ cells in Tg(fabp10a:GFP) embryos at 72 hpf. ***p<0.001, two-tailed Student’s *t*-test. (D) Transgenic zebrafish embryos Tg(fabp10a:DsRed; 5xERE:GFP) were exposed to DMSO and E2 from 24-72 hpf and imaged at 72 hpf. All values represent mean ± SEM, *n* as indicated, all scale bars, 200 μm.
The effect of E2 on liver development is mediated by esr2b

To identify the specific estrogen receptor mediating the effects of E2 on early liver development, embryos were exposed to E2 concomitantly with the specific ESR1 antagonist MPP or ESR2 antagonist PHTPP (24-72 hpf), and fabp10a expression was quantified at 72 hpf (Figure 2.2A,B). Embryos exposed to PHTPP had increased liver-specific gene expression ($p<0.0001$), while MPP exposed embryos were unchanged. Furthermore, PHTPP, but not MPP, blocked the effect of E2 and partially normalized liver gene expression area, indicating that E2 regulates liver development via ESR2. The results of these chemical modulations were confirmed by morpholino (MO)-mediated knockdown of the individual zebrafish estrogen receptors: esr2b morphants exhibited a significant increase in liver size compared to uninjected controls and esr1 and esr2a morphants, as measured by fabp10a expression area (Figure 2.2C,D; $p<0.0001$). Furthermore, in contrast E2 exposure in esr1 and esr2a morphants, which exhibit the expected decrease in liver gene expression, the negative impact of E2 exposure was substantially limited in esr2b morphants, further confirming that E2 acts primarily through esr2b to impact liver development.

These chemical and genetic inhibition studies prompted us to further examine the developmental role of esr2b by generating esr2b knockout zebrafish using transcription activator-like effector nucleases (TALENs) (Sander et al., 2011). TALEN-generated esr2b mutants contain a 5 base pair deletion in the first exon of esr2b, predicted to cause a premature stop codon (Figure S2.3A). Indeed, compared to wild-type siblings, esr2b expression was not detected in esr2b$^{-/-}$ mutant embryos as assayed by WISH at 72 hpf, suggesting complete loss of esr2b transcripts in these embryos (Figure S2.3B). Both esr2b$^{+/-}$ and esr2b$^{-/-}$ embryos exhibited no gross developmental abnormalities
compared to their wild-type siblings (esr2b\textsuperscript{+/+}) and survived to adulthood. esr2b\textsuperscript{-/-} embryos, however, exhibited an \sim50\% increase in fabp10a expression area compared to wild-type siblings at 72 hpf (Figure 2.2E,F); in addition, esr2b\textsuperscript{-/-} embryos completely lacked a response to E2 (Figure 2.2F), compellingly confirming the role of esr2b as a mediator of estrogenic regulation of liver development.
Figure 2.2 Estrogen receptor 2b mediates impacts of E2 on embryonic liver size

(A) Representative images of embryos exposed to DMSO, ERα antagonist (MPP), and ERβ antagonist (PHTPP) alone or together with E2 from 24-72 hpf. (B) Liver size was quantified by ISH for *fabp10a* at 72 hpf and analyzed by ImageJ. **p<0.01, ****p<0.0001, two-tailed Student’s *t*-test. (C) Representative images of liver size in *esr1*, *esr2a*, or *esr2b* morphants with DMSO or E2 exposures from 24-72 hpf. (D) Liver area is quantified by ISH for *fabp10a*. ****p<0.0001, two-tailed Student’s *t*-test. (E) Representative images of *esr2b*−/− mutants and their wild-type siblings upon exposure to DMSO or E2 from 24-72 hpf. (F) Liver size was quantified by ISH at 72 hpf. ns=not significant, ****p<0.0001, two-tailed Student’s *t*-test. All values represent mean ± SEM, *n* as indicated, all scale bars, 200 μm.
E2/esr2b signaling functions during a specific window of liver development

To further delineate the critical developmental period influenced by estrogen signaling, wild-type embryos were exposed to DMSO and E2 at different time intervals designed to specifically target hepatic progenitor specification (18-24 hpf), hepatoblast budding (24-42 hpf), hepatoblast differentiation toward mature hepatocytes (42-72 hpf) (Figure 2.3A). Expression of the endodermal progenitor marker foxa3 and hepatoblast marker hhx were evaluated at 48 hpf, as well as the hepatocyte marker fapb10a at 72 hpf. E2 exposure from 18-24 hpf and 24-42 hpf had minimal to no effect on either endoderm or hepatoblast populations as evaluated by WISH (Figure 2.3B); additionally, no significant effects were observed on hepatocytes during the earliest exposure window, with modest decreases in liver gene expression after 24-42 hpf of E2 treatment. In contrast, later developmental exposure to E2 from 42-72 hpf, focused on the process of hepatocyte differentiation, consistently caused a decrease of fapb10a expression (Figure 2.3C,D; p<0.01), suggesting the most significant impact of E2 during differentiation of hepatocytes. Together, these data indicate that E2 signaling plays a key role during embryonic liver development, specifically impacting hepatoblast differentiation toward mature hepatocytes.

E2/esr2b signaling modulates hepatoblast differentiation

Given that exogenous E2 appeared to alter hepatocyte differentiation, we asked whether esr2b activity specifically impacted the proliferative dynamics or the temporal commitment to hepatic differentiation or both. To begin to address this further, esr2b+/+ and esr2b−/− embryos were treated with either DMSO or ANAS from 24-72 hpf, and liver area was quantified at 54, 60 and 72 hpf by WISH for fapb10a (Figure 2.3E,F). While
both ANAS-exposed as well as esr2b<sup>−/−</sup> embryos exhibited increased fabp10a expression at 72hpf, each showed no alterations in the onset of fabp10a expression compared to wild-type sibling control, indicating that E2/esr2b function is not directly involved in regulation of the timing of hepatic differentiation.
Figure 2.3 E2 exposure inhibits hepatoblast differentiation and maturation

(A) Scheme illustrating E2 exposure time-windows: 18-24 hpf targeting hepatic specification, 24-42 hpf targeting hepatoblast budding, 42-72 hpf targeting hepatoblast differentiation and maturation. (B) Images of endoderm (foxa3) (100% or 25/25 and 100% or 20/20 and 91% or 22/22) that remained unaffected upon E2 exposures from 18-24 hpf or 24-72 hpf. (C) Images of embryonic liver size demonstrating the most severe impact of E2 on liver size during 42-72 hpf exposure time-window. (D) Liver size distribution of embryos exposed to E2 at 18-24 hpf, 24-42 hpf, and 42-72 hpf assessed by ISH for fabp10a at 72 hpf as % of embryos with large (dark grey), medium (light grey) or small (black) livers. (E) Images demonstrating hepatocytes in esr2b+/+ embryos exposed to DMSO or anastrozole (ANAS) and esr2b−/− mutants assayed by ISH fabp10a at 54, 60, and 72 hpf. (F) Liver area quantification by ImageJ. ns=not significant, one-way ANOVA, ns=not significant, ****p<0.0001, two-tailed Student’s t-test, **p<0.01, two-tailed Student’s t-test. n as indicated, all scale bars, 200 μm.
Precursor cell populations do not only undergo differentiation during development to become mature cells but also proliferate to maintain the progenitor pool. Therefore, we sought to delineate whether E2 affects hepatoblast differentiation towards hepatocytes, or proliferation, or both. Embryos were exposed to E2 from 24-72 hpf and the relative area of hepatoblast (prox1) and hepatocyte (sid4, fabp10a) expression was quantified by WISH and ImageJ analysis at 72 hpf. E2 exposure significantly reduced levels of the differentiating hepatocyte marker sid4 similarly to fabp10a at 72 hpf (Figure 2.4A,B), whereas prox1 expression was unchanged. In contrast, in esr2b−/− mutants there was no significant impact on prox1, indicating hepatoblast formation is not impacted. As noted before, the area of sid4 and fabp10a expression were each increased compared to that of esr2b+/+ embryos (Figure 2.4A,B), demonstrating that E2/esr2b signaling is involved in hepatocyte differentiation. Importantly, this negative impact of estrogenic regulation on hepatocyte differentiation was definitively mediated by esr2b, as levels of sid4 and fabp10a were not affected in esr2b−/− mutants upon E2 exposure. Together, these observations indicate that E2/esr2b signaling has an important role in hepatoblast differentiation.
Figure 2.4 E2 signals via esr2b to decrease hepatoblast differentiation and proliferation

(A) Expressions of hepatic progenitors (prox1), differentiating hepatocytes (sid4), and differentiated hepatocytes (fabp10a) of esr2b^{+/+} and esr2b^{-/-} embryos exposed to DMSO or E2 from 24-72 hpf at 72 hpf. (B) Quantification of prox1, sid4, and fabp10a expressions at 72 hpf using ImageJ. All values represent mean ± SEM, n as indicated, all scale bars, 200 μm.
E2/esr2b regulation controls hepatobiliary fate decisions

By 72 hpf, the embryonic liver is comprised of a mixture of hepatic progenitors, endothelial cells, and differentiating cells of the hepatocyte and biliary lineage. Given that modulation of E2/esr2b activity results in altered hepatocyte development, we sought to assess whether other hepatic cell types were also affected. We previously showed that early (12-24 hpf) vascular development and specification can be influenced by estrogenic activity (Carroll et al., 2014). To determine whether alterations in endothelial cell number or function contributed to the impact 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 24-72 hpf. Whereas hepatocytes exhibited reduced GFP expression, no defects were observed in endothelial cell number or vascular structure by fluorescence imaging (Figure S2.4A). Similarly, cloche mutants, which lack all vascular endothelium (Reischauer et al., 2016), demonstrated a decrease in hepatocyte-specific gene expression compared to wild-type embryos exposed to E2 (Figure S2.4B), implying the impact of E2/esr2b signaling on liver development is not mediated by vascular endothelium. Indeed, this finding is in line with the observation that while endothelial cells are important for liver growth, they are not required for early liver specification (Korzh et al., 2008). E2 exposure did not alter endodermal-derived exocrine or endocrine pancreas as assessed by WISH for trypsin and insulin respectively (Figure S2.4C).

During embryonic development, immature hepatoblasts are specified from common endodermal precursors, then subsequently differentiate to become hepatocytes or biliary epithelial cells (BEC), also known as cholangiocytes. Given the
impact of E2/esr2b on hepatocyte-associated gene expression, we further examined the effect of E2 exposure on the specification of BECs. Wild-type embryos were exposed to either E2 or the ESR antagonist fulvestrant (Ful) from 24-72 hpf, and the BEC and hepatocyte populations were assessed at 72 hpf by WISH for the biliary tree progenitor marker sox9b (Delous et al., 2012) and fabp10a, respectively. Interestingly, while inhibition of E2/esr2b signaling with fulvestrant led to an increase in liver area (Figure 2.5A,B, p<0.0001), it significantly decreased sox9b expression (p<0.05). Conversely, E2 exposure resulted in a reduced hepatocyte marker expression area with increased BEC marker expression. Further, expression of the notch ligand deltaC (dlc) was significantly decreased in esr2b/− mutants compared to wild-type siblings (Figure 2.5C,D; p<0.001), indicating reciprocal impact on hepatocyte-versus-biliary fate.
Figure 2.5 E2 signaling affects hepatoblast differentiation to impact hepatic lineages

(A) Biliary tree progenitors marked by sox9b in embryos exposed to DMSO, E2, or Ful from 24-72 hpf at 72 hpf. Ful decreased cholangiocyte formation compared to controls (red arrowheads). (B) Biliary tree and liver size distribution of embryos as assessed by ISH for sox9b and fabp10a at 72 hpf as % of embryos with large (dark grey), medium (light grey) or small (black) biliary tree or liver. *p<0.05, ††††p<0.0001, two-tailed Student’s t-test. * indicates significant difference of % Ful-exposed embryos with small biliary tree compared to that of the controls. † shows significant difference of % Ful-exposed embryos with large liver compared to that of controls. (C) Representative images of embryos ISH for delta C (dlc) in the liver region (red arrowheads). (D) Quantification of dlc staining in the liver region. ***p<0.001, two-tailed Student’s t-test. $n$ as indicated, scale bars, 200 μm., except for higher magnification images of sox9b expressions, scale bar, 100 μm.
The role of E2/Esr2 function is conserved in murine liver development

To investigate whether the role of estrogen in liver development is conserved across vertebrates, we employed a murine embryo explant culture system. Fate mapping studies in mouse embryos revealed the presence of liver bud precursors by 6 somite stage (ss), prior to hepatic differentiation (Angelo et al., 2012). Ex vivo exposure to E2 (20 μM) during hepatic differentiation from 7-8ss decreased hepatocyte commitment as marked by hepatocyte nuclear factor 4α (HNF4α) expression; significantly, expression of the endodermal marker FOXA1 as well as the hepatoblast marker PROX1 were not affected (Figure 2.6). Together these findings suggest that differentiation of mammalian hepatic progenitors towards the hepatocyte lineage is inhibited by physiologically relevant E2 levels.

![Figure 2.6 E2 exposure inhibits hepatic differentiation in murine explant culture](image)

Representative immunofluorescent images of mouse embryos exposed to E2 (20 μM) from 7-8 somite stage (ss) leading to a decrease in expression of hepatocytes marked by hepatocyte nuclear factor 4α (HNF4α) while no effects on endoderm (FOXA1) or hepatic progenitor (PROX1) were observed.
**Human ESR2 is epigenetically regulated during hepatic differentiation**

Finally, to further examine the fundamental role of estrogenic regulation on hepatoblast commitment to hepatocyte differentiation, we examined epigenetic modifications of the human ESR1 and ESR2 coding sequences to determine if ESR expression is forcibly repressed. Specifically, we investigated epigenetic changes across pluripotent embryonic stem cells (ESCs), differentiated endodermal cells, and mature hepatocytes. The pattern of H3K4 methylation (H3K4me1), an activating histone mark, remained relatively unchanged at the ESR1 locus across all cell types (Figure S2.5A). In contrast, H3K4me1 is significantly diminished at the ESR2 locus in differentiated hepatocytes compared to undifferentiated ESCs or endodermal progenitor cells. Importantly, this epigenetic change correlates with decreased expression of ESR2. RNA-seq transcriptome analyses of all three cell populations also revealed that expression of the E2-degrading enzyme Cyp1a2 is augmented, while the E2-synthesizing enzyme Cyp19a1/aromatase is reduced in hepatocytes compared to that of less differentiated precursors (Figure S2.5B), further implying that E2 signaling is specifically downregulated in differentiating liver cells to allow commitment. Collectively, these data indicate that restriction of E2/ESR2 signaling is necessary for hepatic progenitor differentiation to functional hepatocytes across vertebrate species.
2.4 DISCUSSION

In this study, we utilized a chemical genetic screening approach to identify a novel role for estrogen during liver development. Estrogen, signaling via the nuclear hormone receptor *esr2b*, regulates hepatobiliary differentiation. Optimal levels of E2 and *esr2b* activity are essential for normal liver development, specifically during hepatocyte differentiation. Elevated E2 activity results in impaired hepatocyte differentiation with decreased number of hepatocytes and increased biliary lineage marker expression, while inhibition of E2 or loss of *esr2b* directs hepatoblast differentiation towards hepatocytes at the expense of the biliary lineage. Our preliminary investigations indicate that this function of E2 in regulation of fate choice during liver development is conserved in mammals.

**Estrogen regulates hepatobiliary fate decisions**

In the liver, hepatocytes and cholangiocytes arise from the same hepatic progenitor pool (Wilkins and Pack, 2013). While several pathways have been described that are important for the differentiation or either hepatocytes or biliary epithelial cells, no pathway, to our knowledge, has been found to affect hepatobiliary fate decisions of embryonic hepatoblasts. Prior studies, however, suggest the relevance for estrogen signaling in cholangiocyte proliferation: adult vertebrate animal studies have shown differential expressions of ER subtypes in distinct liver cell populations with Esr2 being absent in hepatocytes, but highly expressed in cholangiocytes (Alvaro et al., 2000). Functionally, Esr2 has been shown to promote growth as well as proliferation of biliary cells and its high expression has been implicated in biliary cirrhosis and cholangiocarcinoma (Alvaro et al., 2002). In addition, one of the major pathways
required for biliary cell differentiation is Notch signaling, and Esr2, not Esr1, has been shown to activate Notch components in a variety of other cell types (Froehlicher et al., 2009; Brooks et al., 2014; Dago et al., 2015). These data emphasize the potential role of E2/Esr2 in promoting hepatoblast fate toward biliary cell differentiation, while suppressing hepatocyte differentiation. Indeed, our findings showing that inhibition of E2 signaling or loss of esr2b increased hepatocytes at the expense of cholangiocyte populations suggest that E2/esr2b signaling in hepatoblasts is a key driver in determining hepatobiliary fate.

**E2 signaling in the maintenance of progenitor and stem cell populations**

Molecular pathways guiding early embryonic development and controlling cell commitment and differentiation are important for the maintenance and growth of stem and progenitor cell populations (Takebe et al., 2011). Given that E2 is widely present in the developing vertebrate and activation of E2 signaling inhibits hepatocyte differentiation, E2 regulation may likewise be involved in controlling the homeostasis of multipotent progenitor cells in other organ systems. Indeed, murine studies have revealed a role for E2 in driving hematopoietic stem cell renewal in adult females and in particular during pregnancy (Nakada et al., 2014), in protecting bone marrow-derived mesenchymal stem cells against apoptosis (Ayaloglu-Butun et al., 2012), and in enhancing proliferation of periodontal ligament stem cells to impact bone regeneration (E et al., 2016). Female mice also had a higher capacity of muscle resident stem cell self-renewal (Deasy et al., 2007). Similarly, neural stem cells were found to proliferate according to the estrous cycle in rodents (Pawluski et al., 2009), demonstrating the involvement of E2 in the maintenance and expansion of various stem cell niches.
Interestingly, E2 signaling has been shown to promote stemness in multiple cancer cell types, such as breast cancer (Sun et al., 2014) and thyroid cancer (Xu et al., 2013). These studies support the likelihood of important yet underappreciated roles of E2 function in stem cell biology.

**Embryonic E2 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 (Walker and Ho, 2012). The “fetal origin of adult disease hypothesis” posits that the defects from adverse conditions experienced in utero, such as that from early malnutrition and growth retardation, can extend into adulthood and affect the risk of developing diseases later in life, including diabetes, obesity, coronary heart disease, kidney and liver failure (Gluckman et al., 2008; Stanner et al., 1997; Hallan et al., 2008; Barker, 2007). 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. While our study, examining the enhancer of histone methylation mark H3K4me1 in human cells, suggests that Esr2 expression is suppressed during hepatocyte differentiation, prior studies have noted the plasticity of the ER regulatory region epigenomes as they could be transiently or permanently altered upon prenatal exposure to estrogenic compounds such as BPA (Ramos et al., 2003; Kawai et al., 2007; Maffini et al., 2006). Given that epigenetic modifications take place throughout organ differentiation (Reik, 2007), and that epigenetic reprogramming during organogenesis can affect disease susceptibility in adulthood, it is tempting to speculate that our observed effect of E2 signaling on
hepatocyte differentiation can imprint a long-term influence on adult liver functions and homeostasis.

The present study illustrates the powerful potential of chemical genetic screens during embryogenesis to identify novel regulators of organ development. Our work discovers a novel and unexpected role for estrogen in regulating hepatobiliary fate. These findings are of immediate relevance for mammalian physiology, in particular in light of the increasing 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.
2.5 MATERIALS AND METHODS

Zebrasfish husbandry and generation of esr2b−/− mutant

Male and Female wild-type Tu zebrasfish, Tg(-2.8fabp10a:eGFP)sas3 (Her et al., 2003), abbreviated fabp10a:GFP, Tg(flk:mCherry) (Wang et al., 2010), and Tg(5xERE:GFP) (Gorelick and Halpern, 2011) were used. All zebrasfish were maintained according to the standard Institutional Animal Care and Use Committee guidelines (IACUC) at Harvard Medical School. TALEs targeting endogenous esr2b were generated according to the published protocol (Sanjana et al., 2012; Cong et al., 2012) and were obtained from The Broad Institute Genetic Perturbation Platform. mRNAs of TALEN pairs were synthesized by mMESSAGE mMACHINE kit (Ambion) and were injected into 1-cell stage wild-type larvae. Somatic mutation rate was determined from pooled-larvae genomic DNA extraction and sequencing. Primers used for esr2b PCR: Forward 5'-GCCAGGGTCTCTCTTGTTT-3', Reverse 5'-TGACAGCTGCCACCTAAAGA-3'. Adult TALEN-injected fish (F0) were out-crossed to wild-type, and their progeny (F1) was screened for somatic mutation by PCR and sequencing. 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.

Chemical treatments

Zebrasfish embryos were exposed to chemicals for 48 hours from 24-72 hours post fertilization (hpf) and were analyzed at 72 hpf (unless otherwise specified in the text). Chemicals include: β-Estradiol (Tocris, 2824) (10 μM), MPP dihydrochloride (Tocris, 1991) (80 μM), PHTPP (Tocris, 2662) (8 μM), Anastrozole (Tocris, 3388) (10 μM), Fulvestrant or ICI 182,780 (Tocris 1047) (10 μM).
Morpholino injection

ATG morpholino oligonucleotides (MO) were designed against \textit{esr1}, \textit{esr2a}, \textit{esr2b} (Gene Tools) and were validated (Carroll et al., 2014). MOs were injected into wild-type Tu embryos at one-cell stage. \textit{esr1} MO: 5’-AGGAAGGTTTCCTCCAGGGCTTCTCT-3’ (10 μM), \textit{esr2a} MO: 5’-ACATGGTGAAGGCGGATGAGTTCAG-3’ (10 μM), \textit{esr2b} MO: 5’-AGCTCATGCTGGAGAACACAAGAGA-3’ (10 μM).

Whole mount \textit{In situ} hybridization.

Embryos were fixed with paraformaldehyde (PFA) and performed ISH according to standard protocols (Thisse and Thisse, 2008). Anti-sense and sense RNA probes corresponding to \textit{esr1} and \textit{esr2a} were generous gifts from Dr. Daniel Gorelick (Gorelick and Halpern, 2011), probe for \textit{esr2b} from Dr. Arnaud Menuet (Menuet et al., 2002) and probe for \textit{sox9b} from Dr. John Postlethwait (Yan et al., 2005).

Expressions and fluorescent image analysis.

\textit{In situ} hybridization for liver-specific marker \textit{fabp10a}, endoderm marker \textit{foxa3}, hepatoblast marker \textit{prox1}, biliary tree progenitor \textit{sox9}, endocrine pancreas \textit{insulin}, and exocrine pancreas \textit{trypsin}, as well as transgenic 5xERE:GFP, \textit{flk:mCherry}, and \textit{fabp10a:GFP} embryos were imaged using fluorescent microscopy Zeiss Discover V8/Zxio Cam MRC. Quantification of specific gene expressions were analyzed and quantified with ImageJ.

ESC differentiation and ChIP-seq analysis

Human ESCs were cultured according to published protocol (Bock et al., 2011), and ChIP-sequencing analysis was performed according to the published methods (Gifford et al., 2013). Hepatocytes isolated from adults were used in ChIP-seq experiments.
Embryo culture and E2 treatment

CD-1 (Charles River) females were mated with CD-1 studs and the morning of the copulation plug defined as 0.5 days postcoitum (dpc). To obtain embryos, females were sacrificed early on day 8 of development. Culture of embryos were performed according to the published method (Angelo et al., 2012). Culture of embryos were treated with E2 (20 μM) dissolved in DMSO, directly to the roller culture media from 7-8 somite stage (ss). Immunofluorescent for FoxA1, PROX1, HNF4α were performed according to the protocol (Wang et al., 2015); FoxA1 (1:1000; Seven Hills); HNF4α (1:200; Santa Cruz); PROX1 (1:200; Covance). Secondary antibodies (1:1000, Molecular Probes) were incubated for 1 hour at room temperature. Nuclei were counterstained with DAPI (1:10,000, Molecular Probes).
2.6 REFERENCES


Chapter Three

The hepatic estrogen sensor GPER1 activates PI3K/mTOR to promote gender dimorphism in liver growth and cancer


This chapter contains a prepared manuscript draft. It has been modified to fit the style of this dissertation. Supplemental data can be found in Appendices.

3.1 ABSTRACT

Liver cancer is the fastest-growing cause of cancer death worldwide. Elevated estrogen levels are correlated with liver cancer, particularly in males. However, the mechanism by which estrogen influences hepatic growth is poorly understood. Here, we show that estrogen activates G protein-coupled estrogen receptor 1 (GPER1) to drive hepatocyte proliferation during developmental and reparative growth, and in liver cancer. GPER1 stimulation is necessary and sufficient to activate PI3K/mTOR signaling to promote cell cycle progression and liver growth during larval stages and regeneration after injury in zebrafish. gper1 loss diminishes, whereas estrogen accelerates hepatocarcinogenesis, specifically in males. Importantly, human liver cancer tissue exhibits increased GPER1 levels, and estrogen promotes human primary hepatocyte proliferation. Intriguingly, chemical inhibition of GPER1 signaling significantly reduces gender-specific cancer incidence and progression. Our studies identify GPER1 as a novel mTOR-modulating hepatic estrogen-sensor to regulate gender-dimorphic growth, and as an attractive therapeutic target for liver cancer prevention and treatment.
3.2 INTRODUCTION

The liver is a gender dimorphic organ, with significant differences between males and females in its function and susceptibility to diseases (Molodecky et al., 2011; Sato et al., 2001). Estrogen is thought to be important for liver homeostasis and disease prevalence (Gutierrez-Grobe et al., 2010; Della Torre et al., 2016), but it is currently not fully understood how the liver senses and responds to estrogen to regulate gender-specific physiological growth.

Estrogen has long been postulated to play a significant role in hepatocellular carcinoma (HCC) (Clocchiatti et al., 2016), the second leading cause of cancer death worldwide (Ferlay et al., 2015). Patients with cirrhosis and HCC exhibit elevated serum estrogen levels (Guéchot et al., 1988; Castagnetta et al., 2003). Moreover, long-term estrogen exposure in women using oral contraceptives can cause hepatic neoplasia (Rooks et al., 1979). Indeed, estrogenic compounds used in oral contraceptives increase hepatocyte proliferation and promote chemically induced liver tumor formation in murine studies (Ochs et al., 1986; Yager et al., 1986; Mayol et al., 1991). Clinical observations suggest that estrogenic regulation may also be important during liver regeneration as patients undergoing liver resections have elevated serum estrogen levels (Francavilla et al., 1990). Further, female mice demonstrate enhanced liver regeneration (Harada et al., 2001), suggesting a pro-regenerative role of estrogen signaling. Despite these observations, the mechanisms by which estrogen impacts hepatic growth during repair and carcinogenesis remain undetermined.
17β-Estradiol (E2) is the most abundant and biologically active form of estrogen. Canonical estrogen signaling is mediated through the nuclear hormone family estrogen receptors 1 (ESR1/ERα) and 2 (ESR2/ERβ), resulting in direct transcriptional target gene activation. In addition, E2 can exert non-canonical activity through the G protein-coupled estrogen receptor 1 (GPER1). While roles of ESR1 and ESR2 have been widely studied in reproductive biology and cancer formation (Barros and Gustafsson, 2011), the functional consequences of GPER1 signaling are less understood. In particular, the impact and downstream signaling events of GPER1 activation on hepatocyte proliferation, liver regeneration or cancer progression have not been previously identified.

The mechanistic target of rapamycin (mTOR) pathway is a key regulator of liver growth and size and is implicated in several liver diseases, such as fatty liver and liver cancer (Laplante and Sabatini, 2012). While sexual dimorphism of mTOR activity has been demonstrated in the liver (Drake et al., 2013; Baar et al., 2016; Tsai et al., 2016), it is unknown whether estrogen is sensed by mTOR to indicate a gender-specific environment and regulate liver growth, regeneration, and cancer.

Here, we identify the essential function of GPER1 signaling in embryonic liver development and adult liver growth: E2 increases hepatocyte proliferation, size, and cell cycle progression in larval zebrafish. Surprisingly, these effects are not mediated through the classic nuclear hormone estrogen receptors, but via GPER1 and downstream activation of PI3K/mTOR signaling. Further, GPER1 promotes adult liver growth in a gender-specific manner, and together with mTOR, is required for optimal liver regrowth after injury. In addition, GPER1 directly modulates liver cancer formation:
*gper1* mutants develop significantly fewer and smaller liver tumors than wild-type siblings. Importantly, chemical inhibition of GPER1 significantly diminishes E2-induced tumor progression after DMBA carcinogenesis, particularly in male fish. The role of GPER1 is conserved in humans. We propose that GPER1 senses E2 to regulate mTOR activity and proliferative responses during hepatic development and repair, and is an important therapeutic target for liver cancer prevention and treatment.

### 3.3 RESULTS

**Estrogen enhances liver growth**

To establish the effect of E2 on the mature liver in zebrafish, a transgenic hepatocyte reporter line expressing GFP under the control of the fatty acid binding protein 10a promoter, Tg(fabp10a:GFP), was examined. Fish were exposed daily to E2 or DMSO control for six weeks, and liver weight was assessed (Figure 3.1A). Male livers were smaller than female livers at baseline and responded more significantly to E2 exposure with a 4.5-fold increase in weight, while females exhibited 1.2-fold enhancement. (Figure 3.1B). To discover the signals responsible for the E2 effect on liver size, transcriptome analysis was performed (Figure S3.1A): E2 altered both male and female liver transcriptional profiles beyond that of female-associated genes (Figure S3.1B). Specifically, gene ontology analysis revealed that E2 enhanced cell cycle-related gene expression in male and female livers (46 genes, \( p=3.4\times10^{-5} \)), with a more profound effect on cell cycle pathways in males (Figure 3.1C; 324 genes, \( p=7.4\times10^{-13} \)).

To further define metabolic changes upon E2 stimulation, male and female livers were subjected to steady-state metabolomics profiling (Figure S3.1C). Metabolite set enrichment analysis revealed that E2 exposure altered the metabolome specifically in
male livers to affect pyrimidine and purine metabolism (Figure 3.1D; 55 metabolites; pyrimidine, $p=1.9 \times 10^{-8}$; purine, $p=3.5 \times 10^{-3}$). Together, these findings demonstrate a gender dimorphic response to E2 in adult liver growth: E2 potently stimulates liver growth in males by affecting the transcriptional and metabolic state to induce genes and metabolites involved in cell cycle and nucleotide metabolism.

To directly investigate the effects of E2 on hepatocytes without pre-existing gender influences, livers of zebrafish larvae were examined prior to gender specification. Larvae were exposed to E2 at various time points after hepatocytes are fully differentiated (>96 hours post fertilization; hpf). E2 exposure from 110-115 hpf increased liver size, demonstrated by analysis of liver size at 120 hpf by fluorescent imaging using the hepatocyte reporter and in situ hybridization (ISH) for the hepatocyte-specific fabp10a gene (Figure 3.1E), and confirmed by quantification of liver volume using lightsheet microscopy (Figure 3.1F), total hepatocyte number by fluorescent activated cell sorting (FACS) (Figure 3.1G), and fabp10a expression by qRT-PCR (Figure 3.1H). To define the role of endogenous E2 on liver growth, larvae were exposed to the aromatase inhibitor anastrozole (ANAS). Exposure to ANAS decreased liver size, suggesting that E2 signaling is required for normal liver outgrowth during development (Figure 3.1I,J). These results demonstrate that E2 signaling regulates liver growth in larvae, independent of gender specification.
Figure 3.1 E2 increases liver size

(A) Brightfield and fluorescent images of male and female Tg(fabp10a:GFP) adult zebrafish exposed to DMSO or E2. (B) Dissected liver weight (mg). (C) Transcriptomic analysis showing E2-induced upregulated genes in male and female livers (fold change >10 from DMSO). (D) Polar metabolomics analysis demonstrating significant gender dimorphic differences between DMSO and E2-exposed livers (fold change ≥2 from DMSO). (E) Liver size of DMSO or E2-exposed larvae as determined by Tg(fabp10a:GFP) and in situ hybridization (ISH) for fabp10a at 120 hpf. (F-H) Quantification of liver volume by lightsheet microscopy (F), number of GFP⁺ hepatocytes by FACS (G), and fabp10a expression by qRT-PCR in Tg(fabp10a:GFP) larvae at 120 hpf (H). (I) WT larvae exposed to E2, ANAS, and E2+ANAS from 110-115 hpf. (J) Liver area as assessed by ISH for fabp10a at 120 hpf. All values represent mean ± standard error of the mean (SEM), n as indicated, *p<0.05, **p<0.01, ****p<0.0001, two-tailed Student’s t-test, Scale bars, 200 μm.
Estrogen increases liver size via activation of G protein-coupled estrogen receptor 1 (GPER1)

To identify the mediator of E2 regulation on liver growth, larvae were exposed to selective chemical antagonists for each estrogen receptor, MPP (ESR1), PHTPP (ESR2) and G-15 (GPER1), alone and together with E2. Significantly, the E2-induced increase in liver size as measured by fabp10a ISH was specifically inhibited by co-exposure with G-15 but not by blockade of either nuclear estrogen receptor (Figure 3.2A,B). Selective GPER1 activation with the agonist G-1 increased liver size and hepatocyte number, similarly to E2, while GPER1 inhibition with G-15 had the opposite effect (Figure S3.2A,B). Morpholino (MO)-mediated knockdown of gper1, but not nuclear estrogen receptors esr1, esr2a, esr2b, or esr2a+esr2b blocked E2 effects on liver growth, confirming the chemical genetics results (Figure S3.2C,D). Human GPER1 mRNA injected into gper1 morphants partially rescued the small liver phenotype, demonstrating specificity and functional conservation (Figure 3.2C,D). To demonstrate the absence of nuclear receptor-mediated genomic signaling during the exposure conditions, estrogen response element (ERE) reporter fish Tg(5xERE:GFP) that reflect nuclear receptor binding to DNA were imaged. Fluorescence imaging at 120 hpf after E2 exposure from 110-115 hpf indicated that E2 had minimal effect on nuclear hormone signaling in the liver during this developmental period (Figure S3.2E, F). gper1 expression is dynamically regulated during development, as RT-PCR revealed increased expression from 48 hpf when the earliest differentiated hepatocytes are observed, until 120 hpf, correlating with hepatic outgrowth (Figure S3.3A,B). ISH
directly localized gper1 expression to the liver (Figure S3.3C). Together, these data demonstrate that GPER1 is essential for E2-mediated liver growth during development.

To definitively demonstrate the role of GPER1 in mediating E2 growth effects in larval liver development and adulthood, gper1 mutant zebrafish were generated using transcription activator-like effector nucleases (TALENs), inducing a 29-base pair deletion in exon one resulting in a premature stop codon (Figure S3.3D) and leading to loss of GPER1 protein (Figure S3.3E). To validate the liver-specific role of GPER1, gper1−/− mutants were crossed into the Tg(fabp10a:GFP) reporters. GPER1 loss impaired liver growth after 96 hpf, but not earlier, revealing that E2/GPER1 is required for the outgrowth of differentiated hepatocytes (Figure 3.2E,F). Consistent with these findings, expression of hepatic progenitor markers foxA3 and prox1 was not affected in gper1−/− larvae at 48 and 72 hpf (Figure S3.3F,G). Further, compared to WT, gper1−/− mutants had smaller livers at 120 hpf that did not respond to E2 or G-1 exposure (Figure 3.2G,H), definitively demonstrating that E2 signals via GPER1 to increase liver size.
Figure 3.2 GPER1 mediates the E2 effects on liver growth

(A) Liver size of WT larvae exposed to selective antagonists for ESR1 (MPP), ESR2 (PHTPP) or GPER1 (G-15) alone and together with E2 from 110-115 hpf. (B) Liver area. ****p<0.0001, ns=not significant, two-tailed Student’s t-test. (C) Liver size of WT, gper1 morphants, and human gper1 mRNA injected gper1 morphants. (D) Liver size distribution as the % of larvae with large, medium or small liver. *p<0.05, **p<0.01, two-tailed Student’s t-test. (E) gper1+/− mutants in Tg(fabp10a:GFP) background exhibited progressively impaired liver development from 72 to 120 hpf compared to gper1+/+ siblings. (F) Liver size of gper1+/+ and gper1+/− larvae at 72, 96, and 120 hpf, ****p<0.0001, two-tailed Student’s t-test. (G) gper1−/− mutants failed to respond to E2 or G-1 exposure. (H) Liver area. ***p<0.001, two-tailed Student’s t-test; ****p<0.0001, ns = not significant, one-way ANOVA. Liver area as assessed by ISH for fabp10a at 120 hpf. All values represent mean ± SEM, n as indicated, all scale bars, 200 μm.
Estrogen activates GPER1 to promote cellular proliferation and cell cycle progression

To determine the cellular mechanism by which E2 enlarges liver size, cell cycle analysis was performed on propidium iodide-stained fabp10a:GFP+ hepatocytes by flow cytometry (Figure 3.3A). E2 significantly increased hepatocytes in S and G2/M phase (S 21.8%; G2/M 9.4%) compared to controls (S 14.9%; G2/M 4.2%; p<0.01); importantly, this E2-induced cell cycle alteration was blocked by co-exposure to G-15 (Figure 3.3B; S 16.2%; G2/M 2.9%; p<0.01). In contrast, whole-larvae cell cycle analysis in gper1−/− at 120 hpf revealed impaired cell cycle progression (S 14.8%; G2/M 8.1%; p<0.01) compared to WT (S 23.3%; G2/M 8.5%), demonstrating a requirement for GPER1 signaling in normal cell cycle progression (Figure 3.3C,D). To corroborate these results, cellular proliferation was assessed by bromodeoxyuridine (BrdU) incorporation and staining for proliferating cell nuclear antigen (PCNA). E2-exposed larvae exhibited increased BrdU incorporation (Figure S3.4A) and an increased fraction of PCNA+ cells in the liver (Figure 3.3E,F). Conversely, G-15 exposure decreased PCNA+ hepatocytes and inhibited the E2-induced increase in PCNA+ hepatocytes (Figure 3.3E,F). Any impact of E2 on hepatocyte viability was excluded by TUNEL staining, revealing no changes in the liver after chemical exposures (Figure 3.3E). Changes in cell size as a contributor to organ size were examined by pan-Cadherin immunostaining, revealing a significant 50% increase in hepatocyte size upon E2 exposure (Figure 3.3G,H). These results indicate that E2 promotes hepatocyte proliferation, cell cycle progression and size via GPER1, leading to an increase in liver growth.
Figure 3.3 E2 signals via GPER1 to promote cell cycle progression, cellular proliferation, and cell size increase in the liver

(A) FACS profiles of cell cycle analysis of GFP<sup>+</sup> hepatocytes in Tg(fabd10a:GFP) larvae. (B) Distribution of GFP<sup>+</sup> hepatocytes as % in G1(light grey), S(black), or G2/M(dark grey)-phase. * indicates difference from % S and G2/M-phase in DMSO-exposed hepatocytes, **p<0.01, ***p<0.001, ns=not significant, two-tailed Student’s t-test. (C and D) Cell cycle analysis revealed decreased S-phase in gper1<sup>-/-</sup> mutant at 120 hpf. **p<0.01, two-tailed Student’s t-test. (E) PCNA (top panel) and TUNEL staining (bottom panel) of whole larvae section. Liver is outlined in red. Scale bars, 100 μm, scale bar (inset), 30 μm. (F) % PCNA<sup>+</sup> cells from total hepatocytes in the liver area. ***p<0.001, ****p<0.001, two-tailed Student’s t-test; ****p<0.0001, one-way ANOVA. (G) Liver sections of DMSO or E2-exposed larvae stained with Pan-Cadherin (red). Scale bars, 25 μm. (H) Hepatocyte size quantification. ****p<0.0001, two-tailed Student’s t-test. All values represent mean ± SEM, n as indicated.
**Estrogen signals through GPER1 to stimulate the PI3K/Akt pathway**

To determine the downstream signals by which GPER1 enhances liver growth a targeted approach was employed, based on the observation that other G protein-coupled receptors acting through Gα/Gβγ subunits can activate PI3K signaling (Dorsam and Gutkind, 2007). To define the interaction between GPER1 and the PI3K/Akt pathway, larvae were exposed to the PI3K inhibitor LY292002, the PI3K activator 740 Y-P, and the Akt inhibitor MK-2206. While E2 increased liver size, co-exposure of E2 with either LY292002 or MK-2206 diminished the estrogenic effect ([Figure 3.4A,B; S3.5A]). MK-2206 exposure alone decreased liver size in WT but not in gper1−/− mutants, suggesting that E2/GPER1-PI3K/Akt signaling is essential during larvae liver outgrowth. In contrast, treatment of gper1 morphants or mutant larvae with 740 Y-P rescued liver size towards WT levels ([Figure 3.4A,B; S3.5B]). As PI3K can activate both Akt and MAPK/Erk pathways (Lee et al., 2006), the impact of E2 on MAPK/Erk signaling was investigated. Western blot for phosphorylated Akt (p-Akt) and phosphorylated Erk (p-Erk) indicated that E2 increased p-Akt, but had minimal effects on p-Erk ([Figure 3.4C]). Finally, gper1−/− mutants had decreased baseline p-Akt levels compared to WT, whereas 740 Y-P-treated gper1−/− mutants showed restored p-Akt level similar to that of WT ([Figure 3.4C]). These data demonstrate that E2 activates the PI3K/Akt pathway via GPER1 to increase liver mass.

**Estrogen activates mTORC1 pathway via GPER1 to increase liver size**

To determine the effector of E2/GPER1-mediated activation of PI3K/Akt signaling controlling liver growth, the role of the mechanistic target of rapamycin complex 1 (mTORC1), an established downstream target of Akt, was investigated. Co-exposure
with the mTORC1 inhibitor rapamycin diminished the enlarged liver phenotype induced by E2 or G-1 (Figure S3.5C,D). Similarly, knockdown of mtor alone decreased liver size and blocked E2-effects on the liver as assessed by ISH for fabp10a (Figure S3.5E,F). Hypomorphic mtor mutant larvae (Ding et al., 2011) exhibited smaller liver size that did not change upon E2 or G-1 exposure (Figure 3.4D,E). To investigate the epistatic relationship between GPER1 and mTOR, gper1+/−;mtor+/− double mutants were generated, whose liver size was indistinguishable from mtor+/− mutants (Figure 3.4D,E), demonstrating that GPER1 acts in a linear pathway upstream of mTORC1. Western blot analysis of the mTORC1 target, ribosomal protein S6 (S6), demonstrated increased phosphorylated S6 (p-S6) in E2 and G-1-exposed larvae (Figure 3.4F); similarly, whole-mount immunohistochemical staining localized increased p-S6 to the liver (Figure S3.5G). These effects were abolished in gper1+/− and mtor+/− mutants (Figure 3.4F). Together, our functional and biochemical analyses demonstrate E2/GPER1-mediated regulation of liver size during development to be dependent on activation of the PI3K/mTORC1 pathway.
Figure 3.4 E2 signals via GPER1 to stimulate PI3K/mTORC1 to increase liver size.

(A) Liver size of gper1+/+ and gper1−/− larvae after chemical exposures. E2-induced increase in liver size was blocked by E2+MK-2206 co-exposure (top row arrowheads). Small liver in gper1−/− mutant was normalized by 740Y-P (bottom row arrowheads).

(B) Liver area. *p<0.05, ****p<0.0001, ns=not significant, two-tailed Student’s t-test. (C) Immunoblot analysis of gper1+/+ and gper1−/− whole larvae after chemical exposures. (D) E2 or G-1 exposure increased liver size in gper1+/+, mtor+/+, but not in gper1−/−, mtor−/−, or gper1+/+,mtor−/− larvae (red arrowheads). (E) Liver area. **p<0.01, ****p<0.0001, ns=not significant, one-way ANOVA. (F) Immunoblot analysis of gper1+/+, gper1−/−, mtor+/+, and mtor−/− whole larvae after DMSO, E2, or G-1 exposure. All ††† indicates difference from DMSO-exposed liver, ††p<0.05, †††p<0.001, ††††p<0.0001, ns=not significant, two-tailed Student’s t-test. Liver area was assessed by ISH for fabp10a at 120 hpf. Values indicate mean ± SEM, n as indicated, scale bars, 200 μm.
Estrogen promotes liver regeneration via activation of GPER1 and mTORC1 pathways

Previous work from our group and others has demonstrated that many signals important for liver development are also essential for liver regeneration (Goessling et al., 2008; Goessling et al., 2009). To explore whether the GPER1/mTOR pathway also regulates liver repair, a genetic ablation strategy was utilized, where bacterial nitroreductase is expressed in hepatocytes (Tg(fabp10a:CFP-NTR)), enabling targeted ablation of liver cells upon metronidazole (Mtz) exposure (Curado et al., 2007). Larvae were exposed to Mtz from 84-120 hpf followed by chemical incubation for 5 hrs and liver size assessment at 30 hrs post Mtz treatment (30 hpt) (Figure 3.5A). E2 or G-1-exposed larvae demonstrated significantly larger liver size compared to controls, indicating enhanced repair (Figure 3.5B,C). gper1−/− mutants crossed into the Tg(fabp10a:CFP-NTR) background exhibited significantly reduced regrowth after ablation, which was not enhanced by E2 or G-1 (Figure 3.5B,C). These results indicate that E2/GPER1 signaling mediates the pro-proliferative response during larval liver repair after injury.

To examine the specific involvement of the E2/GPER1-PI3K/mTOR axis during liver repair, compound mtor−/−;fabp10a:CFP-NTR fish were generated. Liver regrowth in mtor−/− mutants was significantly reduced and not impacted by E2 or G-1 exposure (Figure 3.5B,C), indicating a general requirement for mTOR during liver regeneration, and specifically in mediating E2 effects on liver repair. Whole mount immunohistochemistry demonstrated lower levels of p-S6 in the livers of gper1−/− and mtor−/− mutants after injury, indicating that they failed to upregulate mTORC1 signaling.
upon hepatocyte ablation (Figure 3.5D). Collectively, our data reveal an exciting new connection between E2 and mTORC1 to promote liver growth during regeneration.

**Figure 3.5** E2 promotes liver regeneration via GPER1 and mTORC1 pathways

(A) Scheme demonstrating timeline for Mtz exposure and liver regeneration analysis. (B) gper1+/+, gper1−/−, mtor+/+, and mtor−/− larvae all in Tg(fabp10a:CFP-NTR) background at 150 hpf. (C) Liver area (fold from -Mtz WT) as determined by CFP expression. *p<0.05, **p<0.001, ns=not significant, one-way ANOVA. All † indicates significant difference from Mtz-treated WT livers at 30 hpT, ††p<0.01, ††††p<0.0001, two-tailed Student’s t-test. (D) Whole mount p-S6 immunostaining revealed mTORC1 activation in regenerating WT livers but not in gper1−/− or mtor−/− mutant livers (liver is outlined in black). All values represent mean ± SEM, n as indicated, all scale bars, 200 μm.
GPER1 mediates a male-biased response to estrogen on adult liver growth

Given that E2/GPER1 signaling promotes liver growth in zebrafish larvae, we postulated that it may similarly influence adult liver size as observed in Figure 3.1. Liver weight in WT and gper1−/− adults was not significantly different in males, whereas female gper1−/− livers were significantly smaller than WT, indicating the impact of circulating E2 levels and intact GPER1 signaling for gender-specific differences in liver size (Figure 3.6A,B). The observed increase in liver weight upon E2 exposure in WT livers was significantly reduced in gper1−/− males but not in females (Figure 3.6A,B). The higher sensitivity to E2 exposure in male livers can be explained by a six-fold increase in gper1 expression levels in males over females (Figure 3.6C). Additionally, both male and female gper1−/− livers still responded modestly to E2, suggesting that GPER1-independent signals may also contribute to the impact of E2 on liver growth. Western blot analysis demonstrated increased levels of p-Akt after 5 hours of E2 exposure in male WT but not gper1−/− mutant livers (Figure 3.6D), highlighting the persistence of PI3K/Akt signaling as a target of E2/GPER1 in adult livers. These results demonstrate that GPER1 is required for a normal response to circulating E2 in the female liver, while intrinsically higher levels of GPER1 expression sensitize male livers to exogenous E2-induced liver growth.

Finally, to investigate whether these effects could be similarly observed in human hepatocytes, a human primary hepatocyte coculture system was employed (Khetani and Bhatia, 2008; Shan et al., 2013). E2 increased the number of proliferating male hepatocytes doubly labeled with hepatocyte nuclear factor 4 alpha (HNF4α) and ethynyldeoxyuridine (EdU) in a concentration-dependent manner (Figure 3.6E,F;
p<0.01). GPER1 is expressed in human hepatocytes but not in the cocultured support cells (Figure S3.6A), indicating a cell-autonomous effect of E2/GPER1 on hepatocyte proliferation. Together, these results demonstrate the conserved role of E2/GPER1 in promoting hepatocyte proliferation.

**Figure 3.6 E2 acts via GPER1 to promote gender dimorphism in adult liver growth**
(A) Male and female gper1+/+ and gper1−/− treated with DMSO or E2. Scale bars, 2mm. (B) Liver weight (mg). ***p<0.0001, ns=not significant, two-tailed Student’s t-test. (C) gper1 expression in male and female livers. ****p<0.0001, two-tailed Student’s t-test. (D) Immunoblot analysis for p-Akt, GPER1, and β-actin levels in gper1+/+ and gper1−/− male livers exposed to DMSO or E2 for 5 hours. (E) Immunofluorescent staining of DMSO or E2-exposed male donor-derived hepatocytes with EdU (red) and HNF4α (green). White arrowheads indicate EdU and HNF4α double positive cells. Scale bar, 100 μm. (F) % EdU+ hepatocytes. * indicates significant difference from DMSO controls. *p<0.05, **p<0.01, two-tailed Student’s t-test. Values represent mean ± SEM, n as indicated.
GPER1 contributes to gender-dimorphism in cancer formation and progression

Deregulation of signaling pathways controlling embryonic development and adult tissue homeostasis are frequently causative in carcinogenesis (Manning and Cantley, 2007; Laplante and Sabatini, 2012). Given the role of E2/GPER1 in normal liver growth, its impact on cancer formation was examined in a well-established model of chemical liver carcinogenesis, employing dimethylbenzanthracene (DMBA) (Spitsbergen et al., 2000): gper1−/− mutants exhibited significantly and dramatically reduced cancer incidence (Figure 3.7A; 42.8% vs. 22.2%, \( p<0.05 \)) and liver tumor number per fish (Figure 3.7B; 0.7 vs. 0.3, \( p<0.05 \)) compared to WT at 22 weeks post DMBA treatment. Moreover, average liver tumor size was significantly smaller in gper1−/− adults (Figure 3.7C; 0.7 vs. 0.2 mm, \( p<0.01 \)). These results demonstrate a requirement for GPER1 in the formation and progression of liver cancer. To determine if E2/GPER1 is an oncogenic signal in liver carcinogenesis, DMBA-exposed adults were subsequently treated with DMSO, E2, or a combination of E2 and G-15 (Figure 3.7D). Histological analysis of tumors at 33 weeks post DMBA treatment indicated the presence of hepatocellular adenoma (HCA), hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) (Figure S3.7A). E2-treated fish had decreased survival compared to controls and fish concomitantly exposed to E2 and G-15 (Figure 3.7E; \( p<0.0001 \)). Importantly, E2 increased liver tumor growth with larger tumor size (Figure S3.7B; 0.5 vs. 0.9 mm, \( p<0.01 \)). Strikingly, co-treatment of E2 with G-15 blocked the E2-induced increase in tumor progression back to baseline levels (Figure S3.7B; 0.9 vs. 0.4 mm, \( p=0.01 \)). Gender-stratified analysis of treatment response in the cohorts revealed that the E2-induced increase in tumor size and its blockade by G-15 was
predominantly seen in male, but not in female fish (Figure 3.7F,G; p<0.001). Together, these results indicate that GPER1 is an essential component for the sexually dimorphic response to E2 in liver growth and carcinogenesis.

To determine the relevance of GPER1 in the human liver and in cancer, GPER1 expression was quantified in liver tissues from 68 individual patients by immunohistochemistry: 30% of non-cirrhotic livers, but 91% of cirrhotic livers, and 86% of HCC samples exhibited positive patchy to diffuse cytoplasmic GPER1 staining (staining score ≥1+), with some enhanced staining of hepatocytes around portal tracts and fibrous bands (Figure 3.7H;S3.7C,D,E; non-cirrhotic vs cirrhotic; p<0.05). These results demonstrate that GPER1 is increasingly expressed in cirrhotic livers and in HCC, supporting a role for GPER1 in human hepatocarcinogenesis.
Figure 3.7 E2/GPER1 signaling promotes liver cancer initiation and progression

(A) Liver cancer incidence in DMBA-exposed adults was reduced by 50% in gper1−/− mutants compared to gper1+/+ siblings. *p<0.05, one-tailed Chi-Square test. (B) Number of liver tumors per fish in DMBA-exposed gper1+/+ and gper1−/− adults. *p<0.05, one-tailed Mann-Whitney test. (C) Tumor size (mm) per fish in DMBA-exposed gper1+/+ and gper1−/− adults. **p<0.01, two-tailed Student’s t-test. (D) Scheme illustrating DMBA carcinogenesis followed by E2 and G-15 prevention trial. (E) Kaplan–Meier survival plot demonstrating survival of DMBA-treated fish (% of total) after chemical exposures. n = 42, 30, 25 for DMSO, E2, and E2+G-15 treated adults at 249 days post first treatment, p<0.0001, Log-rank (Mantel-Cox) test. (F) Liver tumor size (mm) per fish in DMBA-exposed male and female at 9 mpf. ***p<0.001, ns = not significant, one-way ANOVA. (G) DMBA-exposed Tg(fabp10a:GFP) fish after treatments and their liver histology. NT=non-tumor, scale bars (whole animal), 2mm, scale bar (histology section), 100 μm. (H) Human liver sections immunostained for GPER1 in non-cirrhotic liver, cirrhotic liver, and HCC. Scale bar, 100 μm. All values represent mean ± SEM, n as indicated.
3.4 DISCUSSION

Liver cancer is the second most common cause of cancer death, and it predominantly affects men. In this study, we identified GPER1 as a novel estrogen receptor in hepatocytes that cell-autonomously regulates larval liver growth and is responsible for gender dimorphism in liver cancer. E2 promotes hepatocyte cell cycle progression and proliferation through GPER1 and downstream activation of PI3K/mTOR signaling. Importantly, the GPER1/mTOR pathway remains essential for proliferative expansion of hepatocytes during liver repair. Prospective in vivo longitudinal carcinogenesis assays identify GPER1 as an essential factor to promote E2-mediated liver cancer initiation and progression. Unique gender stratification analysis demonstrates this to be a male-predominant effect. Finally, pre-clinical studies utilizing a selective GPER1 antagonist highlight the therapeutic potential of blocking GPER1 activation for the treatment of liver cancer.

GPER1 as an estrogen sensor regulating mTOR activity in the liver

The mTOR pathway is essential for organ size regulation (Lloyd, 2013), functioning as a growth checkpoint that tightly coordinates cell growth with environmental cues, such as growth factors, cellular stress, oxygen content, energy status, and amino acid availability (Laplante and Sabatini, 2012). Here, we reveal a previously unidentified role of E2/GPER1 acting upstream of mTORC1 to promote hepatocyte proliferation and liver growth. We further identify the impact of E2/GPER1 activation of mTORC1 on pyrimidine and purine metabolism, supporting recent work that mTORC1 is essential for de novo pyrimidine and purine biosynthesis to meet the anabolic demands of rapidly proliferating cells (Ben-Sahra et al., 2013; Robitaille et al.,...
Prior studies have identified gender-specific responses to mTOR modulation with regard to lifespan and cardiac function (Lamming, 2014; Gürgen et al., 2013) and reported the sexual dimorphism of mTOR activity in the liver (Drake et al., 2013; Baar et al., 2016; Tsai et al., 2016). Our study is the first to elucidate how hepatic mTOR senses a gender-specific environment and provides novel mechanistic insight by demonstrating that GPER1 acts as an E2 sensor to regulate mTOR activity and influence liver growth.

**GPER1 and estrogen sensing in liver regeneration**

Several clinical and laboratory studies have shown sexually dimorphic responses of the liver after hepatectomy, with females being more tolerant and having a higher rate of liver regeneration (Imamura et al., 1999; Harada et al., 2001). While E2 has been correlated with a pro-regenerative role in the liver (Kawai et al., 2007), the downstream pathways involved are incompletely understood. Here, we reveal that E2 specifically activates GPER1 and mTORC1 to promote liver regrowth after injury. The importance of this E2 regulatory axis can be gleaned from clinical observations: strikingly, E2 levels have long been known to rapidly increase after hepatectomy (Francavilla et al., 1990). Pregnancy is also associated with hepatomegaly (Dai et al., 2011), and the gestational increase of E2 enhances the liver repair response (Gershbein, 1958), suggesting a physiological requirement for E2 signaling during periods of liver growth. Indeed, gper1−/− mutants exhibit significantly delayed liver outgrowth during development and following injury. Nevertheless, gper1−/− mutants have a greater capacity for liver regeneration compared to mtor−/− mutants, indicating that E2/GPER1 is one among several other inputs integrated by mTOR to regulate liver regeneration. Given the pro-proliferative
effect of E2/GPER1 signaling shown here to act through PI3K/mTOR, which has been reported to be important for liver regeneration (Jackson et al., 2008; Volarevic et al., 2000), we postulate that GPER1 is the conserved sensor that mediates E2 activation of PI3K/mTOR signaling in a gender dimorphic fashion to promote liver regeneration.

**GPER1 as a mediator of estrogen signaling in promoting liver cancer**

Sexual dimorphism in liver cancer has long been documented. While several lines of evidence point to the significance of E2 signaling in HCC (Clocchiatti et al., 2016), a detailed mechanistic understanding has not been established. Current reports have described both protective and tumor-promoting effects for E2: E2-mediated reduction of inflammatory cytokines, such as IL-6, in liver-resident immune cells appears to inhibit tumorigenesis (Naugler et al., 2007; Wei et al., 2016). Li et al. indicated a protective role for E2 via an ESR1-mediated interaction with transcription factor Foxa1/2 (Li et al., 2012); nevertheless, in the absence of Foxa1/2, E2 promoted liver tumorigenesis. Several other studies have shown liver tumor-promoting properties of E2 (Taper, 1978; Yager et al., 1991; Lee and Edwards, 2001). Interestingly, clinical studies in HCC patients using an ESR antagonist, tamoxifen, have been disappointing (Chow et al., 2002; Barbare et al., 2005). In line with our mechanistic analysis, tamoxifen was later found to be an agonist for GPER1 (Prossnitz and Barton, 2011), which can increase the size and number of hepatic lesions (Dragan et al., 1991; Williams et al., 1997). These data reveal the complexity and specificity of E2-mediated signaling in that the response it elicits may depend on the cell type and receptor activated. Our results definitively demonstrate a growth-promoting role for E2 in liver cancer acting through hepatocyte-associated GPER1.
Our finding that the GPER1-PI3K/mTOR axis enhances hepatocyte proliferation prompted us to hypothesize that GPER1 also activates this signaling cascade to promote liver carcinogenesis. Indeed, activation of PI3K/Akt (~70%) and mTORC1 (~45%) pathways is found in HCC patient samples and positively correlates with tumor metastasis, recurrence, and poor prognosis (Villanueva et al., 2008; Chen et al., 2009). Genetic alterations, such as mutations and copy number amplification, however, are found at lower frequencies in PI3K/mTOR pathway components in HCC. For instance, exome sequencing revealed mutation frequencies in PIK3CA (≤ 2%), mTOR (≤ 2%), TSC1/TSC2 (≤ 5%), and PTEN (≤ 3%) (Forbes et al., 2015; Schulze et al., 2015). It is tempting to speculate that PI3K/mTOR activity in HCC may instead depend upon upstream ligand activation, such as that mediated by increased E2 levels and GPER1 expression in cirrhotics. As over 80% of HCC patients are diagnosed at late stages without hope for cure, there is clearly a dire need for targeted therapeutic interventions. The pro-proliferative consequence of E2/GPER1 activation of the PI3K/mTOR pathway, together with our in vivo data showing strong responses to GPER1 antagonist treatment in both cancer initiation and progression clearly indicates that optimizing drugs targeting E2/GPER1 may offer exciting new therapeutic applications in liver cancer treatment.
3.5 MATERIALS AND METHODS

Zebradish husbandry and generation of gper1<sup>−/−</sup> mutant

Male and Female wild-type Tu zebrafish, Tg(-2.8fabp10a:eGFP)<sub>αβ3</sub> (Her et al., 2003), abbreviated fabp10a:GFP, Tg(fabp10a:CFP-NTR) (Curado et al., 2007), and xu015Gl (Ding et al., 2011) were used. All zebrafish were maintained according to the standard Institutional Animal Care and Use Committee guidelines (IACUC) at Harvard Medical School.

TALEs targeting endogenous gper1 were generated using FLASH-assembly and were obtained from Addgene (TAL3272, TAL3273) (Sander et al., 2011). mRNAs of TALEN pairs were synthesized by mMESSAGE mMACHINE kit (Ambion) and were injected into 1-cell stage wild-type larvae. Somatic mutation rate was determined from pooled-larvae genomic DNA extraction and sequencing. Adult TALEN-injected fish (F<sub>0</sub>) were out-crossed to wild-type, and their progeny (F<sub>1</sub>) was screened for somatic mutation by PCR and sequencing (see also Table S3.1). F<sub>1</sub> with mutations at TALEN-targeted site were raised and out-crossed for at least 4 generations to avoid possible TALEN-induced off-target effects.

Chemical treatments

Zebrafish larvae were exposed to chemicals for 5 hours from 110-115 hours post fertilization (hpf), recovered and were analyzed at 120 hpf (unless otherwise specified). Adults were treated for 5-7 hours per exposure with frequency as described in the text. Chemicals are listed in Table S3.5.

Morpholino injection.
ATG morpholino oligonucleotides (MO) were designed against *esr1*, *esr2a*, *esr2b* (Carroll et al., 2014), *gper1*, and *mTOR* (Gene Tools, LLC) and were injected into wild-type Tu larvae along with their mismatch standard morpholino control at one-cell stage (MO sequences and concentrations are listed in Table S3.3).

**mRNA injection.**

Human GPER1 cDNA-containing plasmid was obtained from Dana-Farber/Harvard Cancer Center (HsCD0032896) and was transcribed using mMESSAGE mMACHINE Transcription kit (Ambion). 200 µg of mRNA was injected into each one-cell stage larvae.

**Western blot analysis.**

Pooled larvae (*n*=30-40) per group were homogenized in RIPA lysis buffer with protease and phosphatase inhibitors (Roche). Protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membrane. Membranes were blocked with 5% milk or 5% BSA in TBST according to antibody manufacturer protocol, followed by incubations with primary antibody overnight and HRP-conjugated secondary antibody. Membranes were subjected to ECL and imaged on X-ray films. Antibodies are listed in Table S3.4.

**Whole mount in situ hybridization.**

Larvae were fixed with paraformaldehyde (PFA) and performed ISH according to standard protocols (Thisse and Thisse, 2008). Probe for *gper1* was a generous gift from Dr. David Volz (Jayasinghe and Volz, 2012).

**Liver size analysis.**
Larvae ISH for liver-specific marker *fabp10a* and *fabp10a:GFP* transgenic larvae were imaged using fluorescent microscopy Zeiss Discover V8/Zxio Cam MRC. 3D imaging of *fabp10a:GFP* was carried out by Lightsheet fluorescent microscopy, utilizing a Zeiss Lightsheet Z.1 at the Harvard Center for Biological Imaging (HCBI) facility. Liver images were analyzed and quantified with ImageJ.

**Histology and Immunohistochemistry.**

Larvae and adult zebrafish were fixed with PFA and paraffin embedded. Tissues were then sectioned (5 µm) and stained with hematoxylin & eosin (H&E). De-identified formalin-fixed paraffin-embedded human liver tissue sections were obtained from the pathology files of the University of Utah (IRB 00091019) and from commercially available tissue array (OD-CT-DgLiv01-003, US Biomax). IHC was performed after sections were deparaffinized in xylene, rehydrated with ethanol, and heat-induced antigen retrieval. Sections were incubated overnight in primary antibodies followed by secondary antibodies and DAB/HRP substrate (LmmPACT, Vector Lab) or fluorescent-labeled secondary incubation for detection. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using TACS TdT In Situ Apoptosis detection kit (Trevigen) according to manufacturer’s protocol. GPER1 staining was scored by a pathologist (K.J.E.) as follows, based on the mean amount of cytoplasmic reactivity in hepatoid cells: 0+, minimal or no staining; 1+, faint/mild staining; 2+, moderate staining; 3+, strong staining. Antibodies are listed in Table S3.4.

**Flow cytometry and cell cycle analysis.**

*fabp10a:GFP* larvae were pooled (*n* = 30) and manually dissociated with pestle in 0.25% trypsin for 10 mins. After 10% FCS/DMEM was added, samples were stained
through a 35 µm nylon mesh filter and GFP+ cells were quantified using BD Accuri C6 Plus flow cytometer (BD Bioscience). For cell cycle analysis, dissociated cells were fixed and permeabilized using BrdU Flow kit (BD Bioscience, 559619) and were incubated in propidium iodide (PI) at 50 µg/ml final concentration followed by flow cytometry analysis. All analysis was performed with FlowJo v10.0.7 software.

**qRT-PCR Analysis.**

RNA was isolated from pooled zebrafish larvae with Trizol and was DNase treated. cDNA was synthesized using SuperScript III First-Strand Synthesis kit (Life Technologies). qRT-PCR was performed using iQ SYBR Green Supermix and iCycler (BioRad) and relative gene expression levels were calculated using the ΔΔCt method with *ef1α* as reference gene. Primers are listed in Table S3.2.

**DMBA-induced carcinogenesis.**

*gper1+/* or wild-type gper1+/+ fry were exposed to 3 rounds of 24-hour treatment of 5 ppm DMBA at 3, 4, and 5 weeks (Spitsbergen et al., 2000). Fish were allowed 1-week recovery before subsequent three times/week chemical treatments. Fish were then monitored daily for survivals and tumor formation until 7-9 months post DMBA treatment. Fish were then either analyzed immediately at time of death or at 33 weeks post DMBA exposure, when all remaining fish were sacrificed. Histologic analysis of liver tumors was performed by a board-certified pathologist with expertise in zebrafish liver histology (K.J.E.) in a blinded fashion.

**Steady-state Metabolomics analysis.**

Adult livers were surgical removed from male and female wild-type gper1+/+ or gper1−/− with or without E2 exposures and were subjected to methanol extraction as previously
described (Yuan et al., 2012). Extracted polar metabolites were identified using LC-MS/MS with QTRAP 5500 System (SCIEX) at the Beth Israel Deaconess Medical Center Mass Spectrometry facility. Metabolic pathway analysis was performed with Metabo Analyst (Xia et al., 2015).

**RNA transcriptomic analysis.**

Adult livers were surgically isolated and RNA was extracted in Trizol (Life Technologies), isopropanol precipitated and ethanol washed. RNA was DNase treated using TURBO DNA-free kit (Life Technologies) and RNA quality and quantity were determined using Qubit (Life Technologies) and Bioanalyzer (Agilent). DNA library was created using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB) and sequencing was performed on Illumina NextSeq 500. polyA sequences were aligned onto the ZV9 genomic assembly and differentially expressed genes were determined using TopHat and Cufflinks pipelines. Gene Ontology (GO) terms were analyzed using DAVID (NIAID, NIH) (Huang et al., 2009).

**Primary human hepatocyte culture and treatment.**

96-well glass bottom plates (Greiner Bio-one) were incubated with a solution of type I collagen in water (100 μg/ml; BD Biosciences) for 1 h at 37 °C. A feeder layer of J2-3T3 fibroblasts (gift from H. Green) was plated onto the collagen at a density of 16,000 cells per well at day -1. Primary human hepatocytes (Hu4175:3-yr old male donor, Invitrogen) were plated onto the fibroblasts on day 0 at a density of 4,000 cells per well and maintained under standard culture conditions with daily replacement of the hepatocyte medium (1X DMEM/F12 (phenol red free), 10% fetal bovine serum (FBS), 15.6 μg/ml insulin, 7.5 μg/ml, hydrocortisone, 16 ng/ml glucagon, 1% penicillin-streptomycin) for 6
days. From day 6 onwards, the FBS concentration of the hepatocyte medium was lowered to 2% and the DMSO concentration was set at 0.1%. From day 7-10, cells were pulsed daily with indicated concentration of estrogen for 5.5hr per day, followed by 18.5hr culture in hepatocyte medium with 10µM EdU. On day 11, cells were fixed in 4% PFA for imaging analysis. Fixed cells were stained with Click-iT EdU Alexa Fluor 594 Imaging Kit (Thermo Fisher), with additional antibody staining for HNF4α (ab55223) and goat-anti-mouse 488 (Jackson Immunolabs). Nuclei were visualized with Hoechst stain (Invitrogen). Cells were imaged on a Nikon Eclipse TE200 Microscope, and analyzed in NIS-Elements Viewer 4.0, ImageJ, and Photoshop.
3.6 REFERENCES


Wnt signaling regulates developmental specification of stem cells and regeneration. Cell 136, 1136-147.


CHAPTER FOUR

Conclusion, future directions, and discussion
4.1 CONCLUSION

Estrogen signaling has a biphasic effect on liver growth

Embryonic development involves many different processes, including cell specification, differentiation, and growth. The work presented in Chapter 2 and Chapter 3 define the roles of E2 signaling during both early hepatic differentiation and later during hepatocyte growth and adult liver homeostasis. Intriguingly, the effects of E2 on the liver are biphasic: preventing hepatoblast differentiation and proliferation during early hepatic development, while promoting hepatocyte growth and liver size in differentiated and adult liver (Figure 4.1). During early hepatogenesis, the level of E2 signaling is kept low as evidenced by minimal expression of aromatase enzymes and ERs. Activated E2 signaling negatively impacts hepatic differentiation and proliferation via esr2b. Importantly, while blockade of ERs increased liver size, it negatively impacted biliary tree formation, suggesting the potential fate determination role of E2 in bipotential hepatoblasts to promote biliary tree formation while suppressing hepatocyte differentiation.
Figure 4.1 Estrogen has a biphasic effect on liver size
Embryos were exposed to E2 during different time windows that correlate with different stages of liver development. Red bars represent percentage of embryos with smaller livers and green bars represent percentage of embryos with bigger liver upon E2 exposure. Embryos exposed to E2 prior to 84 hpf had decreased liver size while embryos exposed to E2 after 84 hpf had increased liver size.

Conversely, late E2 signaling activation increased differentiated hepatocyte number and liver size via gper1. Late E2 exposure activates rapid Akt/mTOR signaling pathway to drive hepatocyte proliferation and growth. Defects in developmental pathways are commonly implicated in adult diseases. Indeed, given the importance of E2/gper1 signaling during liver growth, I have shown that elevated E2 accelerated, whereas loss of GPER1 activity in gper1 knockout or GPER1 inhibitor-treated adults blocked, carcinogen-induced liver cancer incidence and progression. These findings can be further translated to human disease, as E2 similarly increased proliferation of
primary human hepatocytes and GPER1 expression further increased in human liver tissues with hepatocellular carcinoma (Figure 4.2).

**Figure 4.2 Estrogen signaling in vertebrate liver**
Early E2 signaling activation via *esr2b* inhibits hepatic differentiation and decreases liver size. Conversely, later activation of E2 signaling in differentiated hepatocytes via *gper1* increases hepatocyte proliferation and liver size. In adult fish, E2 increases liver size via *gper1* particularly in males. E2-*gper1* increases liver cancer initiation and progression in zebrafish and is involved in human cirrhosis and liver cancer progression.
4.2 FUTURE DIRECTIONS AND DISCUSSION

Estrogen signaling: how does the specificity arise and how is it regulated?

Given the data described in Chapter 2 and 3, it is fascinating how E2 signaling involves not only various ERs, but also several compounds that can bind and activate the receptors’ downstream signals. These multiple components increase the complexity and, importantly, the specificity of E2 signaling. How can specificity be achieved in such a complex network?

Emergence of unique estrogen receptor isoforms. From an evolutionary perspective, steroid receptors arose in the base of the vertebrate line, in amphioxus and lamprey fish (Baker, 1997). Of all steroid receptors, ER was the first ancestral receptor and has further evolved into other vertebrate steroid receptors, such as androgen, glucocorticoid, and progesterone receptors (Thornton, 2001; Bridgham et al., 2006). The first alternative isoform of ER discovered was ERβ, identified in 1996 (Kuiper et al., 1997; Mosselman et al., 1996). Even though the sequence of the ligand binding and the DNA binding domains are well conserved between ERα and ERβ, ERβ was shown to originate from a duplication event around 450 million years ago (Kelley and Thackray, 1999), suggesting that the unique functions of ERβ allowed it to survive through natural selection. Indeed, previous findings have demonstrated the distinct biological functions, downstream signaling, and pathology of ERβ (Dechering et al., 2000). A more recently identified ER is G protein-coupled estrogen receptor (GPER1) (Filardo et al., 2002). The sequence and structure of GPER1 are unrelated to that of other ERs, and its downstream cascades are unique, resembling those membrane receptors with rapid signaling (Filardo et al., 2007). GPER1 expression patterns, E2 binding affinities, as well
as pathologies in GPER1 null animals (Revankar et al., 2005; Mårtensson et al., 2009) also differed from those involving ERs. From our results in Chapter 2 and 3, it is apparent that E2 can have unique and divergent impacts on the liver through activation of different receptors. Notably, while we have defined the primary role of GPER1 during hepatic growth in Chapter 3, the presence of other ERs has been reported (Chandrasekar et al., 2010). How E2 selectively or cooperatively signals through these other receptors and how it impacts hepatocytes remains to be elucidated.

Spatial and temporal regulation of individual ERs. The predominant mode of ERα and ERβ signaling regulation is their unique tissue distributions. While ERα is expressed in adult ovarian, uterus, testes, breast, and liver, ERβ is expressed in bone marrow, prostate, and brain (Zhao et al., 2010). Expression of GPER1 was detected in tissues such as breast, blood vessels, and the hypothalamus. Importantly, expression levels of ERα and ERβ can be dynamically regulated transcriptionally and/or epigenetically. For example, ERα expression can be modulated by E2 itself (Ihionkhan et al., 2002), vitamin D3 (Swami et al., 2000), or by DNA methylation (Issa et al., 1994). Expression of ERβ is regulated by promoter methylation and exposure to phytoestrogens, such as genistein (Swedenborg et al., 2009). While regulation of GPER1 expression is less known, recent studies revealed roles of IGF and promoter methylation in GPER1 expression in breast cancer (Vaziri-Gohar and Houston, 2016; Weissenborn et al., 2017).

ER expression can also be temporally regulated. For instance, developmental stage-dependent expression of ERs in B lymphopoiesis (Igarashi et al., 2001), as well as nutrient-dependent expression of hepatic ERα, have been observed (Della Torre et al., 2005).
Specifically, timing of GPER1 expression has also been linked to disease progression, including in lung cancer (Jala et al., 2012). While we have shown that timely expression of hepatic ERβ (Chapter 2) and GPER1 (Chapter 3) allow distinct downstream functions, detailed temporal regulation of these receptors’ expressions (genomic or epigenetic) during liver development, growth, and disease remain to be explored.

**Spatial and temporal regulation of ligand E2.** Invertebrates such as octopus and drosophila also possess relatives of estrogen receptors, similar to the vertebrate estrogen-related receptor (ERR) (Bertrand et al., 2004). These receptors, however, are E2-independent and constitutively active (Ostberg et al., 2003; Baker and Chandsawangbhuwana, 2007), suggesting an increased complexity of vertebrate E2 signaling through the ligand-dependent mechanism. The major enzyme responsible for E2 synthesis is the cytochrome P450 enzyme aromatase encoded by the *CYP19* gene. Expression of aromatase is hormone and tissue-specific through the regulation of alternative splicing in the promoter regions (Simpson et al., 1997). For instance, ovary *CYP19* is regulated by follicle stimulating hormone (FSH), while adipose *CYP19* is regulated by glucocorticoids (Simpson et al., 1997). This hormone-responsive *CYP19* promoter further determines the timely expression of the aromatase enzyme. In Chapter 2, the absence of aromatase expression prior to hepatic differentiation (Hao et al., 2013) may provide another mechanism to ensure that activation of E2 signaling occurs at the right time. Nonetheless, the detailed localization and expression data of aromatase enzyme or other enzymes involved in E2 synthesis throughout liver development are
still lacking. Further investigation will deepen our understanding of E2 pathway regulation and potentially improve aromatase inhibitor-based cancer therapies.

**Estrogen signaling: endocrine or paracrine signaling?**

E2 is a sex steroid hormone mainly secreted from the ovary into the circulating blood stream as part of the endocrine system in premenopausal women. However, in men and post-menopausal women, E2 is synthesized in various organs, including adipocytes, smooth muscles, vascular endothelial, parts of the brain, and the liver (Simpson, 2003). This local biogenesis of E2 demonstrates that E2 can also function as a paracrine signaling molecule. Our results in Chapter 2 revealed the expression of esr2b in the liver after hepatic differentiation, however, the expression of aromatase was not detected in the liver during this time window (Lassiter and Linney, 2007). Likewise, our data in Chapter 3 revealed gper1 expression in the liver, where minimal aromatase expression was observed (Chiang et al., 2001). These data suggest that during early liver organogenesis, E2 signals in an endocrine fashion to impact liver formation.

This mode of E2 signaling may be beneficial during embryogenesis as gestational E2 is required for development of multiple fetal organs. For instance, E2 modulates the activity of glucocorticoids, which are necessary for the formation of fetal lung, thymus, kidney, and liver (Liggins, 1969; Liggins, 1994). Circulating E2 during pregnancy is also essential in suppressing pro-inflammatory responses, allowing healthy fetal development and successful pregnancy (Robinson and Klein, 2012). Circulating E2 can be advantageous as levels of E2 is strictly monitored. Too much or too little E2 can be promptly corrected through the negative and positive feedback loops between placenta and fetal adrenal gland (Albrecht and Pepe, 1999).
During rodent and zebrafish organogenesis, E2 activity in peripheral tissues such as in the liver rely on circulating E2. Uniquely among vertebrates, however, human fetal liver expresses aromatase and produces E2 locally (Toda et al., 1994), suggesting a distinct role of local E2 during human hepatogenesis. This high aromatase expression decreases postnatally and becomes undetectable in healthy adult liver (Castagnetta et al., 2003). Interestingly, primary and metastatic human liver cancers have increased levels of aromatase (Hata et al., 2013), suggesting further that local E2 may be necessary for both the neoplastic growth of liver cancer cells as well as the rapid growth of the liver during embryonic development.

Indeed, our results in Chapter 3 revealed an increase in the expression of human hepatic GPER1 in cirrhotic and HCC patients compared to minimal GPER1 expression in healthy livers, supporting the association between high E2/GPER1 activity and liver carcinogenesis. Multiple studies have similarly demonstrated high levels of locally synthesized E2 in E2-dependent breast cancer despite the low levels of circulating E2 (Miki et al., 2007; Suzuki et al., 2008). High levels of intratumoral aromatase and E2 were also reported in human lung (Verma et al., 2011) and endometrial carcinomas (Takahashi-Shiga et al., 2009). These data point to an apparent advantage of locally produced E2 for cancer cells. One possible explanation may be that high levels of locally synthesized E2 can elude the negative feedback loop established to maintain normal circulating E2 levels. Nevertheless, whether local E2 production and progression of liver cancer are directly connected, still awaits further investigation. Delineating different modes of E2 actions: endocrine (via circulation) and paracrine (via local synthesis), is crucial not only for our data interpretation and experimental designs
(where and how to measure E2: blood vs. tissue) but also for the development of targeted therapies in E2-addicted disorders such as cancer.

**Estrogen signaling: the liver size regulator?**

The processes underlying body and organ size determination are complex and despite much effort, they are not fully understood. Besides its function in detoxification, digestion, and metabolism, the liver plays a major role in organ cellular proliferation, survival and whole body size control via the secretion of insulin-like growth factor (IGF) and its binding proteins (IGFBP) in response to growth hormones (Dupont and Holzenberger, 2003). Interestingly, however, the liver itself is not deemed to be a major target of IGF action due to its low IGF receptor expressions (Zimmermann et al., 2000).

Central to the regulation of liver size are mTOR and Hippo signaling, the pathways known to control cell size and cell proliferation, respectively (Tumaneng et al., 2012). Normal liver size requires tight coordination between cell size and cell proliferation as an individual cell needs to reach a minimum size before it can divide and proliferate. Indeed, increasing studies reveal crosstalk between Hippo and mTOR signaling pathways in vitro and *in vivo* in determining organ growth, including in the heart and in neurons (Xin et al., 2011; Hansen et al., 2015; Tumaneng et al., 2012). We describe in Chapter 3 that E2/GPER1 plays an important role as an upstream activator of mTOR signaling, and its stimulation led to an increase in liver size. Interestingly, we observe that upon E2/GPER1 signaling activation, an increase in liver size did not linearly correlate with the treatment durations (1 week, 2 weeks, or 6 weeks), but instead reached a plateau after a 1-week exposure period (data not shown). These observations suggest that other mechanisms exist to limit the liver size. While mTOR
signaling is known to be a positive regulator of liver size, Hippo signaling is deemed to be a negative regulator, maintaining hepatocytes in a quiescent state when normal liver size is reached (Avruch et al., 2011). Indeed, a recent study in breast cancer cell lines has uncovered the crosstalk between E2/GPER1 and Hippo signaling in regulating cancer cell proliferation (Zhou et al., 2015). It is then tempting to speculate that the crosstalk between E2/GPER1 and Hippo pathways also takes place in the liver. With coordination between signaling pathways required for organ growth control, it would not be surprising that E2/GPER1, mTOR, and Hippo signaling pathways work together to regulate liver size.

**Estrogen signaling: from zebrafish to human liver development and diseases**

Our findings have shed light not only on the developmental mechanisms during hepatogenesis in zebrafish but further facilitate our understanding of human liver organogenesis. Indeed, E2 signaling and its components are largely conserved between fish and human. For instance, the mammalian aromatase enzyme gene cyp19a1 is duplicated in zebrafish to become ovary cyp19a1a and brain cyp19a1b (Goldstone et al., 2010). Mammalian ERα has a zebrafish ortholog esr1, and ERβ is duplicated in zebrafish to become esr2a and esr2b (Tingaud-Sequeira et al., 2004). An ortholog of mammalian sex hormone binding globulins, shbg, is also present in zebrafish (Bobe et al., 2010). Similar to mammalian high E2 environment in utero, zebrafish E2 is known to be maternally provided to the zygote (Pikulkaew et al., 2010). Notably, developmental abnormalities found in newborns and children associated with early E2 exposure have been long reported (Aksglaede et al., 2006), while the direct impact of E2 on organogenesis was unclear. Our findings in Chapter 2 that E2 signals
via esr2b to disrupt the hepatic differentiation deepens our mechanistic understanding of estrogenic influence during liver development. A future direction would be to investigate the underlying downstream signals mediating this process. Candidate pathways may involve Wnt and Notch signaling as they are essential for hepatocyte and biliary epithelial differentiation and maturation processes, respectively. Pinpointing key signals governing hepatic progenitor maintenance and differentiation can further shed light on our knowledge in liver progenitor/stem cell biology.

Our results in Chapter 3 have direct implication for human diseases, specifically for liver cancer. Importantly, transcriptomic data revealed carcinogen (Dimethylbenzanthracene or DMBA) - induced zebrafish liver tumors, a liver cancer model used in our study, to have striking similar molecular mechanisms compared to that of human liver cancer (Lam et al., 2006), suggesting the direct relevance of our findings on GPER1 in human liver cancer progression. Indeed, we have shown the correlation between an increase in GPER1 expression and human liver cancer progression and that GPER1 inhibition can prevent E2-induced increase in HCC incidence, suggesting a novel target for liver cancer treatment.

Our findings in Chapter 3 have elucidated a cell-autonomous impact of E2 on hepatocytes. However, the liver constitutes heterogeneous cell populations including resident macrophage (Kuffer cells), stellate cells, and biliary epithelial cells, and the effects of E2/GPER1 signaling in these populations require further detailed investigation. A recent murine study has reported a protective role of E2 against HCC via suppression of inflammatory factors (Wei et al., 2016). Nevertheless, hepatic GPER1 expression was shown to be different in murine and human livers. Indeed, while
expression of GPER1 in human liver has long been reported (Owman et al., 1996; Carmeci et al., 1997; O'Dowd et al., 1998), GPER1 was undetectable in murine livers (Mårtensson et al., 2009; Otto et al., 2009; Bonini et al., 1997), demonstrating the species difference regarding levels of hepatic GPER1. The fact that GPER1 was expressed in zebrafish liver, as shown in Chapter 3, emphasizes that zebrafish is not only a relevant but can also be an ideal model for studying GPER1 in human liver development and diseases. Together, using pharmacological, molecular and cellular approaches along with quantitative in vivo imaging and modeling in zebrafish, our hope is that our insights will advance current understanding of E2 biology and that these findings can be translated into therapeutic applications such as that for cancer treatments.


Xin, M., Kim, Y., Sutherland, L.B., Qi, X., McAnally, J., Schwartz, R.J., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2011). Regulation of insulin-like growth factor
signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. Sci Signal 4, ra70.


APPENDICES

APPENDIX 1: Supplemental materials for Chapter 2
Figure S2.1 A chemical genetic screen identifies E2 signaling as a negative regulator of embryonic zebrafish liver development.

(A) Chemical genetic screen workflow. Wild type (WT) or fbp10a:GFP transgenic reporter fish were exposed to individual compounds from a library of 2640 known bioactives over the course of embryonic liver development (18-72 hpf). Changes in liver size were detected by fluorescence microscopy and ISH for fbp10a. (B) Seven compounds were identified in the screen. Estrogenic compounds 17β-estradiol (E2), estriol (E3), 17α-ethynylestradiol (EE), diethylstilbestrol, and quercetin decreased liver size, whereas the aromatase inhibitor chrysin and estrogen receptor antagonist tamoxifen increased liver size.
Figure S2.2 Optimal E2 signaling is required for normal liver development

(A) Representative images of transgenic embryos Tg(fabp10a:GFP) exposed to DMSO, E2, or Ful from 24-72 hpf at 72 hpf (top panel). Liver size of DMSO, E2, Ful-exposed embryos from 24-72 hpf as assessed by ISH for transferrin at 72 hpf. E2 decreased liver size (52% or 50/96, 50 larvae with phenotype out of 96 total embryos observed) while Ful decreased liver size (37% or 13/35) (bottom panel).

(B) Embryos exposed to DMSO, E2, or bisphenol A (BPA) from 18-72 hpf developed smaller livers compared to controls. Progesterone and testosterone exposures did not impact liver development as demonstrated by normal fabp10a expressions.
Figure S2.3 Estrogen receptor 2b (esr2b) mediates impacts of E2 on embryonic liver size

(A) Genomic structure of zebrafish esr2b with lengths of introns (lines) and exons (boxes) which includes 5’UTR (white box) and coding exons (black boxes). Sequence alignment of esr2b in esr2b+/+ and esr2b−/− embryos indicating TALEN generated 5 base pair deletion in the first exon. (B) Expression of esr2b in esr2b+/+ and esr2b−/− embryos at 72 hpf, showing esr2b expression in the liver (dotted line and red arrowheads) in wild-type but not in esr2b−/− mutants. Scale bars, 200 μm.
**Figure S2.4 E2 signaling affects hepatic lineages**

(A) Double transgenic with endothelial cell and hepatocyte reporter Tg(flk1:mCherry; fabp10a:GFP) embryos exposed to DMSO, E2, or Ful from 24-72 hpf. (B) Images of WT and cloche-/- embryos exposed to DMSO or E2 from 24-72 hpf. Liver size was analyzed by ISH for fabp10a at 72 hpf. E2 decreased liver size in WT (80% or 28/35) at a similar extent to E2-induced decrease in liver size in cloche-/- embryos (100% or 8/8). cloche-/- mutants had smaller liver compared to WT (73% or 16/22). (C) Embryos exposed to DMSO or E2 from 24-72 hpf were ISH for fabp10a, trypsin, and insulin. E2 decreased liver size but did not affect exocrine or endocrine pancreas. Scale bars, 200 μm.
Figure S2.5 Estrogen receptors are downregulated during hepatic differentiation

(A) Read coverage of chromatin immunoprecipitation followed by sequencing (ChIP–seq) of histone H3 lysine 4 monomethylation (H3K4me1) along human ERα and ERβ showing lower levels of H3K4me1 in ERβ regulatory regions in differentiated liver compared to embryonic stem cells (ESC) or endoderm. (B) RNA expression values of E2 signaling pathway components and hepatic markers in ESCs (teal), endoderm (purple), hepatoblasts (yellow), and hepatocytes (green). Expression values were measured as fragments per kilobase of exon per million fragments mapped (FPKM).
APPENDICES

APPENDIX 2: Supplemental materials for Chapter 3
Figure S3.1 Estrogen increases liver growth and size.
(A) Heatmap representing genes significantly enriched upon E2 treatment compared to gender-matched DMSO control of male and female livers (fold change > 10 from DMSO). n=2 for male, female, male+E2, and female+E2. Scaled expression value is plotted in green-red color scale with green indicating low expression and red representing high expression. Hierarchical clustering analysis is based on Pearson correlation. (B) Venn diagram showing intersection of genes differentially expressed in the livers of male vs male+E2, female vs female+E2, and female vs male. (fold change > 10). Values demonstrate number of genes upregulated by E2 in male liver (299 genes), female liver (125 genes) or female-associated liver genes (75 genes). (C) Heatmap representing polar metabolite abundance as determined by LC–MS/MS of male and female livers with and without E2, n=5 for male, female and female+E2, and male+E2. Scaled metabolite abundance value is plotted in green-red color scale with green indicating low abundance and red representing high abundance. Hierarchical clustering is based on Pearson correlation.
Figure S3.2 GPER1 mediates the estrogenic effects on liver growth.

(A) Representative images of WT larvae with increased liver size upon E2 or G-1 treatments, decreased liver size upon G-15 treatment, and normalized liver size upon E2 and G-15 co-treatment as assayed by ISH for fabp10a at 120 hpf. Scale bar, 200 μm. (B) Quantification of GFP+ hepatocytes in fabp10a:GFP larvae by FACS (fold from DMSO). n=3 independent experiments of 30 pooled larvae, mean ± SEM, **p<0.01, ****p<0.0001, two-tailed Student's t-test; ****p<0.0001, one-way ANOVA. (C) Quantification of liver area as determined by ISH for fabp10a (fold from DMSO). n as indicated, mean ± SEM, **p<0.01, ****p<0.0001, ns=not significant, two-tailed Student's t-test. (D) Representative images of liver size as assessed by ISH for fabp10a at 120 hpf. Scale bar, 200 μm. (E) Representative images of 5xERE:GFP reporter fish exposed to DMSO, 5hrs of E2, 24hrs of E2 at 120hpf. Scale bar, 200 μm. (F) Distribution of % 5xERE:GFP fish with high, low, or no GFP expression upon E2 exposures was plotted. n = 50, 47, 24 for number of larvae exposed to DMSO, 5hrs (110-115 hpf) of E2, and 24hrs (96-120 hpf) of E2 respectively.
Figure S3.3 GPER1 mediates the estrogenic effects on liver growth.
(A) Expression of gper1 transcripts in whole embryo as measured by RT-PCR compared to elf1a control. (B) Expression of gper1 transcripts in whole embryo as measured by quantitative RT-PCR (fold from gper1 expression at 12 hpf). (C) Lateral and dorsal view of gper1 expression in larvae at 72, 96, 120 hpf after ISH for gper1. gper1 is expressed in the liver (red arrowhead) starting at 96 hpf. (D) Genomic organization of zebrafish gper1. Black boxes represent exons with ATG site indicated by the arrow. Sequence alignment of gper1+/+ siblings and gper1−/− mutant showing TALEN generated 29 base pair deletion leading to premature stop codon (*). (E) Immunoblot analysis of GPER1 (red arrow) and β-actin levels (F) Images of gper1+/+ and gper1−/− larvae ISH for foxA3 (endoderm) and prox1 (hepatic progenitor) at 48 hpf, prox1 and fabp10a (hepatocyte) at 72 hpf, fabp10a and deltaC (biliary tree) at 120 hpf. (G) Quantification of marker expression area in gper1+/+ and gper1−/− larvae as assessed by ISH (fold from gper1+/+). n as indicated, mean ± SEM, **p<0.01, ****p<0.0001, ns = not significant, two-tailed Student’s t-test. Scale bars, 200 μm.
Figure S3.4 Estrogen increases cell proliferation in the liver.

(A) BrdU analysis of whole larvae exposed to DMSO or E2 at 120 hpf. Larvae were pulsed with BrdU and stained for BrdU after fixation. Larvae treated with E2 had higher number of BrdU positive cells per fixed liver area compared to DMSO-exposed controls. Scale bar, 400 μm, scale bar (inset), 100 μm, n as indicated, mean ± SEM, ***p<0.001, two-tailed Mann-Whitney test.
Figure S3.5 E2 signals via GPER1- PI3K/mTOR pathway to increase liver size.
(A) Liver size of WT larvae after chemical exposures as assessed by ISH for fabp10a at 120 hpf. E2 exposure increased liver size (27% or 21/78, 21 larvae with phenotype out of 78 total larvae observed) while PI3K inhibitor LY292002 decreased liver size (72% or 55/76). Co-treatment of E2 and LY292002 blocked estrogenic effect on liver size (86% or 59/69). (B) Liver size of WT and gper1MO knockdown larvae after chemical exposures at 120 hpf. (C) Liver size of gper1+/− and gper1−/− larvae at 120 hpf after chemical exposures. E2 or G-1-exposed gper1−/− larvae had increased liver size (red arrowhead) that can be blocked by co-exposure of E2+Rapa or G-1+Rapa. gper1−/− blocked E2 and G-1 effects on liver size. (D) Liver area of gper1+/− and gper1−/− larvae ISH for fabp10a at 120 hpf after chemical exposures. n as indicated, mean ± SEM, ****p<0.0001, ns = not significant, two-tailed Student’s t-test. ns=not significant, one-way ANOVA. † indicates significant difference from DMSO-treated WT controls. ‡‡p<0.01, ‡‡‡‡p<0.0001, two-tailed Student's t-test. (E) Liver size distribution of WT larvae and mtor morphants upon exposure to DMSO or E2 as % of larvae with large (dark green), medium (light green) or small (grey) livers. (F) Liver size of WT and mtor morphants. (G) WT larvae at 120 hpf immunostained for p-Akt and p-S6 upon DMSO or E2 exposure. Liver is outlined in black. All scale bars, 200 μm.
Figure S3.6 GPER1 is essential for male-biased response of estrogen on liver size.

(A) RNA sequencing data showing relative expression in Fragments Per Kilobase of transcription per Million mapped reads (FPKM) of ESR1, ESR2, and GPER1 in human primary hepatocytes and in mouse fibroblast cocultured cells.
Figure S3.7 Activation of E2/GPER1 signaling promotes male liver cancer initiation and progression.

(A) Histological features of adult zebrafish liver stained with H&E. Top panel shows zoomed images from normal male liver (a), normal female liver (b), and livers with hepatocellular adenoma (HCA) (c), hepatocellular carcinoma (HCC) (d), and cholangiocarcinoma (CC) (e). Scale bar, 25 μm. Bottom panel demonstrates low magnification images of normal male liver (f), normal female liver (g), and livers with large tumor (h), small tumor (i) and multi-foci tumor (j). Scale bars (f)-(i), 100 μm, scale bar (j), 250 μm. (B) Liver tumor size (mm) per fish in DMBA-exposed WT fish followed by DMSO, E2 or E2+G-15 exposure up until 9 mpf. n as indicated, mean ± SEM, *p < 0.05, **P < 0.01, two-tailed Student’s t-test. (C) Quantification of GPER1 staining scores of non-cirrhotic livers, cirrhotic livers, adjacent non-tumor (ANT) tissues and HCC tissues. n as indicated, mean ± SEM, *p<0.05, ns=not significant, two-tailed Mann-Whitney test. (D) Images of GPER1 staining scoring system. 0 = minimal or no staining; 1+ = faint/mild staining; 2+ = moderate/strong staining. Scale bar, 50 μm. (E) GPER1 staining scores in non-cirrhotic male and female livers. n as indicated, mean ± SEM.
### Supplementary Table 3.1 PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'- 3')</th>
<th>Reverse Primer (5'- 3')</th>
<th>LTR (5'- 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>gper1</td>
<td>TCAAGTTGCCGTCACAAT GC</td>
<td>GTCATCCTCTCCCTGTGG TT</td>
<td></td>
</tr>
<tr>
<td>ef1α</td>
<td>GCGTCATCAAGAGCGTTG AG</td>
<td>TTGGAACGGTGATGTTGA GG</td>
<td></td>
</tr>
<tr>
<td>mtor</td>
<td>ATAAGAAAAAGAAACCACAT GTCATACC</td>
<td>CTTACCACCTCAGAGAC CAAAG</td>
<td>CCCTAAG TACTTGTA CTTTCACT TG</td>
</tr>
</tbody>
</table>

### Supplementary Table 3.2 RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'- 3')</th>
<th>Reverse Primer (5'- 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>gper1</td>
<td>CTCGTGAATAAAGTGTTGCAG</td>
<td>GCAGTCTTTTCTCCAG</td>
</tr>
</tbody>
</table>
### Supplementary Table 3.3 Morpholinos

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5' - 3')</th>
<th>Type</th>
<th>Amount Injected (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>esr1</td>
<td>AGGAAGGTTCCCTCCAGGGCTTCTCT</td>
<td>ATG</td>
<td>2</td>
</tr>
<tr>
<td>esr2a</td>
<td>ACATGGTGAAAGCCGGATGAGTTCAG</td>
<td>ATG</td>
<td>2</td>
</tr>
<tr>
<td>esr2b</td>
<td>AGCTCATGCTGGAGAACACAAGAGA</td>
<td>ATG</td>
<td>2</td>
</tr>
<tr>
<td>gper1</td>
<td>ACATTGGTAGTCTGCTCCTCCATGC</td>
<td>ATG</td>
<td>2</td>
</tr>
<tr>
<td>gper1</td>
<td>GCTGCAACACCTGTATAAGAGAAA</td>
<td>Splice</td>
<td>2</td>
</tr>
<tr>
<td>mtor</td>
<td>GGTGTGACACATTACCTGAGCATG</td>
<td>ATG</td>
<td>2</td>
</tr>
<tr>
<td>control</td>
<td>CCTCTACCTCAGTTACAATTTATA</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>
### Supplementary Table 3.4 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Application</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>α - PCNA</td>
<td>IHC</td>
<td>1:200</td>
<td>Anaspec, AS-55421</td>
</tr>
<tr>
<td>α - Pan-Cadherin</td>
<td>IF</td>
<td>1:1000</td>
<td>Sigma, C3678</td>
</tr>
<tr>
<td>α - BrdU</td>
<td>Whole-mount IHC</td>
<td>1:500</td>
<td>Sigma, B2531</td>
</tr>
<tr>
<td>α - Akt</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling, 9272</td>
</tr>
<tr>
<td>α - pAKT(Ser473)</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling, 4060</td>
</tr>
<tr>
<td></td>
<td>Whole-mount IHC</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>α - mTOR</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling, 2983</td>
</tr>
<tr>
<td>α - S6</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling, 2217</td>
</tr>
<tr>
<td>α - pS6(Ser240/244)</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling, 2215</td>
</tr>
<tr>
<td></td>
<td>Whole-mount IHC</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>α - β-actin</td>
<td>WB</td>
<td>1:5000</td>
<td>Cell Signaling, 4970</td>
</tr>
<tr>
<td>α-Rabbit (Alexa Fluor®647)</td>
<td>IF</td>
<td>1:500</td>
<td>Abcam, ab150075</td>
</tr>
<tr>
<td>α-Rabbit IgG-HRP</td>
<td>IHC,WB</td>
<td>1:1000</td>
<td>Santa Cruz, sc-2004</td>
</tr>
<tr>
<td>α-GPER1</td>
<td>IHC</td>
<td>1:50</td>
<td>Sigma, HPA027052</td>
</tr>
<tr>
<td>α-HNF4α</td>
<td>IF</td>
<td>1:50</td>
<td>Abcam, ab55223</td>
</tr>
</tbody>
</table>
Supplementary Table 3.5 Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
<th>Supplier, Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Estradiol</td>
<td>10 μM</td>
<td>Tocris, 2824</td>
</tr>
<tr>
<td>MPP dihydrochloride</td>
<td>80 μM</td>
<td>Tocris, 1991</td>
</tr>
<tr>
<td>PHTPP</td>
<td>8 μM</td>
<td>Tocris, 2662</td>
</tr>
<tr>
<td>G-15</td>
<td>60 μM (embryo)</td>
<td>Tocris, 3678</td>
</tr>
<tr>
<td></td>
<td>10 μM (adult)</td>
<td>Tocris, 3678</td>
</tr>
<tr>
<td>G-1</td>
<td>8 μM</td>
<td>Tocris, 3577</td>
</tr>
<tr>
<td>Anastrozole</td>
<td>10 μM</td>
<td>Tocris, 3388</td>
</tr>
<tr>
<td>740 Y-P</td>
<td>2 μM</td>
<td>Tocris, 1983</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>1 μM</td>
<td>Tocris, 1292</td>
</tr>
<tr>
<td>LY294002 HCl</td>
<td>15 μM</td>
<td>Tocris, 1130</td>
</tr>
<tr>
<td>NSC 228155</td>
<td>5 μM</td>
<td>Calbiochem, 530536</td>
</tr>
<tr>
<td>Erlotinib HCl</td>
<td>10 μM</td>
<td>Selleckchem, S1023</td>
</tr>
<tr>
<td>MK-2206 2HCl</td>
<td>5 μM</td>
<td>Selleckchem, S1078</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>10 μM</td>
<td>Sigma, M3761</td>
</tr>
</tbody>
</table>