THE ROLE OF LIVER RECEPTOR HOMOLOG-1 IN NORMAL AND CANCEROUS MOUSE MAMMARY GLAND DEVELOPMENT

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&

Faculty of Life and Social Science

Submitted in total fulfilment of the requirements for the degree of
Doctor of Philosophy

January 2014
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GENERAL DECLARATION

Swinburne University of Technology

Higher Degrees by Research

Declaration for thesis based on conjointly published and unpublished work

In accordance with Swinburne University guidelines PhD including associate papers the following declarations are made:

I, Kyren Aloysious Lazarus hereby declare that this thesis contains no material which has been accepted for award of any other degree or diploma at any university of equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the examinable outcome.

This thesis contains two original papers published in peer reviewed journals and two unpublished manuscript in review with their respective journals. Two additional conjointly authored publications in peer reviewed journals appear in the Appendices. The core theme of this thesis is the role of LRH-1 in mammary epithelial cells. The ideas, development and writing up of all the papers in the thesis were the principle responsibility of myself; the candidate working within Prince Henry’s Institute of Medical Research and through the Faculty of Life and Social Science, under the supervision of Dr Colin Clyne, Dr Ashwini Chand and Dr Lara Grollo.

The inclusion of co-authors reflects the fact that the work came from active collaborations between researchers and acknowledges input into team based research.

In the case of chapters three, four, five and the appendices, my contribution to the work involved the following:
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<td>3</td>
<td>Oestradiol reduces Liver Receptor Homolog-1 mRNA transcript stability in breast cancer cell lines.</td>
<td>Published Biochemical and Biophysical Research Communications (2013), 438 (3): 533-539.</td>
<td>80% overall (100% experiments in paper, 100% contribution to experiments within Addendum).</td>
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<td>Conditional over-expression of LRH-1 in female mouse mammary epithelium results in altered mammary morphogenesis via the induction of TGF-β</td>
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<td>Liver Receptor Homolog-1 over expression increases incidence of DMBA-induced mammary tumours</td>
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<td>Appendix B1</td>
<td>Liver receptor homologue-1 expression in ovarian epithelial and granulosa cell tumours</td>
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Date:

2. **Lazarus KA**, Zhao Z, Knower KC, To SQ, Chand AL, Clyne CD (2013), Oestradiol reduces Liver Receptor Homolog-1 mRNA transcript stability in breast cancer cell lines, Biochemical and Biophysical Research Communications, 438, 3, (533-539)


5. Chand AL, Wijayakumara DD, Knower KC, Herridge KA, Howard TL, **Lazarus KA**, Clyne CD (2012) The Orphan Nuclear Receptor LRH-1 and ERα Activate GREB1 Expression to Induce Breast Cancer Cell Proliferation, PLoS ONE 7(2)


Abstracts (presenting author underlined)

1. Lazarus KA, Chand AL, Clyne CD, 2013, “LRH-1 increases incidence of DMBA-mediated mammary tumours” Australian Breast Cancer Conference, Melbourne


3. Lazarus KA, Chand AL. Clyne CD, 2013, “Altered mammary morphogenesis in Liver Receptor Transgenic Mouse Model is mediated via Transforming Growth factor -1 ” The Endocrine Society 95th Annual meeting, San Francisco.


6. Zhao Z, Lazarus KA, Chand AL, Clyne CD, 2013, “LRH-1 regulates hRNPA1 and A2 expression in order to modulate cell proliferation in breast cancer cells” 25th Lorne Cancer Conference, Australia.


10. **Lazarus KA**, Herridge KA, Clyne CD, Chand AL 2011, “Generation of a doxycycline inducible mammary specific Liver Receptor Homolog-1 knock in mouse model” Jackson Laboratory Course Conference, CA #8, Maine, USA.


13. **Lazarus KA**, Herridge KA, Clyne CD, Chand AL 2011, “Generation of a doxycycline inducible mammary specific Liver Receptor Homolog-1 knock in mouse model” Southern HealthResearch Week, CA #8, Monash Medical Centre, Australia – *Second Place – Cancer research category $250*


15. **Fleming NI, Lazarus KA**, Fuller PJ, Simpson ER, Clyne CD, (2010), “Aromatase is a direct target of FOXL2:C134W in granulosa cell tumours via a single highly conserved binding site in the ovarian specific promoter” Southern Health Research Week. *First place - Cancer research category*

AWARDS AND PRIZES ARISING FROM THIS THESIS

2013
1. Awarded Travel Grant for 56th Annual Scientific Meeting, Endocrine Society of Australia, ($200)

2. Finalist, ESA-Novartis Junior Scientist Award, Endocrine Society of Australia meeting, Sydney

3. Highly Commended Talk, Three Minute Thesis 2013, Prince Henry’s Institute, ($100).

4. Invited to participate in Presidential poster competition, ENDO Society, USA - Rescinded

5. Endocrine Day Training day awards, ENDO Society, USA. Awarded free registration ($600) and $400 toward travel costs to the society meeting.

2012
6. Travel Award, Postgraduate Conference Scheme Award Round 1, Faculty of Life and Social Sciences, Swinburne. Awarded scholarship for accommodation costs ($500).

2011
7. Poster Award – 2nd Prize, Cancer Research, Southern Health Research Week, Monash Medical Centre, ($250)

8. Competitively accepted into world renowned course by Jacksons Laboratory titled “20th Annual Short Course on Experimental Models of Human Cancer”. Awarded full waiver of course fees, ($1,500)

2010
9. Awarded Travel Grant for 53rd Annual Scientific Meeting, Endocrine Society of Australia, $250
10. Poster Award – 2nd Prize, Cancer Research, Southern Health Research Week, Monash Medical Centre, ($250)

11. PhD Research supported by Swinburne University Postgraduate Research Award, Swinburne University of Technology, $22,500 per annum. 
ACKNOWLEDGEMENTS

The acknowledgement section of the thesis is one of the most understated and underrated sections since without the help and support of the numerous unnamed individuals my development as an independent researcher would not have been possible. I am indebted to each and every one of these individuals.

Supervisors and members of the Cancer Drug Discovery lab

First of all, I would like to thank my two principal supervisors Dr Colin Clyne and Dr Ashwini Chand. In 2009, I contacted Ashwini since her proposed projects that involved the use of animal models in elucidating novel therapeutical targets in breast cancer excited me. Since she was about to take maternity leave, she pointed me in the direction of Colin who graciously accepted an honours student with no prior experience with animal models into his lab. His open door policy, willingness to talk through complex ideas and at the same time not intrusive is a style that really allowed me to develop as an independent researcher. He has forever instilled in me the importance of “controls in every experiment” and impeccable grammar. Colin you have been a fantastic supervisor and a great mentor. I honestly believe that your support has been a key factor in my scientific career progress.

My immense gratitude is also extended to my supervisor Ashwini who guided me through my first real-time PCR experiment. Since then, Ashwini has been instrumental to my lab performance where we have shared many intellectual and informal discussions. Like Colin, she also believed in an open door policy, and would frequently accompany me down to the animal house were we would discuss openly about the project and other matters while excising mouse mammary glands. Ashwini you have been a fantastic supervisor and I am forever indebted for your help during my PhD. Her scientific knowledge perfectly complemented that of Colin’s and this provided me with the benefit of a perfect environment in which to learn and develop as an independent researcher.

My associate supervisor, Dr Lara Grollo, was a great deal of help during my candidature. Since she is based at Swinburne University, Lara and I only met three to four times a year during which we would have lengthy conversations about my progress and any problems that arose at PHI. I have enjoyed our discussions and really appreciate the help and guidance you have given me during my PhD, many thanks.
Dr Kevin Knower has been my “brother from another mother” during my PhD. I can never forget the conversation he had with my mum during my first interstate conference in Sydney. Our general interests in scientific, basketball, and music topics have been a source for many a conversation. He introduced me to his basketball team which I then replaced him as the star, while he was shunned to the side-lines due to “injuries”. Thanks for all the help Kevin. Dr Chantal Magne de has been one of my best friends in and out of the lab. She guided me through my first western blot and ever since has been close at hand to monitor my lab experiments. We have had lengthy discussions about a range of topics ranging from LRH-1 and Timeless to the relationship between religion and science. I will be ever grateful for our friendship and hope that it continues for years to come.

I would also like to thank the many members of the Cancer Drug Discovery group who I am privileged to be a part of including Rhiannon Coulson, Tamara Howard, Kerrie Herridge, Vanessa Cheung and Kimmy Zhao.

**Prince Henry’s Institute**

I will be forever grateful for my time at PHI, as I have been able to develop professional relationships with many individuals that walked the corridors of this great institute. At every level of management, I have come across genuinely kind and helpful individuals who have wanted nothing but the best for me. In particular, I would like to thank Dr Peter Stanon who has mentored me through my PhD. Dr Kristy Brown who I have had many conversations about the arduous nature of completing a PhD and has kindly helped me during times of need. I will always cherish those conversations. Finally, I would like to thank Dr Sarah Meachem for your continued support during my candidature. During my time as a research assistant with Sarah, I quickly learnt the nature of politics in science and the effective ways to deal with any situations that arose from professional relationships.

**Monash Institute of Medical Research**

Although not affiliated with this institute, I liaised with many of its staff. In particular, Dr Neil Watkins and his team who have helped me extensively with the DMBA study. The first time I met Neil was actually quite serendipitous. He had judged my poster at a Southern Health Poster competition and a few weeks later I ran into him at the Monash gym. We had a lengthy discussion about the complexities of my animal study. Soon, I found myself in Neil’s office, having a conversation about my project and how he could help me with the oral gavage. Neil has been a
great mentor and I will always be grateful for the time he took out of his busy clinical and research schedule to listen to my project and suggest avenues of research direction. Dr Jason Cain and Samantha Jayasekara have been instrumental in helping me during the DMBA study and in particular Jason for always willing to have a chat. I will always be grateful to this team.

PhD students that have walked the walk with me

I would like to thank all the students who have been on this journey with me. For all the discussions we had in the student rooms, whether it was about our projects, our supervisors or a surprisingly lengthy conversation about thesis binding colour options (Sarah To and Jenna Haverfield). For all the extracurricular activities that we undertook and all the fun we had during this journey together. It has been a real unforgettable privilege. Finally a special mention to Jimmy Ham, who has been a dear friend and colleague during my PhD. He has kept me company during my after hours and weekend stays at work. He has been the undisputed king of immunofluorescence; and whose knowledge and help I am very grateful for.

To my other PhD comrades past and present; Dimuthu Alankarage, Huiting Ho, Niru Samarageewa, Xuyi Wang, Daniel Czech, Rajini Sreenivasan, Justin Chen, Amy Winship, and Peter Nicholls it has been a blast.

My family and friends

I owe everything that I am to my Mum, Debra Lazarus and Dad, Keenan Lazarus. They really have been instrumental in making me the person I am. I have observed the value of hard work, diligence and perseverance that my parents exemplified during my upbringing. They have been extremely supportive and very proud of me during my achievements and failures. Dad has always managed to make light of a bad situation especially when I complained that I was not successful in a competition or grant. I will always be grateful to them for giving me a nurturing environment and every opportunity to establish a career in Australia. Nathan, my brother has been always there and has been extremely proud of my achievements, often boasting about me to his friends. Little does he know that even though he is my younger brother, I often look up to him for strength and guidance. Thank you for your understanding during this process. My friends Will, VJ, Brandon, Bruce, Rahul, Avon, Reuben, Michael and Nalin have kept things in perspective during my PhD.

Lastly my fiancé Agnetta Fernandes, who even though has been 2733 kms away, has been my closest friend. Since embarking this project, she of all people has sadly felt the greatest impact.
Thank you for the tireless support, constant encouragement and a breath of fresh air when I needed it the most. I am grateful for being able to share every achievement and failure with you and the time we spent on the phone, on Skype or together makes me forget about all the stresses of work. You have been my rock and for that I will be forever grateful. In many ways this is as much your achievement as it is mine, lots of love. I can’t wait for the next steps in our journey together.

I will close this little bit with a quote which I have relied on during the writing of this thesis:

“All things are difficult before they are easy”
- Dr Thomas Fuller
The liver receptor homolog-1 (LRH-1) is a transcription factor that is expressed in and plays a functional role in developing and adult tissue of endodermal origin, including liver, pancreas and the ovary. It is an orphan member of the nuclear receptor superfamily. LRH-1 is implicated in cancer where it promotes cell proliferation by inducing key cell cycle genes Cyclin D1/E1. In the breast, LRH-1 synergises with the oestrogen receptor-α (ERα) to promote cancer cell proliferation, motility and invasion.

In order to explore novel LRH-1 targets genes and dependent mechanisms, a microarray was performed using LRH-1 depleted MCF-7 cells blocked by siRNA strategies. LRH-1 blockade in vitro revealed a reduction of TGF-β signalling. This thesis uncovered TGF-β1 as a novel LRH-1 target gene and direct physical interaction between the two factors. To confirm these findings a novel doxycycline inducible mammary epithelial specific LRH-1 transgenic mouse model was employed. A microarray on mammary tissue from dox treated animals confirmed induction of TGF-β isoform and downstream signalling in response to LRH-1 overexpression. In virgin animals, the consequence of LRH-1 overexpression was examined and we observed altered mammary gland morphogenesis. These findings were attributed to the induction of TGF-β in the mouse mammary glands.

Whilst our in vitro studies suggest a clear role and function for LRH-1 in breast cancer cell proliferation via both oestrogen-dependent and -independent mechanisms, they have been limited to cell lines. Hence, using this transgenic mouse model, the role of LRH-1 on mammary tumorigenesis was assessed by challenging the LRH-1 transgenic animals with a chemical (DMBA) carcinogen. We showed that LRH-1 increased incidence of DMBA-mediated mammary tumours. Furthermore, we demonstrate increased β-catenin nuclear localisation coupled with an increase in cyclin D1 and E1 transcript expression, which suggest irregular cell cycle leading to increased mammary epithelial cell proliferation.

Combined, these data suggest that LRH-1 may play a dual role in mouse mammary epithelial cells, where it inhibits mammary ductal development in virgin animals, but promotes mammary carcinogenesis in a challenged environment. These findings provide significance of the potential of LRH-1 as a novel therapeutical target for breast cancer treatment.
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<td>AF-1</td>
<td>Activation Function -1</td>
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<td>AF-2</td>
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<tr>
<td>cAMP</td>
<td>cyclic 3',5' – Adenosine Monophosphate</td>
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<td>DAX-1</td>
<td>The Dosage-sensitive, sex reversal Adrenal hypoplasia congenital critical region on the X chromosome gene 1</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
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<td>DCIS</td>
<td>Ductal Carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>DNA</td>
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<td>Epidermal Growth Factor</td>
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CHAPTER 1:
LITERATURE REVIEW
1.1 Introduction

The liver receptor homolog-1 (LRH-1) is a transcription factor and part of a large family of nuclear receptors (see review (Lazarus et al. 2012)). Unlike other nuclear receptors such as the steroid hormone receptor; oestrogen receptor alpha (ERα), LRH-1 is one of many orphan nuclear receptors which are characterised by the lack of a known physiological ligand. LRH-1 is not only important in cholesterol metabolism, but it also regulates stem cell pluripotency, steroidogenesis. Importantly, LRH-1 is expressed in gastric, pancreatic and intestinal tissue where it is implicated in cancer progression (Fayard et al. 2004, Lazarus et al. 2012). Due to the multiple functions and tissue-specific functions of LRH-1, controlling LRH-1 by specific ligands could have high therapeutic potential. Recently, its role in breast cancer has gained some research interest and examination of LRH-1 immunoreactivity revealed elevated expression in breast epithelial carcinoma tissue (Miki et al. 2006). In breast cancer cells LRH-1 enhances cell motility, invasion and proliferation (Chand et al. 2010, Chand et al. 2012). However, a complete understanding of the function of LRH-1 in the breast is limited by the use of breast cancer cell lines “in vitro”. Generating an in vivo model of LRH-1 overexpression in murine breast will allow one to assess its importance in mammary gland development and tumorigenesis.

Understanding mammary gland development and tumorigenesis in rodent models has been instrumental in advancing the study of the human breast and breast cancer development. Nuclear receptors such as the ERα have been shown to play key roles in mediating mammary gland development. However, the role of orphan nuclear receptors in normal breast development and cancer are less understood. This may be due to the fact that deletion of these receptors often have serious detrimental effects that cause embryonic lethality long before the development of the rudimentary epithelial tree occurs (Pereira et al. 1999, Collins et al. 2004, Pare et al. 2004). To overcome this, mammary gland-specific knockouts or transgenic mice for key orphan receptors have been developed to explore their function in these tissues.

The objective of this thesis is to examine the function and significance of overexpressing LRH-1 in mouse mammary epithelial cells. Therefore its role in normal mammary gland development and in a carcinogenic environment will be explored. In this literature review, I will discuss the current knowledge of LRH-1, its function and its mode of regulation. The importance of nuclear receptors in the breast will also be discussed. This literature review comprises primarily of a review article published in the Journal of Steroid Biochemistry and Molecular Biology.
addition, current opinions and recent findings have been incorporated as appropriate. Refer to Appendix B3 for the original review article.

1.2 Nuclear Receptor Superfamily

Nuclear receptors (NRs) comprise a large superfamily of mammalian transcription factors that, via the regulation of target genes, are essential in embryonic development, differentiation, metabolism and cell death (Gronemeyer et al. 2004, Benoit et al. 2006, Germain et al. 2006, Schweitzer et al. 2008). There are currently forty-eight members in the NR family (Mangelsdorf et al. 1995). Most NRs are ligand activated and these ligands include steroid hormones, fat-soluble vitamins A and D, and thyroid hormones. Hence they provide the critical link between the ligand and specific target genes through the recruitment of a range of positive and negative regulatory proteins, referred to as coactivators and corepressors (Olefsky 2001, Schweitzer et al. 2008). Nuclear receptors have been used as therapeutic targets for many diseases including breast cancers, skin disorders and diabetes. For a number of these nuclear receptors, classical exogenous ligands have not been identified and are currently considered to be orphan nuclear receptors (Benoit et al. 2006). The number of orphan receptors has decreased over the past two decades as excitingly new ligands have been discovered. Due to the significant role they play in the body and the ability to bind to synthetic compounds, orphan nuclear receptors represent a tremendous opportunity in understanding and treating human disease.

1.2.1 Nuclear Receptor Structure

As a family, NRs share a common highly conserved domain structure (Fig.1). The activation function domain (AF-1) is located at the amino terminus, and is a highly divergent region that regulates NR transcriptional activity independent from ligand binding (Kumar and Litwack 2009). The AF-1 domain at the N-terminus of the receptor is one of the two major sites for the binding of nuclear receptor co-regulators and is an important site for post-translational modifications (Lavery and McEwan 2005). The DNA-binding domain (DBD) is defined by two cysteine-rich zinc finger motifs that permit binding of the receptor to regions in the DNA known as hormone response elements (HREs) (Freedman et al. 1988). This region is also essential to the homo- and hetero-dimerization of nuclear receptors (Freedman et al. 1988). Proximal to the DBD, is the flexible hinge region which contains the nuclear localisation signal (NLS) (Picard et al. 1990). Recent studies have demonstrated the presence of two structurally different types of NLS regions that can independently mediate nuclear localisation of LRH-1 by an importin α/β
mechanism (Yang et al. 2011). The carboxyl-terminal ligand binding domain (LBD) and the activation function-2 (AF2) are essential for hormone recognition and further co-regulator binding (Gronemeyer et al. 2004). In some cases, the LBD is also responsible for receptor dimerization and repressing the nuclear receptor (Robinson-Rechavi et al. 2003). Precisely how the LBD is involved in the action of the NR will be discussed below.

Receptors that are activated by known ligands act in a ligand-inducible manner and control a plethora of gene networks during development, differentiation, homeostasis and disease (Olefsky 2001). However, as aforementioned, no known classical nuclear receptor ligands have been identified for orphan NRs and they may respond to a range of signals, including post-translational modifications such as phosphorylation and ubiquitination; and other molecules such as fatty acids and xenobiotics (Gronemeyer et al. 2004). The binding of a ligand to its NR induces its translocation into the nucleus where it either dimerises as homodimers or some NRs form heterodimers with the retinoid X receptor α (RXRα) (Olefsky 2001). The receptor dimer then binds to specific response elements (RE). The REs contain a characteristic DNA sequence motif, that consists of two half site motifs that form either direct or inverted repeats and are separated by a spacer (Mangelsdorf et al. 1995). Almost two decades ago Mangelsdorf proposed four categories of nuclear receptors where Class I receptors include the known steroid hormone receptors, which function as homodimers and bind to the inverted repeats. Class II receptors exist as heterodimers with RXR receptor partners and bind to direct repeats. The second two classes include the orphan NRs which either act as homodimers (Class III) and bind to direct RE repeats or monomers that bind to single REs (Class IV) (Mangelsdorf et al. 1995).

1.2.2 Nuclear Receptor Action

The activation of ligand dependent NRs involves the recruitment of coactivators, during which the AF-2 plays an important role. The so called “mouse-trap” model of NR transactivation where the ligand binding induces a conformational change in the LBD allowing the coactivators to bind (Giguere 1999). In this model, the AF-2 motif folds back against the LBD upon ligand binding, closing the ligand binding pocket and creating a novel interface involving residues from the AF-2 and three other helices (Giguère 1999). The LBD has been the main focus for drug discovery due to its ligand binding and coactivator recruitment. The LBD contains 12 α helices that are arranged around a central hydrophobic pocket, with helices 3, 7 and 10 making up the pocket. Upon engagement of a ligand, there is an alteration in the orientation of the α-helices and β-sheets, most notably in the C-terminal helix 12 (H12) which is associated with the AF-2 (Wurtz
et al. 1996). H12 undergoes a conformational shift exposing a hydrophobic binding groove that recruits coregulators containing one or more LXXLL motifs (Westin et al. 1998). These motifs are a contiguous sequence of five amino acids where L = leucine and X = any amino acid (Plevin et al. 2005). The binding of the ligand induces the recruitment of coactivators or corepressors.

These factors bind to the NR using LXXLL motif within their polypeptides and associate with H12. Functionally, an agonist will enhance the interaction of the LBD and one or more coactivators. In contrast an antagonist positions itself on the H12 in order to block the site of coactivators thereby blocking its transcription. This is the case for oestrogen receptor where the binding of an antagonist to the H12 helix reduces its transactivation by oestrogen. Since NRs play key roles in physiological process such as development, metabolism, growth, reproduction; targeting them have been vital in the discovery of very successful drugs.

1.2.3 Nuclear Receptors as Drug Targets

Due to the large number of roles NRs play in biology and the vast genetic networks influenced by these receptors, NRs are of great interest in modern biomedical research and drug discovery. These drugs are currently administered in clinical settings to treat diseases such hypertension, diabetes, cancer and a huge range of cardiovascular, metabolic and reproductive diseases (Gronemeyer et al. 2004). Over the last few decades, there have been considerable advances in developing drugs against NRs such as oestrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR) and glucocorticoid receptor (GR) to treat various diseases. For example the development of selective oestrogen receptor modulators (SERMs) has been successfully used to treat breast cancer patients.

Technological advances such as high throughput screening, genome-wide association studies, and pathway analysis tools will enable new discoveries which will lead to drugs successfully used to target NRs in complex diseases. A better understanding of ligand-induced transcription that produces tissue-selective effects will enable the development of more efficient drugs with fewer unwanted side effects. In contrast to ligand-regulated NRs, the orphan nuclear receptors typically display constitutive transcriptional activity. This characteristic trait highlights the potential for these orphan nuclear receptors as novel drug targets.
1.2.4 Orphan Nuclear Receptors

Orphan receptors play diverse and important biological roles in development and adult physiology even in the absence of a ligand (Schoonjans et al. 1997, Xie and Evans 2001, Benoit et al. 2006, Sonoda et al. 2008). However, even in the absence of a ligand the H12 is positioned for maximal activation and coregulator recruitment (Greschik et al. 2004, Flaig et al. 2005). Excitingly, recent crystallisation studies have demonstrated natural or synthetic compounds binding and activating these orphan receptors. Since they are known to play crucial roles in development, homeostasis and disease, the knowledge that they could be regulated by synthetic compounds makes them good targets for drug discovery. Compounds with specific activities for specific orphan receptors will be of clinical and pharmacological relevance.

The identification of these orphan nuclear receptors came about from the efforts of low-stringency screening of cDNA libraries with the well conserved DBD as a probe (Giguère 1999). This cloning exercise led to the discovery of proteins with structural features found in nuclear receptors but no known linked ligand. Hence, these receptors were termed “orphan receptors”. A majority of orphan receptors possess all the functional domains of a classical NR; however some receptors lack AF-1 or 2 functional domains. The orphan nuclear receptor dosage sensitive sex reversal-adrenal hypoplasia congenital gene on the X chromosome, gene 1 (DAX-1) and small heterodimer partner (SHP) lack a functional DBD, but are able to bind to DNA via other mechanisms and interacting with other proteins. However, other orphan receptors bind to DNA much like the classical NR, via specific hormone RE (HRE). Additionally, it has become clear that the binding of coregulators is vital to the transcriptional activity of the orphan nuclear receptors (Riggins et al. 2010).

As aforementioned the research into synthetic ligands that may regulate orphan NRs is vital to drug development as these factors play an important role in biology. Also compounds that target regions outside the NR and play an important role in its transactivation, may have a potential as novel therapeutical treatment. The evolution of technology has seen the rise of virtual screening of compound libraries with the crystal structure of the orphan nuclear receptor. This has led to the development of selective ER modulators (SERMs) and antagonists for retinoic acid receptor (RAR) and thyroid receptor (TR) (Schapira et al. 2000, Schapira et al. 2003). This review will focus on the structure, function and modulation of an orphan nuclear receptor, NR5A2 or more commonly liver receptor homolog-1 (LRH-1).
1.3 The Liver Receptor Homolog-1

1.3.1 An Overview of the LRH-1

Liver Receptor Homolog-1 (LRH-1; NR5A2; FTZ-F1; FTF; CPF) is an orphan nuclear receptor belonging to the NR5A subfamily of nuclear receptors that plays vital roles in embryogenesis, nutrient regulation and cancer (Chapter 1, Figure 1). LRH-1 has been shown to be expressed in various species such as drosophila, mouse, frog, chicken, horse, zebrafish and human (Kudo and Sutou 1997, Boerboom et al. 2000, Chai and Chan 2000, Takase et al. 2000, Pare et al. 2001). LRH-1 was initially described as fetoprotein transcription factor, and as an essential activator of the α-fetoprotein gene in hepatocytes (Galarneau et al. 1996). The function of this gene is to protect the embryo from circulating maternal oestrogen by tightly binding to oestradiol (Baker et al. 2006). In addition LRH-1 was demonstrated to transduce early development signals to the albumin gene, which is an essential nutrient carrier to the mammalian embryo (Bernier et al. 1993, Galarneau et al. 1996). Taken together, it is not surprising that homozygous LRH-1 knockouts are embryonic lethal at E6.5-7.5 (Pare et al. 2004). As its name suggests, LRH-1 also plays an important role in early liver differentiation. During the later stages of organogenesis, LRH-1 expression pattern changes from endodermal tissue sites, to predominance in the liver, intestine and pancreatic cells. This expression pattern led to studies that showed that LRH-1 was a key regulator in genes involved in cholesterol homeostasis and bile acid synthesis (Nitta et al. 1999, Goodwin et al. 2000, Lu et al. 2000, Chen et al. 2003, Fayard et al. 2003, del Castillo-Olivares et al. 2004, Pare et al. 2004, Kim et al. 2005, Lee et al. 2008) These roles will be discussed in more detail in 1.3.5.
LRH-1 plays an important role in steroidogenesis and malignancy. It is implicated in gastric, intestinal and pancreatic cancer (Botrugno et al. 2004, Schoonjans et al. 2005, Benod et al. 2011). In breast tumour epithelial cells, it regulates ERα expression and is an ERα target gene (Annicotte et al. 2005, Thiruchelvam et al. 2011). Furthermore, it induces aromatase expression, the enzyme responsible for oestrogen biosynthesis in breast adipose stromal cells (Clyne et al. 2002). Together, these data demonstrate the tight synergy between LRH-1 and oestrogen signalling to promote breast cancer cell proliferation, motility and invasion. These roles will be discussed in 1.3.6.

LRH-1 and its mammalian homolog steroidogenic factor-1 (SF-1) binds to DNA as monomers to nuclear receptor half site sequences (Ueda et al. 1992, Freeman et al. 2004, Benoit et al. 2006). As LRH-1 is constitutively active (Fayard et al. 2004), its regulation of function

1.3.2 The Structure and Activity of the LRH-1

The human gene encoding LRH-1 spans more than 150 kb of chromosome 1q32.11 and has eight exons. Three isoforms of LRH-1 have been identified, implying that LRH-1 undergoes alternative splicing (Fayard et al. 2004). The dominant isoform hLRH-1v1 differs from the smaller isoforms hLRH-1 and hLRH-1v2 by the absence of exon 2. In addition the smallest isoform HLRH-1v2 is truncated in the C-terminus by an alternative splicing in exon 5 and cannot activate transcription (Fayard et al. 2004). Recently 5’- Rapid amplification of complementary DNA ends identified two new variants LRH-1 v4 and LRH-1 v5 (Thiruchelvam et al. 2011). The new variants initiate transcription in the newly named exon 2a and 2b respectively. Findings from this study reveal that LRH-1v4 is the predominant variant in breast cancer cells (Thiruchelvam et al. 2011). The expression patterns of these variants and their roles in other tissues remains to be identified.

Most NRs form either homodimers or some form heterodimers with RXRα to bind to their cognate response elements found in the promoters of the target genes. However, the binding of members of the NR5A subfamily is dictated by the Ftz-F1 box and it binds as a monomer to the extended half site YCAAGG YCR (where Y is any pyrimidine and R is any purine) (Ueda et al. 1992, Lee and Moore 2002, Fayard et al. 2004, Solomon et al. 2005). LRH-1 binding is orchestrated by the Ftz-F1 box which is a stretch of 26 amino acids at the C-terminal of the DBD. The ligand binding domain (LBD) of most NRs consists of twelve α-helical regions, folded into a three-layered, anti-parallel helical sandwich with a conserved β-turn between H5 and H6 (Fayard et al. 2004). The LBD also contains a pocket to which a ligand binds causing a reversible conformational change. This repositions H12 allowing for co-activator recruitment (Fayard et al.
2004, Solomon et al. 2005). Structural and molecular studies may hold the key to understanding the ligand-independent constitutive activity of LRH-1.

For LRH-1, the key to constitutive activation may lie in the N-terminal region of the LBD. This is strongly conserved amongst orphan receptors and shares limited sequence amongst other nuclear receptors (Fayard et al. 2004). The presence of an additional structure pertaining to an extension of an H2 layer may provide an explanation of the stabilised conformation, by stabilising H12 which contains the C-terminal activation function-2 helix (AF-2) (Fayard et al. 2004). The folding over of the AF-2 region allows the LBD to be held in an active conformation even in the absence of a ligand. The importance of the H2 was demonstrated by a decrease in activity and coregulator recruitment following the mutation of a conserved arginine (R352E) in H2 of LRH-1, which destabilizes H12 (Sablin et al. 2003). Currently there is a proliferation in the development of physiological and synthetic ligands as agonists and antagonists that are directed toward the LBD and AF-2 via molecular screening technologies. These will be discussed further.

1.3.3 LRH-1 Modulators as a Novel Therapeutic Target

Since its identification in 1996, there is accumulating evidence of LRH-1 as an important regulator of pathways involved in metabolism, steroidogenesis, cancer and regulation of pluripotency. Given its expression and functions in various tumours including invasive breast carcinomas, ductal carcinomas in situ, colon, gastric and pancreatic cancers, the potential impact of LRH-1 modulators as therapeutic targets should be considered. Recent progress with the identification of small molecule agonists (Whitby et al. 2006, Whitby et al. 2011) and phospholipid ligands will eventually lead to the development of selective LRH-1 modulators. There appears to be therapeutic benefits for both the activation of LRH-1 (in the liver) where it would be beneficial in the treatment of metabolic disease such as diabetes, and in the suppression of LRH-activity in tumour cells (gastric, pancreatic, intestinal, breast) where it would potentially have an anti-proliferative effect (Schoonjans et al. 2005, Wang et al. 2008, Chand et al. 2010, Benod et al. 2011).

As LRH-1 is expressed in inflammatory cells such as lymphocytes (Benod et al. 2011) and in surrounding breast adipose stromal cells, blocking of LRH-1 function would have additional anti-tumour effects such as down-regulating inflammatory pathways and reducing oestrogen synthesis within the tumour milieu. Given the importance of LRH-1 in metabolism and cancer, the regulation of its activity could potentially lead to important breakthroughs in treatment.
of these diseases. The regulation of LRH-1 function occurs via interaction with intracellular phospholipids, transcriptional co-regulators (CoRs) and post-translational modifications such as phosphorylation and sumoylation (Chapter 1 Figure 2). By considering these modes of regulation, new approaches to block or enhance LRH-1 activity may emerge.

Chapter 1 Figure 2: LRH-1 activity can be regulated by various mechanisms in a tissue specific manner. Regulation mechanisms include synthetic ligands, post-translational modifications and phospholipids. Co-regulators can also play a role in regulating LRH-1, which then modulates expression of target genes that are involved in metabolism, proliferation, pluripotency and steroidogenesis.

1.3.4 Regulation of LRH-1 Activity

1.3.4.1 Phospholipids as LRH-1 activators

LRH-1 is classified as an orphan nuclear receptor because 'classical' high-affinity acutely regulated ligands have not yet been identified. Crystallisation of the LRH-1 ligand binding domain (LBD) (mouse and human isoforms) identified bacterial phospholipids in the ligand binding pocket. Subsequently phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidyglycerol were shown to bind with human LRH-1 and SF-1 (Sablin et al. 2003, Krylova et al. 2005, Li et al. 2005, Ortlund et al. 2005, Wang et al. 2005). These findings demonstrate phospholipids as an emerging regulator of LRH-1. Functional studies of mouse LRH-1 LBD show that ligands are dispensable for activity (Sablin et al. 2003). Disruption of the size and shape of the hydrophobic ligand binding pocket does not appear to affect its transcriptional activity. However mutations in the human LRH-1 LBD reduce

In support of this idea, dilauroyl phosphotidylcholine (DLPC) and diundecanoyl phosphotidylcholine DUPC have emerged as strong LRH-1 activators in vitro and in vivo models (Lee et al. 2011). Studies in mice have shown that DLPC stimulates LRH-1 activity, increasing bile acid levels, lowering hepatic lipids and improving glucose homeostasis (Lee et al. 2008, Musille et al. 2012). Recently, structure-based discovery of antagonists for LRH-1 have demonstrated compounds “3” and “3d2” are able to inhibit the transcriptional activity of LRH-1 in a Biacore-based assay (Benod et al. 2013). In addition, these compounds are able inhibit proliferation of LRH-1 positive pancreatic, colon and breast cancer cell lines (Benod et al. 2013). However, addressing the tissue-specificity of these drugs will be a major concern.

1.3.4.2 Co-activators of LRH-1

Structurally LRH-1 is permanently held in an active conformation and binds to DNA as a monomer (Fayard et al. 2004, Freeman et al. 2004, Benoit et al. 2006). LRH-1 activity is primarily regulated by transcriptional co-regulators. LRH-1 can also act as a competence factor by binding to other orphan nuclear receptors and transcriptional complexes to enhance transcription of target genes. Peroxisome proliferator-activated receptor-γ-co-activator-1 α (PGC-1α) is a key co-activator of LRH-1 in the ovary, facilitating LRH-1 mediated differentiation of granulosa cells into progesterone producing luteal cells (Yazawa et al. 2010). This interaction is blocked by a known repressor of LRH-1 activity, DAX-1 in granulosa cells (Yazawa et al. 2010). Since both DAX-1 and PGC-1α can bind to LRH-1 (Yazawa et al. 2010), this indicates that the binding affinity of DAX-1 for LRH-1 is stronger than PGC-1α (Yazawa et al. 2010). Furthermore, the interaction between LRH-1 and PGC-1α is also evident in bile acid homeostasis (Shin and Osborne 2008). However this interaction in the liver is blocked by SHP-1 (Shin and Osborne 2008). Recently a genomic-wide interrogation of hepatic fairesoid X nuclear receptor (FXR), revealed that FXR binds to LRH-1, and LRH-1 is required for the FXR mediated activation of SHP, Rdh9, Pcx and Pemt (Chong et al. 2010).

In human adipose stromal cells, PGC-1α enhances LRH-1 dependent aromatase promoter II transcription (Clyne et al. 2002). The p-160 family members steroid receptor co-activators (SRC-1 and SRC-3) also regulate LRH-1 activity (Xu et al. 2004). These co-activators contain an LXXLL motif in the receptor interaction domain; short peptide sequences derived from these co-
activators are shown to bind LRH-1 LBD (Safi et al. 2005). Multi-protein bridging factor (MBF-1) which also interacts with LRH-1 does not possess the LXXLL motif, typical for most co-activators, but interacts with the TATA-binding protein (TBP) (Brendel et al. 2002). This interaction is either in isolation or through the recruitment of transcription factor IID complex (Brendel et al. 2002).

1.3.4.3 Co-repressors of LRH-1

Nuclear receptors SHP and DAX-1 act as repressors of many NRs including LRH-1 by inhibiting co-activator binding (Francis et al. 2003). The repression of LRH-1 by SHP has been well-defined due to its high interaction with the nuclear receptor (Goodwin et al. 2000, Lu et al. 2000, Lee and Moore 2002). Like most co-repressors, SHP binds to the AF-2 region of LRH-1 (Fayard et al. 2004). The LRH-1–SHP interaction occurs via interaction with LRH-1 residues Arg361 and Glu534 which form an atypical charge clamp (Ortlund et al. 2005). Upon SHP interaction, p160 co-activators are competed out for binding to the CoR domain of LRH-1. In addition to the N-terminal receptor interaction domain, SHP includes a C-terminal domain with autonomous repression function (Lee and Moore 2002). In breast adipose stromal cells and in hepatocytes, SHP represses LRH-1 activity with the blocking of PGC-1α and LRH-1 interactions (Shin and Osborne 2008). This action in hepatocytes is shown to be mediated via the recruitment of SIRT-1 by SHP (Chanda et al. 2010). SHP is a well-known target gene of LRH-1 and recent reports show a cooperative action with FXR to activate SHP transcription (Chong et al. 2010).

DAX-1 colocalizes with LRH-1 and SF-1 in granulosa cells (Yazawa et al. 2010) and in mouse embryonic stem cells (Kelly et al. 2010). Crystallisation of mouse DAX-1 with LRH-1 LBD indicates that its N-terminus LXXLL-related motifs interact directly with LRH-1 (Ito et al. 1997, Suzuki et al. 2002, Suzuki et al. 2003). It has also been reported that the C-terminus end is essential for DAX-1 mediated repression (Lopez et al. 2001). DAX-1 binds to the AF-2 domain as a dimer, binding with high affinity in contrast to most repressors which interact via their single LXXLL motif (Sablin et al. 2008). It is hypothesised that the DAX-1 dimer binds LRH-1 in a “claw-like” fashion, one of the DAX-1 structures extending into the ligand binding pocket. This feature may act as a sensor for ligand binding, and or additional interactions with components of the transcriptional machinery (Suzuki et al. 2003, Sablin et al. 2008).

LRH-1 function is also inhibited by the protein inhibitor of activated signal transducer and activator of transcription-γ (PIASγ) (Hsieh et al. 2009). This inhibition occurs due to the
competitive binding of PIAS γ on the AF2 domain, which is involved in the binding of co-activator SRC-1. However, the over-expression of SRC-1 could partially overcome the LRH-1 mediated induction of CYP11A1 (Hsieh et al. 2009). The silencing mediator for retinoid and thyroid receptors (SMRT) is also shown to represses LRH-1 through an indirect mechanism currently unknown (Fayard et al. 2004).

The role of transcriptional CoRs is critical in the modulation of transcription factor activity. In the case of orphan nuclear receptors, co-regulators may well be the most important mode of functional regulation. Evidence of co-localisation and interaction between CoRs and LRH-1 suggest tissue and cell specific modulation of LRH-1 action. Could targeting the disruption of these CoR interactions provide a means to selectively repressing or activating LRH-1 activity? Due to the presence of SHP and DAX-1 in breast cancer (Conde et al. 2004, Insabato et al. 2009), it is tempting to postulate their roles in repressing LRH-1 in breast cancer cells. However this avenue requires further investigation.

1.3.4.4 Post-transcriptional regulation of LRH-1

In addition to CoRs, the activity of LRH-1 is modulated by post-translational modification. Phosphorylation of the serine residues 238 and 243 located within the hinge region of LRH-1 via phorbol 12-myristate 13-acetate (PMA), was found to be important for LRH-1 transactivation (Lee et al. 2006). Sumoylation of LRH-1 also occurs in the hinge region, allowing for additional control of its activity via regulation of its subcellular localisation (Yang et al. 2009). Sumoylated LRH-1 is localised to transcriptionally inactive nuclear bodies, away from active chromatin. Interestingly, the newly identified phosphatidylycholine agonists of LRH-1, DPLC and DUPC, activated LRH-1 mutants lacking phosphorylation sites (S238, 243A) or the sumoylation site (K270R) (Lee et al. 2011). The double mutants F342W and I426W targeting the ligand binding pocket where not activated by DPLC or DUPC highlighting the importance of the ligand binding pocket in regulating LRH-1 activity.

1.3.4.5 Synthetic ligands

The putative ligand for LRH-1 has been the subject of debate for a number of years. The identification of synthetic agonists for LRH-1 and SF-1 supports the notion of ligand dependent activity of LRH-1 (Whitby et al. 2006). GSK8470, a substituted cis-bicyclo [3.3.0] -oct -2-ene, was identified as a high affinity ligand for both LRH-1 and SF-1. This molecule led to increased expression of the LRH-1 target gene SHP in liver cells (Whitby et al. 2006). Modifications to this
molecule at 3 different sites also led to the identification of other agonists with varying degree of potency and efficacy. One such molecule, RWJ101 was shown to be selective for LRH-1, however further work is being undertaken to improve its efficiency (Whitby et al. 2011). Recently, antagonists for LRH-1 were developed and were identified as three novel benzothiophene derivatives (Rey et al. 2012).

An inverse agonist was identified for SF-1; 4-(heptyloxy)phenol (AC-45594) and its analogues were shown to regulate SF-1 activity and subsequently down regulate SF-1 target genes (Del Tredici et al. 2008). This molecule had no effect on LRH-1 activity indicating that despite their structural similarities, distinct ligands for LRH-1 and SF-1 could be synthesized. Finally, Benod et. al. recently identified small molecule antagonists by screening and molecular studies. These compounds, named (3) and (3d2) antagonise the proliferative activity of LRH-1 (Benod et al. 2013). The future of the development of this drug is bright, and the further characterisation of these synthetic drugs will lead to therapeutics for diseases mediated by LRH-1.

1.3.5 Established roles of LRH-1: Cholesterol Metabolism

A key biological function of LRH-1 is the regulation of cholesterol metabolism via its effect on bile acid homeostasis. LRH-1 regulates enterohepatic development and function via the expression of key genes involved in the regulation of bile acid synthesis, cholesterol homeostasis and transport (Nitta et al. 1999, del Castillo-Olivares and Gil 2000, Goodwin et al. 2000, Lu et al. 2000, Wang et al. 2001, Chen et al. 2003, Fayard et al. 2003, Francis et al. 2003, Fayard et al. 2004, Freeman et al. 2004). LRH-1 activates gene transcription of the rate-limiting enzyme in bile acid biosynthesis, cytochrome P450 family 7A1 or Cholesterol 7 α-hydroxylase (Cyp7A1) (Goodwin et al. 2000, Lu et al. 2000, del Castillo-Olivares et al. 2004, Shin and Osborne 2008). In humans, the loss of function of Cyp7A1 results in a decrease in bile acid excretion and an increase in hepatic and serum cholesterol levels (Pullinger et al. 2002). However, LRH-1+/− heterozygous mice have elevated CYP7A1 and CYP8B1 mRNA levels (Pare et al. 2004). This may be due to a dominant effect of LRH-1 on Cyp7A1 and Cyp8B1 transcription.

Interestingly, hepatocytes and intestinal epithelium (IE) specific LRH-1 knockout mice showed no overt abnormalities (Lee et al. 2008). Furthermore, LRH-1 deficiency in the IE had no effect on Cyp7A1 levels. This discrepancy in observations may be due to the minor role LRH-1 plays in Cyp7A1 regulation, or there might be an alternative redundant factor regulating Cyp7A1 in the absence of LRH-1 (Lee et al. 2008). Further work need to be undertaken in determining the
role of LRH-1 in the feedback mechanism of bile acid synthesis. In contrast to Cyp7A1, Cyp8B1 levels were significantly decreased with LRH-1 deficiency, consistent with previous findings.

LRH-1 positively regulates expression of other enzymes and transporters involved in reverse transport of cholesterol and bile acid synthesis pathways. These genes contain the LRH-1 response element (nuclear receptor half site) in their promoters and they include cytochrome P450 family 8B1 (Cyp8B1) or Sterol 12α hydroxylase, multidrug resistance protein 3 (MRP3), cholesteryl ester transfer protein (CETP), scavenger receptor class B type 1 (SR-B1), mouse apical sodium-dependent bile acid transporter (ASBT) and human apolipoprotein A1 (ApoA1) (Nitta et al. 1999, Goodwin et al. 2000, Lu et al. 2000, Wang et al. 2001, Bohan et al. 2003, Chen et al. 2003, Fayard et al. 2004, Yazawa et al. 2009). Consistently, selective knockout of LRH-1 in hepatocytes results in the significant ablation of expression of these target enzymes (Lee et al. 2008).

The consequence of the activation of these enzymes are outlined. Increase in ApoA1 allows the initiation of high density lipoprotein (HDL) biosynthesis and for ApoA1 to act as an acceptor of cholesterol and phospholipids effluxed from peripheral tissues. Up regulation of SR-B1 receptors allows the transfer of mature HDL particles from plasma into hepatocytes (Schoonjans et al. 2002). CETP transfers the cholesteryl esters from plasma HDL into Apolipoprotein B-containing triglyceride-rich very low density lipoproteins (VLDL). These VLDL particles are acted upon by lipoprotein lipase and the fatty acids are taken up by the adipose and skeletal muscle. The cholesterol-rich remnants are then taken up by the liver (Packard et al. 2000, Chen et al. 2003).

Within hepatocytes, cholesterol and cholesteryl esters are converted to bile acids by Cyp7A1 and Cyp8B1 for secretion out of the liver in bile. Furthermore MRP3 and ASBT are involved in bile acid recycling, indicating that LRH-1 is important for bile acid homeostasis (Inokuchi et al. 2001, Bohan et al. 2003). Major subsets of LRH-1 gene targets are involved in the transfer of cholesterol to the liver and subsequent elimination into bile acids, and in bile acid synthesis, highlighting the importance of LRH-1 in cholesterol metabolism (Pullinger et al. 2002).
1.3.6 Emerging Roles of LRH-1 in Disease

1.3.6.1 Embryonic stem cells: pluripotency and differentiation

As aforementioned, LRH-1 is a critical factor in early embryonic development, and LRH-1 null mice die at embryonic day 6.5-7.5 with features typical of visceral endoderm dysfunction (Pare et al. 2004). One of the key factors essential for maintaining pluripotency in embryonic stem cells (ESCs), Oct4 is regulated by LRH-1 (Gu et al. 2005, Wagner et al. 2010). Its role in Oct4 regulation is confirmed with the loss of Oct4 expression in the absence of LRH-1 (Gu et al. 2005). In addition to Oct4, LRH-1 is known to regulate other factors required to maintain pluripotency (Guo and Smith 2010, Miyamoto et al. 2011).

A report suggests that LRH-1 regulates Oct4 and Nanog induction and its expression is regulated via the Wnt signalling pathway, via binding of β-catenin to activate an embryonic-specific LRH-1 promoter (Wagner et al. 2010) thus identifying a new pathway for the regulation of self-renewal in ESC. In addition a recent study indicates that LRH-1 acts as a transcriptional activator in the regulation of Oct4 gene expression through the direct binding with LRH-1/SF-1 binding sites (Sung et al. 2012).

Somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) with the introduction of Oct4, Sox2 Klf4, and c-Myc (Takahashi and Yamanaka 2006). Of these factors, Oct4 cannot be replaced by other factors to allow for the generation of iPSC from somatic cells (Nakagawa et al. 2008). A study entailing a screen of nuclear receptors for their ability to enhance reprogramming efficiency led to the discovery that LRH-1, and to a lesser extent SF-1 could replace Oct4 in the reprogramming of mouse embryonic fibroblasts to iPSCs further indicating its vital role in the regulation of Oct4 and maintaining pluripotency (Gu et al. 2005, Heng et al. 2010).

A point mutation within the LRH-1 LBD (A368M) that interferes with the binding of endogenous phospholipids (Sablin et al. 2003) did not abrogate its reprogramming capacity, suggesting that phospholipid binding is not essential for this function. However, since LRH-1 is constitutively active, and its activity appears to be regulated primarily by binding of protein co-regulators (see below), further studies into looking at the exact function of phospholipids in terms of regulating LRH-1 function are required. The possibility remains that pharmacological modulation of co regulator interactions could impact LRH-1 function in stem cells.
Recent work suggests that a transcriptional partner of LRH-1 required to activate Oct4 expression is DAX-1. DAX-1 is abundant in ESCs and it plays a role in maintaining pluripotency (Wang et al. 2006). DAX-1 expression is controlled by LRH-1 and Nanog (Kelly and Hammer 2010). In mouse ESC, DAX-1 interaction with LRH-1 does not inhibit but rather activates Oct4 gene transcription [47]. This is thought to be due to the interaction of steroid receptor RNA activator (SRA) with DAX-1 to allow an activation function (Kelly et al. 2010). This interaction of LRH-1 with DAX-1 is evidence of importance of co-regulator interaction in the modulation of LRH-1 activity. It also raises the possibility that co-regulator interaction allows for tissue specific LRH-1 functions.

1.3.6.2 Ovarian function: steroidogenesis and luteinisation

LRH-1 is expressed at relatively high levels in the ovary, with highest expression in granulosa cells and corpora lutea, with no expression observed in theca cells (Hinshelwood et al. 2003). Recent work has shown that LRH-1 is highly expressed in granulosa cell tumours (GCTs) (Chand et al. 2013). Although SF-1 is also present in the ovary its expression is lower in LRH-1 expressing cells and highest in the theca (Hinshelwood et al. 2003). The two nuclear receptors are differentially regulated, SF-1 mRNA and protein increased in granulosa cells by oestradiol, whereas LRH-1 expression was increased by FSH in granulosa cells and by prolactin in luteal cells [64]. Both receptors have been shown to regulate ovarian steroid synthesis although the relative roles of each are still unclear, since they both share common target genes including aromatase, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (HSD3B2) and inhibin α subunit (Falender et al. 2003, Liu et al. 2003, Peng et al. 2003, Kim et al. 2005, Weck and Mayo 2006).

Recent work has suggested that LRH-1 preferentially regulates progesterone over oestrogen production (Saxena et al. 2004, Saxena et al. 2007, Yazawa et al. 2010). Consistent with this, granulosa cell-specific LRH-1 null mice exhibit anovulation due to failure of cumulus expansion, luteinisation, and follicular rupture (Duggavathi et al. 2008). Loss of LRH-1 in granulosa cells also impaired progesterone synthesis due to reduced expression of StAR and cytochrome P450 side-chain cleavage (P450scc). Interestingly aromatase expression was unaltered. This dramatic phenotype highlights the potential of LRH-1 antagonists as novel contraceptives, since loss of LRH-1 inhibits both ovulation and luteinisation (Duggavathi et al. 2008). Furthermore, a recent study demonstrated that corpus luteum-specific LRH-1 null mice exhibit impairment in luteal function, decidualization, placental formation and gestation via its
effect on progesterone production (Zhang et al. 2013). Interestingly, the authors show that Wnt4, which is essential for placental development is a direct target for LRH-1 (Zhang et al. 2013). Recent work has also shown LRH-1 directly binds to and regulates 5-Aminolevulinic acid synthase 1 (ALAS1) which is a rate limiting enzyme for heme biosynthesis in steroidogenic tissues (Ju et al. 2012). In addition, LRH-1 was also shown to play an important role in hypothalamus in regulating the female reproductive axis by regulating the neuropeptide kisspeptin 1 in neurons in the arcuate (Arc) (Atkin et al. 2013).

1.3.6.3 Colon cancer and inflammation

Recent evidence indicates a role of LRH-1 in colon cancer development and progression. LRH-1 is highly expressed in intestinal crypts where it is involved in the control of cell proliferation and renewal (Botrugno et al. 2004). Botrugno et al have demonstrated that LRH-1 promotes intestinal cell proliferation by stimulating the expression of the G1 cyclins D1 and E1 (Botrugno et al. 2004). In these cells, LRH-1 co-activates β-catenin/Tcf4 to induce cyclin D1 and e-Myc expression, and also binds directly to the cyclin E1 promoter to promote transcription in synergy with β-catenin (Botrugno et al. 2004). Consistent with this, haplo-insufficiency of LRH-1 markedly protects against tumour development in both genetic (ApcMin/+) and chemical (azoxymethane) induced models of intestinal cancer (Schoonjans et al. 2005). Alterations in LRH-1 expression and localisation are also seen in human colon cancer, with increased LRH-1 expression observed in surface epithelial cells that are normally LRH-1 negative and non-proliferative (Schoonjans et al. 2005). However, the lack of significant proliferative phenotype in intestinal specific LRH-1 knock-out mice [41] raises questions as to the role of endogenous LRH-1 in vivo.

LRH-1 also plays an important role in glucocorticoid synthesis within the intestine by inducing expression of steroidogenic enzymes (cholesterol side chain cleavage enzyme and 11β-hydroxylase that catalyse the first and last steps in glucocorticoid formation, respectively) in intestinal epithelial cell lines (Mueller et al. 2006). This is likely of physiological relevance since haplo-insufficiency of LRH-1 in mice reduced expression of these enzymes and glucocorticoid synthesis in response to immunological stress (Mueller et al. 2006). Similar effects were seen in mice with an inducible intestinal epithelial deletion of LRH-1 (Coste et al. 2007). Consistent with these findings, expression of both LRH-1 and its steroidogenic target genes were reduced in patients with Crohn’s disease and ulcerative colitis (Coste et al. 2007).
1.3.6.4 Pancreatic cancer

Recent genome-wide association studies have linked mutations in the LRH-1 gene and its up-stream promoter regions with pancreatic cancer (Petersen et al. 2010). In addition, LRH-1 expression is found to be elevated in human pancreatic ductal adenocarcinomas (Benod et al. 2011). In pancreatic cancer cells, LRH-1 also mediates tumour cell proliferation via the up regulation of cyclins D1, E1 and c-Myc. Conversely knockdown of LRH-1 expression resulted in cell cycle arrest but not apoptosis in pancreatic cancer cells. This study implicates an important role for LRH-1 in mediating proliferation and differentiation pancreatic ductal adenocarcinomas and therefore a potential therapeutic target for this cancer (Benod et al. 2011).

1.3.6.5 Metabolic disorders

One of the key sites of LRH-1 expression and function is in the hepatocytes where it regulates transcription of genes such as Cyp7A1, Cyp8B1, encoding enzymes critical in bile acid and cholesterol synthesis; scavenger receptor class B member 1 (SR-B1) important for cholesterol transport and SHP, an LRH-1 co-repressor with known roles in obesity and diabetes (Lee et al. 1999, Goodwin et al. 2000, Schoonjans et al. 2002). Whilst these roles in the liver are well characterised, until recently the effect of LRH-1 in metabolic disorders was not well understood. A recent study (Lee et al. 2011) describes two phosphatidylcholine phospholipids, dilauroyl phosphatidylcholine (DPLC) and diundecanoyl phosphatidylcholine (DUPC) as potent agonists of LRH-1, binding to the ligand binding pocket of the human receptor. This is an extension to previous findings where structural analysis of LRH-1 identified bacterial phospholipids bound in the ligand binding pocket (Krylova et al. 2005, Ortlund et al. 2005). These two lipid ligands, DPLC and DUPC potently activate NR5A receptors: LRH-1 (human and mouse homologues) and SF-1 (mouse homologue), however, show no effects on other nuclear receptor function. Lee and colleagues hypothesised that the activation of liver-specific activity of LRH-1 would increase bile acid production thereby having a positive metabolic effect in diabetic and obese disease models.

In diet induced obese mice, oral administration of DPLC resulted in increased bile acid production, decreased hepatic steatosis and improved glucose utilisation (Lee et al. 2011). Additionally in liver specific LRH-1 knockout animals, DPLC treatment did not show any improvement in the high fat diet induced metabolic phenotype. This suggests a direct effect on LRH-1 receptor activity and/or the downstream activation of LRH-1 dependent pathways in regulating insulin sensitivity. Due to the absorption and clearance of these lipid ligands, the primary site of LRH-1 activation appeared to be the liver and SF-1 activity in the adrenal was not
affected. This study provides an interesting prospect of DPLC and DUPC, which are components of dietary lecithin, as a liver-specific LRH-1 ligand.

1.3.6.6 Breast cancer

In human breast adipose, LRH-1 is expressed in low levels, localising specifically in the stromal fraction (Clyne et al. 2002). However in breast cancer, LRH-1 expression is high and localised both in tumour epithelial cells and intra-tumoural stroma (Annicotte et al. 2005, Miki et al. 2006, Knower et al. 2013). High expression is observed in primary invasive breast carcinoma and ductal carcinoma in situ (Annicotte et al. 2005). Although the effects of LRH-1 in breast cancer are not fully understood, evidence suggests that its roles are tightly integrated with the oestrogen signalling pathway (Chapter 1 Figure 3).

One of the first identified target genes of LRH-1 in breast adipose stromal cells is $CYP19A1$ (Clyne et al. 2002, Clyne et al. 2004, Zhou et al. 2005), encoding for cytochrome P450 family 19A1 or aromatase. Aromatase is a critical enzyme required for the conversion of androgens to oestrogens. After menopause, when the risk of developing oestrogen-dependent breast cancer is greatest, local aromatase expression in adipose tissue represents the major source of oestrogen in women (as well as in men). Aromatase activity is regulated primarily by transcription of the $CYP19A1$ gene via tissue specific promoters (Means et al. 1991, Mahendroo et al. 1993, Simpson et al. 1993). By activating $CYP19A1$ transcription, LRH-1 most likely regulates the availability of mitogenic oestrogen for tumour growth (Clyne et al. 2004). In the tumour context, the aberrant expression of LRH-1 allows the activation of the gonadal-specific aromatase promoter (promoter II). LRH-1 expression in adipose stromal cells is positively regulated by breast tumour derived factors such as prostaglandin $E_2$ (Zhou et al. 2005). In addition to stromal cells, aromatase is present in breast cancer epithelium (Miki et al. 2007), and its expression also regulated by LRH-1 in breast cancer epithelial cells (Bouchard et al. 2005).

A second level of cross-talk is evident in that expression of LRH is itself regulated by oestrogen in breast cancer cells. Annicotte et al reported that the human LRH-1 promoter contains a near-perfect palindromic oestrogen response element (ERE) (Annicotte et al. 2005), to which ER$\alpha$ binds to stimulate promoter activity. This is consistent with the observed correlation between LRH-1 mRNA levels and ER$\alpha$ status in a variety of breast cancer cell lines (Annicotte et al. 2005), as well as the known positive association between LRH-1 positivity and ER$\alpha$ status in primary human breast carcinoma (Miki et al. 2006, Thiruchelvam et al. 2011). Knockdown of LRH-1
expression with siRNA in MCF-7 cells inhibits the proliferative effect of oestrogen (Annicotte et al. 2005), suggesting that the mitogenic effects of oestrogen may be mediated, in part, via LRH-1.

Furthermore LRH-1 regulates expression of ERα in breast cancer cells; siRNA knockdown of LRH-1 in MCF-7 cells inhibits expression of both ERα, and of ERα target genes such as Trefoil Factor 1 (TFF1, pS2), whereas transfection of LRH-1 into these cells stimulates ERα expression (Thiruchelvam et al. 2011). This effect is mediated by LRH-1 binding directly to the major ERα promoter used in breast cancer cells (Thiruchelvam et al. 2011). Therefore, LRH-1 both regulates, and is regulated by ERα in addition to regulating the synthesis of its ligand. In addition, LRH-1 synergizes with ERα to activate oestrogen target genes Growth Regulation by Breast Cancer 1 (GREB1) and pS2 (Chand et al. 2012). Recently, this intimate relationship between LRH-1 and ERα was furthered by a study demonstrating co-regulation of genes by LRH-1 and ERα. This study confirmed previous work showing synergistic binding of LRH-1 and ERα at oestrogen responsive elements controls the expression of oestrogen-responsive genes (Lai et al. 2013).
Chapter 1 Figure 3: LRH-1 has dual functions in the breast cancer cell. In cancer associated fibroblasts, it is capable of activating the \textit{CYP19A1} gene that encodes the enzyme aromatase which is the key steroidogenic enzyme for oestrogen biosynthesis. In tumour epithelial cells, LRH-1 synergises with oestrogen signalling to promote breast cancer cell proliferation, motility and invasion.

Although the above evidence indicates that LRH-1 induces proliferation by stimulating oestrogen signalling, LRH-1 importantly also has oestrogen-independent effects on breast cancer cells. siRNA-mediated knockdown of LRH-1 inhibited breast cancer cell motility, invasion and colony formation in both ER +ve MCF-7 and ER -ve MDA-MB-231, as well as the non-tumourgenic MCF-10A mammary epithelial cell line (Chand et al. 2010). Over-expression of LRH-1 (in the absence of oestrogen) produced the opposite effects.
In addition, over-expression of LRH-1 resulted in the post-translation cleavage of mature 120kDa E-Cadherin to its inactive 97kDa form. This effect may be mediated by matrix metalloproteases (MMPs) since LRH-1 was also shown to induce MMP9 mRNA expression in MCF-7 cells (Chand et al. 2010), consistent with the known role of LRH-1 as a regulator of MMP9 expression in the ovary (Duggavathi et al. 2008). LRH-1 was also shown to influence actin remodelling in breast cancer cell lines (Chand et al. 2010) which, taken with its effects on E-Cadherin and MMP9, may suggest a role for LRH-1 in promoting epithelial to mesenchymal transition (EMT). Given that LRH-1 has well-characterised roles in mouse and human embryonic stem cells (Gu et al. 2005, Da-ming et al. 2006, Zhou et al. 2007, Xie et al. 2009), and that breast cancer stem cells possess many of the characteristics of cells undergoing EMT (Mani et al. 2008, Blick et al. 2010), the potential role of LRH-1 in promoting EMT is an intriguing possibility that merits further investigation.

These findings have proved an intriguing role for LRH-1 in the breast; however a more complete understanding of its role in the breast is limited by the lack of in vivo models. The mouse has been well identified as a viable in vivo model for understanding human breast development and breast cancer. This thesis will aim to understand the importance of LRH-1 in the breast by characterising a novel inducible LRH-1 mammary epithelial cell specific transgenic mouse model. Further details of this work will be explored in chapters 4 and 5.

1.3.7 Conclusion

Since LRH-1 is implicated in important cellular functions and plays a role in various diseases, it represents an attractive therapeutic target in infertility, cancer and metabolic disorders. LRH-1 has dual function in the breast. In cancer associated fibroblasts, it is capable of activating the CYP19A1 gene that encodes the enzyme aromatase which is the key steroidogenic enzyme for oestrogen biosynthesis. In tumour epithelial cells, LRH-1 synergises with oestrogen signalling to promote breast cancer cell proliferation, motility and invasion. Oestrogen is vital hormone for breast development and also is a potent hormonal stimulant for breast tumorigenesis. Current endocrine therapies for treating breast cancer works on the principle of inhibiting oestrogen availability in the breast tissue by either blocking oestrogen action at the receptor (through the use of SERMs) or by blocking the production of oestrogen by inhibiting aromatase (with the use of aromatase inhibitors). However the benefits of these hormone therapies are counter-balanced by acquired or de novo therapeutic resistance, as well as side effects that reduce patient compliance (van Leeuwen et al. 1994, Gail et al. 1999). Additionally, approximately 25%
of breast tumours do not express the ERα, and hormone therapies are therefore ineffective. A better understanding of the complex and intricate mechanisms that are involved in breast cancer development and progression is required in order to identify new therapeutic targets, particularly for patients who do not / no longer benefit from current anti-oestrogen therapies.

Interestingly, LRH-1 is expressed in oestrogen receptor negative breast tissue and studies in our lab have demonstrated LRH-1 protein is expressed in ER- breast cancer cell lines where it has a functional role in cell motility, invasion and proliferation (Miki et al. 2006, Chand et al. 2010, Chand et al. 2012). These findings indicate the importance of targeting LRH-1 in hormone independent tumours. Although these findings provide vital evidence of the importance of LRH-1 in ER- breast cancer, it is limited by the use of breast cancer cell lines. To further understand the importance of LRH-1 in the breast, we sought to develop a novel LRH-1 mammary transgenic mouse model. Understanding the signalling response to LRH-1 overexpression in the breast will provide us clues as to how to target the receptor especially in patients with hormone-independent tumours.

1.4 Questions

This review of the expression pattern of LRH-1 in breast cancer cell lines and its role in breast cancer leaves a number of outstanding questions, which include:

1. It is known from expression studies in vitro that LRH-1 transcript is positively associated with ERα positivity in breast cancer cell lines. Furthermore, LRH-1 transcript levels are known to correlate with ERα transcript levels in breast cancer cell lines. What is the expression pattern of LRH-1 protein in breast cancer cell lines and how does this associate with ERα positivity? What is the significance of this correlation in breast cancer cell lines?

2. What is the role of LRH-1 overexpression in mouse mammary glands? Since, it has an important role in early development; does it have an important role in mouse mammary gland development?

3. LRH-1 promotes breast cancer epithelial cell proliferation, invasion and migration in vitro. What is the role of LRH-1 in mammary epithelial cell proliferation in vivo?
Chapter 1: Literature Review

1.5 Aims of Thesis

Chapter 3: The aim of this chapter is to investigate the expression pattern of LRH-1 protein in breast cancer cells and to establish the significance of its correlation with ER positivity in vitro. Subsequently this chapter aims to identify the mechanisms of LRH-1 protein and transcript discordance observed in ER negative breast cancer cell lines.

Chapter 4: Using a combination of in vitro and in vivo strategies including characterising a novel inducible mammary specific LRH-1 transgenic mouse, the aim of this chapter is to understand the relationship between LRH-1 and TGF-β signalling, thereby exploring the role of LRH-1 in virgin mouse mammary gland development.

Chapter 5: Utilising the newly characterised transgenic mouse and by first examining the proliferative responses in normal mammary development under the influence of LRH-1 overexpression and then employing a chemical carcinogen 7, 12-Dimethylbenz(a)anthracene to explore the tumour promoting role of LRH-1, this chapter aims to elucidate the role of LRH-1 in mammary tumorigenesis.

1.6 Summary

LRH-1 is an orphan nuclear receptor that plays integral roles in development, steroidogenesis and cancer. In the breast, LRH-1 plays dual roles in the stroma and epithelial cells. In cancer associated fibroblasts, LRH-1 is capable of activating CYP19A1 gene that encodes for the enzyme aromatase. Aromatase is a key steroidogenic enzyme that is responsible for oestrogen biosynthesis. In tumour epithelial cells, LRH-1 synergises with oestrogen signalling to promote cell proliferation, invasion and migration. Expression of LRH-1 in breast cancer has been limited to one, relatively small Japanese breast cancer cohort and cell line models. Additionally, the expression of LRH-1 protein in ER- cell lines has not been explored. This thesis will explore the expression pattern of LRH-1 transcript and protein in breast cancer cell lines in order to elucidate its potential as a therapeutical target in hormone independent breast cancers.

Since work done by our lab and others have focused on using breast cancer cell lines to elucidate the role of LRH-1 on breast cancer proliferation, a novel in vivo model of LRH-1 overexpression to explore the role of LRH-1 on mammary gland development and proliferation
is described. In study 1 chapter 3, the results of a publication titled “Oestradiol reduces Liver Receptor Homolog-1 mRNA transcript stability in breast cancer cell lines” are described. These findings explore the expression pattern of LRH-1 in breast cancer cell lines, and analyse protein and transcript stability in order to elucidate discordance between protein and transcript expression. This chapter demonstrates that LRH-1 transcript and protein are highly stable in ER- breast cancer cell lines and its expression in ER- cells merits its potential as a novel therapeutical target for breast cancer.

In study 2 chapter 4, the results of a publication titled “Conditional activation of Liver Receptor Homolog -1 in the mammary epithelium of transgenic mice results in altered mammary morphogenesis via the induction of TGF-β” are described. Here, the novel relationship between LRH-1 and TGF-β in breast cancer cell lines is examined and subsequently a novel LRH-1 transgenic mouse model is utilised to further understand this relationship. This chapter demonstrates that LRH-1 partially inhibits mammary ductal morphogenesis by a decrease in lateral budding and this effect is in part mediated by activated TGF-β signalling.

In study 3 chapter 5, the novel LRH-1 transgenic mouse model that was described in chapter 4 is employed to elucidate the role of LRH-1 in promoting carcinogen induced mammary tumorigenesis in vivo. This chapter demonstrates that LRH-1 overexpression increases incidence of DMBA-mediated mammary tumours. These results suggest a dual role for LRH-1 in the mouse mammary, whereby it plays an inhibitory role mediated by active TGF-β signalling in developing mammary glands, and then promotes tumour development in a challenged environment.

This thesis provides for the first time important information for the role of LRH-1 in the mouse mammary epithelial cells and contributes to the overall knowledge of its potential as a novel therapeutic for breast cancer patients especially for those that have tumours that are hormone-independent.
CHAPTER 2:

MATERIALS AND METHODS

This chapter summarises the materials and methods used for this thesis and should be referred to for all of the following chapters. Any specific methods can be found in the individual results chapters.
## 2.1 General Materials

### 2.1.1 Chemicals and Reagents

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Glycine       Sigma
Histolene      Southern Health Medical Centre
ICI 182-780       Tocris Bioscience
Isopropanol    Merck Bioscience
L-glutamine    Sigma
Mayers Haemotoxilin    Sigma
NaCl           Sigma Aldrich
Nucleofector cell line solution V    Lonza
Oestradiol     Sigma
Oligo dT primers    Promega
Orange G loading buffer    Sigma Aldrich
Phenylmethanesulfonyl fluoride    Sigma Aldrich
Picric Acid    Sigma Aldrich
Random hexamer primers    Promega
Scott’s water    Sigma
Sodium dodecyl sulphate    Sigma Aldrich
TEMED           Biorad
Thymol          Sigma
Tween20         Sigma
Tris base       Sigma Aldrich
Xylene Cyanole FF    Sigma Aldrich
Yeast Extract    Sigma Aldrich

2.1.2 Cell Culture reagents
All reagents were kept under sterile conditions

75cm2 culture flask    Nunc
175cm2 culture flask    Nunc
6-well culture plates    Nunc
12-well culture plates    Nunc
Antimitotic/antibiotic    Gibco
Dubecco’s Modified Eagles Medium (DMEM)    Gibco
Dubecco’s Phosphate Buffered Saline    Gibco
DMEM/F-12 1:1      Gibco
Fetal Calf Serum     Gibco
HEPES buffer        Sigma Aldrich
Kanamycin           Gibco
L-Glutamine         Gibco
Leibovitz L-15      Gibco
Pantothenic acid    Sigma Aldrich
Penicillin/Streptomycin Gibco
RPMI Medium         Gibco
Sodium Pyruvate     Sigma Aldrich
Tryple Express      Gibco
Weymouths Medium    Gibco

2.1.3 Cell lines
All cell lines were sub cultured under suppliers prescribed conditions

COS-7 (ATCC CRL-1651)
HS-578t (ATCC HTB-126)
MCF-7 (ATCC HTB-22)
MCF-10A (ATCC CRL-10317)
MDA-MB-231
MDA-MB-361
MDA-MB-453
T47-D (ATCC HTB-133)
ZR-75

2.1.4 Antibodies
Specific details of all antibodies are stated in Appendix 1

2.1.5 Kits
All kits were utilized as per manufacturer’s instructions

Avidin and Biotin block
Dual-Luciferase kit Promega
2.1.6 Nucleic acid weight standards

1kb Plus DNA ladder  Invitrogen

2.1.7 Bacterial strains

JM109 competent cells  Promega
XL-1 blue competent cells  Agilent Technologies

2.1.8 Bacterial Media

Liquid media
Lysogeny Broth
1% (w/v) NaCl
1% (w/v) tryptone
0.5% (w/v) yeast extract

Solid media
Lysogeny-agar plates: 1.5% (w/v) bactoagar was added to Lysogeny broth prior to autoclaving. Antibiotics were added from sterile stock solutions following autoclaving and cooling to less than 55°C.

2.1.9 Equipment

3550 UV Microplate reader  Biorad
ABI 7900T Sequence Detection System  Applied Biosystems
Chapter 2: Materials and Methods

Confocal Microscope     Nikon
Film Developer         Agfa
Humidifying incubator  NU Air
isCUBE-X thermal cycler Integrated Sciences
Laminar flow hood    Clean air
Light cycler PCR machine Roche
Light Microscope        Olympus
Microcentrifuge      Eppendorf
Nanodrop spectrophotometre Thermo Scientific
Typhoon Variable Mode Imager GE Healthcare
Tissue Lyser            Qiagen
Ultracentrifuge       Beckman
Vortex                           Selby

2.1.10 Common buffers and solutions

PBS (10x)
NaCl 80 g
KCl 2 g
Na2HPO4 14.4g
KH2HPO4 2.4 g
H2O to 1 L

TBS (10x)
Trizma base 24.2 g
NaCl 80 g
H2O to 1 L
pH adjusted to 7.6

TAE (50x)
Trizma base 242 g
Glacial acetic acid 57.1 mL
0.5M EDTA (pH 8.0) 100 mL
H2O to 1 L

Muscle cell lysis buffer (5x)
100% Triton X-100 25 mL
Trizma base 15.15g
CDTA 3.475g
Glycerol 500 mL
H3PO4 adjust pH to 7.8
H2O to 1 L DTT (add before use) to 2mM

Luciferase buffer
(MgCO3)2,Mg(OH)2 1.04 g
Tricine 40 mL
EDTA (0.5 M) 400 μL
MgSO4,7H2O (1M) 5.34 mL
NaOH adjust pH to 7.8
H2O to 2 L

Luciferase reagent
### Chapter 2: Materials and Methods

<table>
<thead>
<tr>
<th>Lucasferase buffer 1.4 L</th>
<th>Laemmli 2X Loading buffer</th>
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<tbody>
<tr>
<td>ATP (0.02M) 4 mL</td>
<td>4% SDS</td>
</tr>
<tr>
<td>DTT (1M) 50 mL</td>
<td>10% β-mercaptoethanol</td>
</tr>
<tr>
<td>CoA 300 mg</td>
<td>20% glycerol</td>
</tr>
<tr>
<td>Luciferin 250 mg</td>
<td>0.004% bromophenol blue</td>
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<tr>
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<td>0.125 M Tris HCL</td>
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**Carnoys Fixative**
- 60% Ethanol
- 30% Chloroform
- 10% glacial acetic acid
- Store at 4°C

**Carmine Alum Solution**
- 1g Carmine
- 2.5g aluminum potassium sulphate
- 500ML distilled water
- Boil for 20 min then adjust to 500ML
- Filter and add crystal of thymol
- Store at 4°C

**Citrate acid buffer**
- 1.92g Citric acid
- 1000ml H2O
- Mix and adjust PH to 6.0 with 1N NaOH
- Add 0.5ml of Tween20 and mix
- Store at 4°C
2.2 General Methods

2.2.1 In vitro studies

2.2.1.1 Isolation of nucleic acids

**RNA extraction from cultured cells:** RNeasy Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer’s protocol to extract RNA from cultured cells. In brief, cells were washed once with PBS in culture dishes before the addition of lysis buffer (Buffer RLT provided with kit; 10 μL BME added for each 1 mL buffer; 350 μL buffer per well in a 6 well plate). The dishes rested on ice for 3 – 5 minutes before contents were collected into chilled eppendorf tubes. Homogenisation was performed using a 19 gauge sterile needle and 1 mL syringe. Equal volume of 70% ethanol was then added and gently mixed using a pipette. The mixture was centrifuged and flow-through discarded. The RNA was subsequently washed with buffer RW1 and RPE (provided with kit) before being eluted using 50 μL of water. RNA clean-up was performed using Ambion DNA-free DNase treatment (Applied Biosystems) as per manufacturer’s protocol.

2.2.1.2 Preparation of nucleic acids

**Reverse transcription:** 500 ng of total RNA was incubated with 250 ng of random hexamers and 1μL 10mM dNTPs at 65°C for 5 minutes and then cooled on ice for at least 1 minute. Then a minimum of 200ng total RNA was reverse transcribed using AMVrt (Promega) according to manufactures protocol. The cDNA was aliquoted into small volumes to prevent freeze-thaw cycles and half was diluted 1 in 4 for further analysis.

**Plasmid preparation:** The Wizard SV Miniprep system (Promega) was used to isolate plasmid DNA according to manufacturer instructions. The Wizard SV Maxiprep system (Promega) was used to isolate plasmid DNA according to manufacturer instructions.

2.2.1.3 Analysis of nucleic acids

**Agarose gel electrophoresis:** The appropriate weight of DNA resolution grade agarose was mixed with 1X TBE and boiled in the microwave until dissolved. The solution was allowed to cool for 5 minutes. 1.5μl of ethidium bromide per 100ml of gel solution was incorporated, then the gel was poured in to the electrophoresis gel apparatus and allowed to set. The gel running buffer was 1X TBE. Plasmid DNA or restriction digested DNA was made up to a final volume of 10μl with 6X orange G loading buffer. A voltage of 100 volts was passed through the gel until
the DNA had migrated toward the positive cathode as desired. The agarose gel was then visualised by exposure to ultraviolet (UV) light.

**Quantification** 2μl of DNA/RNA sample was measured at an absorbance of 260nm against a blank water control in a photo spectrophotometer (Thermo Scientific).

**Sequencing** Automated sequencing was carried out using the ABI Prism 377 automated sequencer according to manufacturer instructions. Specific oligonucleotides for sequencing reactions are outlined in specific chapters.

### 2.2.1.4 Cloning of nucleic acids

**Restriction enzyme digestion** DNA was digested with appropriate restriction enzymes and respective buffers supplied by the manufacturers. To ensure complete digestion, 2-5 units of enzyme was added per 1μg of DNA and the reaction was incubated between 1hr and overnight.

**Isolation of DNA from agarose gels** DNA fragments of interest were visualised under UV light. Fragments were excised with a scalpel and placed in to a 1.5ml eppendorf tube. The DNA was purified from the gel fragment using the Wizard SV Gel Extraction kit (Promega) according to manufacture instructions.

**Ligation of restriction fragments to vector DNA** Ligation reactions were performed in a total 10μl reaction containing 1X ligation buffer (Promega), 1 U of T4 DNA Ligase (Promega), 50ng of prepared vector and 3 molar excess of fragment. Ligations were performed overnight at 4°C or 15°C.

### 2.2.1.5 Transformation of plasmid DNA into bacteria

**Heat shock transformation** 50μl of CaCl2 competent cells were removed from -80°C and allowed to thaw on ice. 50ng of plasmid or 2μl of ligation mix was then added to cells and incubated for 20mins on ice. Cells were then heat shocked at 42°C for 45secs and allowed to recover on ice for 2mins. 1ml of LB media was added to cells and the reaction was incubated for 1.5hr at 37°C on a rotating shaker. Cells were then spun down at 500rpm in a microcentrifuge. Transformed cells were plated on to LB agar plates containing the required antibiotic and incubated overnight at 37°C.
2.2.1.6 Amplification of nucleic acids

Polymerase chain reaction

Block PCR Following reverse transcription, the cDNA product was used for PCR amplification. Separate reactions were carried out for individual genes. The total 25μl was comprised of 1X GoTaq Green Master Mix (Promega), 0.5μM of each specific primer and 1μl cDNA product made up to total volume using PCR grade water. Reactions were amplified in an isCUBE-X thermal cycler (Integrated Sciences). All amplifications were initiated with a single cycle 95°C denaturation step for 2mins before denaturing, annealing and extension cycling phases. Amplifications were ended with a final 5min extension cycle at 72°C. For semi-quantitative comparisons of gene expression, PCR reactions were restricted to linear phase of amplification by limiting the number of PCR cycles. PCR products were visualised by agarose gel electrophoresis.

LightCycler capillary PCR Real time quantification of mRNA levels was conducted using the LightCycler system (Roche) with Fast Start Master SYBR Green I. Standard curves were produced by amplification of DNA, serially diluted to within the pg quantity range, from the gene of interest. Standard curves were used as a reference to determine the amount of mRNA amplified from samples. dCt calculation of crossing point values were also used to calculate absolute fold change gene expression values. All values obtained were standardized to amplification levels of the housekeeping genes 18S and β-ACTIN.

Applied Biosystems PCR Real time quantification of mRNA levels was conducted using the ABI 7900T sequence detection system (Applied Biosystems) with Power SYBR green detection. Standard curves were produced by amplification of DNA, serially diluted to within the pg quantity range, from the gene of interest. Standard curves were used as a reference to determine the amount of mRNA amplified from samples. dCt calculation of crossing point values were also used to calculate absolute fold change gene expression values. All values obtained were standardised to amplification levels of the housekeeping genes 18S and β-ACTIN. Specific TaqMan Gene expression assays for the ABI 7900T sequence detection system were also used as detailed in the specific chapters and appendices.

Oligonucleotides Gene specific oligonucleotides used for amplification of transcripts or plasmids are outlined in specific chapters and appendices. In most instances, the literature was
used as a reference to obtain established primer sets. When primers were unavailable, oligonucleotides were designed through the web-based program Primer3 (http://frodo.wi.mit.edu/primer3/). Primers for the purpose of qRT-PCR were designed to span introns to allow for the detection of genomic contamination through size differentials.

2.2.1.7 Tissue culture

All tissue procedures were performed in laminar flow tissue culture hoods using sterilised materials. All cell lines were grown as an adherent monolayer, and incubated at 37°C with 5% CO2 in a humidifying incubator.

**Thawing and freezing of cells** Cell lines were removed from liquid nitrogen and quickly thawed in a 37°C water bath with agitation. Thawed cells were then spun down and excess media aspirated to limit the carryover of DMSO. Cells were then resuspended and added to flasks containing pre-warmed growth medium. For freezing, cells were taken from flasks and pelleted by centrifugation. Cells were then resuspended in media containing 10% DMSO, aliquoted into 1ml cryogenic tubes and placed in a Cyro 1°C freezing container at -80°C. The following day, cells were transferred for storage in liquid nitrogen.

**Sub-culturing of cells** Visual inspection was carried out daily and subcultivation took place once cells were at 80-90% confluency. Media was aspirated, cells washed with PBS then incubated with 2ml Tryple Express (Invitrogen) for 2-3 minutes at 37°C to digest anchor proteins responsible for attaching cells to the surface of the flask. Cells were then diluted into flasks containing fresh medium.

**Nucleofection** Transfection was performed using electroporation by the Amaxa Nucleofector kit, Amaxa Biosystems (Lonza) using cell line solution V and specific programs as mentioned in the chapters. Each transfection was seeded into a single well of a 6-well plate. In most cases, cells were harvested within 48hrs for assaying.

2.2.1.8 Reporter assays

**Cell lysis** Following 48hrs transfection (unless otherwise stated), culture media was removed from plates and cells were washed with 1ml PBS per well. 120μl of 1X Reporter Lysis Buffer (Promega) was added to each well and placed on to a shaker at room temperature for

~ 39 ~
10mins to lyse cells. Complete collection of lysed cells was ensured by pipetting of lysis buffer. Cells were then collected and placed into a 1.5ml eppendorf tube and spun down at top speed for 2mins at room temperature to collect cell pellet. The supernatant was then used for reporter assays.

**β-Galactosidase assay** 30μl of cell lysate was added to a 96-well flat-bottomed clear microplate. 30μl if 2X β-galactosidase assay buffer was then added and the reaction incubated at 37°C for at least 10mins or until color development was observed. Absorbance was measured at 420nm in a microplate reader for determination of β-galactosidase activity.

**Luciferase assay** 50μl of cell lysate was added to a 96-well luciferase assay plate. 50μl of reconstituted 2X luciferase assay buffer was then added to each well. Luminescence readings at 405nm were instantly measured for determination of luciferase activity.

### 2.2.1.9 Knockdown and over-expression of LRH-1 in MCF-7 cells

To reduce basal LRH-1 expression levels in the MCF-7 breast cancer cell line, the SureSilencing short hairpin RNA (shRNA) plasmids (KH05888G, SABiosciences, Frederick, MD, USA) designed to specifically knockdown the expression of LRH-1 by RNA interference were used, as previously verified (Chand et al. 2010). MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 50U/ml penicillin and 50μg/ml streptomycin at 37°C in 5% CO2. Cells were transfected with either pGeneclip hMGFP-LRH-1 or pGeneclip hMGFP encoding a scrambled shRNA sequence as negative control using the Nucleofector device, Kit V (Amaxa Biosystems), according to the manufacturer’s instructions. GFP-positive cells were collected by fluorescence-activated cell sorting (FACS) 24 hours after transfection. To over-express LRH-1, pcDNA3.1 plasmid expression construct containing the full length sequence of human LRH-1 was transfected using Amaxa transfection as previously described. Control transfections were performed with pcDNA3.1 vector alone and mRNA isolated for qPCR analysis and protein lysates extracted for western blot analysis 24 hours post-transfection.

### 2.2.2 In vivo studies

#### 2.2.2.1 Maintenance, generation and genotyping of transgenic mice

All studies involving the mice were regulated under the protocol approved by Monash Medical Centre; Ethics: MMCB10/12. The pTetOLRH-1 transgene was generated from a construct containing the human LRH-1 open reading frame (Clyne et al. 2002). Briefly, a Kozak
sequence was introduced by site-directed mutagenesis using the QuickChange® II Site-Directed Mutagenesis Kit (Agilent Technologies, USA) and primers F- Kozak: 5’-TTA AGC CAA AGA ACT GCC TAT AAT TTC ACT CAC CAT GGC TTC TAA TTC AGA TAC TGG GGA TTT ACA AG -3’ and R-Kozak: 5’- CTT GTA AAT CCC CAG TAT CTG AAT TAG AAG CCA TGG TGA GTG AAA TTA TAG GCA GTT CTT TGG CTT AA-3’. The LRH-1 open reading frame containing the Kozak sequence was then subcloned into the MCS of the pTRE-Tight-BI-DsRed Express vector (Clontech, USA) after NotI restriction digest. Orientation of the insert was verified by digesting the vector with BamHI. The vector was then linearized using ApaLI and gel purified. The pTetOLRH-1 founder mice were generated by pronuclear injection (MouseWorks Service, Australia) of the purified transgene, and complete transgene insertion was verified using primers that spanned the 3’ end of the DsRed open reading frame (L1) (F: 5’-5’- GCC GAT GAA CTT CAC CTT GT -3’ and R: 5'- CGA GGA CGT CAT CAA GGA GT -3’) and the 3’ end of the LRH-1 open reading frame (L2) (F: 5’- TCG ACC ACA TTT ACC GAC AA -3’ and R: 5’- TGG CTG ATT ATG ATC CTC TGG -3’). MMTVrtTA-pTetOLRH-1 (MMTVtet-LRH-1) double transgenic mice were generated by crossing pTetOLRH-1 founder females with MMTVrtTA males. Genotyping of double transgenic animals was performed using primers for LRH-1 listed above and MTB F- TGC CGC CAT TAT TAC GAC AAG C, R- ACC GTA CTC GTC AAT TCC AAG GG. Only female MMTVtet-LRH-1 mice were selected for this study unless otherwise stated.

2.2.2.2 Induction of LRH-1 transgene expression

To induce LRH-1 transgene, doxycycline hyclate (Clontech, Cat# 631311) was supplied in drinking water at a 2 mg/ml concentration with 3% sucrose. The control cohorts were provided with 3% sucrose in drinking water. The water was replaced every three days and kept under light sensitive conditions.

2.2.2.3 Determination of Stage of Mouse Estrous Cycle

Vaginal smears were performed using saline and a plastic transfer pipette. Sloughed cells were spread on a glass slide, stained with haematoxylin and eosin, and examined by light microscopy to determine stage of the oestrous cycle (proestrus, estrus, metestrus I, metestrus II, or diestrus).
2.2.2.4 Fixation/Tissue preparation

In all experiments, animals were euthanized by CO2 asphyxiation. The mammary glands were then excised for gross morphology by whole mounts (see below), protein detection and localisation by fixing the tissue in 4% Paraformaldehyde for 2-4 hrs and then storing in 70% Ethanol until paraffin embedding, and frozen fixed for RNA and protein analysis by snap freezing the tissue in a cryovial in liquid nitrogen.

2.2.2.5 Mammary gland whole mount analysis

The fourth inguinal mammary glands were harvested and spread onto slides, fixed with Carnoy’s fixative (60% Ethanol, 30% Chloroform and 10% glacial acetic acid) for 2-3 hours. The glands were placed in 70% ethanol for 15 mins followed by 5 mins each in 50% ethanol, 25% ethanol and double distilled H\textsubscript{2}O before staining overnight with Carmine Alum. The mammary glands were dehydrated sequentially for 5 mins each in 70%, 90%, 2x100%, and cleared in histolene for 2x15mins to dissolve fat in the gland. The slides were maintained in DPX mounting media for analysis under light microscope. The number of primary and secondary branches, and ductal ends were counted for the entire gland. The mammary gland tree occupancy was then estimated according to the sections occupied. Data points from all mice in the same group were averaged and plotted.

2.2.2.6 Immunohistochemical (IHC) analysis

Sections of 5 \(\mu\)m thickness were cut from formalin-fixed, paraffin-embedded inguinal mammary glands. Prior to staining, the sections were de-waxed and rehydrated in graded ethanol washes. Antigen retrieval was performed by immersing slides in 600 ml of Target Retrieval Buffer (Dako) or citrate buffer (ph6) and heating in a 1000 W microwave at 100% power for 5 min, standing at room temperature for 5 min and then heating for an additional 5 min at 40% power, before cooling at room temperature for an hour. Sections were then treated with 3% H\textsubscript{2}O\textsubscript{2} for 5 min to block endogenous peroxidase activity. To block endogenous avidin and biotin in the sections, the avidin/biotin blocking kit (Vector Laboratories) was used. The sections were then incubated with specific primary antibodies (see Appendix 1). For antibodies raised in mouse, the mouse on mouse (M.O.M) basic kit (Vector Laboratories) was employed for detection. For antibodies raised in rabbit, the Rabbit IgG Kit, (Vector Laboratories) was utilized for detection. The tissue was then incubated with ABC reagent (Vectastain Elite, Vector, PK-6101) for 30 minutes. Staining was detected by staining with 3, 3′-diaminobenzidine tetrahydrochloride (DAB Liquid Substrate Kit, Dako). The sections were counterstained with Mayer’s haematoxylin.
(Sigma Diagnostics), dehydrated and mounted with DPX mounting medium (Sigma Diagnostics) under 22 × 50 mm cover slips (HD Scientific). Staining was photographed with OLYMPUS microscope. Staining-positive cells were counted in 40 fields and data were expressed as a percentage of total epithelial cells counted.
2.2.3 Methods for in vitro and in vivo studies

2.2.3.1 Western blotting

Protein lysates were prepared by placing the mammary glands or breast cancer cells in protein lysis buffer (5 mM HEPES, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM NaF, 2 mM EDTA, 10 mM Na pyrophosphate, 1% Nonidet P-40, 10% glycerol and protease inhibitors (Roche)). The tissue was then homogenized using the Qiagen Tissue lyser LT. After homogenising, the sample was centrifuged (full speed for 15 mins) and incubated in 4°C for 15 mins on ice. The supernatant was then collected and used for downstream protein applications. Protein concentrations in these lysates were measured by BCA Protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts (40 μg) of total cellular protein extract were electrophoresed on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Hybond – C Super; Amersham, RPN203G). Membranes were blocked with 5% (w/v) milk protein in Tris-buffered saline containing 0.05% Tween-20. Blots were then incubated with primary antibodies as indicated, washed in Tris-buffered saline containing 0.1% Tween-20 and probed with primary antibodies. Secondary IgG-conjugated mouse or rabbit antibodies, and Alexa fluor 700 (Invitrogen) were used at a dilution of 1:10 000 to visualize protein bands. Band intensities were quantified using the Odyssey infrared imaging system and Odyssey 3.0 Software (LI-COR Biosciences, Lincoln, NE, USA). Blots shown are representative of a minimum of three separate experiments.

2.2.3.2 Quantitative reverse-transcriptase PCR (Q-RT-PCR)

Total RNA was prepared from mouse mammary tissue or breast cancer cells using the RNeasy Kit (Qiagen, 74106) or Trizol (Life Technologies, 10296010) as per manufacturers protocol, treated with DNaseI (Ambion, AM1906), and quantified using a NanoDrop 1000 Spectrophotometer. First strand cDNA synthesis using 1.0 μg total RNA was performed using AMV Reverse Transcriptase (Promega, M5101) primed by random hexamers. mRNA was quantified by ABI Prism 7900-HT Real-time PCR system or the LightCycler PCR system. Fold changes in expression of LRH-1 was calculated using the ddCt method (Schmittgen and Livak 2008) with 18s as an internal control. Data represented was collated from RNA collected from three separate experiments.

2.2.3.3 Expression profiling and analysis

Gene expression profiling Illumina (San Diego, CA) human whole genome 6-v2 bead chips (HumanHT-12 v4), containing 46325 probes was conducted in two separate experiments.
(i) in MCF-7 cells where the effects of LRH-1 knockdown on global gene expression was determined and (iii) in mammary tissue where human LRH-1 was over-expressed using the dox-inducible transgenic mouse described above. In MCF-7 cells, ten transfections were performed and pooled per condition, GFP-positive cells isolated by FACS, and RNA isolated.

For the transgenic mouse studies, RNA was isolated from mammary tissue from three month control and doxycycline treated LRH-1 transgenic mice (n=3) and array performed for individual samples to analyse gene expression per animal using Illumina (San Diego, CA) mouse WG6v2 Expression Bead Chip.

Expression profiling was performed at the Australian Genome Research Facility, Parkville, Australia). For the MCF-7 cell microarray, samples were normalized by quantile normalisation using the lumi package in R statistical software and subsequently imported into GeneSpring GX for analysis. In each case, genes were considered differentially expressed between groups if fold change (positive or negative) ≥ 1.4. Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) was used to identify gene networks according to biological functions and/or diseases in the IPA database. The significance value associated with a given function was expressed as a \( P \) value (calculated using right-tailed Fishers exact test) by comparing the number of LRH-1 – regulated genes associated with a given function to the total number of occurrences of those genes in all functional annotations in the IPA database.

For the mouse mammary microarray, raw signal intensity values were subjected to variance stabilization transformation including background correction, log2 transformation and variance stabilization using the lumiR package of R Bioconductor (Du et al. 2008, Lin et al. 2008). ANOVA analysis of normalized probe intensities values was performed in Partek® Genomic SuiteTM software (version 6.6 build 6.12.0420; Copyright © 2003-2012 Partek Inc., St. Louis, MO, USA). ANOVA was used to calculate significance of variation in normalized expression values between sample groups, fold change of gene expressions was calculated as mean ratio. Probes with an unadjusted \( p \)-value of 0.05 or less (no False Discovery Rate correction was applied) and an absolute fold change of 1.5 or more were defined as differentially expressed. For both data sets, we used IPA to assess significant upstream regulators. Activation z-score was calculated as a measure of functional and translational activation in Networks and Upstream regulators analysis. An absolute z-score of below (inhibited) or above (activated) 2 was considered as significant.
2.2.4 Statistical Analysis

Statistical analysis for each data set was performed using GraphPad Prism5 software package (GraphPad Software Inc., San Diego, CA). Means were compared using either unpaired t-tests or one way ANOVA with Tukey’s post hoc test for multiple comparisons, as appropriate. The data is presented as means ± standard error of the mean (SEM). P-values are annotated as: * p<0.05; ** p<0.01 and *** p<0.001.
CHAPTER 3:
OESTRADIOL REDUCES LRH-1 TRANSCRIPT STABILITY IN BREAST CANCER CELL LINES.

Chapter 2 comprises a manuscript that has been published in Biochemical and Biophysical Communications 438 (3) 533-539 along with additional experiments presented within the Addendum
Thesis chapter 3: Declaration

Results chapter 2 entitled “Oestradiol reduces LRH-1 transcript stability in breast cancer cell lines”

In the case of Chapter 3, contributions to the work involved the following

<table>
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<th>Name</th>
<th>% contribution</th>
<th>Nature of contribution</th>
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<tr>
<td>1. Kyren A. Lazarus</td>
<td>80%</td>
<td>Performed all experimental work, data analysis, Preparation of manuscript. Proofreading and submission of submissions. Conceived the study and provided intellectual input. Additional work in the addendum carried out solely by the candidate</td>
</tr>
<tr>
<td>2. Zhe Zhao</td>
<td>3%</td>
<td>Performed experiments</td>
</tr>
<tr>
<td>3. Kevin C. Knower</td>
<td>4%</td>
<td>Performed experiments, proof-reading</td>
</tr>
<tr>
<td>4. Sarah Q. To</td>
<td>3%</td>
<td>Helped with data collection, proof-reading the manuscript.</td>
</tr>
<tr>
<td>5. Ashwini L Chand</td>
<td>5%</td>
<td>Concept, critically revised the manuscript, responsible for submission</td>
</tr>
<tr>
<td>6. Colin D. Clyne</td>
<td>5%</td>
<td>Concept, critically revised the manuscript.</td>
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Declaration by co-authors

The undersigned hereby certify that:

(1) They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) There are no other authors of the publication according to these criteria

(4) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or published of journals of other publications, and (c) the head of the responsible academic unit; and

(5) The original data are stored at the following location and will be held for at least five years from the date at the location:

**Prince Henry’s Institute of Medical Research, Clayton VIC, Australia.**
Chapter 3: Oestradiol reduces LRH-1 transcript stability in breast cancer cell lines

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3.1 Integrative commentary for chapter 3

Previous work has demonstrated that LRH-1 expression is regulated by oestrogen, and consistently, LRH-1 transcript is positively associated with oestrogen receptor alpha (ER) expression in breast cancer cell lines. We have previously shown similar LRH-1 protein levels in ER negative (ER-) MDA-231 and ER+ MCF-7 cell line. Hence, we suggest discordance between LRH-1 transcript and protein expression. The objectives of this study were to: 1) define the expression pattern of LRH-1 protein and transcript in breast cancer cell lines, and 2) analyse the stability of LRH-1 transcript and protein in order to explain this discordance. Eight breast cancer cell lines were used to analyse the expression pattern of LRH-1 transcript and protein using RT-PCR, western blotting and immunocytochemistry. The stability of LRH-1 transcript and protein were assessed by using actinomycin-D and cyclohexamide respectively in a decay study. Subsequently, we analysed the expression and stability of LRH-1 transcript variants, in particular LRH-1v4 which was demonstrated to be highly expressed in breast cancer and highly responsive to oestrogen treatment (Thiruchelvam et al. 2011). Finally we assessed the effect of oestradiol on LRH-1 transcript stability in breast cancer cells. We predicted that after oestrogen stimulation, LRH-1 transcript stability would be reduced and treating cells with ICI 182,780 would rescue this reduction in transcript stability.

This chapter was published in Biochemical and Biophysical Communications 438 (3) 533-539
Chapter 3: Oestradiol reduces LRH-1 transcript stability in breast cancer cell lines

Oestradiol reduces Liver Receptor Homolog-1 mRNA transcript stability in breast cancer cell lines

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1 These authors contributed equally to this article.

Abstract

The expression of orphan nuclear receptor Liver Receptor Homolog-1 (LRH-1) is elevated in breast cancer and promotes proliferation, migration and invasion in vitro. LRH-1 expression is regulated by oestradiol (E2) with LRH-1 mRNA transcript levels higher in oestradiol receptor α (ERα) positive (ER+) breast cancer cells compared to ER− cells. However, the presence of LRH-1 protein in ER+ cells suggests discordance between mRNA transcript levels and protein expression. To understand this, we investigated the impact of mRNA and protein stability in determining LRH-1 protein levels in breast cancer cells. LRH-1 transcript levels were significantly higher in ER+ versus ER− breast cancer cell lines, however LRH-1 protein was expressed at similar levels. We found LRH-1 mRNA and protein was more stable in ER− compared to ER+ cell lines. The tumor-specific LRH-1 variant isoform, LRH-1v4, which is highly responsive to E2, showed increased mRNA stability in ER− versus ER+ cells. In addition, in MCF-7 and T47-D cell lines, LRH-1 total mRNA stability was reduced with E2 treatment, this effect mediated by ERα.

Our data demonstrates that in ER− cells, increased mRNA and protein stability contribute to the abundant protein expression levels. Expression and immunolocalisation of LRH-1 in ER− cells as well as ER+ tumours suggests a possible role in the development of ER+ tumours. The modulation of LRH-1 biactivity may therefore be beneficial as a treatment option in both ER− and ER+ breast cancer.

1. Introduction

Liver Receptor Homolog-1 (LRH-1; NR5A2) is an orphan member of the nuclear receptor superfamily that share substantial structural homology within their DNA and ligand binding domains (for review, see [1,2]). LRH-1 is a key mediator in metabolic pathways involved in bile acid synthesis and cholesterol metabolism via regulation of cytochrome P450 7A1 (CYP7A1) [3]. LRH-1 has also important roles in steroid hormone production in the ovary and testis, through regulation of genes including cytochrome P450 aromatase (CYP19A1), steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (HSD3B2) and inhibit n α subunit [4]. More recently, LRH-1 has been demonstrated to play an important role in early embryonic tissue via its ability to regulate the expression of octamer-binding transcription factor 4 (OCT4) [1,3]. LRH-1 promotes cell proliferation in gastric, colon and pancreatic cancers [1,2]. We have demonstrated LRH-1’s importance in breast cancer, through activation of P450 aromatase, the enzyme that catalyses the conversion of androgens to oestradiol [4].

17β-Oestradiol (E2) promotes cell proliferation and breast cancer development by binding to its receptor oestrogen receptor α (ERα) and ERβ. In the breast, LRH-1 is involved in E2 signaling via various pathways creating a positive feedback loop promoting tumor cell proliferation. Firstly, LRH-1 stimulates transcription of the CYP19A1 gene, primarily occurring in cancer associated fibroblasts (CAFs), thereby contributing to the supply of mitogenic E2 that acts in a paracrine manner to promote neighbouring epithelial cell proliferation [4]. Breast tumor cells secrete factors such as prostaglandin E2 (PGE2), which directly stimulate LRH-1 expression in tumor-associated stroma, and contribute to LRH-1 mediated activation of the CYP19A1 [5]. LRH-1 also directly regulates expression of ERα in breast cancer cells via the activation of a major ERα promoter used in breast cancer cells [6]. In turn, LRH-1 stimulates expression of ERα target genes, such as GREE-1 and p52, by binding to ERα response elements in their promoters [7]. Finally, LRH-1 is itself regulated by E2 via binding of ERα to the LRH-1 promoter [8].
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Therefore, a positive feedback loop is established whereby LRH-1 both regulates, and is regulated by ERα.

Consistent with its role in ERα-signaling and regulation of expression by ERα, LRH-1 transcript is largely confined to ERα breast cancer cell lines, with ERα– lines expressing little or no LRH-1 mRNA [6,8]. Recently, Thiruchelvam et al. identified two novel variants, LRH-1v4 and LRH-1v5 that are highly responsive to ERα treatment. Despite the relative lack of LRH-1 mRNA in ERα– breast cancer cells, a number of observations suggest that LRH-1 has actions in ERα– breast cancer tissues and cells that may be physiologically relevant. For example, LRH-1 protein was found to be expressed in a subset of ERα– human breast cancer tissue by immunohistochemistry [9]. Additionally, LRH-1 protein was expressed at similar levels in both the ERα– MDA-MB-231 cell line and the ERα+ MCF-7 line [10]. Knockdown of endogenous LRH-1 inhibited tumor cell migration and invasion in ERα– MDA-MB-231 cells and cell proliferation in ERα+ MCF-7 cells [7,8,10]. This suggests that expression of LRH-1 in ERα– cells may be relevant to breast cancer proliferation and invasion, for example by directly stimulating cell cycle genes [11], or stimulating ERα target genes such as GREB-1 [7].

In the present study we assessed LRH-1 mRNA and protein expression in a panel of ERα+ and ERα– breast cancer cell lines. LRH-1 protein is expressed at similar levels in all cell lines tested despite strong enrichment of LRH-1 mRNA in ERα+ cell lines. Differences in LRH-1 mRNA and protein stability between ERα+ and ERα– cells were observed. This discrepancy may in part be explained by the negative effect of Estradiol treatment on LRH-1 mRNA half-life in ERα– cells. This data demonstrated stable expression of LRH-1 mRNA and protein in ERα– breast cancer cell lines, suggesting that LRH-1 may have important effects in hormone independent cancers, as it does in ERα+ tumors.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines (MCF-7, ZR-75, T47D, MDA-MB-231, MDA-MB-361, MDA-MB-453, MDA-MB-468 and HS578T) were obtained from American Type Culture Collection (ATCC) and grown in the recommended culture media and conditions.

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Fig. 1. Discordance in LRH-1 mRNA and protein expression levels in breast cancer cell lines: (A) total mRNA was isolated from a panel of breast cancer cell lines. Levels of expression were determined by Q-PCR and normalized to 18S. (B) Representative image showing expression of LRH-1 (red) in protein lysates, β-tubulin used as a loading control (green). (C) Densitometry quantification of immunofluorescence data. (D) LRH-1 immunoactivity was detected in the nucleus and cytoplasm in MCF-7 and MDA-MB-231 cells. Rabbit IgG was used as a control. Images were taken at 100× magnification. Scale bar, 20 μm. Data shown from independent experiments as mean n = 3 ± SEM, **P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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according to ATCC protocols. Cells were incubated for 72 h in medium containing 3% charcoal-stripped serum (CSS) before addition of treatments including 17β-oestradiol (10 nM, Sigma) and/or 10182.780 (100 nM, Sigma) for 24 h (ethanol for vehicle control).

2.2. Real-time PCR

Total RNA was prepared using the RNeasy Kit (Qiagen, 74106), treated with DNase (Ambion, AM1906), and quantified using a NanoDrop 1000 Spectrophotometer. First strand cDNA synthesis using 1.0 μg total RNA was performed using AMV Reverse Transcriptase (Promega, M5101) primed by random hexamers. PCRs were carried out using Taqman probes against LRH-1 (Hs00187067_m1, Applied Biosystems) and Taqman Gene Expression Master Mix (Applied Biosystems). RT-PCR for LRH-1, v1, v4, and 18s was analysed using the Power SYBR Green PCR Mix (Applied Biosystems) using the following primers: sense 5'-CTTCTACGTGGGGATTACAC-3', and antisense 5'-TATCTCCACACAGGGGCAA-3' (V1/V2); sense 5'-CTTCTAAAGACCCGACTACAC-3', antisense 5'-TATCTCCACACAGGGGCAA-3' (V4); sense 5'-CCAGTCCGAGCCATAAAAG-3', and antisense 5'-TATCTCCACACAGGGGCAA-3' (V5) and, sense 5'-CGGCTCTAGAATCCGCGG-3', and antisense 5'-GGCTCTAGATCCGGCGG-3' (18s) [6]; mRNA transcripts were quantified by ABI Prism 7900HT Real-time PCR system. Fold changes in expression of LRH-1 was calculated using the ΔΔCt method [12] using 18s as an internal control.

2.3. Actinomycin D treatment for determination of mRNA stability

MCF-7, T47D, MDA-MB-231 and Hs578T cells were incubated for 72 h in medium containing 3% charcoal-stripped serum (CSS), and then supplemented with 5 μg/ml Actinomycin D (Sigma, A1410) in phenol red free media. The cells were treated with the indicated substances for various time points up to 24 h. RNA was extracted, cDNA synthesised and RT-PCR performed as described above.

2.4. Determination of protein turnover by Cyclohexamide treatment

MCF-7 and MDA-MB-231 cells were incubated for 72 h in medium containing 3% CSS, and then supplemented with 10 mg/ml Cyclohexamide (Sigma, C4849) in phenol red free media. The cells were treated for 0, 4, and 8 h. Protein was extracted, and western blot was performed as below.

2.5. Western blot analysis

Protein extraction and immunoblots for LRH-1 and β-tubulin were performed as described previously [13]. Briefly protein was extracted from cell lysates and separated on a 10% SDS-PAGE gel. The protein was then transferred to a nitrocellulose membrane (Amersham). After blocking with 5% skim milk, the membrane was incubated with anti-LRH-1 antibody (ab18293, Abcam; H2325, R&D systems) for 16 h at 4°C. Anti-β-tubulin (mab3408, Millipore) was used as a loading control. Protein bands were visualised using the Odyssey infrared imaging system and Odyssey 3.0 software (Loric Biosciences).

2.6. Immunocytochemistry

Cells were fixed with 100% cold methanol, permeabilized with PBS + 0.1% Triton X-100 (Thermo, 28318) followed by blocking with 0.3% H2O2, Avidin/Biotin (Vector, SP-2001). The fixed cells

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were blocked using horse serum (Vector, PK-6101), for 20 min at RT according to the manufacturer's protocol. Cells were then incubated with LRH-1 primary antibody (1:200, ab18299, Abcam) at 4 °C O/N. IgG was used as negative control. After washing, the cells were incubated with biotinylated secondary antibody (Vector, PK-6101) for 30 min. Finally, 3,3-diaminobenzidine (Dako, K3468) staining was used for antibody detection and Myer's Hematoxylin solution (Sigma Aldrich, MHS1) was used for nuclei detection. Staining was photographed with OLYMPUS microscope.

2.7. Statistical analysis

All data are reported as mean ± SE for three or more experiments. Statistical analyses for experiments comparing two groups were performed by two-tailed Student's independent t test using GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) and a P value of < 0.05 was considered statistically significant.

3. Results

3.1. LRH-1 expression in breast cancer cell lines

To confirm that LRH-1 transcript levels positively associate with the ER− status of breast cancer cell lines, we first quantified total LRH-1 mRNA expression in several ER+ and ER− breast cancer cell lines by RT-PCR (Fig. 1A). Consistent with previous studies [14,15], we observed significantly higher expression of LRH-1 mRNA in ER+ cells compared to ER− cells (p < 0.0001) (Fig. 1A). We demonstrate LRH-1 protein levels in a panel of ER− and ER+ cell lines (Fig. 1B and C). Two protein bands were detected, at approximately 61 and 56 kDa that correlate to the LRH-1v4 isoform [6]. Interestingly, expression of the 56 kDa isoform was restricted to ER− cells. We also observed a novel predominantly nuclear localization of LRH-1 in MCF-7 and MDA-MB-231 cells by immunochemistry (Fig. 1D).

3.2. LRH-1 transcript and protein stability is reduced in ER+ cells

To address the discrepancy between LRH-1 mRNA and protein expression, we measured the stability of LRH-1 mRNA and protein in ER− and ER+ cell lines. In the presence of the RNA synthesis inhibitor Actinomycin D, LRH-1 total transcript degraded more rapidly in ER+ cells compared to ER− cells (Fig. 2A). The half-life of LRH-1 mRNA in ER+ cells was approximately double that in ER− cells (p < 0.01, Fig. 2B). In ER+ breast cancer cell lines, LRH-1 mRNA half-life was 1.2 h in MCF-7 and 1.3 h in T47-D cells. In ER− cell lines, LRH-1 mRNA half-life was 2.5 h in MDA-MB-231 and 2.1 h in HS578T cells. We also determined the degradation rates of LRH-1 protein in ER+ MCF-7 and ER− MDA-MB-231 cell lines by western blot following treatment with a protein synthesis inhibitor, Cyclohexamide (Fig. 2C). LRH-1 protein was significantly more stable in MDA-MB-231 (half-life: 11.69 h) than in MCF-7 (half-life: 3.8 h) cells (Fig. 2D). The LRH-1 isoform v4 (56 kDa) was highly stable after protein synthesis inhibition in MDA-MB-231 cells (Fig. 2C). Collectively, this data suggests that differences in mRNA and protein stability may explain the discrepancy observed between LRH-1 transcript and protein levels across the panel of breast cancer cell lines.

3.3. Expression and stability of LRH-1 transcript variants

Primers specific for LRH-1 isoforms were used to quantify expression levels of previously described LRH-1 variants [6]. We confirmed LRH-1v4 as the predominant form of LRH-1 in ER− breast cancer cell lines while little or no LRH-1v1/2 and v5 were detected (Fig. 3A). LRH-1v4 degraded at a more rapid rate in ER+ MCF-7 cells (half-life: 1.0 h) compared to MDA-MB-231 cells (half-life: 2.2 h) (Fig. 3B).

3.4. Oestradiol reduces LRH-1 transcript stability via ERα

Steroid hormones control the degradation of specific mRNAs, and regulate transcript stability of a variety of genes (including vitellogenin and ERα [15]). To determine the effects of E2 on LRH-1 total mRNA stability, we treated two ER+ cell lines (MCF-7 and T47D) with E2 and found significant increase in steady state LRH-1 transcript levels with treatment (p < 0.001). Conversely, co-treatment with ERα antagonist ICI 182,780 caused a reduction in mRNA levels (p < 0.001, Fig. 4A and B), and total transcript degradation rates were increased (Fig. 4C and D). LRH-1 mRNA half-life was decreased from 1.28 h to 0.47 h in E2 treated cells (p < 0.01, Fig. 4E and F) and decrease was inhibited by ICI 182,780 in the presence of E2 (p < 0.01, Fig. 4E and F). These findings suggest that the effects of E2 on LRH-1 mRNA stability are mediated via ERα.

4. Discussion

In the breast, LRH-1 regulates oestrogen production in cancer-associated stroma. In tumor epithelial cells, LRH-1 is involved in
Chapter 3: Oestradiol reduces LRH-1 transcript stability in breast cancer cell lines

ERα-mediated gene transcription, cell proliferation, motility and invasion [7,8,16,17]. Expression of LRH-1 in ER+ breast cancer, and its role in oestrogen synthesis, has driven the search for synthetic antagonists to block its oestrogenic and proliferative effects [18–20]. Previous studies have shown that LRH-1 mRNA expression is restricted to ER+ breast cancer cells. However, apparent expression and activity of LRH-1 in ER– tissues and cells prompted us to investigate LRH-1 expression at the protein level. Here we systematically correlated LRH-1 mRNA and protein expression levels in a panel of ER+ and ER– breast cancer cell lines. Despite large differences in mRNA levels between ER+ and ER– cells, LRH-1 protein is present at similar levels across these cell lines. Interestingly, a ~60 kDa isoform is detected in both ER+ and ER– cells while a ~56 kDa isoform was detected exclusively in the ER+ cells; both of which are biactivc. The presence of LRH-1 protein in ER– cells may explain previous studies reporting its functional effects in cancer where siRNA-mediated knockdown of LRH-1 inhibited tumor cell motility and invasion similarly in both ER– MDM-MB-231 and ER+ MCF-7 cells [10]. Immunoblotting indicated similar expression of LRH-1 protein in MCF-7 and MDA-MB-231 cells [10]. In line with clinical immunohistochemistry data, LRH-1 protein is localised in the nucleus of epithelial cells in both ER+ and ER– breast tumor biopsy samples [9].

Tumor cells regulate rapid proliferation by altering the rate of protein synthesis via several mechanisms including gene transcription and changes to mRNA stability. To address the discrepancy observed between LRH-1 mRNA and protein levels, we assessed LRH-1 mRNA transcript and protein stability rates. In ER– cells, mRNA and protein is approximately 2-fold and 3-fold respectively more stable compared to ER+ cells. We identified a stable LRH-1 isoform LRH-1v4 (~56 kDa) in MDA-MB-231. This isoform is likely relevant in breast cancer, and isoform-specific expression studies would clarify its role in ER– tumors.

Our findings contrast with those of a previous report where no change in LRH-1 mRNA stability was measured in MCF-7 treated with E2 [8]. Along with the two newly discovered variants, our study used primers that detected all five variants, while in the previous study probes measured expression of three transcript variants [6,8]. Our observations are in line with another study identifying LRH-1v4 as the dominant transcript in breast cancer.

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Fig. 4. Oestradiol reduces LRH-1 mRNA stability in MCF-7 (A, C and E) and T47D (B, D and F) cells: Cells were serum starved, then treated with E2 (10 nM) ± ICI (100 nM) for 24 h. (A and B) Steady state LRH-1 mRNA expression levels were quantified against 18s. (C and D) Actinomycin-D decay study was then performed. **VC; ○, E2; ○, E2 + ICI (E and F) half-life was interpolated from decay log curve above. Data shown from independent experiments as n = 3 ± SEM. **P < 0.01, ***P < 0.001.
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cells, and its high responsiveness to E2 [6]. We show that LRH-1 transcript is relatively more stable in MDA-MB-231 compared to MCF-7 cells. The increased stability of LRH-1 transcript in MDA-MB-231 cells may contribute to the increased translation to protein in MDA-MB-231 cells. We were unable to detect LRH-1 transcript in MCF-7 cells, therefore we hypothesise that LRH-1 transcript in MCF-7 cells may be highly unstable and possibly modified through post-translational mechanisms. Further studies involving the translation of each transcript isoform may be required to further understand the discordance of LRH-1 transcript and protein in breast cancer cell lines.

Steroid hormones were shown to be the first regulators of mRNA stability and degradation processes [31]. We hypothesised that this may account for the differences in LRH-1 mRNA decay rates observed in ER+ and ER− breast cancer cells. The stability of a large number of mRNA transcripts encoding for proteins such as vitellogenin, ApolVLDL, ovalbumin, conalbumin, renin, proges-
terone receptor and ERα is regulated by steroid hormones [15]. The earliest characterised is the stabilization of vitellogenin mRNA by E2 [21]. Treatment with E2 decreases ERα mRNA stability from 4 h to 40 min in MCF-7 cells [22]. Here we demonstrate that LRH-1 mRNA stability is reduced, like that of ERα, with E2 treatment in MCF-7 and T47D cells. Furthermore, treatment with the ERα antagonist compound ICI 182,780 increased LRH-1 mRNA sta-
bility, implicating an ERα-regulatory mechanism in MCF-7 and T47D cells.

The expression and turnover of mRNAs is a crucial step in post-transcriptional regulation of protein expression and is regulated by cis-acting elements located in the 3′UTR, such as the AU-rich ele-
ments (AREs) and microRNAs (miRNAs) [25]. These are bound by protein factors that in turn recruit deadenylation enzymes causing mRNA decay [24]. An example of this is observed in the regulation of vitellogenin mRNA stability via an E2-regulated cis-acting ele-
ment in the vitellogenin 3′UTR [25]. The analysis of two main LRH-1 transcript variants revealed very long 3′UTRs and sequence analysis of this region revealed a repetition of an AUUAA motif which plays an important role in mRNA decay kinetics [26,27]. Factors binding to these AUUAA motifs may affect the stability of LRH-1 mRNA differently in ER+ and ER− cell lines. Interestingly these two different transcripts of LRH-1 (5.2 kb and 3.8 kb) were differentially expressed in normal liver and a hepatoma cell line; 5.2 kb transcript was predominant in normal liver tissue, while the 3.8 kb transcript increased in hepatoma HepG2 cells [26]. The authors proposed this difference in transcripts and their 3′UTR lengths may impact mRNA stability and LRH-1 isoform expression levels in the different cell lines. Interestingly, we did observe two LRH-1 isoforms of different sizes in the MDA-MB-231 and MCF-7 cells.

Comparatively little is known regarding the regulation of LRH-1 expression and activity in ER− breast cancer cells. In ER− cells, other additional mechanisms may be involved in the stabilisation of LRH-1 mRNA which is reflective of the high pro-
iferative index and metastatic nature of these cells. Protein fac-
tors such as heterogeneous nuclear ribonucleoproteins (hnRNPs) and the HuR proteins, and endonucleases have been documented to affect mRNA stability in tumor cell [28].

In conclusion, we have shown different LRH-1 isoforms are ex-
pressed in ER+ and ER− breast cancer cells, with protein levels con-
sistent across a panel of breast cancer cell lines. We found mRNA transcript and protein stability rates were reduced in ER+ cells, which may in part explain the discordant mRNA and protein expression observed. LRH-1 is a novel therapeutic target in ER− breast cancer [19]. The presence of LRH-1 in ER− cells suggests that targeting its proliferative effects may be beneficial in ER− dis-
ease, as it is in ER+ tumors.

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3.2 Chapter 3 Addendum

Oestradiol reduces LRH-1 transcript stability in breast cancer cell lines.

3.2.1 Introduction

The preceding publication described the expression of LRH-1 transcript and protein in breast cancer cells lines. Prior to this work, the known expression of LRH-1 protein in breast cancer cell lines was limited. This study showed for the first time LRH-1 protein expression in a panel of breast cancer cell lines. The presence of LRH-1 protein in ER- cell lines is consistent with studies localizing LRH-1 in ER- breast tumours (Miki et al. 2006), which elevates its potential as a therapeutical target for breast cancer treatment. The expression profiling of LRH-1 in vitro demonstrated a discrepancy between LRH-1 transcript and protein levels in ER+ and ER- cell lines. This publication went further to explain this discrepancy and demonstrated that in ER-cells, increased transcript and protein stability contribute to the abundant protein expression.

These findings were enhanced by the analysis of a previously characterized LRH-1 isoform LRH-1v4, which are highly responsive to oestrogen treatment and over-expressed in breast cancer cell lines. This study shows that the LRH-1v4 transcript was highly stable in MDA-231 cells. Finally, we demonstrate that treating MCF-7 and T47-D breast cancer cells with
oestradiol reduced LRH-1 stability which was rescued in the presence of ERα antagonist ICI 182,780. These findings indicate ERα regulatory mechanisms involved in regulating LRH-1 stability in these cell lines.

The expression and turnover of mRNAs is a crucial step in post-transcriptional regulation and is regulated by cis-acting elements located in the 3’UTR, such as AU-rich elements (AREs) and microRNAs (Ross 1996). In order to understand the mechanism by which oestradiol decreases LRH-1 stability, we investigated the effect of oestradiol on transcriptional activity of a luciferase reporter gene cloned upstream of the 3’UTR LRH-1 region. The following data describes further experiments conducted subsequent to publication of this paper that provides insight into potential mechanisms by which oestradiol inhibits LRH-1 mRNA stability.

3.2.2 Methods

3.2.2.1 Cell Culture
MCF-7 breast cancer cell lines were obtained from American Type Culture Collections (ATCC) and grown in the recommended culture media and conditions according to ATCC protocols.

3.2.2.2 Cloning of 3’UTR-LRH-1
Nucleotides 1832 to 2763 of accession: NM_205860.1 was extracted from genomic DNA using Wizard SV kit according to manufactures protocol. Subsequently, this region was cloned into the pMIR-Report luciferase (6470 bp, Ambion USA).

3.2.2.3 Cell Treatments
Cells were incubated for 72h in medium containing 3% charcoal-stripped serum (CSS) before treatment with the experimental agents indicated. Treatments included 17β-oestradiol (10nM, Sigma) and/or ICI (100nM, Sigma) for 24h (ethanol for vehicle control). Luciferase and β-galactosidase activity of soluble cell extracts was measured using the Luciferase Assay system (Promega) and β-gal Reporter Gene Assay (Roche).

3.2.2.4 Statistical analysis
Data was analysed using tools within the GraphPad Prism 5 statistical software package. Generally, a one-way analysis of variance (ANOVA) or Student’s T-test were performed. The
data is presented in this thesis as mean ± standard error of means with p<0.05 (*), p<0.01 (**), and p<0.001 (***), considered statistically significant.

3.2.3 Results

The 3’-UTR (corresponding to nucleotides 1832 to 2763 of accession: NM_205860.1) region of LRH-1 was amplified and cloned downstream of a luciferase reporter gene (pMIR-R/LRH-1). MCF-7 cells were co-transfected with pMIR-R/LRH-1 (or empty vector for control) and β-gal to control for transfection efficiency, and treated with 17β-E₂ alone or in the presence of ICI 182780. Activity of the luciferase reporter alone was unaffected by treatment. Addition of the LRH-1 3’UTR reduced luciferase activity by approximately 90%, indicating that sequences within the LRH-1 3’UTR destabilise the luciferase mRNA (Chapter 3 addendum figure 2A). In the presence of 17β-E₂, luciferase activity was further reduced (by 35%). However, this effect was not reversible by ICI 182,780, suggesting an ERα-independent mechanism. We confirmed the anti-oestrogenic effect of ICI 182,780 by monitoring expression of a known ERα-dependent gene, GREB-1 (Chapter 3 addendum figure 2B). GREB-1 mRNA was strongly induced by 17β-E₂ and reversed by ICI 182,780. Together, these data suggest that 17β-E₂ decreases LRH-1 mRNA stability via its 3’UTR, in an ERα-independent manner.

3.2.4 Discussion

The expression and turnover of mRNAs is a crucial step in post-transcriptional regulation of protein expression regulated by cis-acting elements located in the 3’ UTR, such as the AU-rich elements (AREs) and microRNAs (miRNA) (Ross 1996). These recruit protein factors that in turn recruit deadenylation enzymes that cause mRNA decay (Fabian et al. 2010). A study demonstrating the regulation of Vitellogenin mRNA stability by oestrogen revealed an oestrogen-regulated cis-acting element in the Vitellogenin 3’UTR (Nielsen and Shapiro 1990). This indicated that oestrogen could indeed regulate mRNA stability via the 3’UTR. miRNAs cause mRNA decay in human cells, C. elegans, Drosophila S2 cells and zebrafish (Giraldez et al. 2006, Schmitter et al. 2006, Fabian et al. 2010). Many studies support the idea that miRNAs destabilize target mRNAs through deadenylation and decapping (Fabian et al. 2010). We hypothesised that the observed reduction in luciferase reporter activity of the 3’UTR sequence of the LRH-1 gene with E₂ treatment was due to the up regulation of miRNAs.
Chapter 3: Oestradiol reduces LRH-1 transcript stability in breast cancer cell lines

The human genome is predicted to code >400 miRNAs that regulate one third of gene expression and E2 has been shown to regulate miRNAs in animal models as well as cell lines including MCF-7 cells by treating cells with 17β-E2 or ERα antagonist ICI 182780 (Bhat-Nakshatri et al. 2009, Klinge 2012, Masuda et al. 2012). Additionally, it has been reported that miR-206 is able to influence oestrogen regulated gene expression by directly reducing ERα mRNA stability (Adams et al. 2007). Furthermore it was revealed that treatment of MCF-7 cells with 1nM 17β-E2 or ERα agonist PTT reduced miR-206 levels by ~80% (Adams et al. 2007). The authors suggest a negative feedback loop where by which oestrogen temporally reduced ductal/lobuloalveolar proliferation (Adams et al. 2007). Using Targetscan software, two E2-regulated miRNAs, miR-27a/b and miR-200a/b were demonstrated to have predicted binding sites on the LRH-1 3’UTR (Chapter 3 addendum Figure 1) (WIBR 2012). Expression profiling of miRNA expression in human cancers have revealed its expression in various human cancer including breast cancers suggesting common altered regulatory pathways (Calin and Croce 2006, Volinia et al. 2006). In breast cancer miRNAs have been implicated in oncogenesis, invasion and metastasis (Ma et al. 2007, Huang et al. 2008). Interestingly, miRNAs have also been implicated in breast cancer cell tamoxifen resistance (Ward et al. 2012). Although the molecular details as to how these miRNAs regulate LRH-1 transcript stability remains to be elucidated, these new findings have opened up further investigations on to the relationship between miRNAs and LRH-1 transcript stability. Future investigations into specific oestrogen regulated miRNAs that may be involved will allow us to further our understanding of the mechanisms by which LRH-1 transcript is destabilised by miRNAs.

This chapter discusses important regulatory aspects of LRH-1 transcript and protein in breast cancer cell lines. Together with the growing body of evidence, our lab and others have demonstrated the importance of LRH-1 expression in breast cancer progression.
Chapter 3 addendum figure 1: Using TargetScan software, predicted miRNA binding sites were revealed for LRH-1 3'UTR
Chapter 3: Oestradiol reduces LRH-1 transcript stability in breast cancer cell lines

Chapter 3 addendum figure 2

A

![Graph showing luciferase/β-gal activities](image)

B

![Graph showing GREB-1 mRNA expression vs 18s](image)

Chapter 3 addendum figure 2: Oestradiol affects LRH-1 3’UTR: A) The LRH-1 3’UTR was cloned into the pMIR-Report miRNA luciferase reporter system. MCF-7 cells were transfected with this construct, treated with E2 ± ICI and luciferase and β-gal activities were measured. B) Total mRNA from the above cells was extracted and GREB-1 expression was analysed to confirm the activity of E2 and ICI. Data shown as n=3 ± SEM, * P<0.05, **P<0.01 and ***P<0.001.
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Chapter 4:

CONDITIONAL OVER-EXPRESSION OF LRH-1 IN FEMALE MOUSE MAMMARY EPITHELIUM RESULTS IN ALTERED MAMMARY MORPHOGENESIS VIA THE INDUCTION OF TGF-β

The following chapter is presented in manuscript form as it is currently Ahead of Print in Endocrinology
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Thesis chapter 4: Declaration

Results chapter 2 entitled “Conditional over-expression of LRH-1 in female mouse mammary epithelium results in altered mammary morphogenesis via the induction of TGF-β”

In the case of Chapter 4, contributions to the work involved the following

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<th>Name</th>
<th>% contribution</th>
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<tr>
<td>1. Kyren A. Lazarus</td>
<td>81%</td>
<td>Performed all experimental work, data analysis, conceived the study and provided intellectual concept. Preparation of manuscript. Proof-reading of manuscript</td>
</tr>
<tr>
<td>2. Kristy Brown</td>
<td>2%</td>
<td>Provided reagents, Proof-reading the manuscript</td>
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<tr>
<td>3. Morag Young</td>
<td>2%</td>
<td>Assisted in experiment setup</td>
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<td>4. Zhe Zhao</td>
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<td>5. Rhiannon Coulson</td>
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<td>7. Colin D. Clyne</td>
<td>5%</td>
<td>Concept, critically revised the manuscript, responsible for submission</td>
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Declaration by co-authors

The undersigned hereby certify that:

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4.1 Integrative commentary for chapter 4

At the outset of this work it was well established that LRH-1 plays a significant role in breast cancer cell proliferation, motility and invasion. Primarily, LRH-1 has been shown to regulate aromatase, which catalyses conversion of androgens to oestrogen. Aromatase expression and activity is low in breast cancer cells, however there is a marked increase of expression and activity in cancer associated fibroblasts compared to normal fibroblasts. Chapter 2 confirmed previous publications that LRH-1 transcript expression correlated with ER status in breast cancer cell lines. In addition, the study provided evidence of oestradiol regulating LRH-1 expression and transcript stability. LRH-1 synergises with oestrogen signalling, and that LRH-1 and the ER share similar target genes. Our lab and others have shown that LRH-1 is able to bind to ER target genes that have shared binding sites (Chand et al. 2012, Lai et al. 2013), and importantly at these shared sites LRH-1 promotes ER recruitment and vice versa (Lai et al. 2013).

In order to explore novel LRH-1 target genes and dependent mechanisms in breast cancer cell lines, we performed a microarray after depleting LRH-1 in ER+ MCF-7 cells using siRNA strategies. We observed a reduction of TGF-β signalling in response to LRH-1 depletion. Therefore, the objectives of this study were to: 1) define the relationship between LRH-1 and TGF-β in breast cancer cell lines. 2) confirm this relationship in a novel LRH-1 transgenic mouse model. We found genes involved in TGF-β signalling to be highly responsive to LRH-1 knockdown. The expression levels of TGF-β and activation of downstream signalling were subsequently analysed. Moreover, we performed chromatin immunoprecipitation studies to examine the binding of LRH-1 to the TGF-β promoter. To extend our study we then utilised a novel inducible mammary specific LRH-1 transgenic mouse model to explore this relationship further. The expression of TGF-β and activation of downstream signalling in the whole mouse mammary glands was examined. In addition, the consequences of LRH-1 regulating TGF-β in mouse mammary glands were explored.

This chapter is currently Ahead of Print at Endocrinology.
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Full title: Conditional over-expression of Liver Receptor Homolog-1 in female mouse mammary epithelium results in altered mammary morphogenesis via the induction of Transforming Growth Factor-β

Abbreviated title: LRH-1 induces TGF-β expression in mammary epithelial cells

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Abstract

Liver Receptor Homolog-1 (LRH-1) is an orphan nuclear receptor that belongs to the NR5A subgroup of nuclear receptors. LRH-1 induces key genes to regulate metabolic process, ovarian function, cancer cell proliferation and steroidogenesis. In the breast, LRH-1 modulates and synergizes with endogenous estrogen signalling to promote breast cancer cell proliferation. We used siRNA knockdown strategies in order to deplete LRH-1 in breast cancer cells and followed with microarray analysis to identify LRH-1 dependent mechanisms. We identified key genes involved in TGF-β signalling to be highly responsive to LRH-1 knockdown. This relationship was validated in two breast cancer cell lines over-expressing LRH-1 in vitro and in a novel transgenic mouse with targeted LRH-1 over-expression in mammary epithelial cells. Notably, TGF-β signalling was activated in LRH-1 over expressing breast cancer cells and mouse mammary glands. Further analyses of mammary gross morphology revealed a significant reduction in mammary lateral budding after LRH-1 over expression. These findings suggest that the altered mammary morphogenesis in LRH-1 transgenic animals is mediated via enhanced TGF-β expression. The regulation of TGF-β isoforms and SMAD2/3-mediated downstream signalling by LRH-1 also implicates a potential contribution of LRH-1 in breast cancer. Collectively this data demonstrates that LRH-1 regulates TGF-β expression and downstream signalling in mouse mammary glands.
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Introduction

Liver receptor homolog-1 (LRH-1) is a monomeric nuclear receptor that belongs to the NR5A subgroup of nuclear receptors. LRH-1 regulates transcription of key genes to regulate metabolic processes including bile acid synthesis, reverse cholesterol transport and glucose homeostasis (for review, see (Lazarus et al. 2012)) Its high expression in granulosa cells in the ovary and recent functional studies in ovary-specific knockout mice demonstrates that it is essential for both ovulation and pregnancy (Duggavathi et al. 2008, Zhang et al. 2013). LRH-1 is also highly expressed in the intestine, gastric system and pancreas, where it regulates cell proliferation (Schoonjans et al. 2005, Wang et al. 2008, Benod et al. 2011). In these tissues there is growing evidence that LRH-1 contributes to tumour formation, where it is involved in cell cycle progression to promote tumour proliferation (Wang et al. 2008). In ovarian and breast tumour tissue LRH-1 is involved in the up-regulation of enzymes such as steroidogenic acute regulatory protein, and aromatase, both of which regulate rate-limiting processes in the production of steroid hormones (Miki et al. 2006, Saxena et al. 2007).

The best characterized actions of LRH-1 in breast cancer are the modulation of endogenous estrogen signalling: LRH-1 is expressed in breast adipose stromal cells and breast cancer-associated fibroblasts, where it stimulates expression of aromatase, the rate-limiting step in estrogen synthesis (Clyne et al. 2002). In breast cancer cell lines, LRH-1 regulates expression of estrogen receptor alpha (ERα)(Annicotte et al. 2005), and is itself an ERα target gene (Thiruchelvam et al. 2011). LRH-1 can also directly stimulate expression of ERα target genes such as GREB1 and pS2 by binding to estrogen response elements within their promoters (Chand et al. 2012). Thus, LRH-1 expression in breast cancer cells and cancer-associated fibroblasts mediates a positive feedback loop that contributes to increased estrogen synthesis and action.

Localisation of LRH-1 in human breast tissue demonstrates nuclear expression in luminal epithelium of normal mammary glands; and increased levels in both ER-positive and ER-negative tumours and associated stroma (Miki et al. 2006). Functional studies in cell lines demonstrate that over-expression of LRH-1 increases estrogen-mediated cell proliferation (Annicotte et al. 2005). LRH-1 also has direct, estrogen-independent effects on breast cancer cell proliferation (Chand et al. 2012). In addition, over-expression or knockdown or LRH-1 in ER-positive, ER-negative and normal mammary epithelial cell lines demonstrate a role in cell proliferation, migration and invasion associated with remodelling of the actin cytoskeleton, and cleavage of E-Cadherin.
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(Chand et al. 2010). While the mechanisms of these effects are currently unclear, they may involve stimulation of matrix metalloprotease activity, and/or direct induction of cell cycle-associated genes. Aside from genes involved in estrogen signalling, few LRH-1 target genes have been identified in normal or breast cancer epithelial cells. In order to obtain an understanding of LRH-1 dependent mechanisms in breast cancer cells, we used siRNA knockdown strategies followed by microarray analysis to identify novel LRH-1 target genes in MCF-7 cells. We found genes involved in TGF-β signalling to be highly responsive to LRH-1 knockdown. This LRH-1-dependent regulation of TGF-β family members was observed in both human breast cancer cells \textit{in vitro}, and in a transgenic mouse with targeted LRH-1 over-expression in mammary glands. The induction of LRH-1 in mammary glands of adult transgenic mice caused alterations in Smad2/3-mediated TGF-β signalling pathways and abnormal mammary ductal formation. Collectively this data demonstrates the importance of LRH-1 in regulating ductal morphogenesis to promote normal mammary gland formation. The regulation of expression of TGF-β isoforms and Smad2/3-mediated signalling by LRH-1 also implicates a potential contribution in breast cancer.

Methods

\textbf{Generation and genotyping of transgenic mice}

All studies involving the mice were regulated under the protocol approved by Monash Medical Centre; Ethics: MMCB10/12. The pTetOLRH-1 transgene was generated from a construct containing the human LRH-1 open reading frame (Clyne et al. 2002). Briefly, a Kozak sequence was introduced by site-directed mutagenesis using the QuickChange® II Site-Directed Mutagenesis Kit (Agilent Technologies, USA) and primers F- Kozak: 5’-TTAAGCCAAAGAACTGCTATAATTTCACTCACCATGGCTTCTAATTCAGATACTGGGATTTACAAG -3’ and R-Kozak: 5’-CTTGTAAATCCCAAGCAGTATCTGAATTAGAAGCCATGGTGAGTGAAATTATAGGCAGTTCTTTGGCTTAA-3’. The LRH-1 open reading frame containing the Kozak sequence was then subcloned into the MCS of the pTRE-Tight-BI-DsRed Express vector (Clontech, USA) after NotI restriction digest. Orientation of the insert was verified by digesting the vector with BamHI. The vector was then linearized using ApaLI and gel purified. The pTetOLRH-1 founder mice were generated by pronuclear injection (MouseWorks Service, Australia) of the purified transgene, and complete transgene insertion was verified using primers that spanned the 3’ end of the DsRed open reading frame (L1) (F: 5’-GCCGATGAACCTCAGTTCTTGTTAAATCCCAAGCAGTATCTGAATTAGAAGCCATGGTGAGTGAAATTATAGGCAGT -3’) and the 3’ end of the LRH-1 open reading frame (L2) (F: 5’-CGAGGACCGT CATCAAGGAGT -3’).
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5′- TCGACCACA TTTACCGACAA -3’ and R: 5′- TGGCTGATTATGATCCTCTGG -3’). MMTVrtTA-pTetOLRH-1 (MMTVtet-LRH-1) double transgenic mice were generated by crossing pTetOLRH-1 founder females with MMTVrtTA males. Genotyping of double transgenic animals was performed using primers for LRH-1 listed above and MTB F- TGCCGCCATTATTACGACAAGC, R- ACCGTACTCGTAATTCCAAGGG. Only female MMVtet-LRH-1 mice were selected for this study unless otherwise stated.

**Induction of LRH-1 transgene expression**

To induce LRH-1 transgene, doxycycline hyclate (Clontech, Cat# 631311) was supplied in drinking water at a 2mg/ml concentration with 3% sucrose. The control cohorts were provided with 3% sucrose in drinking water. The water was replaced every three days and kept under light sensitive conditions.

**Determination of Stage of Mouse Oestrous Cycle**

Vaginal smears were performed using saline and a plastic transfer pipette. Sloughed cells were spread on a glass slide, stained with haematoxylin and eosin, and examined by light microscopy to determine stage of the oestrous cycle (proestrus, oestrus, metestrus I, metestrus II, or diestrus).

**Mammary gland whole mount analysis**

The #4 inguinal mammary glands were harvested and spread onto slides, fixed with Carnoy’s fixative (60% Ethanol, 30% Chloroform and 10% glacial acetic acid) for 2-3 hours. The glands were placed in 70% ethanol for 15 mins followed by 5 mins each in 50% ethanol, 25% ethanol and ddH₂O before staining overnight with Carmine Alum. The mammary glands were dehydrated sequentially in graded ethanol washed, and cleared in histolene to dissolve fat in the gland. The slides were maintained in DPX mounting media for analysis under light microscope. The number of primary and secondary branches, and ductal ends were counted for the entire gland. The mammary gland tree occupancy was then estimated according to the sections occupied. Data points from all mice in the same group were averaged and plotted.

**Immunohistochemical (IHC) analysis**

Sections of 5 μm thickness were cut from formalin-fixed, paraffin-embedded inguinal mammary glands. Prior to staining, the sections were de-waxed and rehydrated in graded ethanol washes. Antigen retrieval was performed by immersing slides in 600 ml of Target Retrieval
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Solution (Dako) and heating in a 1000 W microwave at 100% power for 5 min, standing at room temperature for 5 min and then heating for an additional 5 min at 40% power, before cooling at room temperature for an hour. Sections were then treated with 3% H$_2$O$_2$ for 5 min to block endogenous peroxidase activity. To block endogenous avidin and biotin in the sections, the avidin/biotin blocking kit (Vector Laboratories) was used. The sections were then incubated with specific primary antibodies (Table 1). For antibodies raised in mouse, the mouse on mouse (M.O.M) basic kit (Vector Laboratories) was employed for detection. For antibodies raised in rabbit, the Rabbit IgG Kit, (Vector, PK-6101) was utilized for detection. The tissue was then incubated with ABC reagent (Vectastain Elite, Vector, PK-6101) for 30 minutes. Staining was detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (DAB Liquid Substrate Kit, Dako). The sections were counterstained with Mayer’s haematoxylin (Sigma Diagnostics), dehydrated and mounted with DPX mounting medium (Sigma Diagnostics) under 22 × 50 mm cover slips (HD Scientific). Staining was photographed with OLYMPUS microscope. Staining-positive cells were counted in 40 fields and data were expressed as a percentage of total epithelial cells counted.

Western blotting

Protein lysates were prepared by placing the mammary glands or breast cancer cells in protein lysis buffer (5mm HEPES, 137mm NaCl, 1mm MgCl$_2$, 1mm CaCl$_2$, 10mm NaF, 2mm EDTA, 10mm Na pyrophosphate, 1% Nonidet P-40, 10% glycerol and protease inhibitors (Roche)). The tissue was then homogenized using the Qiagen Tissue lyser LT. After homogenising, the sample was centrifuged (full speed for 15mins) and incubated in 4°C for 15mins on ice. The supernatant was then collected and used for downstream protein applications. Protein concentrations in these lysates were measured by BCA Protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts (40μg) of total cellular protein extract were electrophoresed on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Hybond – C Super; Amersham, RPN203G). Membranes were blocked with 5% (w/v) milk protein in Tris-buffered saline (TBS) containing 0.05% Tween-20. Blots were then incubated with primary antibodies as indicated, washed in TBS containing 0.1% Tween-20 and probed with primary antibodies. Secondary IgG-conjugated mouse or rabbit antibodies, and Alexa fluor 700 (Invitrogen) were used at a dilution of 1:10000 to visualize protein bands. Band intensities were quantified using the Odyssey infrared imaging system and Odyssey 3.0 Software (LI-COR Biosciences, Lincoln, NE, USA). Blots shown are representative of a minimum of three separate experiments.
Quantitative reverse-transcriptase PCR (Q-RT-PCR)

Total RNA was prepared from mouse mammary tissue or breast cancer cells using the RNeasy Kit (Qiagen, 74106), treated with DNaseI (Ambion, AM1906), and quantified using a NanoDrop 1000 Spectrophotometer. First strand cDNA synthesis using 1.0 μg total RNA was performed using AMV Reverse Transcriptase (Promega, M5101) primed by random hexamers. PCRs were carried out using Taqman probes from Applied Biosystems against LRH-1(Hs00187067_m1), NR4A1 (Hs00374226_m1), TP53 (Hs01034249_m1), ITGB1 (Hs00559595_m1), KRT18 (Hs02827483_g1), ANXA2 (Hs00743063_s1), MMP3 (Mm00440295_m1), ACTA2 (Mm00808218_g1), TPSAB1/2 (Mm00491950_m1), TNFRSF12A (Mm00489103_m1), HTRA1 (Mm00479892_m1) and Taqman Gene Expression Master Mix (Applied Biosystems). Other genes were analysed using the Power SYBR Green PCR Mix (Applied Biosystems) using the following primers, 18s: sense-CGGCTACCACATCCAAGGAA, antisense-GCTGGAATTACCGCGGCT; mTGF-β1: sense-AGCCCGAAGCGGACTACTAT, antisense-TTCAACATTTGCTCCACAC; mTGF-β2: sense-TTTAAGAGGGATCTTGGATGGA, antisense-AGAATGGTCAGTGGTTCCAGAT, mTGF-β3: sense- CGCACAGAGCGAGAATTGA, antisense-GTGACATGGACAGTGATGC. mRNA was quantified by ABI Prism 7900-HT Real-time PCR system. Fold changes in expression of LRH-1 was calculated using the ddCt method (Schmittgen and Livak 2008) with 18s as an internal control. Data represented was collated from RNA collected from three separate experiments.

Knockdown and over-expression of LRH-1 in MCF-7 cells

To reduce basal LRH-1 expression levels in the MCF-7 breast cancer cell line, the SureSilencing short hairpin RNA (shRNA) plasmids (KH05888G, SABiosciences, Frederick, MD, USA) designed to specifically knockdown the expression of LRH-1 by RNA interference were used, as previously verified (Chand et al. 2010). MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 50U/ml penicillin and 50μg/ml streptomycin at 37°C in 5% CO₂. Cells were transfected with either pGeneclip hMGFP-LRH-1 or pGeneclip hMGFP encoding a scrambled shRNA sequence as negative control using the Nucleofector device, Kit V (Amaxa Biosystems), according to the manufacturer’s instructions. GFP-positive cells were collected by fluorescence-activated cell sorting (FACS) 24 hours after transfection. To over-express LRH-1, pcDNA3.1 plasmid expression construct containing the full length sequence of human LRH-1.
was transfected using Amaza transfection as previously described (Chand et al. 2010). Control transfections were performed with pcDNA3.1 vector alone and mRNA isolated for qPCR analysis and protein lysates extracted for western blot analysis 24 hours post-transfection.

Expression profiling and analysis

Gene expression profiling Illumina (San Diego, CA) human whole genome 6- v2 bead chips, containing 46325 probes was conducted in two separate experiments: (i) in MCF-7 cells where the effects of LRH-1 knockdown on global gene expression was determined and (iii) in mammary tissue where human LRH-1 was over-expressed using the dox-inducible transgenic mouse described above. In MCF-7 cells, ten transfections were performed and pooled per condition, GFP-positive cells isolated by FACS, and RNA isolated.

For the transgenic mouse studies, RNA was isolated from mammary tissue from three month control and doxycycline treated LRH-1 transgenic mice (n=3)and array performed for individual samples to analyse gene expression per animal using Illumina (San Diego, CA) mouse WG6v2 Expression Bead Chip.

Expression profiling was performed at the Australian Genome Research Facility, Parkville, Australia). For the MCF-7 cell microarray, samples were normalized by quantile normalisation using the lumi package in R statistical software and subsequently imported into GeneSpring GX for analysis. In each case, genes were considered differentially expressed between groups if fold change (positive or negative) ≥ 1.4. Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) was used to identify gene networks according to biological functions and/or diseases in the IPA database. The significance value associated with a given function was expressed as a $P$ value (calculated using right-tailed Fishers exact test) by comparing the number of LRH-1 – regulated genes associated with a given function to the total number of occurrences of those genes in all functional annotations in the IPA database.

For the mouse mammary microarray, raw signal intensity values were subjected to variance stabilization transformation including background correction, log2 transformation and variance stabilization using the lumiR package of R Bioconductor (Du et al. 2008, Lin et al. 2008). ANOVA analysis of normalized probe intensities values was performed in Partek® Genomic SuiteTM software (version 6.6 build 6.12.0420; Copyright © 2003-2012 Partek Inc., St. Louis, MO, USA). ANOVA was used to calculate significance of variation in normalized expression
values between sample groups, fold change of gene expressions was calculated as mean ratio. Probes with an unadjusted p-value of 0.05 or less (no False Discovery Rate correction was applied) and an absolute fold change of 1.5 or more were defined as differentially expressed. For both data sets, we used IPA to assess significant upstream regulators. Activation z-score was calculated as a measure of functional and translational activation in Networks and Upstream regulators analysis. An absolute z-score of below (inhibited) or above (activated) 2 was considered as significant.

**Chromatin Immunoprecipitation**

The chromatin immunoprecipitation procedures were performed as described previously (Chand et al. 2012). The precipitated chromatin was analysed by quantitative real-time PCR to demonstrate relative occupancy. Primer sequences are as followed LRH-1RE1: sense- ACTCTGCTCCAACGTCACC, antisense- GGCACCGCTTCTGCTCTTC; LRH-1RE2: sense- CAATTACCACCACATCTGATCTACC, antisense- GGGATAGATAAGACGGTGAGGAG; negative: sense- CTCACAGCAATTACCACCATC, antisense- TTTGACTACCAGACTGAGGAGCT amplified at 58°C for 45 cycles. Primer sequences for LRH-RE1 GREB-1 were as previously described (Chand et al. 2012).

**Statistical analysis**

All data are reported as mean ± SE for three or more separate experiments as indicated. Semi-quantification of IHC staining and Sirius red staining was performed by three independent researchers. Statistical analyses for experiments comparing two groups were performed by two-tailed Student's independent t test using GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) and a P value of <0.05 was considered statistically significant.

**Results**

**LRH-1 over-expression induces TGF-β isoforms and downstream signalling in vitro**

To identify LRH-1 regulated genes in breast cancer epithelial cells, Illumina expression profiling was performed using RNA from GFP-sorted MCF-7 cells expressing either LRH-1 siRNA or control siRNA. Significant alterations were observed for 1200 genes, of which 726 were down-regulated and 474 were up-regulated (Fig1A). Genes altered in expression were associated with processes regulating cellular morphology, movement and interaction
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(Supplementary Table 1). Using IPA, we demonstrate a significant inhibition of TGF-β1 as an upstream regulator with a z-score of -2.596. This suggested that LRH-1 might modulate endogenous TGF-β signalling, and we therefore examined this potential relationship in detail. Notably, we observed a marked down-regulation of several genes involved in TGF-β signalling, including transforming growth factor-β2 (TGF-β2), nuclear receptor 4A1 (NR4A1), tumour protein p53 (TP53), integrin-β1 (ITGB1), cytokeratin 18 (KRT18) and annexin A2 (ANXA2) (Fig 1A, n=6, red dots). To validate genes involved in TGF-β signalling, we performed RT-PCR on LRH-1 depleted MCF-7 cells and observed a significant decrease in LRH-1, TGF-β2, NR4A1, TP53, ITGB1, KRT18 and ANXA2 (Fig 1B, red dotted line represents 1.3 fold down regulation).

We transiently over-expressed LRH-1 in two ERα-positive breast cancer cell lines, MCF-7 and T47-D. LRH-1 mRNA levels were significantly increased in each cell line (Fig. 1B), although the absolute levels were higher in MCF-7 cells. Over-expression of LRH-1 significantly increased expression of TGF-β1, 2 and 3 mRNA in MCF-7 and T47D cells (Fig 1C and D). Furthermore, we also examined the expression levels of several genes involved in TGF-β signalling as observed in the microarray. We demonstrate a significant increase in NR4A1, TP53, ITGB1, and KRT18 in LRH-1 overexpressing MCF-7 and T47D cell lines (Fig. 1C and D). A significant increase in ANXA2 was only observed in T47-D cells (Fig 1D). We observe a differential expression of TGF-b isoforms in MCF-7 and T47-D cells.

LRH-1 over-expression increased protein expression of bioactive TGF-β isoforms in MCF-7 cells, and this correlated with reduced expression of their corresponding precursor isoforms (Fig 1E). In addition, we quantified LRH-1 and TGF-β relative intensity and demonstrate increased expression in LRH-1 overexpressing cells (Fig. 1F). Quantification of active/inactive relative intensity revealed increased TGF-β active forms in LRH-1 overexpressing cells (Fig. 1F).

TGF-β isoforms initiate signalling via ligand-induced oligomerization of serine/theronine receptor kinases and phosphorylation of cytoplasmic signalling molecules Smad 2 and Smad 3. Carboxy-terminal phosphorylation of the Smad 2 and 3 results in their partnering with transducer Smad4; which causes subsequent translocation into the nucleus. In order to demonstrate activation of TGF-β signalling pathways we analysed the phosphorylation status of Smad 2/3 in MCF-7 cells. In cells over-expressing LRH-1, an increase in Smad 2 and 3 phosphorylation was observed...
with no change in levels of total Smad 2 and 3 (Fig. 1G). Quantification of phospho/total Smad levels reveals an increase in phosphorylated Smad 2 and 3 in LRH-1 overexpressing MCF-7 cells (Fig. 1H). Taken together, these results identify a novel association between LRH-1 and TGF-β in ERα-positive breast cancer cells.

**TGFB1 is a direct transcriptional target of LRH-1**

Using the UCSC genome browser (http://genome.ucsc.edu) to obtain TGF-β1 promoter sequence and Biobase transcription binding site analysis software Patch (http/www.gene-regulation.com/pub/programs.html), we identified potential LRH-1 consensus half sites (LRH-1REs) within a region spanning 2kb of the TGF-β1 promoter. We selected two LRH-1 REs, LRH-1 RE1 (-682bp) and LRH-1 RE2 (-1406bp) from the start site (Fig. 2A). Furthermore, we selected a region that had no LRH-1 binding site, denoted "negative" (Fig. 2A). We demonstrate LRH-1 interaction on LRH-1 RE1 of the TGF-β1 promoter region (Fig. 2B). However, no interaction was observed on LRH-1 RE2 and the "negative" region. As shown previously, we observed interaction on the LRH-1 RE1 of the GREB-1 promoter region (Fig. 2B, (Chand et al. 2012)).

**Generation of a transgenic mouse that inducibly expresses LRH-1 in mammary gland**

To demonstrate that LRH-1 activates TGF-β signalling in vivo we generated a transgenic mouse in which human LRH-1 is expressed specifically in mammary epithelial cells in response to doxycycline. We first created a line in which human LRH-1 is expressed under the control of a tetracycline-responsible element (TRE), and then crossed this line with the previously described MMTV-rtTA (MTB) line (in which expression of the reverse tetracycline transactivator is directed to mammary epithelial cells by the MMTV promoter ((Gunther et al. 2002), Fig. 3A, 3B). The result double transgenic animals were phenotypically normal, and are termed **MMTV**tet-LRH1. As expected, the human LRH-1 transgene (hLRH-1) was undetectable in mammary RNA isolated from vehicle-treated mice (Fig 3C). However, administration of 2 mg/ml doxycycline (dox) in drinking water from 56 days of age for short (3 week, n=14) or long (3 month, n=16) time frames resulted in significant expression of hLRH-1 at both time points (Fig 3C). Using an antibody that recognizes both mouse and human LRH-1, we confirmed that dox treatment strongly induced LRH-1 protein expression in mammary gland, whereas endogenous expression of LRH-1 in liver and intestine was unaffected (Fig 3D). Immunohistochemistry confirmed that dox-induced hLRH-1 was expressed specifically in luminal epithelial and myoepithelial cells, as expected for MMTV-driven transgene expression (Fig 3E). LRH-1 expression was semi-
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quantitated and we observed a 1.5 fold increase in three week treated animals and a threefold increase in three month treated animals (*p<0.05, **p<0.01, Fig 3F).

**LRH-1 over-expression induces TGF-β signalling in mouse mammary gland**

To determine if LRH-1 activates TGF-β signalling in mouse mammary, as it does in MCF-7 breast cancer cells, we performed Illumina expression profiling using whole mammary RNA isolated from vehicle- and dox-treated *MMTV* tetLRH1 mice (Fig. 4A). 242 genes were significantly altered in the dox-treated group, of which 120 were down-regulated and 122 were up-regulated (Fig. 4A). Similar to MCF-7 cells, genes whose expression were modulated by dox included those associated with cellular movement, interaction, cell death survival, cancer and embryonic development (Supplementary Table 2). A volcano plot was generated and again consistent with the MCF-7 data, genes involved in TGF-β signalling, including matrix metalloproteinase 3 (MMP3) which is involved in epithelial to mesenchymal transition (Radisky and Radisky 2010), smooth muscle actin alpha2 (ACTA2) which is required for breast cancer cell migration (Lee et al. 2013), tryptase alpha and beta 1/2 (TPSAB1/2) which activates TGF-β1 (Zeng et al. 2013), tumour necrosis factor receptor superfamily, member 12A (TNFRSF12a) which is involved in breast cancer cell motility (Rhee et al. 2008) and HtrA serine peptidase 1 (HTRA1) which regulates angiogenesis through TGF-β signalling (Zhang et al. 2011), were significantly up regulated in mammary glands of dox-treated *MMTV* tetLRH1 mice (Fig. 3A, n=5, red dots) (Fig. 4A). Using IPA, we demonstrate a significant activation of TGF-β1 as an upstream regulator with a z-score of 2.524.

We confirmed that dox treatment of *MMTV*tetLRH1 mice increased mRNA levels of TGF-β1, 2 and 3 in mammary gland at both short and long time points (Fig 4B, 4D, 4F), and demonstrated significant correlations between mRNA levels of hLRH-1 and TGF-β1 (r = 0.4917, Fig 4H) and between hLRH-1 and TGF-β2 (r = 0.5661, Fig 4I). Dotted lines show 95% confidence limits. No correlation was observed between hLRH-1 and TGF-β3 (data not shown). Immunohistochemical analysis of mammary glands demonstrated increased immunostaining of TGF-β isoforms 1, 2 and 3 protein in luminal epithelial cells of dox-treated *MMTV*tetLRH1 mice at 3 week and 3 month time points, compared with age-matched control animals (Fig. 4C, E and G, Supplementary Fig. 2).
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The marked genes involved in TGF-β signalling observed in the mouse microarray were validated and we demonstrated increased expression of MMP3, ACTA2, TBSAB1/2, TNFRSF12a and HTRA1 in three month dox treated animals (Fig. 5 A-E). Consistent with this, protein levels of TGF-β1 and -2, as well as of active, phosphorylated Smad 2 and Smad 3, were all significantly increased in mammary lysates from dox-treated MMTVtetLRH1 mice (Fig. 5F,H). Quantification of TGF-β isoform intensity revealed increased expression of TGF-β1 and 2 (Fig. 5G). Also, quantification of phospho/total Smad2 and 3 relative intensity reveals increased phosphorylated Smad 2 and 3 in dox treated animals (Fig. 5I).

Finally, TGF-β is known to induce expression of collagen 1, via activation of the Smad pathway (Chen et al. 1999). Accordingly, we demonstrated increased collagen expression, as determined by Sirius Red stain, in mammary glands of dox-treated MMTVtetLRH1 mice at both short and long time points (1.75-fold and 2.3-fold, respectively), compared to age-matched controls (Fig. 5J and K).

LRH-1 over-expression inhibits lateral bud formation

The primary actions of TGF-β in the regulation of mammary development processes include the regulation of ductal outgrowth, lateral branching, lactation and apoptosis (Ingman and Robertson 2008). Indeed, over-expression of TGF-β in mice inhibits ductal morphogenesis (Silberstein and Daniel 1987). Therefore, we examined mammary gland morphology in control and dox-treated MMTVtetLRH1 adult virgin female mice, in oestrous (Supplementary Fig 1B), to determine if LRH-1 induces a similar phenotype. Gross morphology of mammary glands exhibited no overall structural differences between the two treatment cohorts, with both groups displaying developed mammary tree structures that extended beyond the lymph node and the presence of terminal end buds (data not shown). However, dox-treated MMTVtetLRH1 mammary glands exhibited a poorly branched epithelial tree with rudimentary, thin ducts and a significant decrease in lateral buds compared to control animals (Fig. 5L and N). Quantification of lateral bud numbers revealed a 3-fold reduction at 3 weeks (p<0.01) and a 2-fold reduction at 3 months (p<0.01), compared to age-matched, vehicle-treated mice (Fig 5M and O). Body weights and vaginal smears of animals after three month dox treatment were analysed and showed no difference, suggesting that the phenotype was due to activation of the hLRH-1 transgene, and not increased animal weight or oestrous cycle (Supplementary Fig. 1A and B, control n=4, dox n=4). Quantification of terminal end bud (TEBs) revealed no significant difference with treatment (Supplementary Fig 1C, control n=4, dox n=4). Thus, conditional expression of hLRH-1 in mouse
mammary gland did not affect ductal proliferation but produces a lateral end bud phenotype remarkably similar to that induced by TGF-β over-expression.
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Discussion

LRH-1 functions as a transcription factor to regulate steroidogenesis, cholesterol and bile acid metabolism. It also promotes tumour cell proliferation in gastric, intestinal, hepatic, pancreatic and breast cancers (Botrugno et al. 2004, Schoonjans et al. 2005, Chand et al. 2010, Benod et al. 2011, Chand et al. 2012). In breast cancer, a well-characterized action of LRH-1 is the activation of aromatase transcription in tumour-associated stroma (Clyne et al. 2002). As observed in other tumour epithelial cell types, in the ER-positive MCF-7 breast cancer cell line, LRH-1 stimulates proliferation and the expression of genes such as GREB1, cyclins D1 and E1 (Botrugno et al. 2004, Chand et al. 2012). Our previous work identified additional functions for LRH-1 in mediating cell motility, changes to actin cytoskeleton structure, and promoting invasiveness in normal and tumour breast epithelial cell lines (Chand et al. 2010). This suggested that LRH-1 might be involved in other tumour-promoting pathways.

We identified LRH-1 regulated genes in MCF-7 cells where LRH-1 was knocked-down and in MMTVtetLRH1 mice where human LRH-1 expression was induced by treatment with doxycycline in mammary epithelial cells. We discovered that TGF-β2 and TGF-β-regulated genes were among the genes most significantly altered. LRH-1 expression positively correlated with TGF-β1, -2 and -3 mRNA levels, and active protein levels and increased Smad2/3 phosphorylation both in vitro and in vivo (Supplementary Fig. 3). Integrin αvβ6 requires the interaction of latent TGF-β binding protein to mediate the activation of latent TGF-β (Avveni et al. 2004); Annexin A1, involved in phosphorylation and nuclear translocation (de Graauw et al. 2010); and p53 known to interact directly with Smad2/3 on target gene promoters (Cordenonsi et al. 2003). We observed changes in expression of matrix metalloproteinase 3 and α-smooth muscle actin, known TGF-β targets (Uttamsingh et al. 2007) that have also been demonstrated previously as regulated by LRH-1 (Duggavathi et al. 2008, Chand et al. 2010). Furthermore, we demonstrate TGF-β1 and 2 transcript expression higher in MCF-7 cells compared to T47-D cells. These observations are consistent with previous studies showing lower TGF-β1 protein secreted in T47-D cells compared to MCF-7 cells (Fanayan et al. 2000).

Pathway analysis of LRH-1 regulated genes in MCF-7 cells and MMTVtetLRH1 mammary tissue highlighted that most altered genes were involved in the regulation of cell motility, structure and cell-cell interaction, in line with observations from previous studies (Chand et al. 2010).
together with the phenotype of altered mammary gland morphology in $^{MMTV}_{tetLRH1}$ mice, we conclude that the TGF-β pathway may be an effector of these LRH-1 mediated events. Indeed, reduced lateral branching and budding observed in $^{MMTV}_{tetLRH1}$ with ectopic LRH-1 expression is similar in phenotype with mammary glands from mice implanted with TGF-β1 pellets (Silberstein and Daniel 1987, Silberstein et al. 1992). This suggests that LRH-1 may have an inhibitory effect in lateral budding via activation of the TGF-β signalling pathway. It is well established that TGF-β1 is a key regulator of mammary branching morphogenesis. Specifically, it reduces proliferation and differentiation of ductal end buds, an effect that is reversible to allow for the continual development and involution of mammary glands (Silberstein and Daniel 1987, Daniel et al. 1989, Robinson et al. 1991).

The regulation of branching processes controlling extension of ductal branches and formation of alveolar within the mammary fat pad is crucial for lactation. Altered branching morphogenesis and lateral budding observed in doxycycline treated $^{MMTV}_{tetLRH1}$ also implies that LRH-1 may have other effects in different development contexts such as proliferation of end bud structures during pregnancy. Further work to establish the differential cellular effects on mammary development during pregnancy, lactation and involution using the $^{MMTV}_{tetLRH1}$ is ongoing.

The three known mammalian isoforms TGF-β-1, -2 and -3 elicit similar paracrine responses in regulating mammary development, are co-localized in intracellular regions and detected during all stages of mammary gland development (Robinson et al. 1991). Primarily TGF-βs act similarly in governing ductal morphogenesis by influencing extracellular matrix deposition including collagen as observed in animals treated exogenously with TGFβ-1, -2 and -3 (Silberstein et al. 1990, Silberstein et al. 1992, Silberstein 2001). The increased accumulation of collagen around the mammary ducts in doxycycline treated $^{MMTV}_{tetLRH1}$ animals when compared with untreated animals may be attributable to the activation of TGF-β signalling pathways.

Basal TGF-β2 levels in mammary tissue are low, however its expression is strongly up-regulated during pregnancy-associated mammary gland development (Daniel and Robinson 1992, Daniel et al. 1996). TGF-β3 appears to be the only isoform markedly expressed in myoepithelial cells which form the outermost monolayer of mammary ducts (Daniel et al. 1996). The functional significance of myoepithelial cells, other than its role as a contractile tissue during pregnancy, is
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

unclear. It has been suggested that myoepithelial cells are capable of reforming cap cells during formation of lateral branches and may be involved in amplification of basal lamina components (Silberstein and Daniel 1987, Robinson et al. 1991). The up regulation of TGF-β3 by LRH-1 in myoepithelial cells may increase basal lamina components, contributing to inhibition of lateral branching and budding.

Our findings demonstrate a strong association between LRH-1 and TGF-β activity that has not been identified previously. LRH-1 belongs to the NR5A sub-class of the nuclear receptor family, binding as a monomer to a nuclear receptor (NR) half-site sequence consensus YCAAGGYCR (Fayard et al. 2004). ChIP studies indicate direct interaction between LRH-1 and binding sites on the TGF-β1 promoter region. We also observed an overlap of LRH-1RE and AP1 response elements on the TGF-β1 promoter region. Recent ChIP-seq studies have shown LRH-1 interacting with AP1 factors c-fos and c-jun (Lai et al. 2013). Together with studies showing that AP1 regions are responsible for TGF-β1 auto-induction (Kim et al. 1990), our data suggest a possible interaction between LRH-1 and TGF-β1. Further studies need to be undertaken to explore this interaction. Genes encoding the TGF-β-1, -2 and -3 isoforms are located on separate chromosomes and we analysed the first 5kb region of respective promoters to identify the LRH-1 half-site sequence consensus. We identified eleven putative LRH-1 binding sites on the TGFB2 and six on the TGFB3 gene promoters, however further studies to confirm whether LRH-1 directly binds to these sites to activate transcription are yet to be undertaken.

LRH-1 is a critical factor in early embryonic development, essential for maintaining pluripotency by the regulation of Oct4 and Nanog expression via the Wnt signalling pathway. Interestingly, in breast cancer stem cells, TGF-β signalling appears to modulate stem cell phenotype and maintain pluripotency (Massagué 2008, Polyak and Weinberg 2009). In the context of breast tumours, the association between TGF-β and LRH-1 may be worth exploring in the context of disease progression. TGF-β can alter tight junction formation in mammary epithelium and induce signalling in a number of embryonic signalling pathways, including Wnt, Notch, and Hedgehog pathways (Zavadil et al. 2004), as well as the mitogen-activated protein kinase (MAPK) pathway (Guo and Wang 2009). The complexity of this crosstalk signifies the variety of process that may be regulated during mammary gland development and tumorigenesis; and highlights the importance of further investigation into the role of LRH-1 in promoting mammary tumorigenesis. To date, the functions of LRH-1 in mammary epithelial cells have not
been studied *in vivo*. Our data demonstrates that the ectopic expression of LRH-1 in mouse mammary glands leads to inhibition of lateral mammary ductal branching via the stimulation of TGF-β signalling. The TGF-β pathway was also regulated by LRH-1 in breast cancer cells. How LRH-1 exerts its effects on the processes of proliferation and differentiation in the normal mammary gland and tumour cells has important implications for both development and breast cancer.

**Acknowledgements**

This work was supported by a grant from the National Health and Medical Research Council of Australia (1027707 CDC and ALC), by Swinburne University Postgraduate Award (KAL), by NHMRC Career Development Award (GNT1007714, KAB), US Department of Defence Breast Cancer Research Program (W81XWH-10-1-0913, ALC) and the Victorian Government’s Operational Infrastructure Support Program. PHI Data Audit # 13-29
Figure Legends

Figure 1: Microarray analysis reveals genes involved in TGF-β signalling are down-regulated in MCF-7 LRH-1 siRNA cells. (A) Scatter plot of control siRNA (x-axis) and LRH-1 siRNA (y-axis). Black dots represent regulated genes and red dots represent genes involved in TGF-β signalling. Grey dotted lines indicate 1.3-fold up-regulation or down-regulation. (B) Validation of down regulated genes involved in TGF-β signalling from LRH-1 siRNA microarray. Transcript levels were normalised to 18s and presented as fold change relative to control. Red dotted line represents a 1.3 down regulation as noted in the microarray. LRH-1 was over expressed in MCF-7 (C) and T47-D (D) breast cancer cells and real time quantification of LRH-1, TGF-β1, 2 and 3, NR4A1, TP53, ITGB1, KRT18 and ANXA2. Transcript levels were normalised to 18s and presented as fold change relative to control. (E) Western blot analysis of whole-cell extracts collected 24h post transfection with either LRH-1 pcDNA or empty vector reveals an increase in LRH-1, active TGF-β 1, 2 and 3 protein expressions, normalised to β-tubulin. (G) Analysis of protein levels of phosphorylated Smad 2 and 3 reveals an increase after LRH-1 overexpression, normalised to β-tubulin. (F) Quantification of LRH-1, active TGF-β1 protein expression and quantification of active/inactive TGF-β2 and 3 protein expression and (H) quantification of phospho/total Smad2 and 3 protein expression. Data are presented as mean ± S.E.M., n=3 separate experiments, * p<0.05, ** p<0.01, *** p<0.001.

Figure 2: LRH-1 binds to the TGFB1 promoter. (A) Location of LRH-1 response elements on the TGF-β1 promoter. (B) ChIP results using LRH-1 specific antibodies showing occupancy of LRH-1 on the TGF-β1 promoter. As a positive control LRH-1 binding to the GREB-1 promoter was demonstrated. Figures are representative of three separate ChIP experiments.

Figure 3: Characterisation of LRH-1 mammary specific overexpression mouse model. (A) Doxycycline-inducible (dox) system is a bigenic system allowing inducible expression of the gene of interest. To obtain mammary epithelial-specific, Dox-controlled transgene expression, MMTV-RTTA transgenic mouse line, expressing RTTA transactivator under control of mammary-specific MMTV promoter, is mated with LRH-1 transgenic line, leading to dox inducible and mammary-specific LRH-1 overexpression mouse model. (B) Mouse genotype was confirmed using PCR on DNA extracted from mouse tails. L1 and L2 PCRs were used to confirm the presence of hLRH-1 construct. MTB PCR was used to confirm the presence of MMTV-RTTA construct. Only double transgenic animals that were positive for the L1/L2 and MTB constructs.
were used for experiments. (C) Real-time PCR on whole mammary lysates and using a human LRH-1 probe revealed variable expression of LRH-1 transcript in dox treated animals, n.d. not detected. (D) Analysis of protein expression of LRH-1 protein in liver, intestine and mammary showed an increase in LRH-1 protein in mammary lysate after dox treatment, normalised to β-tubulin (E) Immunohistochemistry on 5um sections reveals an increase in LRH-1 immunostaining after dox treatment and protein localisation to luminal epithelial cells as indicated by the arrow. Rabbit IgG was used as a negative control. (F) Quantification of LRH-1 immunostaining reveals increase in LRH-1 expression after dox treatment. Data are presented as mean ± S.E.M., n=7 per treatment, * p<0.05, ** p<0.01. Scale bar = 50μm.

Figure 4: Microarray analysis revealed genes involved in TGF-β signalling are upregulated in RNA extracted from whole mammary lysates of dox treated animals. (A) Volcano plot of fold change (x-axis) and P value (y-axis). Grey dots represent all regulated genes, black dots represent genes significantly regulated and red dots represent genes involved in TGF-β signalling. Black dotted lines represent a cut-off range of >1.5 fold and p<0.05. Real-time analysis of TGF-β1, 2 and 3 using RNA extracted from whole mammary lysates of control animals or treated with doxycycline for three weeks or three months (B, D, F). Immunostaining of TGF-β 1, 2 and 3 in 5mm sections of mammary tissue from control animals or treated with doxycycline for three weeks (C, E, G). Inset shows IgG negative for each antibody. Correlation of transcript expression levels of LRH-1 and TGF-β1 (H) and TGF-β2 (I). Data are presented as mean ± S.E.M., n=7 per treatment, * p<0.05, ** p<0.01. Scale bar = 200μm.

Figure 5: LRH-1 overexpression leads to activated downstream TGF-β signalling. (A-E) Real-time quantification of marked genes involved in TGF-β signalling revealed increased transcript expression of MMP3, ACTA2, TBSAB1/2 TNFRSF12a and HTRA1 in dox treated animals. (F) Western blot analysis of protein extracted from whole mammary lysates of control animals or three month doxycycline treated animals reveals increase in active TGF-β 1, 2 but not 3. (H) In addition increase in phosphorylated Smad 2 and 3 was observed. (G) Quantification of TGF-β isoforms and (I) quantification of phospho/total Smad 2 and 3 in dox treated animals. (J) Sirius red staining of 5mm sections from mammary tissue of control or three month dox treated animals. (K) Quantification of Sirius red staining reveals increase in collagen type 1 levels in dox treated animals. Scale bar = 200mm. (L, N) Whole mount analysis of mammary glands from animals treated with doxycycline for three weeks and three months reveals impairment in
mammary gland lateral bud formation. (M, O) Quantification of lateral buds reveals a significant decrease in number of side buds in dox treated animals. Scale bar = 2.0mm. Data are presented as mean ± S.E.M., n=7 per treatment, * p<0.05, ** p<0.01.

Supplementary Figure 1: (A) Body weights were not different between double transgenic animals on control or doxycycline treatment. (B) Vaginal smears were obtained and showed no difference in oestrus stage in control or doxycycline treated animals. (C) Quantification of terminal end buds revealed no significant difference between treatments. Data are presented as mean ± S.E.M., n=7 per treatment.

Supplementary Figure 2: (A, B and C) Representative images from 5mm sections of mammary glands of three month control and dox treated animals representing an increase in TGF-β1, TGF-β2 and TGF-β3 immunodetection.

Supplementary Figure 3: LRH-1 positively regulates TGF-β signalling in breast cancer cells. (A) Gene network showing genes that are regulated in MCF-7 cells after LRH-1 depletion and connection to TGF-β signalling. Red molecules are genes that are down regulated after LRH-1 depletion and green molecules are genes that are up regulated after LRH-1 depletion in MCF-7 cells.
### Table 1. Antibodies used in paper

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Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Chapter 4 Figure 1: LRH-1 overexpression in vitro induces TGF-β isoform levels and downstream activation
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Chapter 4 Figure 2: LRH-1 binds to TGF-β promoter

A

-1406
LRH-1 RE2
-682
LRH-1 RE1
TGFβ1
acatGGGTCAtgga gcacAGTGGTCAagag

C

Input LRH-1 IgG No Ab Water
LRH-1 RE1
LRH-1 RE2
Negative
LRH-1 RE1 GREB-1

Chapter 4 Figure 2: LRH-1 binds to TGF-β promoter
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Chapter 4 Figure 3: Mammary epithelial cell specific LRH-1 transgenic mouse model
Chapter 4 Figure 4: LRH-1 overexpression in vivo induces TGF-β isoforms levels
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Chapter 4 Figure 5: LRH-1 overexpression in vivo induces downstream TGF-b activation and reduction in lateral budding
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Chapter 4 Supplementary Figure 1
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Chapter 4 Supplementary Figure 2
Chapter 4: LRH-1 induces TGF-β expression in mammary epithelial cells

Chapter 4 Supplementary Figure 3
### Supplementary Table 1. Ingenuity systems pathway analysis of significantly regulated genes in MCF-7 microarray

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### Supplementary Table 2. Ingenuity systems pathway analysis of significantly regulated genes in mouse microarray

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Chapter 5:
LRH-1 OVEREXPRESSION PROMOTES MAMMARY EPITHELIAL CELL PROLIFERATION AND INCREASES INCIDENCE OF DMBA-MEDIATED MAMMARY TUMOURS
Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

Thesis chapter 5: Declaration

Results chapter 3 entitled “LRH-1 overexpression promotes mammary epithelial cell proliferation and increases incidence of DMBA-mediated mammary tumours”

In the case of Chapter 5, contributions to the work involved the following

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<th>Nature of contribution</th>
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<td>1. Kyren A. Lazarus</td>
<td>70%</td>
<td>Performed all experimental work, data analysis, conceived the study and provided intellectual concept. Preparation of manuscript. Proof-reading and submission of manuscript.</td>
</tr>
<tr>
<td>2. Kristy Brown</td>
<td>3%</td>
<td>Provided reagents, Proof-reading the manuscript</td>
</tr>
<tr>
<td>3. Rhiannon Coulson</td>
<td>2%</td>
<td>Performed experiments</td>
</tr>
<tr>
<td>4. Jimmy Ham</td>
<td>3%</td>
<td>Performed experiments, data analysis</td>
</tr>
<tr>
<td>5. Jason Cain</td>
<td>3%</td>
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<tr>
<td>6. Samantha Jayasekara</td>
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<tr>
<td>7. Beena Kumar</td>
<td>2%</td>
<td>Data analysis</td>
</tr>
<tr>
<td>8. Morag Young</td>
<td>2%</td>
<td>Assisted in experiment setup</td>
</tr>
<tr>
<td>9. Neil Watkins</td>
<td>3%</td>
<td>Data analysis and provided intellectual input into study design</td>
</tr>
<tr>
<td>10. Ashwini Chand</td>
<td>5%</td>
<td>Conceived the study, participated in the design, coordination and helped draft the manuscript</td>
</tr>
<tr>
<td>11. Colin D. Clyne</td>
<td>5%</td>
<td>Conceived the study, participated in the design, coordination and helped draft the manuscript</td>
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Declaration by co-authors

The undersigned hereby certify that:

(1) They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

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Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

(3) There are no other authors of the publication according to these criteria
(4) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or published of journals of other publications, and (c) the head of the responsible academic unit; and
(5) The original data are stored at the following location and will be held for at least five years from the date at the location:

**Prince Henry’s Institute of Medical Research, Clayton VIC, Australia.**

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Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

5.1 Integrative commentary for Chapter 5

Previous studies have demonstrated that elevated levels of LRH-1 are implicated in gastric, colon, pancreatic and breast cancer; particularly in the process of cell proliferation (Botrugno et al. 2004, Schoonjans et al. 2005, Wang et al. 2008, Benod et al. 2011). Our laboratory has shown that LRH-1 is involved in breast cancer cell proliferation, motility and invasion (Chand et al. 2010, Chand et al. 2012). Furthermore, LRH-1 expression is elevated in invasive ductal carcinomas (Miki et al. 2006). However, a key limitation of these studies of LRH-1 in the breast is the lack of a suitable transgenic mouse model to understand its role in mammary epithelial cell proliferation.

In chapter 4, I described the characterisation of a novel dox inducible mammary specific LRH-1 transgenic mouse model. This chapter sought to explore the role of LRH-1 in mammary epithelial cell proliferation by using the previously described LRH-1 transgenic mouse model. Furthermore, to ascertain the role of LRH-1 in tumour growth, the LRH-1 transgenic animals were challenged with a chemical carcinogen 7,12-dimethybenz(a)anthracene (DMBA). Previous studies have indicated that LRH-1 is involved in the Wnt/β-catenin signalling pathway increase gastric cancer cell proliferation (Botrugno et al. 2004). Also studies using embryonic stem cell lines and pancreatic and intestinal cancer cell lines describe this relationship between LRH-1 and Wnt/β-catenin pathway (Wagner et al. 2010, Benod et al. 2011).

The hypothesis for Study 3 (Chapter 5) was that LRH-1 promotes mammary epithelial cell proliferation and increases incidence of DMBA-mediated mammary tumours. The aim of this study was to determine the expression of proliferation markers in LRH-1 over-expressing mammary glands, to identify its role in promoting DMBA-mediated tumours and to elucidate the mechanisms by which LRH-1 increases mammary epithelial cell proliferation and DMBA-mediated tumour growth.

This chapter is currently in review at Breast Cancer Research.
Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence
Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

**Full title:** Liver Receptor Homolog-1 over expression increases incidence of DMBA-induced mammary tumours

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Abstract

Introduction

Liver receptor homolog-1 (LRH-1) is an orphan nuclear receptor that is expressed in enterohepatic tissues where it plays roles in cholesterol and bile acid homeostasis. Elevated levels of LRH-1 are implicated in cancer. LRH-1 has dual functions in breast cancer. In cancer associated fibroblasts it activates the enzyme aromatase which is responsible for estrogen biosynthesis. In tumour epithelial cells, LRH-1 synergizes with estrogen signalling to promote breast cancer cell proliferation, motility and invasion. Although these findings have been instrumental to our understanding of the role LRH-1 plays in breast cancer cell proliferation, they have been limited by the use of breast cancer cell lines. Thus, in order to understand the importance of LRH-1 in the breast, we developed a novel LRH-1 mammary specific transgenic mouse model.

Methods

We examined the proliferative response of LRH-1 overexpression in mouse mammary epithelial cells by analysing Ki-67, phosphor-histone H3 (PH3) and proliferating cell nuclear antigen (PCNA) expression by immunohistochemistry. Furthermore, we examined mice treated with the chemical carcinogen 7, 12-Dimethylbenz(a)anthracene (DMBA) by analysing levels of high proliferating foci, microscopic lesions and mammary tumours to explore the role of LRH-1 as a tumour promoter. In addition co-localization of LRH-1 and PCNA was assessed by immunofluorescence. To understand mechanisms by which LRH-1 induces mammary epithelial cell proliferation, we analysed the expression of β-catenin, cyclin D1 and cyclin E1 transcript levels in mouse mammary epithelial cells.

Results

Here, we report that LRH-1 overexpression in mice promotes mammary epithelial cell proliferation and increased incidence of DMBA-mediated tumour formation. We found an increase in Ki-67, PH-3 and PCNA in LRH-1 overexpressing mammary glands. Furthermore, we observed a strong correlation between expression of LRH-1 and PCNA in mammary glands and tumours from DMBA treated mice. Moreover, our data indicates that LRH-1 overexpression induces β-catenin nuclear localization and increases cyclinD1/E1 levels thereby promoting tumorigenesis.
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Conclusions

These results reveal a novel role for LRH-1 in mammary epithelial cell proliferation, augmenting recent findings of its roles in estrogen signalling and breast cancer cell proliferation. Our findings suggest that LRH-1 may serve as a potential therapeutical target for breast cancer treatment.

Keywords: Breast cancer, LRH-1, mammary gland, Wnt/β-catenin, nuclear receptor, proliferation, NR5A2
Introduction

According to the American Cancer Society’s 2013 statistics, breast cancer is the second leading cause of cancer related death in women. Approximately 1 in 8 women in the US will develop invasive breast cancer during their lifetime. Hormone therapies such as the anti-estrogen tamoxifen have proved efficacious in treating tumours that express estrogen receptor alpha (ERα) and are dependent on estrogen for growth. However, these benefits are counter-balanced by acquired and de novo therapeutic resistance, as well as side effects that reduce patient compliance (van Leeuwen et al. 1994, Gail et al. 1999). Additionally, approximately 25% of breast tumours do not express the ERα, and hormone therapies are therefore ineffective against these tumours. A better understanding of the mechanisms that are involved in breast cancer development and progression is required in order to identify new therapeutic targets, particularly for patients who do not / no longer benefit from current anti-estrogen therapies.

The liver receptor homolog-1 (LRH-1, NR5A2) is an orphan member of the nuclear receptor superfamily (for review see (Lazarus et al. 2012)). LRH-1 is a key transcriptional regulator of genes involved in bile acid synthesis, cholesterol metabolism and steroidogenesis (Lazarus et al. 2012). LRH-1 is also implicated in pancreatic differentiation, function and tumour development (Annicotte et al. 2003, Benod et al. 2011), and also promotes aberrant growth in gastrointestinal tumours (Schoonjans et al. 2005, Wang et al. 2008). LRH-1 induces cell proliferation by activating oncogenes cyclin D1 and E1 in cooperation with β-catenin (Botrugno et al. 2004). These studies suggest that LRH-1 may be a proliferative driver in a variety of cancers. In breast cancer, LRH-1 is elevated in 43% of invasive ductal carcinomas and its expression positively correlates with that of steroid hormone receptors and steroidogenic enzymes (Miki et al. 2006). We previously show that LRH-1 activates aromatase expression in cancer-associated fibroblasts (Clyne et al. 2002). We and others subsequently demonstrated that LRH-1 synergizes with estrogen in tumour epithelial cells where it activates ERα expression, is a target gene of ERα and binds and activates ERα target genes (Annicotte et al. 2005, Thiruchelvam et al. 2011, Chand et al. 2012). In addition, LRH-1 has estrogen-independent effects on breast cancer cell proliferation and invasion (Chand et al. 2010). Whilst these studies suggest a clear role for LRH-1 in breast cancer proliferation via both estrogen-dependent and -independent mechanisms, they have been limited to cell lines in vitro; it’s role in mammary epithelial cell proliferation and tumour progression in vivo has not been addressed.
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We recently developed a novel mouse model that expresses human LRH-1 (hLRH-1) specifically in mammary epithelial cells in response to doxycycline (Lazarus et. al. submitted). Here, we show that doxycycline-mediated hLRH-1 expression leads to increased expression of proliferation markers, and nuclear localization of β-catenin, in mouse mammary epithelial cells. hLRH-1 expression also increased the incidence of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumours. These findings are consistent with a model in which increased LRH-1 expression in mammary epithelial cells - as seen in human breast cancer - activates downstream proliferative and/or oncogenic pathways, and provide further evidence that targeting LRH-1 could be beneficial in breast cancer therapy.

Materials and Methods

**Mouse experimental procedure**

All studies involving the mice were regulated under the protocol approved by Monash Medical Centre; Ethics: MMCB10/12. The pTetOLRH-1 transgene was generated from a construct containing the human LRH-1 open reading frame (Clyne et al. 2002). Briefly, a Kozak sequence was introduced by site-directed mutagenesis using the QuickChange® II Site-Directed Mutagenesis Kit (Agilent Technologies, USA) and primers F- Kozak: 5’-TTAAGCCAAAGAACTGCCTATAATTTCACTCACCATGGCTTCTAATTCAGATACTGGGGATTTACAAG -3’ and R-Kozak: 5’-CTTGTAATCCCCAGTATCTGAATTGAAGCCATGTTGAAATTATAGGCAGTCTTTGGCCTAA-3’. The LRH-1 open reading frame containing the Kozak sequence was then subcloned into the multiple cloning sequence of the pTRE-Tight-BI-DsRed Express vector (Clontech, USA) after NotI restriction digest. Orientation of the insert was verifed by digesting the vector with BamHI. The vector was then linearized using ApaLI and gel purified. The pTetOLRH-1 founder mice were generated by pronuclear injection (MouseWorks Service, Australia) of the purified transgene, and complete transgene insertion was verified using primers that spanned the 3’ end of the DsRed open reading frame (L1) (F: 5’-GCGGATCACTTCCTTCTTG -3’ and R: 5’-CGAGGACGT CATCAAGGAGT -3’) and the 3’ end of the LRH-1 open reading frame (L2) (F: 5’-TCGACCACA TTTACCGACAA -3’ and R: 5’- TGGCTGATTATGATCCTCTGG -3’). MMTVrTA-pTetOLRH-1 (MMTVtet-LRH-1) double transgenic mice were generated by crossing pTetOLRH-1 founder females with MMTVrTA males. Genotyping of double transgenic animals was performed using primers for LRH-1 listed above and MTB F- TGCCGCCATTATTACGACAAGC, R-
ACGTACTCGTCAATTCCAAGGG. Only female$^{MMTV}$ tet-LRH-1 mice were selected for this study unless otherwise stated.

To induce LRH-1 transgene, doxycycline hyclate (Clontech, Cat# 631311) was supplied in drinking water at a 2mg/ml concentration with 3% sucrose. The control cohorts were provided with 3% sucrose in drinking water. The water was replaced every three days and kept under light sensitive conditions. To induce mammary tumours, animals were exposed to six weekly doses of 1mg DMBA (in 100µl sesame oil vehicle) delivered by oral gavage from 11 weeks of age. Tumours were detected by manual palpitation and sacrificed when tumours reached >0.5cm in diameter or mice was moribund at an earlier age.

**Whole mount analysis, histology, and immunohistochemistry analysis of mammary tissue**

Whole mounts of inguinal mammary glands were performed as previously described (Lazarus et. al submitted). Dense epithelial foci of greater than 300µm were counted (Gonzalez-Suarez et al. 2010). Haematoxylin and cosin stained sections (5µm) of inguinal mammary glands from MMTV-LRH-1 and WT animals were used to analyse for the presence of pre-neoplastic lesions and overall mammary epithelial morphology.

For immunohistochemistry, antigen heat retrieval as previously described was used on paraffin-embedded sections and sections were incubated with primary antibodies (Table 1). Antigen-antibody complexes were detected as previously described (Lazarus et. al. submitted).

Immunofluorescence of dual PCNA and LRH-1 expression was performed by de-waxing sections as previously described (Lazarus et. al submitted). Specific details are noted below. Antigen retrieval was performed by heating sections in sodium citrate buffer. To block nonspecific staining, sections were treated with 10% normal goat serum (Chemicon) diluted into neat CAS block (Invitrogen) for 20 minutes, before incubation in primary antibody overnight at 4°C. Secondary antibody incubation was performed in the dark for 1 hour (goat anti-rabbit Alexa-546, or goat anti-mouse 488, final dilution 1:200; Molecular Probes) alongside with Hoechst nuclear marker (final dilution 1:1000). Sections were mounted with FluorSave (Calbiochem), and immunofluorescence was visualized on a confocal microscope (Fluoview FV300; Olympus). To control for the possible variations in staining between different immunofluorescent runs, a quality control section was employed in each run; no discernible variations between the runs were observed.
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Quantitative RT-PCR

Whole mammary gland total RNA was extracted and cDNA was produced as previously described (Lazarus et al. submitted). RT-PCR were carried out using the Power SYBR green PCR mix (Applied Biosystems) using mouse Cyclin D1 and E1 primers as previously described (Western et al. 2008). mRNA levels were normalized to 18s as previously described (Lazarus et al. 2013).

Data Analysis and Statistics

Data show the mean ± S.E.M of the number of animals stated. Ki-67, PH3, PCNA and nuclear β-catenin data was expressed as percentage of positive luminal epithelial cell nuclei. Metamorph (Molecular Devices) software analysis of nuclear LRH-1 and PCNA staining intensity was performed by creating a mask using Hoechst staining to delineate epithelial and stromal populations in mammary tumours. Statistical analyses for experiments comparing two groups were performed by two-tailed Student's independent t test using GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) and a P value of <0.05 was considered statistically significant. For survival curve, Kaplan-meier survival analysis was performed and Mantel-Cox test was used to show significance (GraphPad, La Jolla, CA, USA).

Results

hLRH-1 expression induces expression of proliferation markers

Elevated LRH-1 expression is implicated in pancreatic and gastrointestinal cell proliferation contributing to tumorigenesis (Botrugno et al. 2004, Benod et al. 2011). To evaluate a role for LRH-1 in mammary epithelial cell proliferation we utilized a novel mouse model in which human LRH-1 is expressed specifically in mammary epithelial cells in response to doxycycline. We first created a line in which human LRH-1 is expressed under the control of a tetracycline-responsive element (TRE), and then crossed this line with the previously described MMTV-rtTA (MTB) line, in which expression of the reverse tetracycline transactivator is directed to mammary epithelial cells by the MMTV promoter (Gunther et al. 2002). This model is well defined to characterize the role of transgene overexpression in mouse mammary epithelial cells (Gunther et al. 2002). The result double transgenic animals were phenotypically normal, and are termed MMTVtet-LRH1 as previously described (Lazarus et al., submitted). As expected, the human
LRH-1 transgene (hLRH-1) was undetectable in mammary RNA isolated from vehicle-treated mice as previously described (Lazarus et. al., submitted). However, administration of 2 mg/ml doxycycline (dox) in drinking water from 56 days of age for short (3 week) or long (3 month) time frames resulted in significant expression of hLRH-1 at both time points as previously described (Lazarus et. al., submitted). We observed mammary specific induction of LRH-1 protein in dox treated animals as previously described (Lazarus et. al., submitted). Eight week old MMTVtetLRH-1 mice treated with doxycycline for three weeks demonstrated a marked increase in LRH-1 protein in mammary luminal epithelial cells, as expected for MMTV-driven transgene expression (Figure 1A and B). To determine the effects of increased LRH-1 expression on mammary epithelial cell proliferation, we assessed key proliferative markers. Immunohistochemical analysis demonstrated a significant increase in: i) the broad proliferative marker, Ki67 (2.5 fold, p<0.001, Figure 1C and D); ii) the mitosis marker, phosphorylated-histone H3 (6 fold, p<0.001, Figure 1E and F); and iii) the G1/S phase specific marker, proliferating cell nuclear antigen (PCNA) (3 fold, p<0.001, Fig 1G and H) in luminal epithelial cells of mouse mammary glands. MMTVtetLRH-1 mice treated with doxycycline for three months (long term) exhibited similar observations (data not shown). These findings indicate that LRH-1 stimulates mammary epithelial cell proliferation in vivo. Although we did observe reduced mammary lateral branching (Lazarus et. al., submitted), other characteristics such as ductal length, TEB number and ductal outgrowth were examined, but were observed to be normal in dox-treated animals (data not shown).

**hLRH-1 overexpression increases incidence of DMBA-induced mammary tumours**

To determine if increased mammary epithelial proliferation in the presence of hLRH-1 contributes to mammary tumour development, we utilized a 7,12-dimethylbenzathracene (DMBA) carcinogen-induced mammary tumour model 7,12-dimethylbenzathracene (DMBA). Eight week old female MMTVtetLRH-1 and WT animals treated with control or doxycycline for three weeks, received six weekly doses of 1mg DMBA by oral gavage and monitored for mammary tumour development. No difference in DMBA-treated MMTVtetLRH-1 and WT animal body weights were detected confirming that this level of DMBA dosing was not toxic to the animals (Additional File 1A).

Pre-neoplastic mammary morphological changes were detected in DMBA treated animals. Whole mount analysis revealed a significant increase of dense pre-neoplastic epithelial foci (early ductal and alveolar gross lesions) (Gonzalez-Suarez et al. 2010) in MMTVtetLRH-1
animals treated with doxycycline \((p=0.0267, \text{Figure 2A and B})\). Haematoxylin and eosin staining revealed a significant increase in microscopic lesions \(\text{(intraductal hyperplasias)}\) in \(\text{MMTV}^{tet}\text{LRH-1}\) animals treated with doxycycline \((p=0.0003, \text{Figure 2C and D})\). In addition, we observed Ki-67 immunostaining in hyperplastic regions in mammary glands adjacent to mammary tumours \(\text{(Additional File 1B)}\).

Whilst no tumours were observed in mice that did not receive DMBA and that were on dox treatment for up to 6 months \(\text{(Figure 2F)}\), \(\text{MMTV}^{tet}\text{LRH-1}\) and WT mice maintained in the presence or absence of doxycycline presented with lymphoma, skin and gastrointestinal malignancies following 18 to 33 weeks following DMBA administration \(\text{(Table 2)}\). Remarkably, 25\% of \(\text{MMTV}^{tet}\text{LRH-1}\) mice maintained on doxycycline demonstrated the developed palpable mammary tumours as early as 18 weeks following completion of DMBA administration \(\text{(Figure 2E and F, Table 2)}\). Importantly, mammary tumours were not observed in DMBA-treated WT mice. However, the lack of mammary tumours in WT mice may have been due to the animals succumbing from other tumour or complications. Thus, mammary luminal epithelial cell proliferation via hLRH-1 expression leads to the development of mammary tumours in a carcinogen-induced mouse model \(\text{(Mantel cox test, } p=0.0437, \text{Figure 2F)}\).

Histopathological analysis indicated that all mammary tumours were all poor to moderately differentiated squamous cell carcinomas \(\text{(scc, Additional File 2A and B)}\). The scc were characterized by patches of CK8 positive luminal epithelial cells with the SMA positive myoepithelial cells surrounding the squamous epithelial layers \(\text{(Additional File 2C and D)}\). Typically for squamous epithelial differentiations, the basal epithelial layers were highly proliferative as indicated by a Ki-67 index of 5\% \(\text{(Additional File 2G)}\); sccs were negative for ER\(\beta\) and PR protein \(\text{(Additional File 2E and F)}\). LRH-1 protein was detected in all tumours and localized to luminal epithelial cells with a finely, granular nuclear staining \(\text{(Additional File 2H)}\). Although the relative contribution to specific pathway-mediated tumorigenesis is not clear at this stage, our results show that the overexpression of LRH-1 increases incidence of DMBA-mediated mammary tumours.

**LRH-1 expressing mammary cells correlates with proliferation phenotype**

Our present study indicates that LRH-1 promotes tumour cell proliferation \textit{in vivo} in dox treated \(\text{MMTV}^{tet}\text{LRH-1}\) animals, leading to increase incidence of DMBA-mediated mammary tumours. These findings extend previous publications of the role of LRH-1 in cell proliferation.
We analyzed expression of LRH-1 and proliferation marker PCNA in mouse mammary tumours. LRH-1 immunostaining was observed as discrete, finely granular nuclear staining in the luminal epithelial patches of the mammary glands and tumours. Firstly, dox treatment increased LRH-1 expression in DMBA treated mammary glands (Figure 3A and C). In the DMBA treated tumours, PCNA and LRH-1 immunostaining was markedly increased in the DMBA-mediated mammary tumours (Figure 3E). Higher magnifications show LRH-1 and PCNA co-localizing in luminal epithelial cells (Figure 3B, D, and F). This data demonstrates LRH-1 staining in proliferating luminal epithelial cells. No staining was observed in the negative control (Figure 3 G and H). Correlation between LRH-1 and PCNA were observed in all tumours analysed (Figure 3I).

**hLRH-1 overexpression up-regulates nuclear localization of β-catenin in mouse mammary luminal epithelial cells**

LRH-1 has been shown to crosstalk with the Wnt/β-catenin signalling pathways in an intestinal tumour model where LRH-1 synergizes with β-catenin to induce cyclin D1/E1 dependent cell proliferation (Botrugno et al. 2004). In an embryonic stem cell model, β-catenin induces LRH-1 expression which is required for proper maintenance of Oct4, Nanog, and TBX3 dependent pluripotency (Wagner et al. 2010). We demonstrate an increase in β-catenin nuclear localization in dox treated animals in the presence or absence of DMBA (Figure 4A and B). Nuclear β-catenin immunoreactivity was quantified and we observe a significant 25-fold increase in dox treated animals and a 4-fold increase in a dox+DMBA treated animals (Figure 4C). In addition we observe strong nuclear β-catenin staining in luminal epithelial cells of DMBA-mediated mammary tumours (Figure 4D).

**hLRH-1 overexpression increases cyclin D1/E1 transcript levels in mouse mammary glands**

Cyclin D1 and E1 are known oncogenes in breast cancer and are expressed in proliferating cells in the G1 phase of cell cycle. Furthermore, a LRH-1/β-catenin complex can induce CycD1 and E1 expression (Botrugno et al. 2004). We observed an increase in Cyclin D1 and E1 transcript levels after dox treatment in MMTVtetLRH-1 mouse mammary glands (p<0.01, p<0.05, Figure 5A and B). Levels of transcript expression were normalized to 18s expression. In addition, to determine the effect of LRH-1 on breast oncogenes Cyclin D1 and E1 after DMBA insult, we demonstrate an increase in both Cyclin D1 and E1 transcript in dox treated animals (Fig 4C and D). In addition we show no correlation between Cyclin D1 and LRH-1 (p=0.1449, Figure
4E), however a strong significant correlation was observed between Cyclin E1 and LRH-1 (p=0.0014, Fig 4F).
Discussion

The present study provides the first direct in vivo evidence that LRH-1 over-expression in mouse mammary epithelial cells promotes mammary epithelial cell proliferation and increases incidence of DMBA-mediated mammary tumorigenesis. This is consistent with its role in gastric, pancreatic and intestinal tumour progression (Schoonjans et al. 2005, Wang et al. 2008, Benod et al. 2011). LRH-1 is elevated in 43% of invasive breast ductal carcinomas where it strongly correlates with ERα and other steroidogenic enzymes (Miki et al. 2006). We designed the $^{MMTV}_{tet}$LRH-1 model to mimic this elevated expression and activity of LRH-1 as observed in human breast tumours (Miki et al. 2006).

We did not utilize any external hormonal supplementation such as progesterone and our observations were on a hormone-free background. Progesterone supplementation causes a 10-15% increase in mammary tumour incidence compared to using DMBA alone and caused an inhibition of DMBA-mediated gastrointestinal tumours (Jabara et al. 1979). Here we show that LRH-1 promotes DMBA mediated tumour growth independent of progesterone supplementation. Furthermore, $^{MMTV}_{tet}$LRH-1 animals without DMBA do not present with any mammary tumours, suggesting that LRH-1 alone is not sufficient to induce mammary tumour formation.

The tumours we observed were classified as mammary sccs and were ERα and PR negative, which is consistent with studies showing that approximately 50% of DMBA-induced mammary sccs are triple negative tumours (Zinser et al. 2005, Murialdo et al. 2009). Strong LRH-1 positivity was observed in these tumours, along with colocalization of LRH-1 with proliferating epithelial cells in DMBA-mediated tumours, which suggest the potential merit for targeting LRH-1 particularly in hormone-independent tumours. Since the tumours observed in our study are not a true representative of human breast cancer, this highlights the need for clinical studies to determine the associations between LRH-1 expression and breast cancer stage, grade, subtype and outcome.

To evaluate activated signalling pathways in the DMBA treated mammary glands, we analysed levels of nuclear β-catenin. LRH-1 is able to bind and activate β-catenin in an intestinal and ESC model (Botrugno et al. 2004, Wagner et al. 2010). We demonstrate increased nuclear β-catenin staining in dox treated animals. Furthermore in mammary tumours, we observed strong nuclear β-catenin staining in luminal epithelial cells and basal layer of the keratin pearls in the
squamous cell carcinomas. LRH-1 forms a complex with β-catenin to induce Cyclin D1/E1 and cMyc gene transcription (Botrugno et al. 2004, Wagner et al. 2010). Furthermore, LRH-1 binds directly to β-catenin suggesting its direct involvement in the Wnt/β-catenin signalling pathway (Yumoto et al. 2012).

To evaluate cell cycle regulators known to be important in mammary tumorigenesis, we measured mRNA levels of the cyclin D1 and E1 oncogenes which are downstream targets of the Wnt/β-catenin pathway (Sutherland and Musgrove 2004). We observed an increase in cyclin D1/E1 transcript levels in \textit{MMTV}_{tet}LRH-1 treated with doxycycline. Importantly β-catenin and cyclin D1 co-localization significantly correlated with poor prognosis in breast cancer patients (Lin et al. 2000). LRH-1 was shown to act as a coactivator for β-catenin/Tcf4 to drive expression of cyclin D1 (Botrugno et al. 2004). Also, LRH-1 can bind directly to the promoter of cyclin E1 and this transactivation is enhanced by β-catenin (Botrugno et al. 2004). These findings are consistent with our data that show a strong positive correlation between LRH-1 transcript expression and cyclin E1 transcript expression, but not with cyclin D1. Activation of Wnt signalling components are observed in DMBA-mediated mammary tumours (Currier et al. 2005). In addition to the cyclins, expression of cMyc that is also regulated by Wnt/β-catenin is controlled by LRH-1 (Heng et al. 2010, Benod et al. 2011).

Also, matrix metalloproteinase-3 (MMP3) has been recently found to sequester noncanonical Wnt signalling ligand Wnt5b and thereby promote hyper branching in mammary epithelial cells (Kessenbrock et al. 2013). Thus when MMP3 activity was high, Wnt5b was inactivated and the canonical Wnt pathway was activated by one of the several mammary-resident Wnts (Kessenbrock et al. 2013). LRH-1 regulates MMP-3 in mouse mammary glands (Lazarus et. al, submitted). These findings suggest LRH-1 activates components of the Wnt/β-catenin signalling pathway to mediate increase in cell proliferation. Further work needs to be undertaken to understand this relationship.

In summary, our study demonstrates that LRH-1 overexpression promotes mouse mammary epithelial cell proliferation and increases incidence of DMBA-mediated mammary tumours. The elevation of oncogenes cyclin D1 and E1 via the activation of Wnt/β-catenin signalling pathway after LRH-1 overexpression may be responsible for the increase in epithelial
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proliferation observed. These findings suggest that LRH-1 may serve as a potential target for breast cancer treatment.

Competing interests

The author(s) declare that they have no competing interests.

Author’s contributions

KAL carried out all animal studies and downstream applications. Laboratory work and analysis for all figures, preparation of manuscript and proof-reading of submissions. KAB designed the LRH-1 construct for the transgenic animal. RLC helped to take care of some animals. SJH carried out double immunostaining. JEC and WSN helped with the DMBA administration and tumour monitoring. BK helped to analyse mammary tumours. MY was responsible for the initial breeding strategies. DNW helped to analyse mammary tumours. ALC and CDC conceived the study, participated in its design and coordination and helped to draft the manuscript.

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Figure Legends

Figure 1: LRH-1 overexpression induces proliferation markers in mouse mammary epithelial cells. 5μm sections of Formalin fixed paraffin embedded mammary glands from control and dox treated animals were processed for (A) LRH-1 immunostaining, (C) Ki-67 immunodetection, (E) phospho-histone H3 and (G) proliferating cell nuclear antigen. Staining was quantified and represented as a percentage of positive cells/total cells. An increase in (B) LRH-1 (D) Ki-67, (F) PH3 and (H) PCNA staining were observed in dox treated mammary glands. Data are presented as mean ± S.E.M., n=7 per treatment, * p<0.05, ** p<0.01.
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Figure 2: The DMBA-induced mammary carcinogenesis summary. (A) The DMBA carcinogenesis model generated pre-neoplastic lesions or high proliferating foci detectable at whole mount analysis as greater than 300.0 μm (arrows). (B) These foci were quantified and observed to be increased in dox + DMBA treated animals. (C) Microscopic lesions were also observed by H&E staining as indicated by infiltrating luminal epithelial cells (arrow). (D) These microscope lesions were quantified and observed to be increased in dox + DMBA treated animals. (E) Palpable mammary tumours. (F) %Breast Tumour-free Survival curve. Survival curves were based on mice culled due to mammary tumours within a given week post last DMBA treatment. Representative images of n=7 per treatment.

Figure 3: LRH-1 co localizes with PCNA in DMBA-mediated mammary tumours. LRH-1 and PCNA immunostaining of mammary glands and tumours were performed. Shown are representative photomicrographs from (A) mammary gland (MG) from control + DMBA treated animals, (C) increase in LRH-1 immunostaining in MG from dox + DMBA treated animals, (E) increase in LRH-1 and PCNA immunostaining in mammary tumours from dox + DMBA treated animals and (G) negative control for LRH-1 and PCNA immunostaining. Arrowhead represents patches of luminal epithelial cells where LRH-1 co localizes with PCNA. Higher magnification photographs of merged images in which arrows indicate discrete nuclear LRH-1 staining co localizing with nuclear PCNA staining (B, D, F and H). Representative photomicrographs of n=7 per treatment. Scale bar = 100μm, higher magnification scale bar = 10μm.

Figure 4: LRH-1 overexpression induces β-catenin nuclear localization. (A) Doxycycline induces β-catenin nuclear expression in luminal epithelial cells. Inset shows magnified ducts. (B) DMBA treatment with doxycycline induces β-catenin nuclear expression in luminal epithelial cells. (C) Quantification of nuclear β-catenin reveals increase nuclear localization in mammary glands from dox treated animals. (D) Strong nuclear β-catenin expression in luminal epithelial patches in DMBA-mediated mammary tumours. Representative images of n=7 per treatment. Data are presented as mean ± S.E.M., n=7 per treatment, * p<0.05, ** p<0.01.

Figure 5: LRH-1 overexpression induces Cyclin D1/E1 transcript. (A,B) Real-time analyses on mammary lysates from control or dox treated animals reveals a significant increase in cyclinD1/E1 transcript levels. (C,D) This increase was confirmed in DMBA treated animals. (E) Correlation of LRH-1 and CyclinD1 transcript reveals no correlation. (F) Correlation between

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LRH-1 and CyclinE1 transcript reveals a positive significant trend. Data are presented as mean ± S.E.M., n=7 per treatment, * p<0.05, ** p<0.01.
Tables

Table 1. Antibodies used in paper

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Table 2: Summary of LRH-1 transgenic animals challenged with DMBA

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Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

Additional Files

Additional File 1
File format: TIFF
Title of data: Morphology of DMBA treated animals
Description: (A) Bodyweights were not different between DMBA treated double transgenic animals on control or dox treatment. (B) Representative image of Ki-67 immunostaining in micro lesions observed in DMBA-treated mammary glands.

Additional File 2
File format: TIFF
Title of data: Histopathology of DMBA-induced mammary tumours
Description: Histopathological analysis was performed for all tumours (A, B) H&E staining of representative mammary tumour samples from DMBA-treated double transgenic mice. The mammary tumours were mainly squamous cell carcinomas with some characteristics of adenocarcinomas. The squamous cell carcinomas were characterized by patches of (C) CK8 positive luminal epithelial cells and (D) SMA positive myoepithelial cells. (E,F) The tumours were negative for ERa and PR staining typical for squamous cell carcinomas. (G) The tumours had a 5% Ki-67 index, and (H) stained positive for LRH-1.
Chapter 5 Figure 1: LRH-1 overexpression induces proliferation markers in mouse mammary epithelial cells
Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

Chapter 5 Figure 2: The DMBA-induced mammary carcinogenesis summary
Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

Chapter 5 Figure 3: LRH-1 co localizes with PCNA in DMBA-mediated mammary tumours
Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

Chapter 5 Figure 4: LRH-1 overexpression induces β-catenin nuclear localization
Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

Chapter 5 Figure 5: LRH-1 overexpression induces Cyclin D1/E1 transcript
Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

Chapter 5 Additional File 1: Morphology of DMBA treated animals
Chapter 5: LRH-1 promotes mammary epithelial cell proliferation and tumour incidence

Chapter 5 Additional File 1: Histopathology of DMBA-induced mammary tumours
Chapter 6:
INTEGRATIVE DISCUSSION OF FINDINGS AND FUTURE DIRECTIONS
6.1 Study Rationale

Liver receptor homolog-1 (LRH-1) is an orphan nuclear receptor that plays crucial roles in development, steroidogenesis and cancer. LRH-1 has dual functions in the breast. In cancer associated fibroblasts it is capable of activating the CYP19A1 gene that encodes the enzyme aromatase, which is the key steroidogenic enzyme that is responsible for oestrogen biosynthesis. In tumour epithelial cells, LRH-1 synergises with oestrogen signalling to promote breast cancer cell proliferation, motility and invasion.

Oestrogen is a potent hormonal stimulant for breast tumorigenesis and much of its effects are mediated by the oestrogen receptor alpha (ERα). Current endocrine therapies for treating breast cancer works on the principle of inhibiting oestrogen availability in the breast tissue either by blocking oestrogen action at the receptor (through use of SERMs) or by blocking the production of oestrogen by inhibiting aromatase (with the use of aromatase inhibitors). However the benefits of these hormone therapies are counter-balanced by acquired or de novo therapeutic resistance, as well as side effects that reduce patient compliance (van Leeuwen et al. 1994, Gail et al. 1999). Additionally, approximately 25% of breast tumours do not express the ERα and therefore hormone therapies are ineffective. A better understanding of the complex and intricate mechanisms that are involved in breast cancer development and progression is required in order to identify new therapeutic targets, particularly for patients who do not / no longer benefit from current anti-oestrogen therapies.

Interestingly, LRH-1 is expressed in oestrogen receptor negative breast tissue and studies in our lab have demonstrated LRH-1 protein is expressed in ER- breast cancer cell lines where it has a functional role in cell motility, invasion and proliferation (Chand et al. 2010, Chand et al. 2012, Lazarus et al. 2012, Lazarus et al. 2013). Furthermore, LRH-1 is able to bind to and activate oestrogen response elements, thereby effectively replacing the ERα in ER- cells and promoting breast cancer proliferation (Chand et al. 2012). Since there are very limited treatment options, these findings indicate the importance of targeting LRH-1 in ER- tumours. However, our understanding of the importance of LRH-1 in the breast is limited by the use of breast cancer cell lines. To further understand the role of LRH-1 in the breast, we sought to develop a novel LRH-1 mammary transgenic mouse model. LRH-1 expression is low in normal breast; and hence to mimic the increased expression seen in human breast cancer (Miki et al. 2006), our laboratory generated a doxycycline-inducible mammary epithelial cell specific human LRH-1 over expression transgenic mouse model.
Since the late 1980s, genetically engineered mice (GEM) have proven extremely beneficial for studying normal breast development and cancer (Cardiff and Wellings 1999). This is due to the striking similarities between mammary gland morphology and lesions that occur in mice and humans (Cardiff and Wellings 1999). The aim of this thesis was to elucidate the role of LRH-1 in normal mouse mammary gland development and tumorigenesis (Chapter 6, Figure 1). The purpose of this chapter is to place this study in the context of existing knowledge and suggest future directions in light of the data presented here.

6.2 Summary of key findings

This thesis characterises several novel roles and mechanisms of LRH-1 function in the mammary epithelial cell using in vitro and in vivo models. Firstly, a comprehensive study profiling the expression of LRH-1 in breast cancer cell lines was undertaken (Chapter 3). Most studies of LRH-1 have been restricted to ER+ cells, however its expression is clearly abundant in ER-ve cells. Observations from experiments presented in Chapter 3 identified discordance between LRH-1 protein and transcript levels in ER- cell lines. An increase in transcript and protein stability in ER- cells may attribute to this discordance. Importantly, the presence of LRH-1 in ER- breast cancer cells identifies its potential as a novel target for hormone-independent tumours and therefore controlling LRH-1 activity by specific ligands could have high therapeutic potential (discussed in section 6.6).

The mechanistic basis of the role LRH-1 plays in breast cancer cell proliferation is unknown. Microarray analysis using MCF-7 cells depleted of LRH-1 was performed and altered expression of genes associated with processes regulating cellular morphology, movement, interaction and breast cancer were observed (Chapter 4). Moreover, we have previously shown the involvement of LRH-1 in breast cancer cell motility, invasion (Chand et al. 2010). Most importantly, this study identified a novel LRH-1 target gene TGF-β1. LRH-1 blockade in breast cancer cell lines resulted in a reduction in TGF-β signalling. The downstream activation of TGF-β signalling was validated in breast cancer cell lines. This thesis demonstrates a novel relationship between LRH-1 and TGF-β.

Although these findings shed light on the importance of LRH-1 in breast cancer, it is limited by the use of breast cancer cell lines, in vitro. Our lab generated a novel doxycycline inducible mammary epithelial specific LRH-1 transgenic mouse model (MMTV tet-LRH-1) to better understand the role of LRH-1 in mouse mammary gland. The relationship between LRH-1 and
TGF-β was confirmed in mouse mammary glands. Subsequently, the consequence of TGF-β activation in response to LRH-1 was examined and abnormal pubertal mammary gland development was observed. LRH-1 overexpression in mouse mammary epithelial cells resulted in impairment of mammary lateral budding via induction of TGF-β gene expression (discussed in section 6.3).

Additionally, increased expression of key proliferation markers in response to LRH-1 overexpression was observed in the \textsuperscript{MMTV}tet-LRH-1 animals, but these animals failed to present with mammary tumours (Chapter 5). Studies have shown its involvement in pancreatic and intestinal cancers (Botrugno et al. 2004, Benod et al. 2011); therefore the \textsuperscript{MMTV}tet-LRH-1 animals were challenged with a chemical carcinogen (DMBA) to determine if LRH-1 overexpression is associated with mammary tumorigenesis. Increased incidence of DMBA-mediated mammary tumours accompanied the activation of β-catenin and cyclin D1/E1 gene expression was observed in DMBA challenged \textsuperscript{MMTV}tet-LRH-1 animals suggesting that LRH-1 plays a role in promoting mammary tumour formation (Chapter 5). β-catenin has been identified as a potential transcriptional coregulator and LRH-1 promotes intestinal tumour formation by inducing G1 cyclin-mediated cell proliferation through direct synergistic effect with β-catenin (Botrugno et al. 2004). These findings strongly support a functional interaction between these factors (discussed in section 6.4). While the results from these studies have helped provide further insights into the function of LRH-1 mammary development and breast cancer; it raises further questions into the implications of LRH-1 in mouse mammary development during pregnancy and involution; and tumorigenesis using other genetic tumour models. Taken together with the findings from the current thesis project, this identifies LRH-1 as a promising therapeutical target for breast cancer treatment.
Chapter 6 Figure 1: Summary of key findings of thesis. This thesis characterises several novel roles and mechanisms of LRH-1 function in the mammary epithelial cell using \textit{in vitro} and \textit{in vivo} models. 

(A) The discordance observed between LRH-1 transcript and protein expression in ER- cells may be attributed to the discordance observed between LRH-1 transcript and protein expression. (B) LRH-1 overexpression in mouse mammary epithelial cells results in impairment of mammary lateral budding via induction of TGF-\(\beta\) gene expression. (C) LRH-1 overexpression also promoted mammary epithelial cell proliferation and (D) increased incidence of DMBA-mediated mammary tumours via the increased \(\beta\)-catenin nuclear localisation and cyclin D1/E1 gene expression.
6.3 Effect of LRH-1 on mouse mammary gland lateral budding

This thesis describes a novel LRH-1 dependent phenotype in virgin developing mammary glands where during early stages of mammary gland development, the overexpression of LRH-1 leads to an inhibition of mammary lateral budding. Lateral buds form from main ducts of the mammary tree and can either develop as a terminal end bud or cleave to form alveoli. The development of the lateral bud is crucial to form a well-spaced mammary tree and subsequently these give rise to alveolar buds that produce milk during pregnancy and lactation. However, no changes in end bud motility or ductal elongation was observed in the MMTV tet-LRH-1 mice. Although it is reasonable to predict that mechanisms that affect end bud motility, also affect side branching in mouse mammary glands, this may not always be true.

Netrin-1 is a factor which is secreted by the body cells of mammary TEBs, and its receptor neogenin is expressed on adjacent cap cells (Srinivasan et al. 2003). The absence of either Netrin-1 or neogenin resulted in impaired end bud formation (Srinivasan et al. 2003). However, this absence had no effect on lateral branching and ductal patterning indicating that different mechanisms are affected (Sternlicht 2006). Another example is Hedgehog signalling, which is mediated by the binding of Indian, Sonic or Desert Hedgehog to Patched receptors can affect several mammary gland signalling pathways including FGF, Wnt, Notch, TGF-β and PTHrP (Lewis 2001, Lee et al. 2013). Mammary transplants lacking the Indian, Sonic or Desert Hedgehog members branch normally in WT-fat pads, however conditional haploinsufficiency of Patched-1 causes defects in duct and TEB histology but the overall branching pattern is unaffected (Lewis 2001, Lee et al. 2013). These studies indicate that there are factors that can induce different mechanisms to affect branching or ductal morphogenesis during mammary gland development. Similarly, LRH-1 overexpression alters lateral budding, but not ductal growth. These findings suggest that LRH-1 induces a specific gene expression set that is specifically responsible for lateral budding. The possibility that LRH-1 could alter stem cell activity in the lateral bud is also promising. This is discussed in section 6.4.2.

Seminal work by Daniel C et. al. showed that transplanting even the smallest fragment of mammary duct anywhere within an epithelium-free fat pad results in vigorous growth into a full ductal tree (Daniel et al. 2009). Others have replicated this finding coming to the conclusion that the mammary stroma promotes mammary epithelial growth. The mammary ducts control their own patterning by inhibiting its own growth pattern. Decades of research have proven the strong inhibitory role of TGF-β of mammary ductal elongation and lateral branching. Chapter 4 explores the relationship between LRH-1 and TGF-β isoforms in breast cancer cell lines and in
Integrative discussion of findings and future directions

Studies on TGF-β and mammary gland development have demonstrated its effect on the stroma via downstream targets. TGF-β seems to perform these effects through both autocrine feedback mechanisms and paracrine interactions that may involve stromal TGF-β receptors (Sternlicht 2006). The current understanding is that TGF-β may aid in the continuance of mammary tree spacing by enabling neighbouring ducts to avoid one another (Daniel et al. 2009). Little is known about the exact mechanisms by which TGF-β inhibits early mammary ductal morphogenesis, but there are a few factors known to be regulated by TGF-β and also involved in mammary gland morphogenesis.

The parathyroid hormone-related protein (PTHrP) is positively regulated by TGF-β and inhibits ductal elongation and lateral branching when overexpressed in pubertal mice (Wysolmerski et al. 1995). Moreover, PTHrP up regulates matrix metalloproteinase-3 (MMP-3) expression which plays an important role in inhibiting lateral branching but keeps ductal elongation intact (Kawashima-Ohya et al. 1998, Wiseman et al. 2003). This thesis has demonstrated an increase in MMP-3 expression in response to LRH-1 overexpression in mouse mammary glands indicating the importance of the relationship between LRH-1 and MMPs. Further understanding this relationship between LRH-1, TGF-β, PTHrP and MMPs will allow us to explore the mechanisms by which LRH-1 affects mammary gland morphogenesis.

In the developing mammary glands, we observed an induction of TGF-β isoforms and downstream signalling in response to LRH-1. LRH-1 is a transcription factor that binds to DNA as a monomer to the conserved nuclear receptor binding site YCAAGGYCR (for review see (Lazarus et al. 2012). A recent CHIP-seq study demonstrated LRH-1 binds to genes such as GREB-1, pS2, JUN and FOS (Lai et al. 2013). Interestingly, JUN and FOS form an AP-1 complex that is responsible for the regulation of TGF-β gene expression. Furthermore, LRH-1 binds to the AP-1 complex to induce steroidogenic enzymes (Kim et al. 1990, Shen et al. 2006, Dube et al. 2009). This raises the possibility of LRH-1 activating an AP-1 complex to induce TGF-β gene expression. Furthermore, as a consequence of TGF-β induction we notice impairment in mammary gland lateral budding along with the accumulation of collagen I around mammary gland (Chapter 6 Figure 2). This accumulation of collagen I has been well established in models of mammary ductal grown inhibition induced by TGF-β (Daniel et al. 1989). These findings are indicative of increased TGF-β in mammary glands.
To understand the effect of LRH-1 on mouse mammary gland development, a mammary epithelial cell specific LRH-1 knockout needs to be developed. The target depletion of other key nuclear receptors in mammary gland development such as ERα and PR, have demonstrated impaired mammary gland development (Bocchinfuso and Korach 1997, Conneely et al. 2003). Furthermore, the vitamin D receptor (VDR) knockout mice exhibit enhanced mammary ductal development (Zinser et al. 2002). These studies indicate the importance of nuclear receptors in mammary gland development, and taken together with the findings from this thesis, suggest a possible role for LRH-1 in mammary gland development. Examining the LRH-1 mammary epithelial cell specific knockout through the stages of pregnancy, involution and lactation will further define its role in mammary gland development. During pregnancy, prolactin (PRL) and its receptor are the key hormones modulating mammary alveolar and lobular development (Ormandy et al. 1997, Brisken et al. 1999). In addition, LRH-1 was shown to be regulated by prolactin in luteal cells of pregnant mice (Falender et al. 2003). These findings indicate an interaction between prolactin and LRH-1 in the mammary gland of pregnant mouse, which warrants further justification for examining LRH-1 in mammary development.

6.4 Effect of LRH-1 on mouse mammary gland tumorigenesis

Little is known about the mechanisms of LRH-1 action in breast cancer. LRH-1 is elevated in 43% of invasive ductal carcinomas and correlates with ER positivity (Miki et al. 2006). Our lab has shown that via the activation of oestrogen target gene growth regulation by oestrogen in breast cancer 1 (GREB1), LRH-1 can induce cell proliferation in ER+ and ER- breast cancer cell lines (Chand et al. 2012). Using a novel LRH-1 transgenic mouse model and the chemical carcinogen DMBA, Chapter 5 examines the role of LRH-1 in mammary epithelial cell proliferation and tumour incidence. In this model, a significant increase in mammary epithelial cell proliferation and DMBA-induced tumour incidence was observed. Importantly, this phenotype was accompanied by the increase in β-catenin nuclear localisation and cyclinD1/E1 transcript levels. This finding identifies a molecular mechanism through which LRH-1 induces mammary epithelial cell proliferation. This relationship between LRH-1 and β-catenin/cyclin D1/E1 is supported by two studies demonstrating cross talk between LRH-1 and Wnt/β-catenin signalling pathways (Botrugno et al. 2004, Wagner et al. 2010).

6.4.1 Regulation of β-catenin by LRH-1

β-catenin plays a significant role in the regulation of mammary development and breast cancer. Furthermore, β-catenin governs mammary stem cell biology during mammary
development and tumorigenesis (Incassati et al. 2010). One of the major upstream regulators of β-catenin signalling is the canonical Wnt pathway that stabilises β-catenin in the cytoplasm prompting its nuclear translocation where it associates with T-cell factor/Lef DNA binding proteins and displaces transcriptional repressors to induce gene expression (for review see (van Amerongen and Nusse 2009)). Several orphan nuclear receptors such as Nur77, Nur1, RORα and LRH-1 are involved in the regulation of Wnt/β-catenin signalling.

LRH-1 is expressed in intestinal crypts and in collaboration with β-catenin, participates in intestinal cell self-renewal (Botrugno et al. 2004). Moreover, LRH-1 promotes cell proliferation via the induction of cyclin D1/E1 gene expression by (i) binding directly to the cyclin E1 promoter with β-catenin as a coactivator and (ii) by acting as a cofactor for β-catenin/TCF4 on the cyclin D1 promoter (Botrugno et al. 2004). Crystallography studies show direct binding between LRH-1 LBD and β-catenin (Yumoto et al. 2012). The authors propose that LRH-1 might displace one of the TCF α-helices that bind to β-catenin, hence allowing the formation of a LRH-1/TCF/β-catenin complex as seen in intestinal cells (Botrugno et al. 2004, Yumoto et al. 2012). LRH-1 overexpression in mammary epithelial cells causes an increase in β-catenin nuclear localisation. Together with previous findings, this data suggests a novel mechanism by which LRH-1 induces mammary epithelial cell proliferation in vivo.

6.4.2 LRH-1 and stem cells in mammary development and cancer

LRH-1 expression in embryonic stem cells (ESC) allows for the activation of Oct4, which is essential for early embryonic differentiation of primitive endoderm/mesoderm ortrophectoderm (Gu et al. 2005). LRH-1 induces the differentiation of mesenchymal stem cells into somatic cells (Yazawa et al. 2009). Furthermore LRH-1 can replace Oct4 during the programming of induced pluripotency stem cells and increases reprogramming efficiency (Gu et al. 2005, Heng et al. 2010, Kelly et al. 2010). These findings indicate the importance for LRH-1 in maintaining pluripotency and stem cell programming.

In the breast, the Wnt signalling pathway is critical for stem-cell renewal and multipotency (Zeng and Nusse 2010). β-catenin regulates mammary stem cells and progenitor cells to play a role in mammary development and tumorigenesis (Incassati et al. 2010). Specifically, during pubertal development progesterone and oestrogen expression correlated with β-catenin target gene expression in both luminal and basal cells with increased stem cell activity (Joshi et al. 2010). In breast cancer, activated β-catenin signalling increases basally located
multipotent stem cells at the expense of differentiated myoepithelial cells. Interestingly, β-catenin is required for maintaining pluripotency in embryonic stem cells by regulating LRH-1, which in turn is crucial for maintaining proper levels of key stem cell genes Oct4, Nanog and Tbx3 (Wagner et al. 2010). Similarly, the regulation of β-catenin by LRH-1, suggests the importance of LRH-1 in stem cells. Stem cells share attributes with cells undergoing epithelial to mesenchymal transition (EMT) (Mani et al. 2008). LRH-1 induces breast cancer cell motility and invasion and this was associated with the regulation of E-cadherin and MMP9 (Chand et al. 2010). Together, these findings warrant further analysis of LRH-1 expression in breast cancer stem cells and the possibility of its ability to differentiate luminal or basal lineage populations in breast cancer.

6.4.3 Other methods to analyse the role of LRH-1 on mouse mammary gland tumorigenesis

This thesis demonstrates that LRH-1 over expression can increase incidence of DMBA-mediated mammary tumours (Chapter 6 Figure 1). However, we are yet to conclusively prove that LRH-1 is primarily responsible for the increase in DMBA-mediated tumorigenesis we observed (Chapter 5). The supplementation of DMBA-challenged with progesterone has proven to increase mammary tumour specificity. Studies have shown that progesterone supplementation reduces DMBA-induced gastrointestinal tumour formation and increases mammary tumour formation in mice (Jabara et al. 1979).

Apart from utilising progesterone supplementation, other genetic models of breast cancer progression can be employed. The LRH-1 transgenic mouse model can be crossed with other models that develop mammary tumours such as the MMTV-HER2 transgenic line that develops tumours within 28 weeks (Finkle et al. 2004). This transgenic model shares features with human breast cancer and will allow one to explore the role LRH-1 plays in promoting tumour incidence and latency. Furthermore, advancements in 3D culture methods by Bissell and colleagues have provided invaluable models to dissect the complex signalling interactions that are difficult to investigate in vivo (Kenny et al. 2007). An exciting application of transgenic animals is the isolation of primary mammary epithelial cells or tumour cells in 3D or 2D cultures that allow the delineation of signalling mechanisms that are difficult to study in vivo. Tumours derived from LRH-1 overexpressing animals can be isolated and grown in 3D culture to study the specific mechanisms and pathways that are altered or activated during mammary tumour progression.
To further explore the role of LRH-1 in promoting mammary epithelial cell proliferation and mammary tumorigenesis, a mammary epithelial cell specific LRH-1 knockout mouse needs to be utilised. This mouse model will give us a definite answer as to the role of LRH-1 in the mouse mammary. Understanding the mechanisms and signalling pathways down regulated in LRH-1 knockout animals treated with chemical carcinogens or crossed with well-known transgenic mouse models of breast cancer is of paramount importance.

6.5 Mammary cancer and development

During the complex developmental cycling of the mammary ducts, the luminal epithelial cells undergo many rounds of proliferation, remodelling and cell death (Lanigan et al. 2007). These basic process are conscripted by the cancer environment to promote tumorigenesis (Dickson et al. 2000, McGee et al. 2006). It is well known that the very hormones such as oestrogen, progesterone and prolactin that are essential for mammary gland development are also crucial for breast tumorigenesis (Brisken and O’Malley 2010). Therefore understanding the mechanisms that govern normal mammary gland development may help us understand how tumours hijack these normal mechanisms to promote tumorigenesis.

This thesis demonstrates a tumour promoting role of LRH-1 in the mammary gland via the nuclear accumulation of β-catenin and increase in cyclin D1/E1 transcript levels. However this phenotype contrasts my previous reports that LRH-1 plays an inhibitory role in early mammary gland development via the induction of TGF-β isoforms. We are yet to identify the links between LRH-1, the TGF-β and Wnt/β-catenin pathways. However, TGF-β has the potential to function as a tumour suppressor and as a tumour promoter. The hypothesis that TGF-β functions as a tumour suppressor early in carcinogenesis but switches to a tumour promoter in late-stage disease is an attractive proposition to reconcile the otherwise conflicting body of evidence that exists for both the roles. In breast cancer, TGF-β has a clear dichotomous function where it is a potent inhibitor in breast epithelial cells (Knabbe et al. 1987), but its levels are elevated in advanced stages of tumour progression (Gorsch et al. 1992).

Similarly in normal mammary epithelial cells, LRH-1 overexpression leads to an accumulation of collagen and inhibition of mammary lateral budding. However in a tumour environment with the introduction of DMBA, mammary epithelial cells encounter drastic alterations in signalling patterns (Chapter 6 Figure 2). In some breast cancers mutations or loss of expression of members of the TGF-β signalling pathway including TGF-BRI and TGF-BRII have
been observed (Lücke et al. 2001). Also, mutations in SMAD4 have been observed in tumours (Miyaki and Kuroki 2003, Fleming et al. 2013). Therefore, with the lack of key SMAD members in the TGF-β signalling pathway; non-SMAD pathways downstream to TGF-β are activated in tumours, including the oncogenic RAS, PI3K and MAPK pathways (von Lintig et al. 2000, Santen et al. 2002, Baselga 2011). In prostate cancer cells, TGF-β increases PGE_2 secretion, which in turn mediates TGF-β effects on cell migration and invasion through the activation of PI3K/AKT pathway (Vo et al. 2013). Additionally, TGF-β dependent MAPK pathways promote cell invasiveness and in vivo mammary adenocarcinoma tumorigenesis (Daroqui et al. 2012). The induction of these oncogenic pathways in response to TGF-β suggests its importance in tumour progression.

Furthermore, p-SMAD3 shuttles β-catenin into the nucleus forming a well-known protein complex between Smad3/β-catenin/Tcf; thereby promoting c-myc and cyclinD1/E1 transcription (Zhang et al. 2010). Aberrant expression of c-myc in tumour cells represses the expression of cell cycle regulators p15 and p21, thus abolishing the growth suppressive effects of TGF-β (Pardali and Moustakas 2007). Moreover, cyclinD1 cooperates with p21 to mediate the effect of TGF-β on breast cancer cell progression (Dai et al. 2013). Previous findings indicate that LRH-1 can induce c-myc gene expression (Annicotte et al. 2005). Together these findings suggest that in early epithelial cells LRH-1-mediated TGF-β induction may induce cell cycle inhibitor p21 to inhibit lateral branching and restrict increased cell proliferation induced by LRH-1. However in a tumour environment, activation of the Wnt/β-catenin pathway and aberrant c-myc expression might be responsible for the dual role LRH-1 plays in mammary epithelial cells (Chapter 6, Figure 2).

We hypothesise that like TGF-β, LRH-1 has dual roles in mammary epithelial cells where it partially inhibits ductal morphology in virgin glands, but in a tumour milieu it increases incidence of carcinogen induced mammary tumours by enhancing tumour cell proliferation. We are yet to identify the genetic or epigenetic mechanism behind the switch, however exploring these differences will paramount in further understanding the roles of LRH-1 in normal breast development and cancer.
Chapter 6 Figure 2: In a normal mammary epithelial cell, I propose that LRH-1 induces TGF-β signalling which via pSMAD2/3 and SMAD4 promotes Smad target genes and inhibits mammary ductal branching. In a cancerous environment, I propose that LRH-1 can still induce TGF-β signalling, but since SMAD4 is mutated in many cancers, Smad target genes that inhibit mammary ductal branching are not induced. However Smad independent pathways are activated namely RAS, PI3K and MAPK pathways that promote cell proliferation and tumours. In addition, pSmad3 can bind to β-catenin and shuttle it into the nucleus where it is known to induce CycD1/E1 and cMyc gene transcription. Together, LRH-1 can promote cell growth, proliferation and tumour incidence.
6.6 Can LRH-1 be used as a therapeutic for breast cancer patients?

6.6.1 The relevance of targeting LRH-1 in breast cancer?

LRH-1 has oestrogen dependent and independent effects on breast cancer cells to promote breast cancer cell proliferation, motility and invasion (Annicotte et al. 2005, Chand et al. 2010, Chand et al. 2012). Although these studies have been instrumental in our understanding of the role of LRH-1 in breast cancer cell proliferation, it has been limited by the use of breast cancer cell lines in vitro. Our findings have confirmed our in vitro observations in mice, where we demonstrate an increase in cell proliferation and mammary tumour incidence in response to LRH-1. Its relevance to human breast cancer stems from an expression profile performed on a small Japanese cohort that demonstrated elevated levels of LRH-1 in 43% of invasive ductal carcinomas and 28% of ductal carcinoma in situ (Miki et al. 2006). This study showed that LRH-1 positively correlated with sex-steroid receptors, steroidogenic acute regulatory protein, P450 side-chain cleavage and 3beta-hydroxysteroid dehydrogenase (Miki et al. 2006). These findings suggest an important role for LRH-1 in breast carcinoma and steroidogenesis.

A recent study profiling the expression of nuclear receptors in stroma adjacent to breast tumours found that LRH-1 was up regulated in cancer associated fibroblasts compared to normal associated fibroblasts (Knower et al. 2013). However studies exploring the potential for LRH-1 as a therapeutical target in breast cancer are limited. Recently studies of genome-wide analysis of DNA methylation in colorectal cancer have revealed hypermethylation of LRH-1 in tissue from colorectal cancer patients and colon tissue from cancer-free donors (Naumov et al. 2013). Furthermore a genomic wide association study in human pancreatic cancer identified five SNPs that map to LRH-1 causing its down regulation in pancreatic cancer (Petersen et al. 2010). The first genetic event that leads to invasive pancreatic cancer is the mutational activation of KRAS (Collins et al. 2012). In the pancreas, LRH-1 cooperates with pancreatic acinar specific transcription factor complex, pancreas-specific transcription factor 1 (PTF1) to directly bind and activate acinar-specific genes involved in digestion (Holmstrom et al. 2011). Recently two studies validated the pancreatic GWAS study and showed that acinar cells down regulate LRH-1 during recovery from pancreatitis (during which they are susceptible to Kras), pancreas-specific deletion of LRH-1 leaves acinar cells in a metastatic state after pancreatitis and reduced LRH-1 dramatically increases Kras-induced pancreatic intraepithelial neoplasia (Flandez et al. 2013, von Figura et al. 2013). These studies suggest that LRH-1 could be a tumour suppressor in pancreatic cancer, however this is contradictory to LRH-1 overexpression promotes PDAC cell lines in vitro (Benod et al. 2011). Possibly, LRH-1 restrains tumour growth early by inhibiting Kras, while having a tumour promoting role during PDAC progression. These observations of LRH-1 in
human pancreatic cancer may also be valid in human breast cancer. Germline mutations in well-known breast cancer oncogenes BRCA1 and BRCA2 are associated with an increased risk of pancreatic cancer (Mocci et al. 2013). Additionally, breast tumours with Kras mutations present a worse prognosis (Pereira et al. 2013). These studies warrant larger expression profiling of LRH-1 in human breast cancer and a GWAS study examining potential SNPs in human breast cancer.

The action of LRH-1 has been proposed to be independent of a ligand (Sablin et al. 2003). However this study was performed with mouse LRH-1 and two subsequent studies that reported the crystal structure of human LRH-1, demonstrate the presence of a phospholipid molecule bound in the pocket of the putative ligand binding domain (Ortlund et al. 2005, Wang et al. 2005). The establishment that LRH-1 is a ligand dependent nuclear receptor indicates that small molecule compounds that regulate its activity may be effective as a novel breast cancer treatment. Recently, small molecule compounds have been shown to antagonize the effect of LRH-1 on human pancreatic, colon and breast cancer cell lines (Benod et al. 2013). Together with the proven efficacy of nuclear receptor inhibitors, LRH-1 antagonists are a novel therapeutical target for breast cancer treatment.

6.6.2 Implications of blocking LRH-1 in breast cancer

The search for LRH-1 modulators has revealed compelling evidence that LRH-1 could bind regulatory ligands and dilauroyl phosphotidylcholine (DLPC) are potential hormone candidates for this receptor (Krylova et al. 2005, Lee et al. 2011). Moreover, synthetic and naturally occurring agonists have been reported for this receptor (Krylova et al. 2005, Whitby et al. 2006, Busby et al. 2010, Whitby et al. 2011, Rey et al. 2012). The treatment of the DLPC infers antidiabetic effects on mice, indicating that DLPC via LRH-1 agonist activity is a potential therapeutic agent for treatment of metabolic disorders (Lee et al. 2011). In breast cancer, LRH-1 is implicated in increasing breast cancer cell proliferation. Therefore discovery of a LRH-1 antagonist is of paramount importance.

Recently, the discovery of small molecule antagonists for LRH-1 were reported (Benod et al. 2013). However the actions of the compounds discovered in this study were not specific to the breast. Treating breast, colon and pancreatic cells with these compounds resulted in the inhibition of cell proliferation (Benod et al. 2013). While this may be a favourable outcome, a key characteristic of drug development is specificity. At this stage, these compounds are useful for understanding the roles of this receptor in different physiological and pathophysiological
processes. Once fully characterised and optimized, these compounds could be developed into promising therapeutical agents specifically for breast cancer treatment.

6.8 Conclusions

The focus of this thesis is to delineate the role of LRH-1 in breast cancer by examining the functional consequences of LRH-1 overexpression in the developing virgin mammary gland and in a cancerous environment. Firstly, we identify that the discordance of LRH-1 transcript and protein in breast cancer cell lines, may be attributed to increased protein and transcript stability in ER- breast cancer cell lines.

Blockade of LRH-1 in MCF-7 cells revealed a novel relationship between LRH-1 and TGF-β which was confirmed using a novel dox-inducible mammary epithelial cell specific LRH-1 transgenic mouse model. In addition, activation of LRH-1 in this model produces a phenotype remarkably similar to that of the TGF-β over-expressing mouse – impairment of mammary lateral bud formation.

Using the LRH-1 transgenic model, a potential role for LRH-1 in mammary epithelial cell proliferation was identified. LRH-1 overexpression induced mammary epithelial cell proliferation and increased incidence of DMBA-mediated mammary tumours. These findings were accompanied by the elevation of breast oncogenes cyclinD1, cyclinE1 and β-catenin. We conclude that LRH-1 like TGF-β may serve opposing roles in the mammary gland, where it inhibits early development albeit partially, but can promote mammary tumorigenesis. Understanding mouse mammary development and tumorigenesis will provide insights into the improved management of breast cancer.

This work contributes to the overall understanding of LRH-1 – dependent mechanisms in in vitro and in vivo; and has identified novel functions of LRH-1 in a transgenic mouse model that affect mammary epithelial cell proliferation. This is of particular importance to the aetiology of breast cancer, and the development of novel treatments.


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Transforming Growth Factor-β (TGF-β) and the Type II TGF-β Receptor. "Journal of Biological Chemistry 275(50): 39146-39151.


Chapter 7: References cited


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Appendix A: General protocols and optimised reagents

A1: Mouse mammary gland work up

Solutions and materials

1. 70% Ethanol

2. Carmine Alum
   - Carmine 1.0g
   - Alum 2.5g
   - Water 500mL
   - Mix and bring to boil for 15-20 minutes. Add one crystal of thymol as a preservative.
   - Store in 4°C for up to three months

3. Carnoy’s Fixative
   - Glacial acetic acid 10mL
   - Chloroform 30mL
   - Ethanol, absolute 60mL
   - Mix and store in 4°C

4. 10% Formaldehyde

5. Surgical equipment
   - Forceps
   - Scissors

6. Microscope slides superfrost plus

7. Liquid nitrogen

8. Camelaks
Appendix A: General protocols and optimised conditions

Protocol

Killing of mice, preparation for surgery and mammary gland removal

1. The animal is placed in a CO$_2$ chamber that is prepared for CO$_2$ asphyxiation.
2. Turn CO$_2$ gas on
3. Monitor animal progress during asphyxiation
4. The animal was laid on its back and pinned by its hands and feet to a soft board. 70% EtOH was sprayed on the animal to wet the fur.
5. Using forceps, pull up the abdomen skin at the midline and make a sharp incision using the scissors
6. Starting from the incision, cut along the midsection up toward the neck of the animal, while avoiding puncturing body cavity
7. Cut the skin along the front leg of the animal resulting in a Y-shape
8. Cut the skin along the rear limbs in the same way
9. Using forceps gently peel back skin and pin on the soft board.
10. Using forceps lift mammary gland and make small sharp incisions to remove mammary gland.
11. These glands can be used for any downstream applications.

Mammary whole mount preparation

1. The 4$^{th}$ inguinal mammary gland is removed and spread on a glass slide
2. The slide is placed in carnoy’s fixative for 2-4 hours at R.T.
3. Slide is washed in 70% EtOH for 15min
4. Change gradually to distilled water – 50% EtOH for 5min, 25% EtOH for 5min, H$_2$O for 5min
5. Stain in carmine alum O/N
6. Wash in 70% EtOH for 15min
7. Wash in 95% EtOH for 15min
8. Wash in 100% EtOH for 15min
9. Finally clear in histolene for 2x30min and mount with DPX

Formalin fixed paraffin embedded mammary gland sections

1. The 4$^{th}$ inguinal mammary gland is removed and spread on a piece of cardboard
2. The mammary gland is placed in 10% formaldehyde for 2-4 hours at R.T.
3. The gland is removed and then stored in 70% EtOH

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Appendix A: General protocols and optimised conditions

4. When ready, the gland is dehydrated to paraffin and embedded in paraffin blocks
5. Cool block O/N at 4°C to ensure optimal cutting
6. Trim paraffin blocks to optimal cutting surface
7. Cut 5mm slices and place in 37°C water
8. Fish out sections using microscope slide and brush
9. Dry slide at 37°C O/N and store at room temperature until use

Snap freezing
1. Remove thoracic glands and place in cryovial
2. Immediately drop cryovial in liquid nitrogen
3. When ready, remove cryovial using long forceps and place at -80°C
Appendix A: General protocols and optimised conditions

A2: Tissue Homogenisation protocol

1. Remove snap-frozen tissue from freezer and immediately place on dry ice
2. Prepare cutting surface by placing ice over a esky lid and covering up with foil
3. Prepare cutting tools by spraying with 70% EtOH and wrapping in Kimwipes
4. Place cutting tools in dry ice
5. Weigh out 30 - 50mg of tissue
6. Place tissue in Round Bottom sample tube (Qiagen) with one stainless steel ball (Qiagen)
7. Place in either Trizol or RLT buffer for RNA extraction, or protein lysis buffer for protein extraction
8. Place on ice and transport to Qiagen Tissue Lyser
9. Place in Lyser and set to 50 oscillations/second for 2 mins until tissue is homogenised
10. Centrifuge at max speed for 10 minutes at 4 °C
11. Remove supernatant and follow with protein or RNA extraction protocol
Appendix A: General protocols and optimised conditions

A3: General Immunohistochemistry Protocol

Solutions and Materials

1. Histolene and Ethanol
2. Dako antigen retrieval: Prepare as per manufacturers protocol
3. Citrate buffer for antigen retrieval:
   - Citric acid 1g
   - Distilled Water 1000mL
   - Mix to dissolve, adjust to PH 6.0 with NaOH, then add 0.5mL Tween20
   - Store at 4°C
4. PBS
5. ABC Rabbit or Mouse kit
6. DAB chromagen: Prepare as per manufacturers protocol
7. Mayers Haemotoxilin
8. Scott’s tap water
9. DPX

Protocol

Day one

1. Dewax through series of ethanol (7mins for 2x histolene; 7mins for 2x 100%EtOH; 5mins for 90%, 70%, 50%, 25% and H₂O)
2. Antigen retrieval (check A5 antibody table for optimal solution). Microwave 5min 90%, 5min 40%. Cool for 1hr
3. PBS wash for 2x 5min
4. Blocking solution – 10% serum in PBS for 60min (the serum must be the species the secondary antibody is raised in. Use ABC kit for this solution)
5. 1º Ab in 10% serum. Check A4 for antibody dilution details. For negative control add IgG as same as species used to grow 1º Ab and incubate 4°C O/N

Day two

6. PBS wash 2x 5min
7. Biotinylated secondary antibody (from ABC kit) 1 hr R.T.
8. PBS wash 2x 5min
9. ABC amplification (from ABC kit) 30min R.T.
10. PBS wash 2x 5min

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Appendix A: General protocols and optimised conditions

11. DAB 3min
12. Wash 2xdips dH₂O
13. Mayers Haemotoxilin for 5mins
14. Wash 2xdips dH₂O
15. Scotts tap water for 2mins
16. Check Haemotoxilin color
17. Dehydration 70% - 1min, 100% 2x 1min each
18. Histolene 2x 1mins
19. Mount in DPX

A4: General Immunocytochemistry Protocol

Solutions and Materials

1. Methanol 100%
2. PBS 1x
3. Blocking buffer
   - 2.5mL 10x PBS
   - 1.25mL normal serum from same species as the secondary antibody
   - 21.25mL distilled water
   - Add 75μl Triton X-100 while stirring
4. Antibody dilution buffer
   - 4ml 10x PBS
   - 36ml distilled water
   - Add 0.4g BSA and 120μl Triton X-100 while stirring

Protocol

Note: Cells must be grown, treated, fixed and stained directly on chamber slides or coverslips

1. Aspirate liquid from cells, then wash cells in 1x PBS 2x5min
2. Cover cells with ice-cold methanol for 15 mins at -20 °C
3. Aspirate fixative and rinse 3x5min with PBS
4. Block cells in blocking buffer for 60mins
5. Aspirate blocking buffer and apply diluted primary antibody
6. Incubate O/N at 4°C
7. Rinse with PBS 3x5mins

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8. For IHC – proceed with protocol (A), for IF – proceed with protocol (B)

(A) IHC
1. Use ABC kit to perform further steps
2. Add biotynylated secondary antibody and incubate for 60mins
3. Rinse with PBS 3x5mins
4. Add ABC reagent and incubate for 30mins
5. Rinse with PBS 3x5mins
6. Add DAB and watch
7. Stop reaction by dipping in tap water
8. Add Mayer’s Haemotoxilin for 5mins
9. Add Scott’s tap water for 2mins
10. Mount in DPX

(B) IF
1. Incubate in fluorochrome-conjugated secondary antibody diluted in Antibody dilution buffer (1in500) for 1hr in dark
2. Rinse with PBS 3x5mins
3. Coverslip slides in Dapi-Dako Fluorescent mounting media mix 3ul (20ug/ml DAPI) in 100ul of Dako Fluorescent media
4. Place nail polish around coverslip if required
### A5: Primary antibodies

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## Appendix A: General protocols and optimised conditions

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### Appendix B: Publications arising during this thesis

#### B1: LRH-1 expression in ovarian epithelial and granulosa cell tumours

**Declaration**

Appendix B1 entitled “Liver Receptor Homologue-1 expression in ovarian epithelial and granulosa cell tumours

In the case of Appendix B1, contributions to the work involved the following

<table>
<thead>
<tr>
<th>Name</th>
<th>% contribution</th>
<th>Nature of contribution</th>
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<tbody>
<tr>
<td>1. Kyren A. Lazarus</td>
<td>20%</td>
<td>Performed experiments, wrote the manuscript</td>
</tr>
<tr>
<td>2. Ashwini L. Chand</td>
<td>30%</td>
<td>Performed experiments, wrote the manuscript</td>
</tr>
<tr>
<td>3. Niro Pathiarage</td>
<td>15%</td>
<td>Designed the study, performed experiments and Data analysis</td>
</tr>
<tr>
<td>4. Simon Chu</td>
<td>10%</td>
<td>Provided samples</td>
</tr>
<tr>
<td>5. Ann E. Drummond</td>
<td>5%</td>
<td>Data analysis</td>
</tr>
<tr>
<td>6. Peter J. Fuller</td>
<td>10%</td>
<td>Provided clinical samples, manuscript preparation</td>
</tr>
<tr>
<td>7. Colin D. Clyne</td>
<td>10%</td>
<td>Designed the study, Data analysis, Manuscript preparation</td>
</tr>
</tbody>
</table>

**Declaration by co-authors**

The undersigned hereby certify that:

1. They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria
4. Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or published of journals of other publications, and (c) the head of the responsible academic unit; and
5. The original data are stored at the following location and will be held for at least five years from the date at the location:

**Prince Henry’s Institute of Medical Research, Clayton VIC, Australia.**
## Appendix B1: LRH-1 expression in ovarian epithelial and granulosa cell tumours

<table>
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<tr>
<td>Kyren A. Lazarus</td>
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<tr>
<td>Ashwini L. Chand</td>
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<td></td>
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<tr>
<td>Colin D. Clyne</td>
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</table>
Appendix B1: LRH-1 expression in ovarian epithelial and granulosa cell tumours

Liver receptor homologue-1 expression in ovarian epithelial and granulosa cell tumours

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Steroidogenic factor-1
Arenatase

A B S T R A C T
Granulosa cell tumours of the ovary (GCT) express aromatase and produce oestrogens. The ovarian-specific aromatase promoter (gsE) is regulated by members of the group 5A nuclear receptor family, SF-1 and LRH-1. Since both SF-1 and LRH-1 are implicated in proliferation and cancer, we hypothesised that alteration in the expression of either or both receptors may be associated with GCT. We therefore determined the expression of LRH-1, SF-1 and aromatase in a cohort of GCT, mucinous and serous cystadenocarcinomas, and normal ovaries. LRH-1 mRNA was present at low level in normal ovary and serous cystadenocarcinoma, but was elevated approximately 30-fold in GCT, and 8-fold in mucinous cystadenocarcinoma, compared to normal ovary. LRH-1 protein expression was confirmed in GCT by immunohistochemistry. SF-1 mRNA was significantly lower than that of LRH-1 in all samples and not significantly altered in GCT, compared to normal ovary. Aromatase mRNA was present at low level in normal ovary and serous and mucinous cystadenocarcinoma, and significantly elevated (18-fold) in GCT compared to normal ovary. Despite the coordinate over-expression of both LRH-1 and aromatase in GCT versus normal ovary, their levels did not correlate in individual patients; rather, aromatase expression correlated with that of SF-1. Finally, although both LRH-1 and SF-1 activated aromatase promoter activity in transient transfection studies, gel-shift and chromatin immunoprecipitation data indicated that SF-1, but not LRH-1, bound to the aromatase promoter. We conclude that SF-1 regulates aromatase expression in GCT; over-expression of LRH-1 suggests that this receptor may be involved in the pathogenesis of GCT by mechanisms other than the regulation of aromatase. Its role in this disease therefore warrants further investigation.

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1. Introduction
Sex cord tumours of the ovary represent approximately 10% of all ovarian malignancies, with granulosa cell tumours (GCT) accounting for most of these tumours [11]. Although GCT may arise at any age they are most common in 50–60 year old women, ~10% of GCT arise earlier in life and are classified as juvenile GCT. Late detection, strong malignant potential and tendency for late recurrence contribute to their clinical significance. The mechanisms by which granulosa cells undergo malignant transformation are poorly understood, but are thought to involve a disruption to regulatory pathways that function during normal ovarian development, folliculogenesis, and ovulation. Previous studies have focused on activation of endocrine pathways, such as FSH signalling [2], loss of function of components of the inhibin signalling pathway [3,4], hyper-secretion of luteinizing hormone [5,6], oestrogen signalling [7,8], and nuclear receptors [9] (reviewed in [10]). Although GCT express the FSH receptor and exhibit a phenotype resembling FSH-stimulated granulosa cells, their growth appears to be FSH-independent. This suggests that other mitogenic pathways may contribute to the growth of GCT.
Several such pathways have been investigated; an analysis of anti-Mullerian hormone (AMH), inhibin-α subunit, steroidogenic factor-1 (SF-1), and GATA transcription factors 2, 4 and 6 in GCT demonstrated positive protein expression, however only GATA-4 expression was higher in GCT (44%) and correlated positively with clinical stage [11]. In addition, patient follow-up after 10 years indicated that high GATA-4 expression was significantly associated with risk of disease recurrence [11]. Recently, a genetic mutation was identified as a key factor in the aetiology of GCT. Shah et al. reported that ~97% of adult GCT contain a somatic minisence mutation (C134W) in the Forkhead Box L2 (FOXL2) gene [12], a
finding that has subsequently been confirmed by two independent groups [13,14]. Although the mutation would appear to be aetologic or pathogenic, the mechanisms of action of FOXL2 remain to be elucidated.

Liver receptor homologue-1 (LRH-1) is a member of the NRSF subfamily of nuclear receptors. It plays important roles in embryonic development, metabolism and steroidogenesis, and is expressed in tissues of endodermal origin such as liver, intestine, pancreas and ovary (reviewed in [15]). In the ovary, LRH-1 is expressed in granulosa and luteal cells, and is significantly increased during pregnancy [16–18]. LRH-1 is involved in progesterone synthesis [19], and mice with ovary-specific deletion of LRH-1 are infertile due to a failure of ovulation [20]. This ovulation failure arises through multiple mechanisms including reduced progesterone biosynthesis, disruption to prostaglandin and hyaluronic acid cascades, and impaired expression of extracellular matrix proteins, highlighting the critical role of LRH-1 in ovarian function [20].

LRH-1 is implicated in cancer development through its ability to stimulate cell cycle genes including cyclins D1 and E1, in cooperation with β-catenin in colon cancer [21,22]. LRH-1 is also highly expressed in the eozoic carcinoma, and involved in the development and progression of pancreatic adenocarcinoma [23]. Given the established roles of LRH-1 in tumour development in intestine and pancreas – two tissues that express LRH-1 at relatively high levels – and the known role of LRH-1 in regulating steroidogenesis in breast cancer [24,25], we hypothesised that LRH-1 over-expression may also be relevant in tumours arising from the steroidogenic cells of the ovary. We therefore established the expression profiles of LRH-1 and its homologue SF-1 in a cohort of CHT, and compared this with expression in epithelial tumours of the ovary, as well as in normal human ovary. In addition we studied the expression of aromatase, a key enzyme involved in steroidogenesis, as a potential LRH-1/SF-1 target gene in CHT.

2. Materials and methods

2.1. Patients and tissue acquisition

Ovarian CHT (n = 9), mucinous cystadenocarcinomas (n = 8), serous cystadenocarcinomas (n = 9) and normal pre- and postmenopausal ovarian tissues (n = 20) were obtained in a study of serum inhibin levels in ovarian tumours, as described previously [3,4]. The normal tissues were obtained from women who had undergone elective hysterectomy with oophorectomy for a range of conditions not associated with ovarian malignancy. The study protocol was approved by the Research and Ethics Committee of Monash Medical Centre, and all women gave written informed consent for the studies.

2.2. Reverse transcription and real time RT-PCR

RNA was extracted using the guanidinium thiocyanate/cesium chloride method as described previously [34]. 1 μg of total RNA was reverse transcribed using random primers (Promega) and AMV-RT (Promega) according to the manufacturer’s recommendations. The PCR reactions were performed using the Light Cycler® technology (Roche Diagnostics) and SYBR Green-based detection system (DNA Master SYBR Green I, Roche). All PCR products were characterised using an internal standard curve of amplicons of known concentration. Water minus the DNA template served as the negative control. PCR products were verified by melting curve analysis using the Light Cycler 3.5 software (Roche) and product identity was confirmed by direct sequencing. Each sample was normalised on the basis of its 18S expression. Primers used were as follows: LRH-1 (sense, GCTGATAGCCAAACTTTGAAA and antisense, TCTATTTGGCTGAAACTTCT); SF-1 (sense 5′-GGATTTG TTCGTCAAGCACTCA-3′, antisense 5′-GCTTTTCA CAGGATGG TG-3′; GATAF (sense 5′-GGTCTACCCGCAAGA-3′ and antisense 5′-GCTTGGAA TATCCGGC-3′), aromatase (sense 5′-TGG TGAATGTCACGGC-3′, antisense 5′-CGAGAGTCTGCGTG-3′); aromatase promoter II specific (sense, GCAACACGGGATGAT and antisense, CAGGAAATGCCGGGACA). 

2.3. Electrophoretic mobility shift assay (EMSA)

A double stranded nucleotide probe encompassing the aromatase promoter II nuclear receptor half-site (underlined, 5′-GACCT TACAAAGTGCAAG-3′) was generated by annealing complementary single-stranded oligonucleotides and labelling with [α-32P] JcTP. Recombinant proteins were transcribed/translated using the TNT Quick-coupled transcription/translation system (Promega). The protein binding reactions were carried out in 15 μl buffer (20 mM HEPES, pH 8; 1 mM EDTA; 50 mM KCl; 10 mM DTT; the total volume was 1 μg/ml BSA) with 40,000 cpm of labelled probe, 5 μg nuclear proteins or 2 μl of transfected and translated in vitro SF-1 protein or LRH-1 protein, and 1 μg poly (dI-dC) (Roche). The mixtures were incubated at room temperature for 30 min. Antibodies used include SF-1 rabbit antisem (provided by Dr. Morohashi, National Institute for Basic Biology, Okazaki, Japan) and LRH-1 antisem (provided by Dr. Luc Belanger, Laval University, Canada). For LRH-1 antibody the reaction mixture was incubated with this antibody at 4 °C for 1 h before addition of labelled probe. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.5X Tris borate-EDTA for 3 h at 150 V. Gels were dried and composites analysed by phosphor-imaging and ImageQuant 5.0 software (STORM scanner, Molecular Dynamics Inc., CA, USA).

2.4. Chromatin immunoprecipitation

The chromatin immunoprecipitation assay was performed using the ChIP-IT kit (Active Motif, Carlsbad, CA) following the manufacturer’s instructions using KGN cells grown to 70–80% confluency in 10-cm dishes. Antibodies used for immunoprecipitations include (a) anti-LRH-1 (AbCam) (b) anti-LRH-1 (R&D Systems, Inc., MN) (c) SF-1 antibody (ABR Affinity BioReagents) (d) anti-SF-1 serum (Dr. Morohashi) (e) Polyclonal rabbit IgG antibody (Sigma) served as the non-specific control. After elution and purification, the recovered immunoprecipitated DNA samples were used for PCR (GoTaq polymerase; Promega) using primers upstream (sense 5′-GACTAGCGGAACAGACCTGTT-3′, antisense 5′-GCTA CTCGGCACCA-3′) or within the PI of the human CYP19 gene (sense 5′-TTTCCCATACTACGGTGCCG-3′; antisense 5GGAATCTTCTTCTC TGAAGC-3′). Products were separated and visualised on agarose-ethidium bromide gels.

2.5. Immunohistochemistry

5 μm sections were cut from formalin-fixed, paraffin-embedded tissues. Prior to staining, the sections were de-waxed and rehydrated in graded ethanol washes. Antigen retrieval was performed by immersing slides in 600 ml of Target Retrieval Solution (Dako) and heating in a 1000W microwave at 100% power for 5 min, standing at room temperature for 5 min and then heating for an additional 5 min at 40% power, before cooling at room temperature for an hour. Sections were then treated with 3% H2O2 for 5 min to block endogenous peroxidase activity. To block endogenous avidin and biotin in the sections, the avidin/biotin blocking kit (Vector Laboratories) was used. Mouse monoclonal antibody directed against LRH-1 (R&D systems, cat# H2252, and dilution 1:300) was used for immunohistochemistry. The mouse on mouse
Appendix B1: LRH-1 expression in ovarian epithelial and granulosa cell tumours

(M.O.M) basic kit (Vector Laboratories) was employed for detection. LRH-1 staining was detected by staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB Liquid Substrate Kit, Dako). The reactions were stopped in water and sections were counterstained with Mayer’s hematoxylin (Sigma Diagnostics), dehydrated and mounted with DPX mounting medium (Sigma Diagnostics) under 22 × 50 mm coverslips (HD Scientific).

2.6. Statistical analysis

Realtime RT-PCR data was analysed for each data set using GraphPad Prism software version 5.04. One-way anova was performed followed by Newman–Keuls test, comparing each data set to the normal premenopausal ovarian tissue. Spearman’s rank correlation coefficient was used to analyse whether transcript levels for the different genes were correlated. A P-value of less than 0.05 was considered statistically significant.

3. Results

Realtime RT-PCR was used to quantify LRH-1 mRNA levels across the various tissue samples (Fig. 1A). LRH-1 mRNA was readily detectable in normal pre- and post-menopausal ovaries, consistent with previous reports of LRH-1 expression in ovarian tissues of rodents and humans [16,17,26–29]. LRH-1 mRNA was approximately 30-fold higher (P<0.001) in GCT compared to normal premenopausal ovary. LRH-1 mRNA was also detected in serous and mucinous cystadenocarcinomas, although levels were not significantly different to those in normal ovary.

Aromatase is a well characterised target gene of LRH-1 in breast cancer [24,25], that is also expressed in GCT [29]. We therefore examined aromatase mRNA levels across the tissue samples as a potential marker of LRH-1 transcriptional activity (Fig. 1C). Aromatase mRNA was detectable across all samples, but was significantly elevated (P<0.01) in the GCT panel, compared to normal ovary. The aromatase gene possesses a number of alternative first exons,

![Graphs showing expression levels for LRH-1 and aromatase](image)

Fig. 1. Realtime RT-PCR quantification of mRNA transcripts for total (A) LRH-1, (B) SF-1, (C) aromatase and (D) promoter I-specific aromatase in a panel of ovarian biopsies: premenopausal (PRE, n = 8) and postmenopausal (POST, n = 6) normal ovaries, granulosa cell tumours (GCT, n = 9), mucinous (MUC, n = 10) and serous (SER, n = 9) ovarian tumours. All transcripts are normalised for 18S housekeeping gene expression. Statistical significance is indicated at "*" P < 0.01 and "**" P < 0.001 versus normal premenopausal ovary. (E and F) Spearman’s rank correlation coefficient was used to analyse whether LRH-1 and SF-1 transcript levels were correlated with that of aromatase in GCT samples.

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Appendix B1: LRH-1 expression in ovarian epithelial and granulosa cell tumours

Each associated with a unique promoter, that are expressed in a tissue-specific manner [30]. Aromatase transcripts in normal human ovary contain untranslated exon II, and its associated promoter II is known to be regulated by LRH-1 [24]; we therefore performed exon-specific RT-PCR to detect exon II-containing aromatase transcripts across the tissue panel (Fig. 1D). The pattern of expression was similar to that observed for total aromatase: exon II-containing transcripts were detected in all samples examined, with levels in GCT significantly higher (~6-fold, $P < 0.001$) than in normal ovary.

The NR5A family consists of two closely related orphan nuclear receptors: LRH-1 and SF-1. As promoter II is also regulated by SF-1 [24], we next examined mRNA levels of SF-1 and discovered that compared to LRH-1 levels, SF-1 transcripts were relatively low in all samples examined. As for LRH-1, SF-1 mRNA levels were significantly higher in GCT (~10-fold, $P < 0.001$) than in normal post-menopausal ovary (Fig. 1B). Additionally, SF-1 expression was significantly higher in GCT compared with mucinous (~10-fold, $P < 0.001$) and serous cystadenocarcinomas (~6-fold, $P < 0.001$) (Fig. 1B).

We correlated the transcript expression of LRH-1 and SF-1 with total aromatase levels. While a positive correlation between LRH-1 and aromatase was not observed (Fig. 1E), a significant positive correlation was observed between SF-1 and aromatase expression (Spearman's rank correlation coefficient $R = 0.62, P < 0.001$) (Fig. 1F).

As both LRH-1 and SF-1 are able to activate promoter II transcription, we compared the efficiency of LRH-1 and SF-1 in stimulating aromatase promoter II activity in human granulosa cells. We used KGN cells, a human granulosa cell line derived from a granulosa cell carcinoma [31]. Cells were transfected with a luciferase reporter construct driven by the proximal 516 nucleotides of promoter II, along with expression constructs for LRH-1 and SF-1, in the presence or absence of forskolin, to activate cAMP. Forskolin treatment alone induced promoter II activity 1.5-fold, transfection with LRH-1 stimulated luciferase activity 3-fold and 6-fold with LRH-1 over-expression combined with forskolin treatment (Fig. 2A). Similarly, luciferase activity was stimulated approximately 5-fold in the presence of SF-1 and 13-fold in the presence of both SF-1 and forskolin (Fig. 2A). Mutation of a consensus LRH-1/SF-1 nuclear receptor half site within promoter II abolished the ability of forskolin, LRH-1 and SF-1 to induce reporter luciferase activity (Fig. 2A). Quantification of aromatase, LRH-1 and SF-1 mRNA in these cells (Fig. 2B) confirmed that (i) forskolin stimulated endogenous expression of aromatase via promoter II, and (ii) LRH-1 mRNA was present at significantly higher levels than SF-1, consistent with the expression profile of the GCT tissues.

![Image](image_url)

**Fig. 2.** (A) LRH-1 and SF-1 stimulate expression of aromatase promoter II luciferase reporter genes in KGN cells. Cells were transfected with p5.16 (either wild-type (black bars) or harbouring a mutation in the SF-1/LRH-1 promoter half site (white bars)); pCMVβ-gal, and expression constructs encoding SF-1 or LRH-1, and incubated in the presence or absence of forskolin (25 μM) for 24 h (black bars). Luciferase activity was normalised to β-galactosidase. (B) mRNA levels of total CYP19, promoter II-derived CYP19 transcripts, SF-1 and LRH-1 in KGN cells in the absence (open bars) or presence (solid bars) of forskolin (25 μM, 24 h treatment). The mean ± SD for three independent experiments is shown, and statistical significance is indicated at *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control treated cells.

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4. Discussion

LRH-1 is a key nuclear receptor involved in the regulation of steroidogenic pathways in the normal ovary. Here we demonstrate higher levels of LRH-1 and aromatase transcripts in granulosa and epithelial tumours compared to normal pre- and post-menopausal ovaries. LRH-1 is the key transcriptional regulator of aromatase expression in breast cancer tissue, specifically in tumour-associated fibroblasts. LRH-1 binds to its nuclear receptor half site in promoter II to upregulate aromatase expression [24]. To test whether this relationship existed in GCT, we assessed the expression of LRH-1 and aromatase and promoter II transcripts in GCT samples. As expected, in GCT samples, we observed significantly higher promoter II transcript levels than that in normal ovary. Despite this observation, functional in vitro studies demonstrated that rather than LRH-1, its homolog SF-1 regulated aromatase promoter II transcription in GCT granulosa tumour cells. SF-1 bound the nuclear receptor half site preferentially when both NR5A receptors are present. The relative expression of SF-1, although distinctly lower than that of LRH-1, was higher in GCT samples compared to normal tissue. Furthermore a significant correlation was observed for SF-1, and not LRH-1 levels, to that of aromatase. Therefore LRH-1 is likely to have other roles in the pathology of GCT.

The actions of LRH-1 in the regulation of steroidogenesis, lipid metabolism and tumour proliferative pathways in breast, colon and pancreatic cancers are well established [15,33]. Although LRH-1 is highly expressed in granulosa cells of ovarian follicles, to date there is no evidence of its regulation of aromatase transcription in these cells. The strong expression patterns of LRH-1 in follicles, especially in lutinising follicles and the corpus luteum; its regulation of 3β-hydroxysteroid dehydrogenase and cholesterol side-chain cleavage cytochrome P450 (CYP11A1) demonstrate a defining role in the regulation of progesterone synthesis in human granulosa cells [10,26–28,34]. In addition, targeted knockout of LRH-1 in granulosa cells in the mouse ovary, results in infertility due to failure of ovarian luteinisation, follicular rupture and oocyte release [20]. This mouse exhibited deregulated expression of key LRH-1 target genes including steroidogenic acute regulatory protein (STAR), CYP11A1, progestin and glucocorticoid synthase 2 (CYP11B2) and hystronic acid receptor, CD44. However no changes were observed in the expression of aromatase. Collectively, these studies indicate a key role for LRH-1 progesterone production and ovulation in the normal ovary. We suggest that it is likely that the elevation of LRH-1 levels in GCT affects progesterone, and not estrogen synthesis pathways.

LRH-1 regulates tumour cell proliferation with the regulation of c-Myc [23] and cyclin D1 and E1 [21,22,23] in breast and intestinal cancers. LRH-1 and β-catenin interact in a transcriptional complex [36] to regulate the expression of cyclins D1 and E1 in tumour cells [21]. GCT biopsies show nuclear localisation of β-catenin [37]. Furthermore, over-expression of β-catenin in mouse granulosa cells results in the formation of multiple ovarian lesions that give rise to GCT, suggesting an activation of the Wnt pathway in GCT
Appendix B1: LRH-1 expression in ovarian epithelial and granulosa cell tumours

Fig. 4. Immunohistochemistry of LRH-1 localization in (A–C) paraffin embedded GCT biopsy sections and (D) control ovarian tissue (mouse). Arrowheads indicate positive LRH-1 localization in the nucleus of granulosa cells (GC), and absence in thecal cells (T) and the oocytes (O) in mouse ovary sections. In the GCT cells arrowheads indicate positive LRH-1 nuclear localization. Arrows show negative control.

[37,38]. A link between β-catenin and LRH-1 may provide another LRH-1 dependent mechanism of cell proliferation in GCT. In this context, the heterogeneity of LRH-1 positive cells observed in GCT biopsy sections may reflect cells at different phases of the cell cycle.

In conclusion, we have shown that LRH-1 is over-expressed in GCT compared to normal human ovary. Although LRH-1 can regulate aromatase expression in other tissues, our evidence suggests that aromatase is not a target of LRH-1 in GCT. Since pharmacological modulators of LRH-1 have recently been developed, the role of this receptor in GCT warrant further investigation.

Acknowledgements

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References


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Appendix B1: LRH-1 expression in ovarian epithelial and granulosa cell tumours


Appendix B2: LRH-1 and ERα activate GREB1 expression

B2: Declaration

Appendix B2 entitled “The orphan nuclear receptor LRH-1 and ERα activate GREB1 expression to induce breast cancer cell proliferation

In the case of Appendix B2, contributions to the work involved the following

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<tr>
<td>1. Kyren A. Lazarus</td>
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Declaration by co-authors

The undersigned hereby certify that:

(1) They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(2) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(3) There are no other authors of the publication according to these criteria

(4) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or published of journals of other publications, and (c) the head of the responsible academic unit; and

(5) The original data are stored at the following location and will be held for at least five years from the date at the location:

Prince Henry’s Institute of Medical Research, Clayton VIC, Australia.

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Appendix B2: LRH-1 and ERα activate GREB1 expression

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<tr>
<th>Name</th>
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Appendix B2: LRH-1 and ERα activate GREB1 expression

The Orphan Nuclear Receptor LRH-1 and ERα Activate GREB1 Expression to Induce Breast Cancer Cell Proliferation

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Abstract

Background: Liver Receptor Homolog-1 (LRH-1, NR5A2) is an orphan nuclear receptor that is over-expressed in cancers in tissues such as the breast, colon and pancreas. LRH-1 plays important roles in embryonic development, steroidogenesis and cholesterol homeostasis. In tumor cells, LRH-1 induces proliferation and cell cycle progression. High LRH-1 expression is demonstrated in breast cancers, positively correlating with ERs status and aromatase activity. LRH-1 dependent cellular mechanisms in breast cancer epithelial cells are poorly defined. Hence in the present study we investigated the actions of LRH-1 in estrogen receptor α (ERα) positive breast cancer cells.

Results: The study aimed to investigate LRH-1 dependent mechanisms that promote breast cancer proliferation. We identified that LRH-1 regulated the expression of Growth Regulation by Estrogen in Breast Cancer 1 (GREB1) in MCF-7 and MDA-MB-231 cells. Over-expression of LRH-1 increased GREB1 mRNA levels while knockdown of LRH-1 reduced its expression. GREB1 is a well characterised ERα target gene, with three estrogen response elements (EREs) located on its promoter. Chromatin immunoprecipitation studies provided evidence of the co-localisation of LRH-1 and ERs at all three EREs. With electrophoretic mobility shift assays, we demonstrated direct binding of LRH-1 to EREs located on GREB1 and Trefoil Factor 1 (TFF1, p52) promoters. LRH-1 and ERs co-operatively activated transcription of ERE luciferase reporter constructs suggesting an overlap in regulation of target genes in breast cancer cells. Over-expression of LRH-1 resulted in an increase in cell proliferation. This effect was more pronounced with estradiol treatment. In the presence of ICI 182,780, an ERs antagonist, LRH-1 still induced proliferation.

Conclusions: We conclude that in ER-positive breast cancer cells, LRH-1 promotes cell proliferation by enhancing ERs mediated transcription of target genes such as GREB1. Collectively these findings indicate the importance of LRH-1 in the progression of hormone-dependent breast cancer and implicate LRH-1 as a potential avenue for drug development.

Introduction

Exposure of breast tissue to circulating hormones is a key risk factor in breast cancer incidence [1,2,3]. Therefore understanding the mechanisms of hormonal actions is critical in progress towards better treatment options. In this report we analysed the effect of the orphan nuclear receptor NR5A2 (also termed Liver Receptor Homolog-1, LRH-1) on the transcriptional regulation of Growth Regulation by Estrogen in Breast Cancer (GREB1) and breast cancer proliferation.

LRH-1 belongs to the NR5A subclass of nuclear receptors and regulates gene transcription by binding as a monomer to an extended nuclear receptor half-site, consensus YCAAGGCYCR [4]. LRH-1 has well established roles in metabolic pathways involved in bile acid synthesis [5,6] and reverse cholesterol transport [7,8].

It is highly expressed in the ovary where it is vital for the regulation of steroidogenesis [9,10]. In embryonic tissue it causes the differentiation of chondrocytes and pluripotency in embryonic stem cells [13,14]. In addition LRH-1 has a role in gastric, colon, pancreatic and breast cancers [15,16,17,18,19].

LRH-1 contributes to breast cancer development and progression through its ability to induce aromatase expression in cancer associated stromal fibroblasts (CAFs) [17,18,20,21]. In postmenopausal breast cancers, aromatase in adipose is the major source of mitogenic estrogen for growth of ER-positive breast tumors [22].

Aromatase activity is regulated primarily by transcriptional changes of its gene (CYP19A1), via various tissue-specific promoters. Malignant breast epithelial cells secrete prostaglandin E2 (PGE2), allowing increased LRH-1 expression and LRH-1 mediated binding and transcriptional activation of aromatase promoter.
Appendix B2: LRH-1 and ERα activate GREB1 expression

LRH-1 regulates GREB1 expression in breast cancer

PL3/FH [18,20,21,23]. The LRH-1 induced increase in local estrogen cells has a paracrine effect on neighboring tumor cells causing an elevation in LRH-1 expression via the direct binding of ERα to its promoter [15]. LRH-1 can also regulate ERα expression in breast cancer cell lines [24] providing evidence of a possible feedback loop between LRH-1 and ERα within tumor epithelial cells.

Although LRH-1 is not basally expressed in normal mammary tissue, high expression has been demonstrated in the epithelial compartment of both invasive ductal carcinoma and ductal carcinoma in situ [15,17,18]. LRH-1 expression in human tumors correlated with that of other genes involved in steroid synthesis, including P450 side-chain cleavage, 3β-hydroxysteroid dehydrogenase and the Steroidogenic Acute Regulatory protein, suggesting that LRH-1 may influence in vitro steroidogenesis in breast cancer [17].

LRH-1 mediates the mitogenic effect of estrogen in breast cancer cells since siRNA-mediated knockdown of LRH-1 inhibits regulation by LRH-1 in MCF-7 cells proliferation [13]. Recently, we have demonstrated that LRH-1 not only enables the migration and invasion of breast cancer cell lines but also increases the tumorigenic potential of the normal mammary epithelial cell line MCF-10A [25].

To identify cellular pathways regulated by LRH-1 in breast cancer epithelial cells, we performed microarrays to identify genes which were transcriptionally regulated by LRH-1 in MCF-7 cells which had LRH-1 over-expressed or knocked down (data not shown). Interestingly one of the most significantly altered genes, caused by modulation of LRH-1 expression, was Growth Regulation by Estrogen in Breast Cancer (GREB1). Therefore we aimed to elucidate mechanism via which LRH-1 regulated GREB1 transcription.

In the present study we demonstrate co-localisation of LRH-1 and ERα on three critical ERα response elements (ERE) on the GREB1 promoter to activate transcription and cell proliferation. LRH-1 bound directly to ERE sequences present on the promoters of two well characterised, estrogen responsive genes GREB1 and Treflector Factor 1 (TFF2 or p52). These findings indicate that LRH-1 acts synergetically with ERα to induce transcription of GREB1 and unravels a new mechanism of action for LRH-1 in inducing breast cancer cell proliferation.

Results
Effects of LRH-1 on GREB1 expression in MCF-7 cells
MCF-7 cells were transfected with either an LRH-1- specific shRNA, a control shRNA, an expression vector encoding full-length human LRH-1 cDNA or an empty expression vector. Transfection with LRH-1- expression vector increased LRH-1 mRNA (12-fold) (Figure 1A) and protein expression (Figure 1B) compared to vector only transfected cells. Transfection with an expression plasmid encoding an LRH-1- specific shRNA reduced endogenous LRH-1 mRNA levels in MCF-7 cells by approximately 5-fold compared to control shRNA - transduced cells 24 h post transfection (Figure 1A). This was reflected in a reduction in LRH-1 protein by western blot analysis (Figure 1B). Having confirmed over- and under-expression of LRH-1 in MCF-7 cells, we next measured expression of GREB1, a gene previously identified in microarray data sets as significantly regulated by LRH-1 (data not shown). In LRH-1-overexpressing cells, GREB1 expression was elevated 40-fold while in LRH-1 knock down cells, a 2-fold decrease in GREB1 expression was observed (Figure 1C). These results correlated with the microarray findings. GREB1 is a well characterised ERα target gene [26,27]. To determine whether the increase in GREB1 expression could be attributed to LRH-1 mediated changes in ERα expression, we assessed protein expression by western blot. There was an increase observed in ERα levels in LRH-1 over-expressing cells, however the knockdown did not demonstrate a notable decrease (Figure 1B).

Estrogen regulation of GREB1 transcription is mediated by 3 estrogen response elements (EREs) located in the distal and proximal promoter regions of GREB1 [27] (Figure 2A). Analysis of the GREB1 promoter sequence for LRH-1 nuclear receptor half sites (LRHRHRE) containing the YCAAGGTCGCR motif (where Y is a pyrimidine and R is a purine) identified three potential LRHRE sites (Figure 2A). Interestingly these putative LRHREs were located within the ERα nuclear receptor recognition sites (EREs) found in the distal and proximal GREB1 promoter [27,28]. DNA binding sites for ERα and LRH-1 demonstrated significant sequence similarity (highlighted in Figure 2A), raising the possibility that LRH-1 binding could occur within the palindromic ERERE sequence (PoGGTCAnntTGACC(2)) as depicted directly to the EREs of these LRH-1-ERα target genes. To address this hypothesis we examined the promoter regulation of GREB1 in more detail.

LRH-1 is recruited with ERα on the GREB1 promoter
To demonstrate the interaction between LRH-1 and the ERα at the GREB1 promoter, we used chromatin immunoprecipitation (ChIP). We observed interaction of endogenous ERα and LRH-1 on these three ERE sites (Figure 2B) suggesting a direct role of LRH-1 in its transcriptional activation of GREB1. The first ChIP experiment involved ChIP with the LRH-1 antibody first, followed by a second ChIP with the ERα antibody. PCR of the resulting DNA with primers specific to GREB1 indicated co-occupancy of LRH-1 and ERα on the proximal ERE under basal conditions (Figure 2D). A validation, a second ChIP where ERα antibody was used in the first ChIP, followed by LRH-1 antibody for a second ChIP, to demonstrate the same result. Band intensity for the first ChIP (for both LRH-1 and ERα antibodies) was greater, when compared to results in Figure 2B, as the amount of chromatin introduced to the reaction was altered. Collectively, these results demonstrate the occupancy of both ERα and LRH-1 at the EREs located in the GREB1 promoter region, and suggest a direct relationship between LRH-1 and ERα in the regulation of GREB1 gene expression.

LRH-1 binds directly to EREs on the GREB1 and p52 promoters
As ChIP localises transcription factor binding only to the general vicinity of target sequences within genomic DNA (200–300 bp), we next confirmed that LRH-1 can bind directly to these EREs using competition electrophoretic mobility shift assays (EMSA, Figure 3A and 3B).

LRH-1 bound to all 3 GREB1 ERE probes and this LRH-1 DNA complex was supershifted with addition of an LRH-1 antibody (Figure 3A). Furthermore displacement of binding of
Transcriptional activation of ERE-containing luciferase reporters by LRH-1 reflects a synergistic action with ERα.

In order to determine if LRH-1 could increase transcription of ERE-containing reporter constructs, expression plasmids encoding either ERα or LRH-1 were transfected with a luciferase reporter driven by two copies of either a consensus palindromic ERE (2×ERE), or the GREB1 ERE2 (GREB-ERE2). These reporter constructs were chosen for their sequence variability. The consensus ERE lacks the 5’ nucleotides known to support LRH-1 binding; while the GREB-ERE2 sequence contains the LRHRE motif (Figure 2A). Hence the reporter with the consensus ERE would not be expected to respond to LRH-1.

Transfection with ERα increased activities of both reporters, and as expected, this induction was significantly enhanced by 10 nM estradiol treatment (Figures 4A and 4B). Transfection of
LRH-1 alone (with or without estradiol treatment) did not show a difference in 2×ERE reporter activity (Figure 4A). However, LRH-1 alone caused a slight increase on GREB-EER2 promoter activation but this was not altered by estradiol treatment (Figure 4B). The difference in sequence of the ERE palindrome between the two reporter constructs (with the GREB-EER2 having a conserved LRHRE) could explain the small increment of LRH-1 induced reporter activity and sequence specific affinity of LRH-1 binding to EREs.

Co-transfection of ERα and LRH-1 caused a 2-fold increase in activity of both reporter constructs (Figures 4A and B). Interestingly with estradiol treatment there was a significant increase in reporter activity when compared promoter activity with ERα alone and estradiol treatment (Figures 4A and B). These results suggest that LRH-1 may enhance ligand-dependent activity of ERα on transcriptional regulation of a subset of target genes.

Effects of LRH-1 on GREB1 expression and estradiol-dependent cell proliferation

We next determined the effects of LRH-1 on estrogen-dependent cancer cell proliferation (Figure 5), and correlated cell proliferation to changes in transcript levels of LRH-1, ERα and GREB1 (Figure 6). LRH-1 over-expression in estrogen-depleted cells resulted in a significant 2-fold increase in cell proliferation (Figure 5). Estradiol treatment caused a 12-fold increase, while the additive effect of LRH-1 and estradiol treatment caused a 37-fold increase. In these cells, the combined increase LRH-1 and GREB1 expression positively correlates to proliferation.

 Estradiol treatment caused a 6-fold increase in LRH-1 expression consistent with previous studies [13] (Figure 6A). In the stably transfected, serum-depleted cells, a 4-fold LRH-1 over-expression compared to basal levels was observed. Interestingly, the addition of 17β-estradiol in LRH-1 over-expressing MCF-7 cells caused a 24-fold increase in LRH-1 compared to basal expression (Figure 6A). Similar expression patterns were observed for GREB1 under these treatment conditions (Figure 5C). Estradiol treatment induced a 4-fold increase in GREB1 expression, while a 32-fold increase was observed in estradiol treated, LRH-1 over-expressing cells (Figure 5C). These results show a clear positive correlation of LRH-1 and GREB1 transcript expression. ERα transcript levels remained relatively unchanged in response to the above treatment conditions. A two fold increase in ERα transcript was observed in response to estradiol treatment (Figure 3B). Over-expression of LRH-1 and estradiol treatment did not demonstrate a cumulative increment in ERα mRNA, as was observed for LRH-1 and GREB1 (Figure 3B). This lack of change in ERα expression is in concordance with previous reports [29]. Ligand activation of ERα and the increase in LRH-1 expression reflects the synergistic effects of LRH-1 and ERα observed on reporter transactivation assays (Figures 4A and 4B). These observations indicate the growth promoting role of LRH-1 and GREB1 in an ERα dependent manner.

To examine the effect of LRH-1 on cell proliferation independent of estrogen signalling we also treated cells with a combination of estradiol and an ERα antagonist, ICI 182,780 (Figure 5). The presence of ICI 182,780 reduced estradiol-induced
Appendix B2: LRH-1 and ERα activate GREB1 expression

Figure 3. LRH-1 binds to specific ERE sequences of the GREB1 and pS2 promoters. (a) EMSA showing binding of LRH-1 to the EREs present in the GREB1 promoter. Radiolabeled ERE1-GREB1, ERE2-GREB1 and ERE3-GREB1 probes were incubated with in vitro translated LRH-1 protein. In vitro translation of the empty vector was used as a negative control. Anti-LRH-1 antibody was added in addition to the probe and the LRH-1 protein to indicate specificity of protein binding. (b) EMSA showing binding of LRH-1 to the EREs present in the GREB1 and pS2 promoters. Radiolabeled LRHRE probe containing the LRH-1 response element derived from the aromatase promoter, whole cell nuclear extracts infected with a LRH-1 viral construct were incubated with various oligonucleotides (as listed in the figure) including unlabeled LRHRE, mutated LRHRE, ERE1-GREB1, ERE2-GREB1, ERE3-GREB1 and pS2 which were added in 200 fold excess. Anti-LRH-1 antibody and IgG were also added in addition to the probe and the nuclear extract to indicate specificity of protein binding.

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proliferation significantly 2-fold. In LRH-1 over-expressing cells treated with estradiol and ICI 182,780, a 2 fold decrease was also observed. However estradiol-mediated proliferation was significantly higher in LRH-1 over-expressing, ICI 182,780 treated cells compared to basal MCF7 cells (Figure 5). This implicates a role for LRH-1 in mediating a positive effect on tumour cell proliferation treated with ERα antagonists.

LRH-1 regulation of GREB1 expression occurs independently of ERα expression

In the ER-negative breast cancer cell line MDA-MB-231 cells, LRH-1 over-expression caused a significant, 26-fold increase in GREB1 expression (Figure 7C). Co-transfection with LRH-1 and ERα with estradiol treatment demonstrated the synergetic effects on GREB1 expression as observed in MCF7 cells. This data demonstrates that LRH-1 is able to stimulate GREB1 expression independent of ERα signaling.

Discussion

Little is known about the mechanisms of LRH-1 action in breast tumors. LRH-1 is abnormally expressed in 45% of all breast carcinomas and is positively correlated with tumor ER status [17]. Our finding suggests a novel association of LRH-1 and ERα in the regulation of GREB1 transcription and cell proliferation.

The gene encoding GREB1 was first identified as one of the key transcripts up-regulated in MCF-7 cells upon estradiol treatment and thus named after its function [30]. Its expression is positively correlated to ER-positive breast cancer in several clinical studies [26,30, Ras, 2005 #406]. The role of GREB1 in cell proliferation was demonstrated with the suppression of GREB1 with siRNA, causing a significant reduction in cell proliferation [26]. Recently a monoclonal antibody for GREB1 was created, allowing the detection of a 216 kDa protein whose expression positively correlated to ERα expression in breast cancer cell lines and tumor samples, as well as to GREB1 mRNA transcript levels [31]. While expression of GREB1 protein appears predominantly nuclear, some cytoplasmatic staining was also observed. GREB1 protein was expressed in tumor epithelial cells and CAIs [31]. As commercially available antibodies report variations in size of detected band in western blots and variability in cell localisation, the current study focussed on transcript expression analysis.

LRH-1 binds as a monomer to a specific sequence YCAAG-GYCR. Analysis of DNA sequence motifs of ERE and LRH-1 nuclear receptor half site (LRHRH) indicated a sequence overlap suggesting that LRH-1 could bind to EREs of the GREB1 promoter. Our studies show direct binding of LRH-1 to the EREs of known ERα target genes GREB1 and pS2 (TFF1). We demonstrate specificity of LRH-1 binding to all 3 ERE sequence motifs with EMSAs. The recruitment of ERα and LRH-1 on distal and proximal GREB1 promoter EREs by ChIP was also demonstrated. By SeqChip experiments LRH-1 and ERα co-occupancy under basal conditions, to ERE1, the ERE most proximal to the start site was evident. ERα is thought to function as an underlying core transcriptional scaffold for interaction with other transcription factors such as the forkhead protein, FoxA1

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Appendix B2: LRH-1 and ERα activate GREB1 expression

LRH-1 Regulates GREB1 Expression in Breast Cancer

Figure 4. LRH-1 acts synergistically with ERα to activate ERE containing promoters. Transcriptional activation of (a) 2×ERE and (b) GREB-ERE2 luciferase reporters by ERα and LRH-1 with vehicle (veh) or 10 nM 17β-estradiol (E2). Estrogen-deprived MCF-7 cells were overexpressed with LRH-1 or ERα alone, or in combination with the appropriate reporter construct. Cells were treated with 17β-estradiol for 16 h prior to luciferase assays. Data is presented as mean±SE, n=3 separate experiments, treatments in triplicate per experiment. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle control unless indicated by reference line.

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Figure 5. LRH-1 induces cell proliferation in 17β-estradiol andICI 182,780 treated cells. Cell proliferation was measured in pcDNA alone transfected, estrogen-deprived MCF-7 cells (control) or LRH-1 over-expressing (tLRH-1) MCF-7 cells treated with vehicle, 10 nM 17β-estradiol (E2) or 10 nM 17β-estradiol and 1 nM ICI 182,780, an ERα antagonist for 5 days. Data is presented as mean±SEM, n=3 separate experiments, triplicate treatments per experiment. ***P<0.001 compared to control transfected cells, a,b P<0.001 compared to vehicle control.

doi:10.1371/journal.pone.0031593.g005

[32]. Furthermore its actions are triggered by various cellular stimuli including growth factors such as Epidermal Growth Factor [32,33]. This scaffolding function is observed in the regulation of GREB1 (located on chromosome 2) and pS2 (TFF1, located on chromosome 21), where estrogen stimulated ERα DNA binding resulted in the interaction or looping between the 2 chromosomal regions and significant enhancement of gene expression [34]. Our findings raise the possibility that LRH-1 may contribute to the variable actions of ERα mediated transcription in breast cancer cells.

In reporter transactivation assays, LRH-1 over-expression caused a modest increase in GREB-ERE reporter activity and no change on 2×ERE activity. This discrepancy in response reflects the importance of flanking sequences required for selectivity of LRH-1 binding to EREs. The two distal EREs located ~20 kb upstream of the GREB1 transcriptional start site perform vital enhancer functions; ERα binding to these EREs allows chromatin looping and interaction with the proximal ERE to initiate GREB1 transcription [28]. Therefore the use of two copies of a single GREB1 ERE may not be sufficient to demonstrate the impact of LRH-1 and ERα on GREB1 transcription. However, the finding of most interest in the current study is the co-operative effect of ERα and LRH-1 expression in estradiol-treated conditions. This additive effect is observed consistently in mRNA expression analysis, promoter assays and ChIP experiments suggesting that LRH-1 may be associating with the ligand-activated ERα within a transcriptional complex at the EREs of GREB1 promoter.
Appendix B2: LRH-1 and ERα activate GREB1 expression

LRH-1 is known to induce cancer cell proliferation [35, 16, 25, 35]. In breast cancer cells, LRH-1 enhances cell proliferation as a downstream effector of estradiol treatment [15]. In the present study, basal LRH-1 over-expressing MCF-7 cells when treated with estradiol demonstrated a dramatic increase in GREB1 expression, and this correlated with increases in cell proliferation.

The pattern of LRH-1 mRNA mirrored that of GREB1 while ERα mRNA levels were relatively unchanged under these treatment conditions. The expression data indicates that the activation of ERα, combined with the increase in the constitutively active LRH-1 expression, correlates to elevated GREB1 levels. These transcriptional changes are reflected in increased cell proliferation.

Figure 6. Synergistic effects of LRH-1 and 17β-estradiol treatment on GREB1 expression. Quantification of (a) LRH-1, (b) GREB1 and (c) ESR1 mRNA expression in estrogen-deprived MCF-7 cells (control) or LRH-1 over-expressing (+LRH-1) MCF-7 cells treated with vehicle (veh) or 10 nM 17β-estradiol (E2) for 16 h. Data is presented as mean±SE, n=3 separate experiments, triplicate treatments per experiment, **P<0.01, ***P<0.001 compared to vehicle control.

doi:10.1371/journal.pone.0031593.g006

Figure 7. LRH-1 regulation of GREB1 expression in ER negative breast cancer cells. MDA-MB-231 cells were transfected with empty vector (C) or expression vectors for LRH-1 alone (L), ERα alone (E) or both LRH-1 and ERα (L+E). Cells were treated with vehicle or 10 nM 17β-estradiol (E2) for 16 h. Quantification of (a) LRH-1, (b) ERα and (c) GREB1 mRNA expression. Data is presented as mean±SE, n=3 separate experiments, ***P<0.001 compared to vehicle control.

doi:10.1371/journal.pone.0031593.g007
Appendix B2: LRH-1 and ERα activate GREB1 expression

proliferation in LRH-1 over-expressing, estradiol-treated MCF-7 cells. In ER-positive breast cancers, GREB1 expression is correlated with high circulating estradiol levels [30]. In addition, LRH-1 expression is regulated by estradiol, reflected by the positive correlation of its expression in ER-positive tumors [15,17]. In MCF-7 cells co-treated with the ERα inhibitor ICI 182,780, the presence of LRH-1 maintained increased proliferation indicating an ERα independent effect on cell proliferation previously not reported. This implies that the presence of LRH-1 in tumours treated with selective ER modulators may account for estrogen independence proliferation. Hence the regulation of GREB1 expression by LRH-1 identifies a novel mechanism for tumor cell proliferation.

Sun et al. (2007) showed that the three ERα isoforms of the GREB1 locus exhibit different degrees of ERα, coactivator, and polymerase II binding and suggested that different transcriptional regulators may be involved in modulating the ERα driven transcription from the 3 different GREB1 core promoters. Thus LRH-1 may be acting as a co-repressor for some of these GREB1 core promoters, and it may possess different binding affinities to the ERs. We tested this hypothesis and demonstrated that LRH-1 was able to regulate ERα target genes such as GREB1 in ER-negative cancer cells such as MDA-MB-231 cells. LRH-1 also stimulated GREB1 expression significantly; however, this effect was lower than that induced by ERα. The synergistic actions of LRH-1 and ERα appeared to have the most potent effect on GREB1 expression and proliferation. It is a distinct possibility however that other ERα target genes may be transcriptionally regulated by LRH-1 in ER-negative tumor cells.

Another effect LRH-1 may confer is to maintain the expression of GREB1 target genes for longer durations post estradiol treatment. GREB1 expression rapidly induced within 2 h of estradiol treatment and maintained over a 48 h period [26,30]. Could LRH-1 be a mechanism for the maintenance of ERα target gene expression? Assessing transcript levels of GREB1 in LRH-1 over-expressing and basal MCF-7 cells as varying time points post estradiol treatment would answer this question.

The synergistic increase in cell proliferation in LRH-1 over-expressing, estradiol-treated cells could also reflect increased LRH-1 activity in addition to an increase in expression. While LRH-1 is constitutively active, it requires coactivators such as SRC1 and SRC3 to further activate its functions [37,38]. As estrogen is needed to increase levels of SRC1 and SRC3 and has been shown to associate with the three GREB1 ERα [27], this is likely to cause activation of both ERα and LRH-1 at the transcriptional complexes as the proximal and distal promoters.

In summary, our findings provide a molecular mechanism for LRH-1 induced cell proliferation in ER-positive breast cancer cells via the up-regulation of GREB1 expression. LRH-1 was able to induce GREB1 expression independent of ERα expression suggesting an estrogen independent effect on proliferation. This is the first evidence of LRH-1 binding to a subset of ERE palindrome motifs, as observed in GREB1 and pS2 gene promoters. In addition LRH-1 can colocalise with ERα (basally and in response to estradiol treatment) on proximal and distal promoter regions critical for the activation of GREB1 expression. As these proximal and distal regions connect via chromatin looping and may suggest a possible role for LRH-1 in aiding this transcriptional process. Whether LRH-1 partners with ERα as a heterodimer and whether LRH-1 can regulate ERα target genes in ER-negative tumor cells remains to be investigated.

Approximately 60% of premenopausal and 75% of postmenopausal breast cancer patients have ER-positive tumors. LRH-1 expression is positively correlated with tumor ERα status and here we demonstrate a cooperative effect of LRH-1 and ERα on GREB1 expression and cell proliferation. As some of the most successful therapies for breast cancer target the inhibition of ERα actions or of aromatase activity, the blockade of LRH-1 action in ER- positive tumors may provide further efficacy to current treatment regimes.

Materials and Methods

Plasmids

The human LRH-1 expression vector was generated as described previously [25]. The LRH-1 shRNA vector was constructed by cloning a double-stranded oligonucleotide targeting the appropriate sequence (GGATCCATGTTCTCTGGTTA corresponding to nucleotides 1925-1947 of Genbank NM_205980) into pGeneclip lMGFP (Promega). The use of these constructs has been previously verified [25].

Cell culture and transfection

All cells used were obtained from ATCC and grown in the recommended culture media and conditions. MCF-7 cells were cultured in DMEM (Gibco) supplemented with 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C in 5% CO2 (all reagents obtained from Invitrogen). For LRH-1 knockdown, cells were transfected with either pGeneclip lMGFP-LRH-1 or pGeneclip lMGFP encoding a scrambled shRNA sequence as negative control (SA Biosciences). For overexpression, cells were co-transfected with either pcdNA3.1+ or pcdNA3.1+LRH-1, and lGFP. Cells were transfected using the Nucleofector Kit V (Amaxa Biosystems), according to the manufacturer’s instructions. GFP-positive cells were collected by fluorescence-activated cell sorting (FACS); 48 hours after transfection and replated or protein and RNA extracted as required. MDA-MB-231 cells were transfected with pCDNA containing constructs for LRH-1 and ERα using the Nucleofector Kit V as described previously (Chaud et al., 2010).

17β-estradiol (Sigma) treatments were performed in MCF-7 cells on plates that stably expressed the pERE-LRH-1 construct. This allowed inducible treatment of LRH-1 with treatment with 1 µg/ml doxycyclin (Sigma). These cells were deprived of estradiol in phenol red free culture media supplemented with 5% charcoal stripped calf serum for 72 h prior to treatments with vehicle (ethanol) or 10 nM 17β-estradiol (Sigma) for 16 h. Doxycyclin treatment was performed for the same duration as vehicle or estradiol treatments to induce LRH-1 expression. Cells were then used for cell proliferation assays or RNA extracted for further studies.

RNA extraction and quantitative real time PCR (qPCR)

Total RNA was extracted from cultured cells using the RNeasy kit (Qiagen), treated with DNaseI (Ambion), and quantified using a NanoDrop 1000 (Thermo Scientific). First strand cDNA synthesis was accomplished using AMV reverse transcriptase (Promega) and random hexamers. For qPCR, cDNAs were diluted 1:10 in water and amplified using SYBR Green chemistry on the LightCycler system (Roche), as previously described (Clyne et al. 2002). Primer sequences are outlined in Figure S1. Fold changes in expression of each gene were calculated using the delta delta C method [39] using 18S as the internal control.

Western blot analysis

Protein extraction and western immunoblots for LRH-1 and β- tubulin were performed as described previously [25]. Protein
Appendix B2: LRH-1 and ERα activate GREB1 expression

Luciferase reporter assays

HEK293 cells were plated into 48-well plates and transfected with 10 ng pLRH-1 pcDNA and or pERα pcDNA construct with 200 ng 2xERE-luc (donated by Dr S Chua) or GREB1ERE2-constructs. The 2xERE-luc reporter contains the palindromic ERE sequence: AGGGTACAGTGACTGAGGTCA, TGACCCCTTACGTACGACTGTGAG

To construct the GREB-ERE reporter construct, complementary oligonucleotides encoding the GREB ER2 (5'-3': sense: TTCTAAAAGGTCTCATGACCTTATTGG, and antisense: ACAATAGGTCAATGTGACCTTTTGAAGA) were annealed and ligated to form concatamers using T4 kinase and T4 ligase. DNA corresponding to 2 copies of the ERE sequence were purified, ligated into pGEM-T Easy, sequenced to confirm correct orientation, and then subcloned into the luciferase reporter vector pGL2Basic. For transfections, DNA amounts were equalized by the addition of the empty vector (pcDNA3.1) construct and a 1:3 DNA: Lipofectamine ratio used for the transfection as specified by manufacturers (Invitrogen). Cells were maintained in media supplemented with 5% charcoal stripped serum overnight after which cells were treated overnight with 10 nM 17 βestradiol (Sigma).

Cell proliferation assay

Transfected and treated cells, as detailed previously, were washed with PBS, subjected to trypsinization. Cells were suspended in media supplemented with 10% FCS and Trypan blue stain was added to the cell suspension. Viable cell number was quantitated using the Countess Automated Cell Counter according to the manufacturer’s instructions (Invitrogen).

Statistical analysis

All data are reported as mean ± SE for three or more experiments. Statistical analyses for experiments comparing two groups were performed by two-tailed Student’s independent t test using GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) and a P value of <0.05 was considered statistically significant.

Supporting Information

Figure S1 Sequences for qPCR Primers and EMSA probes.

[DOC]

Author Contributions

Conceived and designed the experiments; CDC. Performed the experiments: ALC DDW TLH KAH KJK KAL. Analyzed the data: ALC CDC. Wrote the paper: ALC KJK CDC.

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Appendix B2: LRH-1 and ERα activate GREB1 expression

LRH-1 Regulates GREB1 Expression in Breast Cancer


Appendix B3: Therapeutical potential of LRH-1 modulators

B3: Therapeutical potential of LRH-1 modulators

Appendix B3 entitled “Therapeutical potential of Liver Receptor Homolog-1 modulators”

In the case of Appendix B3, contributions to the work involved the following

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<td>2. Dhilushi Wijayakumara</td>
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<td>3. Ashwini L Chand</td>
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<td>4. Evan R. Simpson</td>
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<td>5. Colin D. Clyne</td>
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Declaration by co-authors

The undersigned hereby certify that:

(1) They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) There are no other authors of the publication according to these criteria
(4) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or published of journals of other publications, and (c) the head of the responsible academic unit; and
(5) The original data are stored at the following location and will be held for at least five years from the date at the location:

Prince Henry’s Institute of Medical Research, Clayton VIC, Australia.

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Appendix B3: Therapeutical potential of LRH-1 modulators

Review

Therapeutic potential of Liver Receptor Homolog-1 modulators

Kyren A. Lazarus, Dhillushi Wijayakumara, Ashwini L. Chand, Evan R. Simpson, Colin D. Clyne

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ABSTRACT

Liver Receptor Homolog-1 (LRH-1; NURSAR) belongs to the orphan nuclear receptor superfamily, and plays vital roles in early development, cholesterol homeostasis, steroidalogenesis and certain diseases, including cancer. It is expressed in embryonic stem cells, adult liver, intestine, pancreas and ovary. It binds to DNA as a monomer and is regulated by various ligand-dependent and -independent mechanisms. Recent work identified synthetic ligands for LRH-1; such compounds may yield useful therapeutics for a range of pathologic conditions associated with aberrant expression and activity of LRH-1.

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1. Introduction

Nuclear receptors (NRs) comprise a large superfamily of mammalian transcription factors that are essential in embryonic development, differentiation, metabolism and cell death [1–3].

NRs mediate cellular signals to the nucleus by directly interacting with DNA sequences known as hormone response elements (HREs) [3–4]. Unlike most NRs, orphan NRs regulate transcription independent of known ligands. Orphan receptors play a diverse and important biological role in development and adult physiology [1,5–7].

Liver Receptor Homolog-1 (LRH-1; NURSAR; FEZ1; FFT; CPE) is one such orphan nuclear receptor that plays vital roles in early development and is important for bile acid synthesis, cholesterol metabolism and steroidalogenesis in the adult [8–14] (see Fig. 1).
LRH-1 and its mammalian homolog Steroidogenic Factor-1 (SF-1) binds to DNA as monomers to nuclear receptor half site sequences [1,15,16]. As LRH-1 is constitutively active [8], its regulation of function occurs via interactions of co-activators and co-repressors [17–19]. Furthermore, interactions with other orphan receptors including small heterodimer partner (SHP) [9,20–24] and dosage sensitive sex reversal-adrenal hypoplasia congenital gene on the X chromosome, gene 1 (DAX-1) cause the repression of LRH-1 transcriptional activity [25,26]. Ligands identified for LRH-1 and its homologue, SF-1 include small drug-like molecules [8] and endogenous phospholipids [27–30]. With its wide implication for disease progression in various organs, this review will focus on the therapeutical potential of LRH-1 modulators.

2. LRH-1: Structure

The human gene encoding LRH-1 spans more than 150kb of chromosome 1q32.11 and has eight exons. LRH-1 binding is dictated by the Fos-F1 box and it binds as a monomer to the extended half site YCAAGG YCR (where Y is any pyrimidine and R is any purine), the recognition motif for the NRSA subfamily of nuclear receptors [8,16,31,32]. The ligand binding domain (LBD) of most NRs consists of twelve α-helical regions, folded into a three-layered, anti-parallel helical sandwich with a conserved β-turn between H5 and H6 [8]. The LBD also contains a pocket to which a ligand binds causing a reversible conformational change. This repositions H12 allowing for co-activator recruitment [8,32]. However, in orphan receptors, the key to constitutive activation may lie in the N-terminal region of the LBD. This is strongly conserved amongst orphan receptors and shares limited sequence amongst other nuclear receptors [8]. The presence of an additional structure pertaining to an extension of an H2 layer may provide an explanation of the stabilised conformation, by stabilising H12 which contains the C-terminal activation helix (AF-2) [8]. The folding over of the AF-2 region allows the LBD to be held in an active conformation without the need for a ligand.

3. Established roles of LRH-1: cholesterol metabolism

A key biological function of LRH-1 is the regulation of cholesterol metabolism via its effect on bile acid homeostasis. LRH-1 regulates enterohepatic development and function via the expression of key genes involved in the regulation of bile acid synthesis, cholesterol homeostasis and transport (Table 1) [8,10,15,20,33–38]. LRH-1 activates gene transcription of the rate-limiting enzyme in bile acid biosynthesis, cytochrome P450 family 7A1 or Cholesterol 7α-hydroxylase (Cyp7A1) [20,33,39,40]. In humans, the loss of function of Cyp7A1 results in a decrease in bile acid excretion and an increase in hepatic and serum cholesterol levels [41]. However, LRH-1 Δ heterozygous mice have elevated Cyp7A1 and Cyp8B1 mRNA levels [42]. This may be due to a dominant effect of LRH-1 on Cyp7A1 and Cyp8B1 transcription. Interestingly, hepatocytes and intestinal epithelium (IE) specific LRH-1 knockout mice showed no overt abnormalities [43]. Furthermore, LRH-1 deficiency in the IE, had no effect on Cyp7A1 levels. This discrepancy in observations may be due to the minor role LRH-1 plays in Cyp7A1 regulation, or there might be an alternative redundant factor regulating Cyp7A1 in the absence of LRH-1 [43]. Further work need to be undertaken in determining the role of LRH-1 in the feedback mechanism of bile acid synthesis. In contrast to Cyp7A1, Cyp8B1 levels were significantly decreased with LRH-1 deficiency, consistent with previous findings.

LRH-1 positively regulates expression of other enzymes and transporters involved in reverse transport of cholesterol and bile acid synthesis pathways. These genes which contain the LRH-1 response element (nuclear receptor half site) in their promoters...
include cytochrome P450 family 8B1 (Cyp8B1) or Sterol 12α hydroxylase, multidrug resistance protein 3 (MRP3), cholesteryl ester transfer protein (CETP), scavenger receptor class B type 1 (SR-BI), mouse apical sodium-dependent bile acid transporter (ASBT) and human Apolipoprotein A1 (ApoA1) [8,20,33,34,37,44,38,45]. These findings are consistent with the selective knockout of LRH-1 in hepatocytes where expression of these targets were significantly ablated [43]. By activating gene expression, LRH-1 impacts various functions outlined. Increase in ApoA1 allows the initiation of high density lipoprotein (HDL) biosynthesis and for ApoA1 to act as an acceptor of cholesterol and phospholipids effused from peripheral tissues. Up regulation of SR-BI receptors allows the transfer of mature HDL particles from plasma into hepatocytes [46]. CETP transfers the cholesteryl esters from plasma HDL into Apolipoprotein B-containing triglyceride-rich very low density lipoproteins (VLDL). These VLDL particles are acted upon by lipoprotein lipase and the fatty acids are taken up by the adipose and skeletal muscle. The cholesterol-rich remnants are then taken up by the liver [37,47].

Within hepatocytes, cholesterol and cholesteryl esters are converted to bile acids by Cyp7A1 and Cyp8B1 for secretion out of the liver in bile. Furthermore MRP9 and ASBT are involved in bile acid recycling, indicating that LRH-1 is important for bile acid homeostasis [44,48]. Major subsets of LRH-1 gene targets are involved in the transfer of cholesterol to the liver and subsequent elimination into bile acids, and in bile acid synthesis, highlighting the importance of LRH-1 in cholesterol metabolism [41].

4. Emerging roles of LRH-1 in disease

4.1. Embryonic stem cells: pluripotency and differentiation

LRH-1 is a critical factor in early embryonic development, and LRH-1 null mice die at embryonic day 6.5–7.5 with features typical of visceral endoderm dysfunction [42]. One of the key factors essential for maintaining pluripotency in embryonic stem cells (ESCs), Oct4 is regulated by LRH-1 [49,50]. Its role in Oct4 regulation is confirmed with the loss of Oct4 expression in the absence of LRH-1 [49]. In addition to Oct4, LRH-1 is known to regulate other factors required to maintain pluripotency [51,52].

A recent report suggests that LRH-1 regulates Oct4 and Nanog induction and its expression is regulated via the Wnt signalling pathway, via binding of β-catenin to activate embryonic-specific LRH-1 promoter [50] thus identifying a new pathway for the regulation of self renewal in ESC.

4.2. Ovarian function: steroidogenesis and lutetisation

LRH-1 is expressed at relatively high levels in the ovary, with highest expression in granulosa cells and corpora lutea, with no expression observed in theca cells [60]. Although SF-1 is also present in the ovary its expression is lower in LRH-1 expressing cells and highest in the theca [60]. The two nuclear receptors are differentially regulated, SF-1 mRNA and protein increased in granulosa cells by estradiol, whereas LRH-1 expression was increased by FSH in granulosa cells and by prolactin in luteal cells [63]. Both receptors have been shown to regulate ovarian steroid synthesis although the relative roles of each are still unclear, since they both share common target genes including aromatase, steroidogenic acute regulatory protein (STAR), 3β-hydroxysteroid dehydrogenase (HSD3B2) and inhibin α subunit [61-65]. Recent work has

Table 1

LRH-1 target genes and functions.

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suggested that LRH-1 preferentially regulates progesterone over oestrogen production [12,66,67]. Consistent with this, granulosa cell-specific LRH-1 null mice exhibit anovulation due to failure of cumulus expansion, luteinisation, and follicular rupture [68]. Loss of LRH-1 in granulosa cells also impaired progesterone synthesis due to reduced expression of 3βHSD and cytochrome P450 side-chain cleavage (P450scC). Interestingly, aromatase expression was unaltered. This dramatic phenotype highlights the potential of LRH-1 antagonists as novel contraceptives, since loss of LRH-1 inhibits both ovulation and luteinisation [68].

4.3. Colon cancer and inflammation

Recent evidence indicates a role of LRH-1 in colon cancer development and progression. LRH-1 is highly expressed in intestinal crypts where it is involved in the control of cell proliferation and renewal [69]. Botruge et al. have demonstrated that LRH-1 promotes intestinal cell proliferation by stimulating the expression of the G1 cyclins D1 and E1 [69]. In these cells, LRH-1 co-activates β-catenin/Tcf4 to induce cyclin D1 and c-Myc expression, and also binds directly to the cyclin E1 promoter to promote transcription in synergy with β-catenin [69]. Consistent with this, haplo-insufficiency of LRH-1 markedly protects against tumour development in both genetic (Apc<sup>Min−/−</sup>) and chemical (azoxymethane) induced models of intestinal cancer [70]. Altered in LRH-1 expression and localisation are also seen in human colon cancer, with increased LRH-1 expression observed in surface epithelial cells that are normally LRH-1 negative and non-proliferative [70]. However, the lack of significant proliferative phenotype in intestinal specific LRH-1 knock-out mice [41] raises questions as to the role of endogenous LRH-1 in vivo.

LRH-1 also plays an important role in glucocorticoid synthesis within the intestine by inducing expression of steroidogenic enzymes (cholesterol side chain cleavage enzyme and 11β-hydroxylase that catalyse the first and last steps in glucocorticoid formation, respectively) in intestinal epithelial cell lines [71]. This is likely of physiological relevance since haplo-insufficiency of LRH-1 in mice reduced expression of these enzymes and glucocorticoid synthesis in response to immunological stress [71]. Similar effects were seen in mice with an inducible intestinal epithelial deletion of LRH-1 [72]. Consistent with these findings, expression of both LRH-1 and its steroidogenic target genes were reduced in patients with Crohn’s disease and ulcerative colitis [72].

4.4. Breast cancer

In human breast adipose, LRH-1 is expressed in low levels, localising specifically in the stromal fraction [13]. However in breast cancer, LRH-1 expression is high and localised both in tumour epithelial cells and intra-tumoural stroma [73,74]. High expression is observed in primary invasive breast carcinoma and ductal carcinoma in situ [74]. Although the effects of LRH-1 in breast cancer are not fully understood, evidence suggests that its roles are tightly integrated with the oestrogen signalling pathway (Fig. 2).

One of the first identified target genes of LRH-1 in breast adipose stromal cells is CYP19A1 [13,14,75], encoding for cytochrome P450 family 19A1 or aromatase. Aromatase is a critical enzyme required for the conversion of androgens to oestrogens. After menopause,

![Fig. 2. LRH-1 acts in a breast cancer cell. LRH-1 activates aromatase transcription in cancer associated adipose fibroblasts leading to increased oestrogen synthesis, thus promoting tumour growth. The oestrogen receptor (ERs) activates LRH-1 expression in malignant epithelial cells. LRH-1 in turn further up regulates ERs and aromatase expression. Cyclin D1 a known target gene is also up regulated by LRH-1. LRH-1 is positively regulated by co-activator PGC-1α, leading to the activation of target genes such as ERs and aromatase. Therefore through its involvement in ERs expression and co-regulators and repressors also act on the ER, suggesting that the LRH-1 signalling pathway is tightly linked with the oestrogen signalling pathway.](image-url)
when the risk of developing oestrogen-dependent breast cancer is greatest, local aromatase expression in adipose tissue represents the major source of oestrogen in women (as well as in men). Aromatase activity is regulated primarily by transcription of the CYP19A1 gene via tissue specific promoters [76-78]. By activating CYP19A1 transcription, LRH-1 most likely regulates the availability of mitogenic oestrogen for tumour growth [14]. In the tumour context, the aberrant expression of LRH-1 allows the activation of the gonadal-specific aromatase promoter (promoter II). LRH-1 expression in adipose stromal cells is positively regulated by breast tumour derived factors such as prostaglandin E2 [75]. In addition to stromal cells, aromatase is present in breast cancer epithelium [79], and its expression also regulated by LRH-1 in breast cancer epithelial cells [80].

A second level of cross-talk is evident in expression of LRH-1 itself is regulated by oestrogen in breast cancer cells. Annicotte et al. reported that the human LRH-1 promoter contains a near-perfect palindromic oestrogen response element (ERE) [74], to which ERα binds to stimulate promoter activity. This is consistent with the observed correlation between LRH-1 mRNA levels and ERα status in a variety of breast cancer cell lines [74], as well as the known positive association between LRH-1 positivity and ERα status in primary human breast carcinoma [11,81]. Knockdown of LRH-1 expression with siRNA in MCF-7 cells inhibits the proliferative effect of oestrogen [74], suggesting that the mitogenic effects of oestrogen may be mediated, in part, via LRH-1.

Furthermore LRH-1 regulates expression of ERα in breast cancer cells; siRNA knockdown of LRH-1 in MCF-7 cells inhibits expression of both ERα, and of ERα target genes such as pS2, whereas transfection of LRH-1 into these cells stimulates ERα expression [81]. This effect is mediated by LRH-1 binding directly to the major ERα promoter used in breast cancer cells [81]. Therefore, LRH-1 both regulates, and is regulated by ERα in addition to regulating the synthesis of its ligand.

Although the above evidence indicates that LRH-1 induces proliferation by stimulating oestrogen signalling, LRH-1 also has oestrogen independent effects on breast cancer cells. siRNA-mediated knockdown of LRH-1 inhibited breast cancer cell motility, invasion and colony formation in both ER+ MCF-7 and ER-ve MDA-MB-231, as well as the non-tumorigenic MCF-10A mammary epithelial cell line [82]. Over-expression of LRH-1 (in the absence of oestrogen) produced the opposite effects.

In addition, over-expression of LRH-1 resulted in the post-translation cleavage of mature 120kDa E-Cadherin to its inactive 9kDa form. This effect may be mediated by matrix metalloproteinases (MMPs) since LRH-1 was also shown to induce MMP9 mRNA expression in MCF-7 cells [82], consistent with the known role of LRH-1 as a regulator of MMP9 expression in the ovary [86]. LRH-1 was also shown to inhibit forest inducers and MMP9, which, with its effects on E-Cadherin and MMP9, may suggest a role for LRH-1 in promoting epithelial to mesenchymal transition (EMT). Given that LRH-1 has well-characterised roles in mouse and human embryonic stem cells [80,83-85], and that breast cancer stem cells possess many of the characteristics of cells undergoing EMT [86,87], the potential role of LRH-1 in promoting EMT is an intriguing possibility that merits further investigation.

4.5. Pancreatic cancer

Recent genome-wide association studies have linked mutations in the LRH-1 gene and its up-stream promoter regions with pancreatic cancer [88]. In addition, LRH-1 expression is found to be elevated in human pancreatic ductal adenocarcinomas [89]. In pancreatic cancer, LRH-1 also mediates tumour cell proliferation via the up-regulation of cyclins D1, E1 and c-Myc. Conversely knockdown of LRH-1 expression resulted in cell cycle arrest but not apoptosis in pancreatic cancer cells. This study implicates an important role for LRH-1 in mediating proliferation and differentiation pancreatic ductal adenocarcinomas and therefore a potential therapeutic target for this cancer [89].

4.6. Metabolic disorders

One of the key sites of LRH-1 expression and function is in the hepatocytes where it regulates transcription of genes such as Cyp7A1, Cyp8B1, encoding enzymes critical in bile acid and cholesterol synthesis; scavenger receptor class B member 1 (SR-B1) important for cholesterol transport and SHP, an LRH-1 co-repressor with known roles in obesity and diabetes [20,46,80]. Whilst these roles in the liver are well characterised, until recently the effect of LRH-1 in metabolic disorders was not well understood. A recent study [91] describes two phosphatidylicoline phospholipids, dl-α-tocopheryl phosphatidyicholine (DPLC) and diunsaturated phosphatidyicholine (DUPC) as potent agonists of LRH-1, binding to the ligand binding pocket of the human receptor. This is an extension to previous findings where structural analysis of LRH-1 identified bacterial lipids as ligands for the ligand binding pocket [24,92]. These two lipid ligands, DPLC and DUPC potentially activate NKS1A receptors: LRH-1 (human and mouse homologues) and SR-1 (mouse homologues), however show no effects on other nuclear receptor function. Lee and colleagues hypothesised that the activation of liver-specific activity of LRH-1 would increase bile acid production thereby having a positive metabolic effect in diabetic and obese disease models. In diet induced obese mice, oral administration of DPLC resulted in increased bile acid production, decreased hepatic steatosis and improved glucose utilisation [91].

Additionally in liver specific LRH-1 knockout animals, DPLC treatment did not show any improvement in the high fat diet induced metabolic phenotype. This suggests a direct effect on LRH-1 receptor activity and/or the downstream activation of LRH-1 dependent pathways in regulating insulin sensitivity. Due to the absorption and clearance of these lipid ligands, the primary site of LRH-1 activation appeared to be the liver and SR-1 activity in the adrenal was not affected. This study therefore reveals a novel, LRH-1 dependent phosphatidylicholine pathway that could be targeted for metabolic disorders such as diabetes.

5. LRH-1 modulators as a novel therapeutic target

Since its identification in 1996, there is accumulating evidence of LRH-1 as an important regulator of pathways involved in metabolism, steroidogenesis, cancer and regulation of pluripotency. Given its expression and functions in various tumours including invasive breast carcinomas, ductal carcinomas in situ, colon, gastric and pancreatic cancers, the potential impact of LRH-1 modulators as therapeutic targets should be considered. Recent progress with the identification of small molecule agonists [93,94] and phospholipid ligands will eventually lead to the development of sensitive LRH-1 modulators. There appears to be therapeutic benefits for both the activation of LRH-1 (in the liver) where it would be beneficial in the treatment of metabolic disease such as diabetes; and in the inactivation of LRH-1 in tumour cells. Suppression of LRH-1 activity in tumour cells (gastric, pancreatic, intestinal, breast) would potentially have an anti-proliferative effect [70,82,89,95]. As LRH-1 is expressed in inflammatory cells such as lymphocytes [89] and in surrounding breast adipose stromal cells, blocking of LRH-1 function would have additional anti-tumour effects such as down-regulating pro-inflammatory pathways and reducing oestrogen synthesis within the tumour milieu.

Given the importance of LRH-1 in metabolism and cancer, the
Appendix B3: Therapeutical potential of LRH-1 modulators

Fig. 3. LRH-1 activity can be regulated by various mechanisms in a tissue specific manner. Regulation mechanisms include synthetic ligands, post-translational modification, phospholipids. Co-regulators and co-repressors also play a role in regulating LRH-1, which then modulates expression of target genes that are involved in metabolism, proliferation, pluripotency and steroidogenesis.

5.3. Regulation of LRH-1 activity

5.3.1. Phospholipids as LRH-1 activators

Crystallisation of the LRH-1 ligand binding domain (LBD) (mouse and human isoforms) identified bacterial phospholipids in the ligand binding pocket. Subsequently phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol were shown to bind with human LRH-1 and SF-1 from both mouse and human [24,27,56,92,96] identifying them as endogenous ligands. Functional studies of mouse LRH-1 LBD show that ligands are dispensable for activity [56]. Disruption of the size and shape of the hydrophobic ligand binding pocket does not appear to affect its transcriptional activity. However mutations in the human LRH-1 LBD reduce phospholipid binding, and prevent recruitment of co-activators causing an inhibition of transcriptional activity of LRH-1 [24,96]. In support of this idea is the recent identification of DPLC and DUPC which act as strong LRH-1 activators in vitro and in vivo models [91].

5.3.2. Co-activators of LRH-1

Structurally LRH-1 is permanently held in an active conformation and binds to DNA as a monomer [1,18,15]. LRH-1 activity is primarily regulated by transcriptional co-activators. LRH-1 can also act as a competence factor by binding to other orphan nuclear receptors and transcriptional complexes to enhance transcription of target genes. Peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) is a key co-activator of LRH-1 in the ovary, facilitating LRH-1 mediated differentiation of granulosa cells into progesterone producing luteal cells [12]. This interaction is blocked by a known repressor of LRH-1 activity, DAX-1 in granulosa cells [12]. Since both DAX-1 and PGC-1α can bind to LRH-1 [12], this indicates that the binding affinity of DAX-1 for LRH-1 is stronger than PGC-1α [12]. Furthermore, the interaction between LRH-1 and PGC-1α is also evident in bile acid homestasis [9]. However this interaction in the liver is blocked by SHP-1 [9]. Recently a genomically wide interrogation of hepatic foamsoid X nuclear receptor (FXR), revealed that FXR binds to LRH-1, and LRH-1 is required for the FXR mediated activation of SHP, Rdh9, Pcxf and Perm [97].

In human adipose stromal cells, PGC-1α enhances LRH-1 dependent aromatase promoter II transcription [13]. The p160 family members steroid receptor co-activators (SRC-1 and SRC-2) also regulate LRH-1 activity [98]. These co-activators contain an LXXLL motif in the receptor interaction domain; short peptide sequences derived from these co-activators are shown to bind LRH-1 LBD [19]. Multi-protein bridging factor (MBF-1) which also interacts with LRH-1 does not possess the LXXLL motif, typical for most co-activators, but interacts with the TATA-binding protein (TPB) [99]. This interaction is either in isolation or through the recruitment of transcription factor IID complex [99].

5.3.3. Co-repressors of LRH-1

Nuclear receptors SHP and DAX-1 act as repressors of many NRs including LRH-1 by inhibiting co-activator binding [35]. The repression of LRH-1 by SHP has been well-defined due to its high interaction with the nuclear receptor [20,31,33]. Like most co-repressors, SHP binds to the AF-2 region of LRH-1 [8]. The LRH-1–SHP interaction occurs via interaction with LRH-1 residues Arg361 and Glu534 which form an ATPylyl charge clamp [24]. Upon SHP interaction, p160 co-activators are competed out for binding to the CoT domain of LRH-1. In addition to the N-terminal receptor interaction domain, SHP includes a C-terminal domain with autonomous repression function [100]. In breast adipose stromal cells and in hepatocytes, SHP represses LRH-1 activity with the blocking of PGC-1α and LRH-1 interactions [40]. This action in hepatocytes is shown to be mediated via the recruitment of SIRT-1 by SHP [21]. SHP is a well known target gene of LRH-1 and in recent reports show a cooperative action with FXR to activate SHP transcription [97]. DAX-1 colocalises with LRH-1 and SF-1 in granulosa cells [12] and in mouse embryonic stem cells [59]. Crystallisation of mouse DAX-1 with LRH-1–LBD indicates that its N-terminus LXXLL-related motifs interact directly with LRH-1 [26,101,102]. It has also been reported that the C-terminus end is essential for DAX-1 mediated repression [103]. DAX-1 binds to the AF-2 domain as a dimer, binding with high affinity in contrast to most repressors which interact via their single LXXLL motif [25]. It is hypothesised that the DAX-1 dimer binds LRH-1 in a “claw-like” fashion, one of the DAX-1 structures extending into the ligand binding pocket. This feature may act as a sensor for ligand binding, and or additional interactions with components of the transcriptional machinery [25,26].

LRH-1 function is also inhibited by the protein inhibitor of activated signal transducer and activator of transcription-γ (PASγ) [104]. This inhibition occurs due to the competitive binding of PASγ on the AF2 domain, which is involved in the binding of co-activator SRC-1. However, the over-expression of SRC-1 could
Appendix B3: Therapeutical potential of LRH-1 modulators

partially overcome the LRH-1 mediated induction of CYP11A1 [104]. The silencing mediator for retinoid and thyroid receptors (SMRT) is also involved in regulating LRH-1 through an indirect mechan-
imism currently unknown [8].

The role of transcriptional CoRs is critical in the modulation of transcription factor activity. In the case of orphan nuclear receptors, co-regulators may well be the most important mode of functional regulation. Evidence of co-localisation and interaction between CoRs and LRH-1 suggest tissue and cell specific modulation of LRH-
1 activity. Could targeting the disruption of these CoR interactions provide a means to selectively repressing LRH-1 activity? Due to the presence of SHP and DAX-1 in breast cancer [105,106], it is tempting to postulate their roles in repressing LRH-1-in breast cancer cells. However this possibility requires further investigation.

5.1.4. Post-transcriptional regulation of LRH-1

In addition to CoRs, the activity of LRH-1 is modulated by post-
translational modification. Phosphorylation of the serine residues 236 and 243 located within the hinge region of LRH-1 via phor-
bol 12-myristate 13-acetate (PMA), was found to be important for LRH-1 transcription [107]. Sumoylation of LRH-1 also occurs in the hinge region, allowing for additional control of its activity via regulation of its subcellular localisation [108]. Sumoylated LRH-1 is localised to the transcriptionally inactive nuclear bodies, away from active chromatin. Interestingly, the newly identified phosphatidyli
choline agonists of LRH-1, DPLC and DUCP activated LRH-1 mutants lacking phosphorylation sites (S236A, 243A) on the sumoylation site (K270R) [21]. The double mutants F342 W and I426 W targeting the ligand binding pocket where not activated by DPLC or DUCP high-
lighting the importance of the ligand binding pocket in regulating LRH-1 activity.

5.1.5. Synthetic ligands

The identification of synthetic agonists for LRH-1 and SF-1 supports the notion of ligand dependent activity of LRH-1 [93]. GSX470, a substituted cis-bicyclo[3.3.0]oct-2-ene, was identified as a high affinity ligand for both LRH-1 and SF-1. This molecule led to increased expression of the LRH-1 target gene SHP in liver cells [93]. Modifications to this molecule at 3 different sites also led to the identification of other agonists with varying degree of potency and efficacy. One such molecule, RWL101 was shown to be selective for LRH-1, however further work is being undertaken to improve its efficiency [94]. Recently an inverse agonist was identified for SF-1: 4-(heptyl)phenol (AC-45594) and its analogues were shown to regulate SF-1 activity and subsequently downregulate SF-1 target genes [109]. This molecule had no effect on LRH-1 activity indicating that despite their structural similarities, distinct ligands for LRH-1 and SF-1 could be synthesized.

6. Future perspectives

Since LRH-1 is implicated in important cellular functions and plays a role in various diseases, it represents an attractive therapeu-
tic target in infertility, cancer and metabolic disorders. NRs have been targeted by pharmaceutical agents for decades, although the majority of drugs act at the ligand binding pocket to mimic or antag-
onise endogenous ligands. For orphan receptors, the challenges are greater. Since co-regulators exert profound effects on LRH-1 activity, the possibility of modulating LRH-1- co-regulator interac-
tions should be considered in addition to searching for compounds that bind to the phospholipid/ligand binding pocket. In addition to tissue-specific coregulators, there may be tissue-specific endoge-
nous ligands adding to the complexity of the regulation of orphan nuclear receptors such as LRH-1. Identification of ligands for LRH-1 will not only uncover its true biological roles but provide an avenue for new treatment opportunities for a range of diseases.

References

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Appendix B3: Therapeutical potential of LRH-1 modulators
Appendix B3: Therapeutic potential of LRH-1 modulators
Appendix C: Animal ethics information

All conditions pertaining to the clearance were properly met, and annual/final reports have been submitted.

APPLICATION FORM FOR THE USE OF ANIMALS FOR SCIENTIFIC PURPOSES IN RESEARCH AND TEACHING

MMCB

ANIMAL ETHICS COMMITTEE

AEC NUMBER MMCB 2010/12

Project Type ☑ Research ☐ Undergraduate Teaching ☐ Training in Procedural Techniques

Project Title The role of LRH-1 in breast cancer proliferation

Animal Use Categories (Refer to List of Categories attached)

1.1, 2.1, 4.7, 4.9, 4.10, 5.1, 5.2

Standard Operating Procedures ☐ No ☑ Yes: Title/AEC Number:

SOPs indicated are to be read in conjunction with the application. Detail any variations from the SOP.

Proposed Start Date 01-04-2010

Proposed Finish Date 20-12-2013

Actual start determined at time of AEC approval

Actual finish determined at time of AEC approval

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<th>Title &amp; Full Name</th>
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<tr>
<td>Chief Investigator</td>
<td>Dr Colin Clyne, PhD, Group Head</td>
<td>Cancer Drug Discovery/Prince Henry’s Institute</td>
</tr>
<tr>
<td>Person to act in Chief Investigator’s absence</td>
<td>Dr Morag Young, PhD, Group Head</td>
<td>Cancer Drug Discovery/Prince Henry’s Institute</td>
</tr>
<tr>
<td>Investigator responsible for animal care</td>
<td>Kerrie Herridge, Research Assistant</td>
<td>Cancer Drug Discovery/Prince Henry’s Institute</td>
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ANIMAL USE: include all animals used eg embryos, neonates, pregnant animals and any expected animal by-catch in trapping protocols.

DECLARATION BY CHAIRPERSON OF AEC

I certify that the procedures/ personnel/location in this project has been considered and approved by the Animal Ethics Committee for the period 18/6/2010 to 17/6/2013

Chairperson’s signature

Print Name

Approval is subject to the following conditions:
1. An Annual Report must be provided each February.
2. A Final Report must be submitted within six months of the completion of the project.
3. Unexpected or adverse effects which impact on the welfare of the animals must be reported to the AEC Chair immediately.

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