A Diversified Approach to Improving Fertility Outcomes: Understanding Women's Fertility Knowledge through Apps and Primordial Follicle Activation in Granulosa Cells

Emmalee A. Ford

B. Science (Hons I)

University of Newcastle

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Declarations

Statement of originality

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

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I hereby certify that this thesis is in the form of a series of papers. I have included as part of the thesis a written declaration from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers.

By signing below, I confirm that Emmalee Ford contributed upward of 50% towards data collection/analysis and manuscript preparation for the publications and manuscripts in this thesis for which I am a co-author.

Jessie M Sutherland

19/10/2020

…… ……

Signature Date

Alexandra E Peters

09/09/2020

…… ……

Emily R Frost 10/09/2020

…… ……

Emma L Beckett

14/09/2020

…… ……

Shaun D Roman 14/09/2020

…… ……

Eileen A McLaughlin

14/09/2020

…… ……

Lesley MacDonald-Wicks

Assistant Dean Research and Training

..

15/12/2020

…… ……

Signature Date

I would like to acknowledge the traditional custodians of the country. This thesis was researched and written on Awabakal land. Always was, always will be.

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Publications arising from work in this thesis

Chapter 2: Literature Review | Published

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Sutherland, J. M., Frost, E.R., **Ford, E. A.**, Peters, A. E., Reed, N. L., Seldon, A. N., Mihalas, B. P., Russel, D. L., Dunning, K. R., McLaughlin, E. A. (2018) Janus kinase JAK1 maintains the ovarian reserve of primordial follicles in the mouse ovary. *Molecular Human Reproduction*, **24**(11), 533-542. DOI: 10.1093/molehr/gay041

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Ford, E. A., Beckett, E. L., McLaughlin, E. A., and Sutherland, J. M. Understanding female fertility and Taking Control of reproductive health: preliminary data from a fertility knowledge survey. Annual Scientific Meeting of the Society for Reproductive Biology, Adelaide, Australia, August 2018. *Poster presentation*

Ford, E. A., Beckett, E. L., McLaughlin, E. A., and Sutherland, J. M. Understanding female fertility and taking control of reproductive health. Australian Society for Medical Research Satellite Scientific Meeting, Newcastle, Australia. June 2018. *Oral presentation*

Ford, E. A., Beckett, E. L., McLaughlin, E. A., and Sutherland, J. M. Understanding premature ovarian failure and taking control of fertility. School of Biological Sciences Annual higher degrees in research conference, Newcastle, Australia. November 2017. *Oral presentation*

Contents

Aims and Objectives of thesis

The aims and objectives explored in this thesis are raised in the chapter outlines and introductions sections, but are compiled here too for clarity.

Primary aim 1: To identify factors involved in the regulation of primordial follicle activation using next generation sequencing

Secondary aims:

- Evaluate the most recent literature pertaining to primordial follicle activation in humans, addressing the connection between accelerated activation and the diagnosis of premature ovarian insufficiency
- Isolate and analyse the role of granulosa cells before and during the process of primordial follicle activation to gain insights into their gene expression during primordial follicle activation
- Use gene ontology analysis to identify novel processes, pathways, genes and/or proteins commonly occurring in granulosa cells undergoing activation

Primary aim 2: To explore the viability of reproductive health smartphone applications as tools for future reproductive health messaging

Secondary aims:

- Consider the current academic literature published on women's reproductive health app content to evaluate how fertility information is reported across a range of study types. Including the accuracy of fertility information within app literature, and the impact this information has on the users of apps
- Determine if there is a knowledge difference between those who did or did not use women's reproductive health apps

Abstract

Infertility is a global public health issue which affects up to 15% of people, with female factors contributing to 50% of all known cases of infertility. 1 in every 100 women become infertile from premature ovarian insufficiency (POI), a condition characterised by the early onset of menopause and subsequent infertility due to premature oocyte depletion. In POI, oocytes can be depleted through accelerated primordial follicle activation, where immature, dormant oocytes encapsulated within granulosa cells, or primordial follicles, are selectively activated for growth and development. Beyond the characterisation of a few dominant signalling pathways, there is limited evidence to explain the process and mechanisms controlling primordial follicle activation. Compounding the implications of female infertility, is compelling evidence to suggest that a general gap in fertility knowledge among Australian women. Studies confirm a lack of knowledge about factors that may influence fertility, and a misconception that assisted reproductive technology can solve these issues. Effective educational intervention strategies have not yet been discovered, but the accessibility and popularity of reproductive health smartphone applications (apps) makes them a worthwhile avenue to explore.

This thesis takes a diversified approach to improving fertility outcomes in women by successfully merging discovery-based laboratory science with public health research. This is achieved through analysing the role of granulosa cells in primordial follicle activation, in conjunction with investigating the efficacy of reproductive health smartphone applications to provide accessible fertility education to women. Herein, I have developed a novel method to collect mass quantities of mouse granulosa cells originating from primordial, activating and primary follicles and conducted a transcriptomic study of isolated granulosa cells. This study was instrumental in providing a deeper understanding of primordial follicle activation, through highlighting pre-existing factors linked to POI and follicle activation that are understudied.

Moreover, my transcriptomic investigations yielded the Wnt pathway antagonist FRZB as a potential upstream regulator of activation through its interaction with WNT3A. A scoping review was used to identify the peer-reviewed evidence of information about fertility in smartphone apps. Through this review, I determined that fertility information reported in studies on apps was very limited, and even fewer studies measured the impact of this information in the comprehension of users. Furthermore, I validated evidence of a fertility knowledge gap in Australia women, through the development and delivery of an online survey to over 600 participants. Through the detailed analysis of my survey results, I identified an association between women who used reproductive health smartphone application and increased likelihood of correctly identifying the most fertile time in the menstrual cycle. Interestingly, I found most app users in this study tracked their cycles, which may account for the increased knowledge of this aspect.

Taken together, the work in this thesis contributes to improving the foundational understanding of granulosa cell-directed primordial follicle activation, emphasising the importance of elaborating on the knowledge of already existing pathways involved in primordial follicle activation, and introducing a novel role for FRZB in this process. Additionally, this thesis identified a unique opportunity for reproductive health smartphone apps to be used to increase fertility awareness in the general public, and constitutes detailed recommendations for all stakeholders in the process of app research and development to enhance their effectiveness. The intersection of public health and molecular biology in the reproductive health perspective in Australia is unique in this project. Ultimately, this thesis will create a platform from which meaningful and relevant content can be conveyed to the public, and foster a greater interest in reproductive health throughout a person's lifetime and empower women's family planning and lifestyle decision making.

Chapter 1: Introduction

1.1 – Female infertility is multifactorial and is increasing in Australia

Infertility is a global public health issue recognised by the World Health Organisation (Ombelet, 2011). The prevalence of infertility is estimated to be between 9 and 15% globally, with male-factor and female-factor contributing equal (Boivin et al., 2007; De Kretser and Baker, 1999; Petraglia et al., 2013). Data from the Australian longitudinal study on women's health has identified even higher rates nationally, with 17% of women aged 28-33, who were trying to conceive, or had been pregnant, reporting experiencing infertility (Herbert et al., 2009), though more recent data from this study would be enlightening. Exacerbating the global rate of infertility is advancing maternal age; the average age of first time mothers in Australia has risen to 29.2 years of age, with 24% of mothers over the age of 35 in 2017 (AIHW, 2019). Maternal age is the largest limiting factor in female fertility, as women age, the number and quality of remaining oocytes drops remarkably, from the age of 35 (Balasch, 2010).

As maternal age is increasing, it is not surprising that the use of assisted reproductive technologies (ART) has also increased by 45% over the decade between 2008 and 2018 (Newman et al., 2020; Wang et al., 2010), yet the take home baby success rate has remained relatively stagnant at 18% (Figure 1). Unfortunately, ART is unable to mitigate the effects of maternal age on fertility, as the number and quality of oocytes within the ovary drastically decreases from the age of 35 in the vast majority of women (Balasch, 2010; Balasch and Gratacos, 2012). Furthermore, the likelihood of miscarriage and birth abnormalities increases substantially with advanced maternal age (Andersen et al., 2000; Jones, 2007). This finite window of fertility makes family planning especially difficult for women, who report the desire to achieve other professional or personal goals before parenthood (Lampic et al., 2006; Prior et al., 2018). However, increased rates of ART treatments may also be attributed to

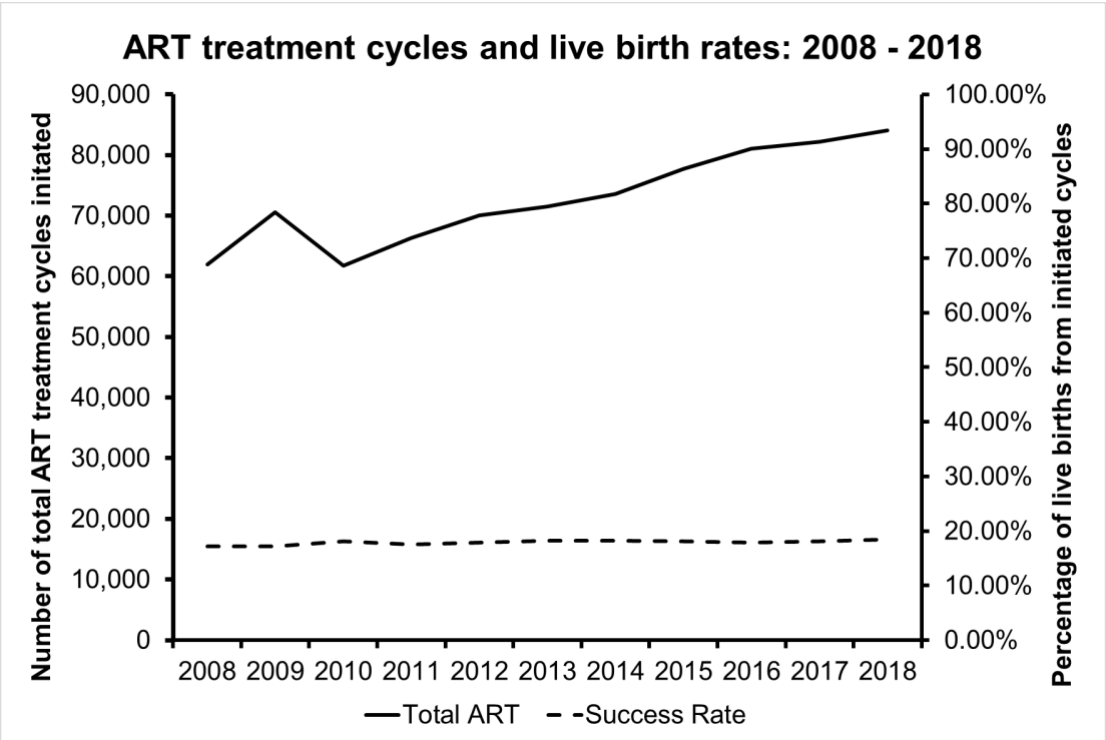

Figure 1: ART treatment cycles and live birth rates in Australia and New Zealand from 2008 to 2018. The total number of assisted reproductive technology (ART) treatment cycles initiated in the years 2007 to 2017 (solid line) is reported. The live births achieved over this period is reported as a percentage of the number of cycles initiated (dashed line). Data obtained from the annual Assisted Reproductive Technology in Australia and New Zealand reports from the National Perinatal Epidemiology and Statistics Unit years 2008 to 2018 (Newman et al., 2020; Wang et al., 2010).

improvements in cancer survival rates, which also has implications on the function and viability of reproductive material (Gorman et al., 2012).

1.2 – Premature ovarian insufficiency may be caused by rapid primordial follicle activation

Unfortunately, there is a proportion of women whose reproductive years are even further reduced or eliminated due to premature ovarian insufficiency (POI). Women with POI experience a reduced pool of oocytes from which to ovulate. POI occurs in over 3% of women, and is characterised by amenorrhea at ≤ 40 years of age, hypoestrogenism and increased gonadotrophin levels (Golezar et al., 2019; Shelling, 2010). Women with POI are not able to utilise the full range of ART options to have their own biological children due to a lack of retrievable oocytes, and must often resort to using donor oocytes (ESHRE, 2015). POI can be

induced by a range of factors which culminate in dysfunctional folliculogenesis, including environmental factors, chemotherapy and other iatrogenic causes (Ford et al., 2020; Spears et al., 2019). Along with infertility, POI patients face negative health consequences, including increased risk of cardiovascular disease, osteoporosis, urogenital atrophy, and mental health conditions like depression and anxiety (Podfigurna-Stopa et al., 2016; van der Stege et al., 2008).

A common cause of oocyte loss in POI is an acceleration in the rate of primordial follicle activation (however, the development of POI may be a consequence of increased follicle loss and destruction by other means (Ford et al., 2020)). Primordial follicles contain a single oocyte enclosed by a layer of flattened granulosa cells (somatic support cells), and are the quiescent, immature unit from which future fertility is drawn. Primordial follicles undergo the process of primordial follicle activation to become a primary follicle, and thus entering the growth phase for eventual ovulation. It is important to note that once primordial follicles are activated, they are destined for ovulation or destruction, with only around 0.5% of follicles developing through to ovulation (Baker, 1963; Findlay et al., 2015; Hansen et al., 2008). At a molecular level, the mechanisms that cause rapid activation of primordial follicles to induce POI in humans is largely unknown (reviewed in Chapter 2 of this thesis), and thus there is a heavy reliance on the use of animal models to investigate this process for the eventual goal of early diagnosis and improvement treatment options.

In mice, the current understanding of primordial follicle activation is that mammalian target of rapamycin complex 1 (mTORC1) is activated in the flattened granulosa cells of primordial follicles, and then KIT ligand, produced by activated granulosa cells, activates the oocyte via the phosphatidylinositol 3-kinase (PI3K) signalling cascade (Zhang and Liu, 2015). However, it has been shown in mice that when mTORC1 signalling is removed, there was no effect on

follicle development (Gorre et al., 2014), suggesting other molecular factors can also contribute to primordial follicle activation. The role of the granulosa cell is critical in primordial follicle activation; providing access to the oocytes, and promoting follicular survival, and growth (El-Hayek et al., 2018; Eppig, 2018; Xiong et al., 2017). Thus, elucidating the means by which granulosa cells initiate primordial follicle activation in humans, has the potential to vastly improve diagnostics and treatment for many women suffering with POI.

To untangle the role of the granulosa cell, investigations at the cellular transcript and protein level may provide promising novel candidates for further analysis. This approach has seen success in previous work, such as the mouse ovary transcriptome study (Holt et al., 2006) which lead to the eventual identification of the roles of stromal-derived factor-1 (SDF1)/CXCR4 and janus kinase/signal transducers (JAK/STAT) in primordial follicle activation. When cultured *in vitro* with SDF1, mouse ovaries were shown to have fewer activating follicles (Holt et al., 2006), and when JAK1 was inhibited *in vitro*, the rate of primordial follicle activation accelerated (Sutherland et al., 2018; Sutherland et al., 2012). New evidence indicates the potential for a conserved role for JAK/STAT signalling in human ovaries, when a granulosa-like cell line was utilised (Frost et al., 2020). This thesis aims to build upon these studies by isolating the granulosa cells from the ovaries to investigate their contribution to mouse primordial follicle activation for the first time. Increased understanding of the role of granulosa cells in follicle cell activation may contribute to improvements to the diagnosis and treatment of POI but would notably facilitate the future risk assessment and prevention of POI. In Chapter 3, I present a new method for isolating mouse granulosa cells originating from primordial, activating and primary follicles. The transcriptome of these cells is explored in Chapter 4, in which I link our existing knowledge of activation and POI to cellular function, through characterising proteins previously unidentified in primordial follicle activation.

1.3 – Lack of knowledge about fertility may be adding to the number of infertile people

Research in model systems is critical for the eventual treatment of infertility, yet there is potential for more rapid interventions at the societal level to improve infertility rates. The ability of the public to understand components of health advice to incorporate into their daily routines is more complicated, but arguably essential, in a comprehensive approach to fertility and reproductive health. Increasing the fertility knowledge and awareness of the general public is also of concern as there is evidence to suggest that women who are less informed, are less likely to seek health advice addressing their infertility problems. In a population of women in the UK searching for conception information online, 56% had not sought health advice, and 20% of these 'non-seekers' fit the clinical criteria for infertility (Bunting and Boivin, 2007). Indeed, among a population of subfertile women in the USA, those that wanted treatment more readily sought information about fertility (Greil and McQuillan, 2004), which raises concerns for non-seekers in accessing vital information about fertility. The delay or avoidance in seeking help for fertility can lead to an increased burden of stress or mental illness, contributing to the economic burden of infertility (Balasch and Gratacos, 2012; Bunting and Boivin, 2007). Both of these factors already disproportionately affect women, with infertility responsible for >8,000 years of life lost due to disability (YLD) for women in Australia in 2003 (Begg et al., 2007).

Knowledge about fertility and reproductive health is critical for identifying when health advice or intervention is required. Improving awareness and understanding may be beneficial in the interests of POI patients, whose health management strategy is typically centred around a regime of hormone replacement therapy and symptomatic treatment (ESHRE, 2015). Indeed, evidence shows that Australian patients with POI do not have sufficient knowledge of the risks and benefits of hormonal medications, nor the risks and benefits of complementary or alternative medicines for which there is widespread use among patients (Gibson-Helm et al., 2014; Goh et al., 2019). The informed choice of medication or treatment is critical for patients,

especially considering the reduced rate of hormone therapy over alternative methods in the context of potential adverse health outcomes of POI (Main and Robinson, 2008). There is clearly a need for robust health education for patients living with chronic reproductive illnesses for improvements in wellbeing and healthcare regimens, with similar levels of misunderstanding observed in studies of health-related knowledge of women with PCOS (Colwell et al., 2010).

Fertility knowledge is also important for lifelong wellbeing, family planning and decision-making, as lifestyle factors and other avoidable factors impacting fertility can be recognised by the individual. Health literacy is associated with good reproductive health knowledge and is related to positive reproductive health behaviours (Kilfoyle et al., 2016). Consequently, improving reproductive health literacy and knowledge is necessary for improving good practice reproductive health habits for all women. For women following health advice that do eventually make it to the treatment stage and undergo ART, the psychological and financial pressures can affect success outcomes and increase the likelihood of dropout in successive cycles which are commonplace in age-related infertility (Klonoff-Cohen et al., 2001; Turner et al., 2013). Studies across the globe report a knowledge gap in the fertility education of women (reviewed in Pedro et al. (2018)), commonly observing in participants; an overestimation of the number of fertile years in women (reviewed in García et al. (2018)), underestimating of the detrimental impacts of lifestyle factors (like cigarette smoking and a sedentary lifestyle) (Hammarberg et al., 2013), and an expectation that ART has the ability to resolve age-related infertility (Bunting et al., 2012; Daniluk and Koert, 2013; Mac Dougall et al., 2013).

1.4 – Smartphone apps for women's health may be an educational opportunity

There have been many approaches to bridging the fertility knowledge gap in women of reproductive age, such as comprehensive web pages, discussions with healthcare professionals

during contraceptive consultations, surveys, and awareness campaigns. While these strategies have had varying levels of success, there are limitations with regards to retention of knowledge (Daniluk and Koert, 2014), information-seeking behaviours (discussed above), population bias (attending fertility clinics, university students) (Boivin et al., 2019; García et al., 2016; Sylvest et al., 2018), time constraints (Hampton et al., 2016) and, in some cases, the overwhelming knowledge of fertility limitations can cause anxiety (Maeda et al., 2016). Another method of educating women of reproductive age about fertility is via the use of reproductive health smartphone applications (apps). A key advantage that apps may have over typical fertility awareness intervention strategies is that apps, by design, often utilise attractive elements to encourage repeated use (called gamification), which incorporates components of learning and behaviour theory to create effective changes in the education and habits of app users (Lister et al., 2014; Zhao et al., 2016). These features have been harnessed in other areas of health behaviour and monitoring such as physical activity and diabetes (Dennison et al., 2013; Direito et al., 2014; Knight et al., 2016), and are beginning to be conducted in reproductive health (meta-analysis protocol developed by Musgrave et al. (2019)). Reproductive health apps are widely used around the globe, and cover an array of topics which includes but is not limited to conception, contraception, family planning, monitoring pregnancy, and tracking menstrual cycles. Some apps are designed to cover multiple areas of reproductive health-related functions, but apps which specifically cover fertility can contain overlapping purposes for their users that do not always correlate with trying to get pregnant (Epstein et al., 2017; Hamper, 2020). This creates an opportunity to explore themes of reproductive health with women of reproductive age who are not currently trying to conceive and reducing the selection bias of health information seekers. However, the variability of current fertility apps may also pose a risk when it comes to the accuracy or reliability of the information contained with them, as it has been shown that only a small proportion of apps contain health professional involvement

(Moglia et al., 2016), and inconsistencies arise in studies reporting the use of apps in terms of the sourcing of apps to study, the participants, and the observations recorded (reviewed in Earle et al. (2020)). Additionally, the extent to which reproductive health apps currently provide information about fertility to users is unclear (reviewed in Chapter 5). This is investigated further in Chapter 6 through the comparison of the fertility knowledge of reproductive health app users and non-app users to identify if current apps have the ability to provide unique educational interventions for women.

1.5 – Conclusion

This thesis takes a multifactorial approach to improving fertility outcomes, through both a fundamental molecular science, and a public health perspective. Together this thesis presents an investigation of mechanisms that contribute to accelerated activation and loss of primordial follicles via a unique animal model, and an exploration of improving the fertility knowledge of women through app use. By undertaking research at different entry points in the field of women's health, this body of work provides a platform for which strong advocacy for women's reproductive health issues can be brought toward policy makers, academia, industry, and app developers on the pursuit toward greater understanding and awareness about fertility.

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Chapter 2: Advances in human primordial follicle activation and premature ovarian insufficiency

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Chapter overview

The aim of this literature review was to evaluate the most recent literature pertaining to primordial follicle activation in humans, addressing the connection between accelerated activation and the diagnosis of premature ovarian insufficiency. In addition, I discuss the current clinical research focus on the control of primordial follicle activation *in vitro* for the preservation of female fertility. I highlight evidence showing that, despite the breadth of knowledge obtained from animal models, the molecular understanding of primordial follicle activation remains largely unexplored in humans. Indeed, where mechanisms established in animal models have progressed to investigation in humans, results were sometimes unexpected. Therefore, this review evaluates the known biological pathways, genetic, and environmental factors that contribute to the regulation of primordial follicle activation explicitly in humans and highlights the role of emerging research techniques in expanding our understanding of the already-established pathways in primordial follicle activation.

Overall, this review emphasises that beyond small sample *in vitro* studies and patient cohort and case studies, the knowledge of excessive primordial follicle activation in the induction of premature ovarian insufficiency is limited, and requires significantly more groundwork in establishing viable studies to take from animal models toward the clinic. Moreover, we argue that future research on primordial follicle activation, as a gonadotrophin-independent event, should focus on the intraovarian signalling between the oocyte, granulosa cells, and extracellular environment.

Original review article

REPRODUCTION
REVIEW

Advances in human primordial follicle activation and premature ovarian insufficiency

Emmalee A Ford[1,2], Emma L Beckett[2,3], Shaun D Roman[1,2,4], Eileen A McLaughlin[1,5,6] and Jessie M Sutherland[1,2]

[1]Priority Research Centre for Reproductive Science, Schools of Biomedical Science & Pharmacy and Environmental & Life Sciences, University of Newcastle, Callaghan, New South Wales, Australia, [2]Hunter Medical Research Institute, New Lambton Heights, New South Wales, Australia, [3]School of Environmental & Life Sciences, Faculty of Science, University of Newcastle, Callaghan, New South Wales, Australia, [4]Priority Research Centre for Drug Development, University of Newcastle, Callaghan, New South Wales, Australia, [5]School of Science, Faculty of Science & Technology, University of Canberra, Bruce, Australian Capital Territory, Australia and [6]School of Biological Sciences, Faculty of Science, University of Auckland, Auckland, New Zealand

Correspondence should be addressed to J M Sutherland; Email: jessie.sutherland@newcastle.edu.au

Abstract

In women, the non-growing population of follicles that comprise the ovarian reserve is determined at birth and serves as the reservoir for future fertility. This reserve of dormant, primordial follicles and the mechanisms controlling their selective activation which constitute the committing step into folliculogenesis are essential for determining fertility outcomes in women. Much of the available data on the mechanisms responsible for primordial follicle activation focuses on a selection of key molecular pathways, studied primarily in animal models, with findings often not synonymous in humans. The excessive induction of primordial follicle activation may cause the development of premature ovarian insufficiency (POI), a condition characterised by menopause before age 40 years. POI affects 1–2% of all women and is accompanied by additional health risks. Therefore, it is critical to further our understanding of primordial follicle activation in order to diagnose, treat and prevent premature infertility. Research in primordial follicle activation has focused on connecting new molecules to already established key signalling pathways, such as phosphatidylinositol 3-Kinase (PI3K) and mammalian target of rapamycin (mTOR). Additionally, other aspects of the ovarian environment, such as the function of the extracellular matrix, in contributing to primordial follicle activation have gained traction. Clinical applications are examining replication of this extracellular environment through the construction of biological matrices mimicking the 3D ovary, to support follicular growth through to ovulation. This review outlines the importance of the events leading to the establishment of the ovarian reserve and highlights the fundamental factors known to influence primordial follicle activation in humans presenting new horizons for female infertility treatment.

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Introduction

The total number of immature primordial follicles that reside in a woman's ovaries are established *in utero* from germ cell nests and is termed the ovarian reserve. Moreover, an estimation of a woman's ovarian reserve is considered a fundamental indicator of fertility. Of equal importance to fertility is the rate at which these immature primordial follicles are recruited for growth through a process known as 'activation'. Of these original primordial follicles, numbering between 500,000 and 1,000,000 in total at birth, only approximately 400 will fully mature into primary oocytes capable of being ovulated and fertilised during a woman's reproductive years (Hansen *et al.* 2008, Findlay *et al.* 2015). The overwhelming majority are fated to undergo atresia (ovarian-mediated cell death) (Baker 1963, Tilly 2001, Marcozzi *et al.* 2018).

Primordial follicle activation involves the recruitment of primordial follicles into folliculogenesis for the eventual selection of one oocyte for ovulation. As women age, the pool of primordial follicles, from which to source candidates for activation, plummets. When there are ~1000 primordial follicles remaining, this results in the cessation of fertility and the onset of menopause (Faddy *et al.* 1992, Hansen *et al.* 2008). A key aspect of our understanding of the depletion of the ovarian reserve is that primordial follicles cannot be regenerated or replaced. This concept has been contested by the identification of oogonial stem cells able to produce new oocytes within the mouse ovary, with emerging evidence for their presence in human ovaries (Clarkson *et al.* 2018), but there is still conjecture over a physiological role for these cells based on definitive evidence (reviewed in Horan & Williams 2017).

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Thus, the challenge for research is to elucidate the mechanisms controlling the depletion of the ovarian reserve via primordial follicle activation, so that we may provide solutions for those faced with the threat of early fertility loss, such as in the case of premature ovarian insufficiency (POI).

POI may also be referred to as premature ovarian failure, but for the purpose of this review, either term applies interchangeably. POI is an infertility condition diagnosed when menopause occurs prior to the age of 40, due to a significant reduction in, or absence of, a woman's pool of primordial follicles. This condition occurs globally in 1–2% of all women (Coulam *et al.* 1986). POI is defined as the premature cessation or absence of ovarian function and is characterised by amenorrhea, hypoestrogenism and an increase in gonadotrophin levels (Bachelot *et al.* 2009, Shelling 2010). A common cause of POI is an acceleration in the rate of primordial follicle activation, thereby resulting in a depletion of the ovarian reserve (Nelson 2009). However, the ovarian reserve may also be depleted via primordial follicle loss (Depalo *et al.* 2003) or from iatrogenic causes such as chemotherapeutics (Ben-Aharon & Shalgi 2012). Women often receive a diagnosis of POI when they are already in significant reproductive decline, and as a consequence of their diagnosis may face psychological impacts in addition to the physiological symptoms and infertility of this condition (Van Der Stege *et al.* 2008, Welt 2008). Long-term physical consequences of POI may be severe, even with traditional hormone replacement therapies to ameliorate the negative effects of a loss of ovarian hormonal support. Women with POI have an increased risk of cardiovascular disease, osteoporosis, urogenital atrophy and neurodegenerative disorders (reviewed in Podfigurna-Stopa *et al.* 2016). Thus, it is critical that we continue towards understanding the process of primordial follicle activation as a means of intervention to preserve the ovarian reserve.

Both the establishment of the ovarian reserve and the initial wave of primordial follicle activation occur *in utero,* prior to sexual maturity and gonadotrophic input, thus relying largely upon regulation by intraovarian factors (Lew 2019). Primordial follicle activation dictates the growth and development of the oocyte as well as the differentiation and proliferation of the surrounding somatic granulosa cells, both essential processes for the ultimate goal of ovulation. However, our understanding of the molecular and biochemical processes underpinning follicular activation in humans remains limited. Herein we review the current knowledge and explore future advances towards controlling primordial follicle recruitment and consequently preserving or prolonging female fertility. This is particularly pertinent for those women diagnosed with POI who experience accelerated activation and early depletion of the ovarian reserve.

Early events within the ovary establish the ovarian reserve that sustains fertility

During human embryo sex determination, the primitive gonads are endowed with the primordial germ cells (Tam & Snow 1981) (Fig. 1). The germ cells migrate to the genital ridge of the primitive gonad, then rapidly proliferate until they number 5–6 million (Motta *et al.* 1997, Mamsen *et al.* 2011, Myers *et al.* 2014). This proliferation occurs rapidly and as cytokinesis is not wholly completed, clusters of germ cells (termed germ cell nests) connected via cytoplasmic bridges remain (reviewed by Pepling 2006). Germ cell nest breakdown directly precedes primordial follicle formation and is a major factor influencing the initial size of the ovarian reserve. During this process, pre-granulosa cells (flattened, squamous granulosa cells prior to differentiation into cuboidal granulosa cells) are primed to encapsulate the oocytes via signalling originating from oocytes and intracellular communication between other pre-granulosa cells (De Felici *et al.* 2005, Grive & Freiman 2015, Suzuki *et al.* 2015*a*). Only a small fraction of the original population of germ cells go on to form primordial follicles, while the remaining oocytes, estimated between one and two thirds, are targeted for coordinated degradation via classical apoptotic mechanisms (Albamonte *et al.* 2008). The role of autophagy in protecting germ cells from apoptosis has been established in mice (Rodrigues *et al.* 2009), with preliminary evidence this occurs in humans too (Sun *et al.* 2017, Zhou *et al.* 2019). The cause for such a substantial loss of oocytes is still unknown, but it is possible that a quality control mechanism exists through which faulty nuclei are lost, and healthy oocytes are preferentially encapsulated into primordial follicles (Tilly 2001, Sun *et al.* 2017). Additionally, a self-sacrifice mechanism previously established in *Drosophila* (de Cuevas *et al.* 1997), and later observed in mice may also be responsible for mammalian germ cell nest breakdown (Grive & Freiman 2015, Pepling 2016). In this mechanism, essential cellular factors are transported via cytoplasmic bridges from neighbouring germ cells within a cluster to the germ cells that will survive and become oocytes in primordial follicles, but this mechanism has not been confirmed in humans (Lei & Spradling 2016).

At the completion of germ cell nest breakdown, the oocytes are each surrounded by a layer of pre-granulosa cells and termed primordial follicles (Maheshwari & Fowler 2008). A surge of retinoic acid released from the mesonephros (primitive kidney) positioned adjacent to the immature ovaries, drives all primordial germ cells to enter meiosis, where they pause arrested at the diplotene stage of prophase I (Borum 1961, Peters 1969). Shortly before birth, primordial follicle activation commences with most follicles developing to the preantral follicle stage (Himelstein-Braw *et al.* 1976). It has been reported that in humans, some follicles will continue onto the antral stage prior to birth (Peters *et al.* 1978).

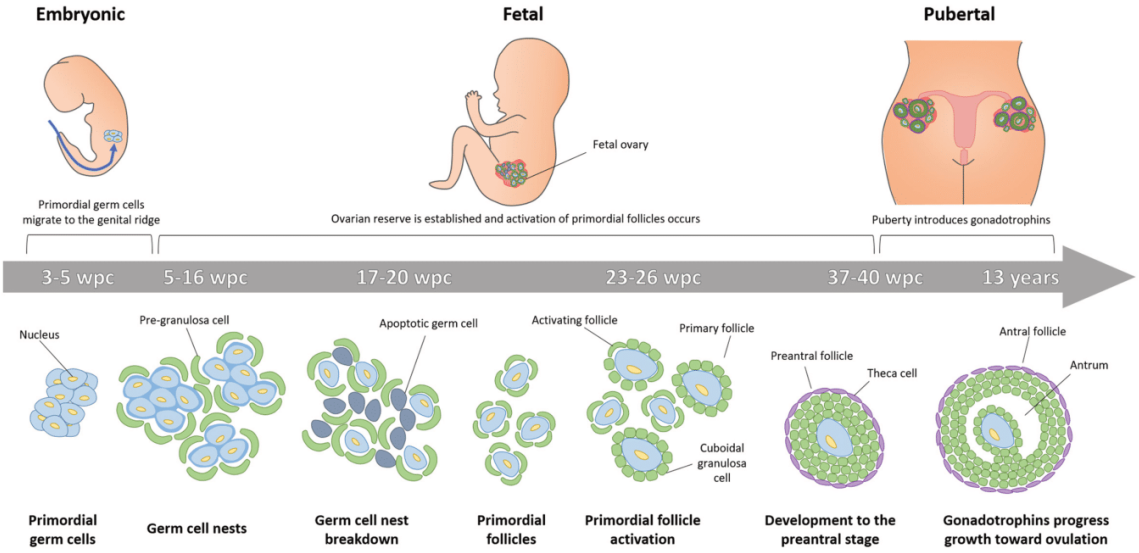

Figure 1 The development of the human ovarian reserve. The timeline of development of the ovarian reserve is depicted, with representations of the germ cells and somatic cells residing in the ovary, and the stage of life of the female at the point which this occurs. Beginning at 3–5 weeks post conception (wpc) in the embryo, the primordial germ cells migrate to the genital ridge. By 5–16 wpc in the foetus, the primordial germ cells proliferate rapidly and vastly increase in number, resulting in germ cell nests as cytokinesis is not wholly complete. The germ cell nests begin to break down at 17–20 wpc, with many germ cells undergoing apoptosis as the pre-granulosa cells start to encapsulate the germ cells to form primordial follicles. Primordial follicles undergo meiosis after a surge of retinoic acid from the mesenephros, and they remain arrested at prophase I of the cell cycle. From 23–26 wpc the first wave of primordial follicle activation commences, as the oocyte grows and granulosa cells grow and differentiate from their flattened, squamous shape to a cuboidal structure. At around the time of birth at 37–40 wpc, a small proportion of preantral and antral follicles are present. However, until puberty occurs at around 13 years of age, activated follicles can only grow until the early antral stage. At puberty, the input of gonadotrophin allows progression beyond early antral stage through to ovulation.

Increased gonadotrophic production at puberty enables successive follicle growth and ovulation (Dungan *et al.* 2006, Choi & Yoo 2013). Continued activation of primordial follicles occurs dynamically throughout the reproductive years until the onset of menopause. Primordial follicle activation constitutes the committing step into folliculogenesis, as the primordial follicles that are activated throughout a woman's life succumb to one of two fates; to be ovulated or destroyed.

Since the endowment of the ovarian reserve contains the potential for future fertility, and primordial follicle activation is responsible for regulating follicle progression beyond this point, it is important to consider how activation is so precisely controlled so that we may identify those women at risk of accelerated activation and POI and develop practices to preserve or prolong fertility.

Excessive activation of primordial follicles can lead to premature ovarian insufficiency

When the rate of primordial follicle activation is accelerated, and control over the size of the ovarian reserve is lost, POI can result (Kalantaridou *et al.* 1998). POI may be induced by aberrations in other vital ovarian processes which are outside the scope of the current review, including but not limited to meiosis, DNA repair and gonadotrophin control (Huhtaniemi *et al.* 2018). POI is largely idiopathic (50–70%) (Chapman *et al.* 2015), but known causes include iatrogenic factors (such as chemotherapeutics), genetic factors, environmental factors or autoimmunity. Autoimmunity is responsible for 10–30% of POI cases and is typically related to adrenal disease (Hoek *et al.* 1997, Ebrahimi & Akbari Asbagh 2015). Additional autoimmune diseases that may cause POI include Addison's disease, hypothyroidism, Whitaker syndrome and diabetes mellitus (Conway *et al.* 1996, Ebrahimi & Akbari Asbagh 2015, Komorowska 2016). Yet, the mechanism by which these diseases result in POI is not usually through excessive or uncontrolled primordial follicle activation but through follicular oocyte destruction (Persani *et al.* 2009 for a review). Thus, it is critical that the factors affecting primordial follicle activation be understood in order to identify those at risk of POI early so that fertility can be preserved.

Iatrogenic factors

Amongst the number of iatrogenic factors able to induce POI (radiation, surgery, physical damage to the ovary), it is chemotherapeutics that have been particularly linked

to changes in primordial follicle activation and/or the depletion of the ovarian reserve. Other studies have also linked damage to the vasculature of the ovarian cortex to depletion of primordial follicles (reviewed in Ben-Aharon & Shalgi 2012). In each of these scenarios, patients are at significant risk of developing POI, though it has not yet been confirmed if gonadotoxic treatment is due to accelerated activation or an increase in follicular atresia (Nguyen *et al.* 2019). Typically, chemotherapeutics target proliferating cells and depending on the dose, duration and treatment, granulosa cells are significantly compromised by these treatments, resulting in primordial follicle loss (Abir *et al.* 2008). After treatment with the alkylating agent cyclophosphamide, women have a 40% chance of developing POI, but the cellular mechanisms resulting in premature menopause remain unclear (Cox & Liu 2014). Human ovarian tissue sections cultured in the active metabolites of cyclophosphamide have a decreased primordial follicle population and a concomitant increase in developing follicles (Lande *et al.* 2017). However, in a combined *in vitro* treatment of chemotherapeutics (adriamycin, bleomycin, vinblastine and dacarbazine), the density of non-growing follicles in human ovarian tissue samples was increased (Mclaughlin *et al.* 2017).

For women undergoing chemotherapy, fertility preservation through cryopreservation of ovarian tissue, followed by transplantation or assisted reproductive technologies is practised, but further developments to ensure the widespread success of this procedure are still underway (Donnez & Dolmans 2017, Fisch & Abir 2018). New research in mice has identified another chemotherapeutic, dacarbazine, that contributes to primordial follicle depletion (Winship *et al.* 2018), which urges further research into its effect on the ovarian reserve in humans.

Genetic factors

Gene alterations, loss-of-function mutations, and whole chromosomal abnormalities, all demonstrate an ability to alter the rate of primordial follicle activation and thus affect female fertility. Abnormalities of the X chromosome such as deletions, duplications or complete ablation (i.e. Turner syndrome 45XO karyotype) contribute substantially to defects in ovarian development and later problems in fertility (Cordts *et al.* 2011). Some estimates suggest that up to 12% of cases of genetic POI are induced by errors within the X chromosome gene complement (Goswami & Conway 2005, Qin *et al.* 2014). While particular regions on the X chromosome have been identified as vulnerable with regard to the development of ovarian disorders such as POI, it appears that the complex interplay of these genes during folliculogenesis makes it difficult to define one particular causative agent (Chapman *et al.* 2015). Molecular and cytogenetic analyses on the types of genetic abnormalities present in POI patients have identified a 'critical' region on the long arm of the X chromosome (Xq13-27) that is frequently associated with this disease (reviewed in; Persani *et al.* 2009). Additionally, a number of genes have been associated with POI that regulate primordial follicle recruitment and the gonadotrophin-independent phase of follicular growth (reviewed in Huhtaniemi *et al.* 2018). Several of these have already been established in primordial follicle activation literature as discussed above and include PI3K/AKT/mTOR signalling, FOXL2 and TGFB signalling.

Despite the range of genetic factors known to cause a rapid depletion in available oocytes and the subsequent onset of POI, more genomic interrogation and cellular research is required to reveal the interactions between these factors and determine how they regulate the ovarian reserve through primordial follicle activation.

Environmental factors

Environmental factors with demonstrated impacts on fertility are numerous and are often linked to systemic effects not specifically related to the depletion of the ovarian reserve. However, there are multiple factors that have been directly linked to a decrease in the size of the primordial follicle pool or an increase in the rate of recruitment – both with implications for the development of POI.

Cigarette smoking has the capacity to directly affect the mammalian follicle reserve as evidenced through animal studies (Jurisicova *et al.* 2007, Gannon *et al.* 2012); yet, findings from human studies on primordial follicle populations affected by smoking remain inconsistent (Caserta *et al.* 2013, Peck *et al.* 2016). However, in a cohort of POI patients in Korea, cigarette smoking was strongly associated with an increased risk of the development of POI (Chang *et al.* 2007). In the human fetal ovary, maternal smoke exposure was found to activate aryl hydrocarbon receptor (AHR) and reduce germ cell proliferation, with concomitant implications for germ cell loss via downstream promotion of apoptosis (Mamsen *et al.* 2010, Anderson *et al.* 2014). However, further studies in humans are required to determine if AHR-driven depletion of the primordial follicle pool occurs as a direct result of exposure to cigarette smoke constituents.

Phthalates are toxicants commonly used as plasticising agents and prone to leaching into the environment (Hannon & Flaws 2015). Human fetal ovaries exposed *in vitro* to mono-(2ethylhexyl) phthalate displayed altered lipid synthesis (Muczynski *et al.* 2012). Another phthalate, butyl benzyl phthalate has been demonstrated to decrease the viability of granulosa cells through AHR activation, which, as outlined above, is detrimental to follicular survival rates (Chen *et al.* 2012). These findings suggest ovarian dysfunction that may contribute to a loss in fertility as a consequence of phthalate exposure.

Bisphenol A (BPA) is another mass-produced toxicant that is widely present by virtue of its use in plastics for packaging and resins. BPA affects the ovaries as it has a similar molecular structure to estrogens and can bind to estrogen receptor alpha (Craig *et al.* 2011). Several animal studies have strongly linked BPA exposure to follicle depletion, and this effect is observed regardless of whether exposure occurs *in utero*, postnatally or during adulthood (reviewed in Richardson *et al.* 2014). Consequently, some countries have implemented bans on BPA use based on these animal studies, as human data on the reproductive consequences of BPA remain scarce (Richardson *et al.* 2014, Mathew & Mahalingaiah 2019). Coincidently, in a study of infertile women, those with higher than average urinary BPA were identified as having a decreased ovarian reserve, and another study explored IVF outcomes for women with higher serum BPA and observed lower pregnancy rates and higher association with miscarriage (Sugiura-Ogasawara *et al.* 2005, Lamb *et al.* 2008).

Cohorts, case studies and genome-wide association studies of POI have been instrumental in our understanding of human primordial follicle activation; yet, there are still substantial gaps in the knowledge of this process. Research is currently being focused towards extrapolating this genomic knowledge into further upstream or downstream influences in the molecular pathways implicated to contribute to the future treatment or even prevention of early depletion of the ovarian reserve.

Humans utilise the classical primordial follicle activation pathways differently

To achieve the preservation of female fertility, the mechanisms that control the size of the ovarian reserve, and the rate of primordial follicle recruitment must be determined. Both these events occur in the absence of gonadotrophic regulation and thus are likely to be wholly reliant on intrinsic signalling mechanisms. The complex network of signalling between the oocytes and the granulosa cells, and between neighbouring granulosa cells is critical in maintaining the size of the ovarian reserve and successive follicle development (Edson *et al.* 2009). Granulosa cells are vital for ensuring the development of the follicle, and their contribution whilst substantial is not yet fully elucidated. The status of granulosa cells is a substantial determinant of follicle survival as follicular atresia can occur if insufficient numbers of granulosa cells surround the oocyte or if these cells do not correctly transition to a cuboidal phenotype upon activation (Gougeon & Chainy 1987, Matsuda *et al.* 2012). There exists considerable literature on the signalling networks and molecules known to be involved in primordial follicle activation in model animal studies (see reviews Adhikari & Liu 2009, Kim 2012, Zhang & Liu 2015). For most of the factors discussed below, a regulatory role in primordial follicle recruitment was initially established using rodent 'loss-of-function' studies. In humans, an analogous phenotype to rodent studies demonstrating ovarian reserve depletion via accelerated primordial follicle activation is frequently observed in cases of POI (Fig. 2). The current hypothesis of primordial follicle activation in mice follows that mammalian target of rapamycin complex 1 (mTORC1) is activated in the flattened granulosa cells of primordial follicles, and then KIT ligand produced by activated granulosa cells then activates the oocyte via phosphatidylinositol 3-kinase (PI3K) signalling (Zhang *et al.* 2014). However, the model for primordial follicle activation remains unclear. The following section discusses the factors identified as playing a role in human primordial follicle activation and to what extent that role is similar to rodent literature (Table 1).

The PI3K/AKT/mTOR pathway

The phosphatidylinositol 3-kinase/AKT serine/threonine kinase/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway is involved in cell survival, growth and migration in various tissues, via the modulation of transcription factors (Cantley 2002). In the mammalian ovary, the PI3K/AKT/mTOR pathway is essential for the regulation of primordial follicle activation, with multiple activators and suppressors identified (reviewed in Zhang & Liu 2015). The negative regulator, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), has been established in human primordial follicle granulosa cells at the protein and gene expression level (Goto *et al.* 2007, Makker *et al.* 2014, Zhang *et al.* 2018). In the ovaries, AKT is a prominent kinase in the PI3K/AKT/mTOR pathway and is expressed in both oocytes and granulosa cells of human follicles (Goto *et al.* 2007, Mclaughlin *et al.* 2014). AKT has a wide range of substrates with both direct and indirect roles in follicle activation (Cecconi *et al.* 2012). For women at risk of POI following chemotherapy, primordial follicle activation was stimulated via AKT promotion/PTEN inhibition in ovarian cortical fragments *in vitro* prior to transplantation, and this technique was able to achieve two live births thus far (Kawamura *et al.* 2013, Suzuki *et al.* 2015*b*, Zhai *et al.* 2016). However, the use of PTEN inhibition to initiate primordial follicle activation has been demonstrated to affect follicular survival (Lerer-Serfaty *et al.* 2013, Mclaughlin *et al.* 2014), while in an animal model, prevent DNA repair (Maidarti *et al.* 2019). Thus, this warrants further attention before the technique should be used routinely in human *in vitro* culture pre-transplantation.

The TSC1/mTORC1 subsection of the PI3K/AKT/mTOR pathway was implicated through mouse studies to have a function in driving primordial follicle activation via the differentiation and developmental fates of the

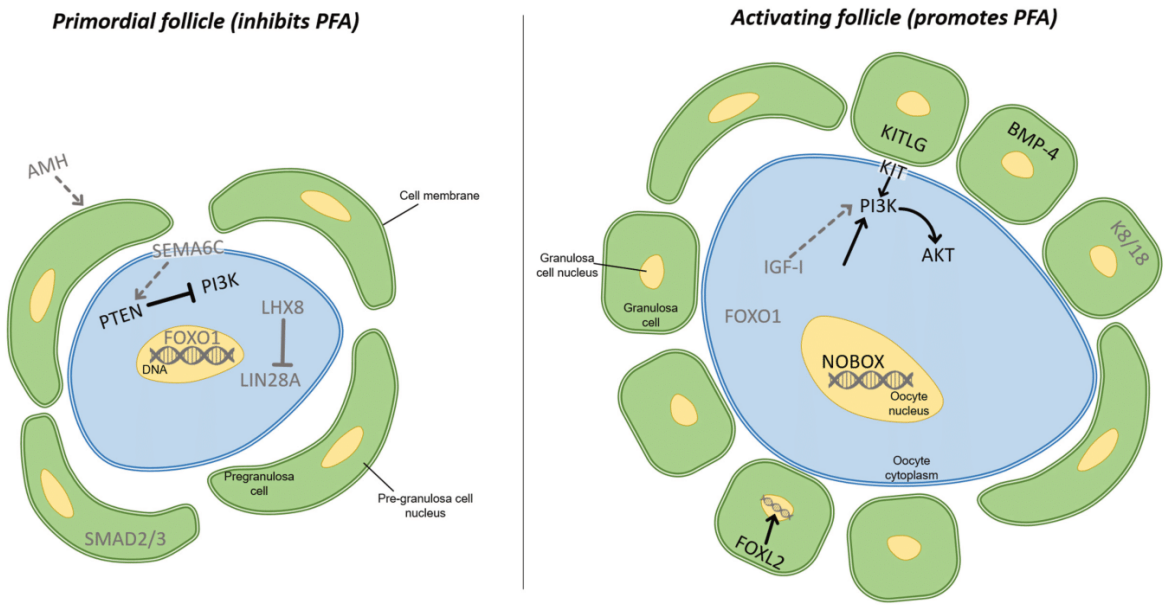

Figure 2 The inhibition and promotion of human primordial follicle activation. Schematics of the factors influencing the maintenance of primordial follicles (the inhibition of primordial follicle activation, PFA) and the activation of primordial follicles (promoting primordial follicle activation). The primordial follicle (left) is an oocyte (blue), surrounded by a single layer of flattened, pre-granulosa cells (green). Once the primordial follicle is activated (right), the oocyte begins to increase in size, and the granulosa cells proliferate and differentiate unto a cuboidal morphology. A number of molecules from classical primordial follicle activation pathways established in animal models, like PI3K/PTEN pathway, signal between oocytes and granulosa cells (black arrows indicate stimulation and interaction, blunt end arrow indicates inhibition) through the cell membrane (represented by double lines) where some proteins reside (i.e. SEMA6C and KIT). Additionally, a suite of transcription factors, like NOBOX, and FOXL2 have been established to interact within the nucleus (yellow) affecting the transcription of their target genes. Several factors have been speculated to influence primordial follicle activation (SEMA6C, K8/18), while others have demonstrated effects with no definitive influence (AMH) and these speculative or putative links are indicated by grey, dotted lines.

granulosa cells (Zhang *et al.* 2014), but additional evidence has shown that TSC1 in oocytes is dispensable for primordial follicle activation (Gorre *et al.* 2014). This suggests that mTORC1 is required for primordial follicle activation in the granulosa cells, but not within the oocyte. Within growing mouse oocytes, however, mTORC2 has been demonstrated to be an essential component of follicular development (Chen *et al.* 2015); yet, its role remains uncharacterised in human oocytes. A recent transcriptome study of human follicles identified an upregulation of the pathway's inhibitor *TSC1* in the oocytes of primary follicles when compared to primordial follicles (Zhang *et al.* 2018), which may suggest a similar role in primordial follicle activation, but whether this role functionally redundant as in animal models is yet to be determined. Furthermore, human follicles treated *in vitro* with an mTORC1 inhibitor exhibited a partial reduction in follicle growth and subsequent decrease in TSC1 mRNA (Grosbois & Demeestere 2018), thus providing supporting evidence for a role in primordial follicle activation.

Despite the PI3K/AKT/mTOR signalling pathways having extensive connections in both primordial follicle activation and the maintenance of quiescence, our fundamental understanding of these processes is still very limited, particularly in the human. Indeed, there are numerous other cellular mechanisms in addition to this pathway known to be involved in primordial follicle activation, and these include transcription factors, growth factors and cytokines.

Transcription factors

Transcription factors specific to the ovaries also contribute to the regulation of primordial follicle activation. A is a known suppressor of mammalian primordial follicle activation, an inducer of follicle atresia and a known substrate of AKT in the PI3K/AKT/mTOR pathway. Human studies using donor cells and tissue samples from women attending fertility clinics have primarily focused on FOXO3A in ovarian cancer, where it has roles in the apoptosis of follicles through AKT activation (Ding *et al.* 2015), granulosa apoptosis *in vitro* (Ono *et al.* 2014) and tumour progression as a positive regulator of p27 (Fei *et al.* 2009). However, aside from apoptosis in ovarian cancer, a defined role of FOXO3A in primordial follicle activation is lacking from human studies, despite evidence in mice that supports this transcription factor

Table 1 Comparison of factors influencing primordial follicle activation between human and animal models.

Molecule	Species evidence	Role in primordial follicle activation	Cell type gene expressed	Cell type protein localised	References
PI3K		Promotes			
	Mouse		Oocyte	Oocyte	Adhikari & Liu (2009)
	Human		Oocyte	Oocyte	Makker *et al.* (2014), Sun *et al.* (2015), Zhang *et al.* (2018)
PTEN		Inhibits			
	Mouse		Oocyte	Oocyte	Adhikari & Liu (2009)
	Human		Oocyte	Oocyte	Goto *et al.* (2007)
AKT		Promotes			
	Mouse		Oocyte and granulosa	Oocyte and granulosa	Adhikari & Liu (2009)
	Human		Oocyte	Oocyte and granulosa	Goto *et al.* (2007), McLaughlin *et al.* (2014), Ernst *et al.* (2017)
FOXO3A					
	Mouse	Inhibits	Oocytes	Oocyte	Castrillon *et al.* (2003), John *et al.* (2008)
	Human	Unknown	Unknown	Unknown	Tarnawa *et al.* (2013)
mTORC1		Promotes			
	Mouse		Oocyte and granulosa	Oocyte and granulosa	Gorre *et al.* (2014), Zhang *et al.* (2014)
	Human		Oocyte and granulosa	Oocyte and granulosa	Ernst *et al.* (2017), Ernst *et al.* (2018)
mTORC2					
	Mouse	Promotes	Oocyte	Oocyte	Chen *et al.* (2015)
	Human	Unknown	Unknown	Unknown	
TSC1		Inhibits			
	Mouse		Oocyte and granulosa	Oocyte and granulosa	Gorre *et al.* (2014), Zhang *et al.* (2014)
	Human		Not specified	Not specified	Grosbois & Demeestere (2018), Zhang *et al.* (2018)
LHX8		Inhibits			
	Mouse		Oocyte	Oocyte	Pangas *et al.* (2006), Ren *et al.* (2015)
	Human		Oocyte	Not specified	Kristensen *et al.* (2015)
AMH					
	Rat and mouse	Inhibits	Granulosa	Granulosa	Baarends *et al.* (1995), Durlinger *et al.* (2002)
	Human	Unknown	Granulosa	Granulosa	Schmidt *et al.* (2005), Carlsson *et al.* (2006)
BMP-4		Promotes			
	Mouse		Granulosa and oocyte	granulosa and oocyte	Chang *et al.* (2002), Knight & Glister (2006)
	Human		Granulosa	Oocyte and Granulosa	Ikeda *et al.* (2016), Pierre *et al.* (2016)
BMP-15		Promotes			
	Mouse		Oocyte and granulosa	Oocyte and Granulosa	Chang *et al.* (2002), Knight & Glister (2006), Persani *et al.* (2014)
	Human		Oocyte and granulosa	Oocyte and granulosa	Margulis *et al.* (2009), Manavella *et al.* (2019)

as a key molecular regulator (Castrillon *et al.* 2003, John *et al.* 2008, Chang *et al.* 2015). While a few *FOXO3A* mutations have been identified in POI patients, the causative contribution these mutations may have on the condition requires investigation (Watkins *et al.* 2006), especially considering *FOXO3A* is not expressed in human primordial oocytes, unlike its mouse counterpart (Tarnawa *et al.* 2013). Thus, it is unlikely that FOXO3A maintains the quiescence of primordial follicles in humans as in mice.

The transcription factor Forkhead box L2 (FOXL2) is essential for squamous to cuboidal granulosa cell differentiation and subsequent formation of secondary follicles in animal models (Schmidt *et al.* 2004, Uda *et al.* 2004, Uhlenhaut & Treier 2006). Many studies in human cell lines, and in mice, have reported FOXL2 targets are also involved in apoptosis, differentiation and the cell cycle (as reviewed in Georges *et al.* 2014), further demonstrating the critical functions of this transcription factor. Notably, a study of human granulosa cells demonstrated that FOXL2 transcripts were less abundant in granulosa cells from primary follicles compared to granulosa cells from primordial follicles (Ernst *et al.* 2018), providing preliminary evidence that FOXL2 downregulation between these stages may coincide with a role in primordial follicle activation in humans. Further investigation of the role of this transcription factor in primordial follicle activation is warranted to confirm the role of FOXL2 in human primordial follicles.

A binding partner of FOXL2 is NOBOX, which is involved in folliculogenesis and the regulation of oocyte-specific gene expression (Huntriss *et al.* 2006, Bouilly *et al.* 2014). In mice, NOBOX is critical for early ovarian development and indirectly participates in

primordial follicle activation via its transcriptional targets (Rajkovic *et al.* 2004, Lechowska *et al.* 2011). Recently, NOBOX transcriptional targets in both humans and mice have been identified and include essential oocyte developmental factors such as growth differentiation factor 9 (GDF9) and octamer-binding transcription factor 4 (OCT4) (Choi & Rajkovic 2006, Bayne *et al.* 2015). Additionally, a novel, loss-of-function *NOBOX* mutation was identified in a POI patient (Li *et al.* 2017), providing further evidence of a key role for this gene in primordial follicle activation. These existing studies justify further investigation of the role that NOBOX has in human primordial follicles.

The LIM homeobox 8 (LHX8) transcription factor has long been associated with the suppression of mouse primordial follicle activation by blocking the expression of RNA-binding protein LIN28A, an upstream activator of the PI3K/AKT/mTOR pathway (Pangas *et al.* 2006, Ren *et al.* 2015). In humans, *Lhx8* transcripts were reported to have decreased expression in early primary follicle oocytes compared to primordial follicle oocytes, thus suggesting a role in the primordial to primary transition (Kristensen *et al.* 2015). This finding highlights the importance of identifying the extent of the roles of currently established pathways in primordial follicle activation.

Growth factors of the TGFB superfamily

The TGFB family of growth factors are involved in a range of cellular processes throughout the body, but in the ovaries specifically, they have roles in early ovarian development and follicle growth (Drummond 2005). Anti-Mullerian-inhibiting substance (AMH) is known, via animal models, to be expressed in the growing follicles, localising specifically to the granulosa cells, and has been established as a suppressor of primordial follicle activation (Visser & Themmen 2005). Corroborating studies in human ovaries have shown that when cultured *in vitro*, AMH could inhibit the proportion of primordial follicles being activated (Carlsson *et al.* 2006). However, conflicting evidence from a 4-week culture of human ovarian tissue supplemented with AMH demonstrated that significantly more follicles were activated to enter the growing phase (Schmidt *et al.* 2005). The mechanisms behind AMH's effect on the ovarian reserve remain unclear as even rodent primordial follicles do not express AMH receptors (Baarends *et al.* 1995, Durlinger *et al.* 2002). In mice, it has been established that *Amh* is a transcriptional target of FOXL2, and thus suggesting they operate in conjunction to maintain the ovarian reserve of primordial follicles (Park *et al.* 2014). Recent evidence in humans has identified that FOXL2 controls AMH indirectly through the transcriptional activation of steroidogenic factor-1, which is a regulator of AMH (Jin *et al.* 2016). Further research has established an inverse relationship whereby AMH is able to modulate the amount of FOXL2 in human granulosa cells (Sacchi *et al.* 2017).

Bone morphogenetic proteins (BMPs) 4 and 15 may participate in early-stage folliculogenesis in humans, in line with several animal studies (see reviews Chang *et al.* 2002, Knight & Glister 2006, Persani *et al.* 2014). Preliminary studies in human gonadal cell culture models have identified BMP4 activity in both the somatic and germ cells during the establishment of the ovarian reserve via the promotion of primordial germ cell apoptosis and the differentiation of pre-granulosa cells (Childs *et al.* 2010, Bayne *et al.* 2016). Studies have also identified the involvement of BMP4 in facilitating the primordial to primary follicle transition in both mouse (Ding *et al.* 2013) and human ovary culture (Ikeda *et al.* 2016). *In vitro* treatment of cultured human GCs with BMP4 and/or 15 induced AMHR2 gene expression (Pierre *et al.* 2016) and this work built on that in sheep models (Estienne *et al.* 2015).

This section has provided an overview of the critical pathways, transcriptional regulators and growth factors that contribute to either the maintenance of the primordial follicle reserve or its activation and subsequent depletion in humans. Many of the factors traditionally associated with primordial follicle activation research have been studied extensively in rodent model systems, with a large portion as yet unstudied in humans or contributing inconsistencies between species. These contrasting studies highlight the importance of continued research in this field to determine if current animal-based research is consistent in humans or warrants the development of more suitable models.

Current research into primordial follicle activation

New insights into primordial follicle activation have arisen primarily from transcriptomic studies, in addition to conditional expression and knockdown studies in animals. Within this research, new factors are identified that link to already established pathways and events. Attention is also being focused towards how intraovarian factors derived from the granulosa cells and the extracellular matrix contribute to the activation status of the follicle.

A family of histone deacetylases, sirtuins, maintain homeostasis throughout the body, responding to changes in metabolism, inflammation and ageing (Vachharajani *et al.* 2016, Grabowska *et al.* 2017). Early work identified that sirtuin 1-null mice were infertile, with no exposition of the underpinning mechanism (Mcburney *et al.* 2003). The presence of sirtuins (SIRTs) has been confirmed in the ovaries of humans, including in the oocyte and granulosa cells, with little evidence, as yet, of their function and how they may contribute to the maintenance of the ovarian reserve or folliculogenesis (Tatone *et al.* 2018). However, SIRTs have recently been linked to the mTOR signalling pathway in rat ovaries,

capable of targeting the activation inhibitor FOXO3a in order to control primordial follicle activation in response to environmental cues like nutrient status (reviewed in Tatone *et al.* 2018). An upstream regulator of the PI3K/AKT/mTOR pathway, Semaphorin 6C (SEMA6C) (Fig. 2), has been newly identified to suppress primordial follicle activation in mice (Zhou *et al.* 2018). Semaphorins were traditionally characterised in the brain, but after links to extensive biological functions in other tissues, they were also found to be functional in human ovaries (Borgbo *et al.* 2013).

A recent study of the transcriptome of human primordial and primary follicles has revealed some novel proteins that may be associated with this developmental transition. Notably, an additional Forkhead transcription factor, *FOXO1*, which exhibited a transcriptional decrease during the primordial to primary transition (Ernst *et al.* 2017). This decrease was accompanied by a subsequent relocation of FOXO1 protein from the oocyte nucleus to cytoplasm indicating a similar role to the well-established function of FOXO3A. Members of the eukaryotic translation initiation factor 2 (EIF2) signalling pathway were also identified to be upregulated during the primordial to primary transition. In particular, the EIF4E gene was observed to increase at the transcript and protein level (Ernst *et al.* 2017). EIF4E traditionally facilitates the translation of stored mRNA in oocytes (Henderson *et al.* 2009).

There has also been growing interest in the TATA-binding proteins (TBPs) and the oocyte-specific TBP2. Emerging evidence in mice has identified the ontogeny of the expression patterns of this protein, which suggests it has a role in the primordial to primary transition – contributing to transcription status of the follicle (Schultz *et al.* 2018). However, studies in human POI cohorts reveal conflicting data, with overexpression observed in some cases, and other studies contend that TBPs do not contribute to POI (Tsuiko *et al.* 2016, Wang *et al.* 2016). A new potential marker of primordial follicles about to undergo activation is the expression status of keratin. The presence of the keratin 8/18 heterodimer (K8/K18) in granulosa cells was strongly correlated with survival status in addition to granulosa cells undergoing the squamous to cuboidal transition (Gaytan *et al.* 2018). Indeed, transcriptional silencing of K8/K18 using siRNA interference in human granulosa cell-like KGNs induced apoptosis (Trisdale *et al.* 2016). This evidence, while still in preliminary stages, prompts further investigation into the role of K8/18, and other typically epithelial-related proteins in granulosa cell physiology. Insulin-like growth factors (IGFs) are traditionally connected to proliferation and antiapoptotic signalling pathways in the ovaries (reviewed in Amutha & Rajkumar 2017) and have now been demonstrated to promote follicle growth via the PI3K/AKT/mTOR pathway in sheep (Bezerra *et al.* 2018), with further evidence demonstrating that transcripts from members of IGF1 signalling were differently expressed in human primordial and primary granulosa cells (Ernst *et al.* 2017, Steffensen *et al.* 2018). The cause for differential IGF1 expression between these two cell stages requires further investigation to determine if IGF1 expression is indeed linked to the activation of primordial follicles. However, the role of IGFs and whether they can modulate activation *in vivo* through androgens or PI3K signalling is yet to be validated.

The extracellular matrix within the ovary is known to be essential for granulosa cell survival and proliferation, and as such is an important consideration in ovarian developmental abnormalities (Berkholtz *et al.* 2006). An appropriately rigid ovarian extracellular environment may be a necessary requirement for follicle survival and has been implicated in the induction of conditions such as POI via the loss of factors essential for maintaining stromal thickness, like FOXL2 (Woodruff & Shea 2011). A recent finding in cat ovarian studies has identified a potential link to primordial follicle activation within a group of extracellular matrix enzymes, the matrix metalloproteinases (MMPs) (Fujihara *et al.* 2018). The activity of MMP9 was stimulated *in vitro* via a high dose of retinoic acid, which led to an increase in the number of follicles undergoing primordial follicle activation (Fujihara *et al.* 2018). Indeed, other growth factors involved in primordial follicle activation (including TGFB superfamily members) are capable of binding to ECM components (Smith *et al.* 1999), and thus, the availability of these growth factors may be able to be regulated by the extracellular matrix and ensure the survival of primordial follicles. Recent evidence in the mouse has linked the expression of other TGFB signalling pathway members, the downstream SMAD2/3, to the inhibition of primordial follicle activation by preventing granulosa cell proliferation (Hardy *et al.* 2018). While current laboratory research is focused on dissecting primordial follicle activation pathways, in clinical research, the challenges lie in diagnostics and controlling the growth and maturation of captured primordial follicles.

Future clinical directions towards harnessing the primordial follicle

By understanding the factors controlling primordial follicle recruitment, we aim to provide POI patients with targeted intervention to prolong their reproductive lifespan. The development and validation of markers and tests that will enable practitioners to identify at risk women are crucial, and with new advancements in technology, there is hope for novel diagnostics and treatments in the coming decades. The technique of optical coherence tomography imaging, used commonly in ophthalmology, was utilised to assess accurately (when compared to histological controls) cortical ovarian tissue sections of chemotherapy patients (Wang *et al.* 2015), indicating its future potential as a non-invasive method for primordial follicle assessment.

A technique for the preservation of fertility still regarded as experimental, but used by clinicians nonetheless, particularly in patients undergoing chemotherapeutic treatment or who have a reduced ovarian reserve, is the surgical removal of small fragment(s) of the ovarian cortex, where primordial follicles reside (Oktay *et al.* 1997, Mclaughlin *et al.* 2015). Intact whole ovaries can also be removed and preserved to excise fragments for later use (Gellert *et al.* 2018). Fragments can be cryopreserved until such time that they are transplanted back into the patient, with follicle activation stimulated *in vivo* (post transplantation), and this strategy has now been successful in producing over 130 live births (Demeestere *et al.* 2015, Donnez & Dolmans 2017, Shapira *et al.* 2018). However, the revascularisation of this transplanted cortical tissue remains a limiting factor in treating infertility. Despite these limitations, promising evidence has emerged through the use of engineered endothelial cells expressing AMH. In a mouse model, the co-transplantation of these engineered epithelial cells with cryopreserved tissue has revealed both the promotion of quiescence in primordial follicles and increased perfusion (Man *et al.* 2018). Alternate efforts to promote angiogenesis of transplanted human ovarian tissue using a mouse model has also been achieved via the assistance of adipose tissue-derived stem cells. The adipose-derived stem cells were preseeded onto the grafting site prior to transplantation of human ovarian tissue and were able to differentiate into human blood vessels and support ovarian survival (Manavella *et al.* 2019).

Ovarian cortical tissue fragments may also be used to grow follicles *in vitro* via an 'artificial ovary' – typically a biological matrix of materials like fibrin, collagen and alginate (Telfer & Fauser 2016, Kallen *et al.* 2018). These models are able to elicit patterns of hormonal fluctuations and growth of human follicles to the antral stage in a manner closely resembling those observed *in vivo* (Skory *et al.* 2015). Ovarian cortical tissue fragments can be directly placed within the matrix and cultured to grow mature follicles (Laronda *et al.* 2014). Primordial follicles can also be isolated from the tissue before being placed in the artificial ovary for activation; they may also be activated *in vitro* prior to being placed in the matrix (Mclaughlin *et al.* 2011, Chiti *et al.* 2017). Alternatively, primary or secondary follicles can be removed from the tissue fragment and cultured successfully in a hydrogel matrix, with ovulation observed in mice, and a small number of meiotically competent metaphase II stage oocytes achieved in human follicles after IVM (Skory *et al.* 2015, Xiao *et al.* 2015). While these methods are still in early development, it is hoped that they will maximise the survival and retention of primordial follicles obtained from patients for future *in vitro* maturation and subsequent IVF.

Despite these novel developments, the fact remains that *in vitro* control over the activation of primordial follicles and future developmental competency is yet to be realised in human oocytes, and this is fundamentally linked to our limited understanding of the process of primordial follicle activation. Ovarian cortical tissue culture usually leads to mass spontaneous, uncontrolled primordial follicle activation, and thus future challenges lie in advancing the culture media and 3D support structures to include the necessary inhibitors to allow the timing of activation to occur in an appropriate and controlled manner (reviewed in Bertoldo *et al.* 2018). This mass activation that occurs *in vitro* has recently been tied to disruptions in Hippo signalling caused by cortex fragmentation, specifically by the movement of Hippo pathway effector, yes-associated protein (YAP), into the nucleus of granulosa cells in humans and mice. The translocation of YAP subsequently introduced growth factors and apoptosis inhibitors, which resulted in follicle growth, indicating a positive influence on primordial follicle activation (Grosbois & Demeestere 2018). This activity was subsequently found to be mediated via AKT of the PI3K/AKT/mTOR pathway (Hu *et al.* 2019), thus demonstrating roles for Hippo-yap signalling in regulating primordial follicle activation, and new potential targets for future drug developments *in vitro* fertility preservation.

Conclusion

The committing step of primordial follicle activation and the regulated depletion of the ovarian reserve remain barriers to current attempts to preserve fertility, particularly in cases of POI. Previous studies have focused on dissecting intraovarian pathways involved in the growth and differentiation of the follicle. However, the reliance on animal models has resulted in some limitations, with findings in human studies not always synonymous. While the aetiology of POI is complex and inducible by internal and external factors, future research into controlling the rate of activation may provide strategies for early diagnosis or prevention. The clinical need for solutions to maintain the primordial follicle pool, particularly in cases where girls and young women must undergo chemotherapy, requires a greater focus in human studies, coupled with the development of robust modelling systems such as those discussed in this review. Enhancing the knowledge of primordial follicle activation, and the factors that facilitate the entry to this process will not only improve outcomes for those at risk of premature fertility loss but may provide the key to preventing these conditions altogether.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Chapter 3: Navigating the role of the granulosa cell during primordial follicle activation

3.1 – Section overview

During the project design phase for the molecular component of this thesis, it was clear that there was a need for the development and optimisation of a technique for the mass collection of granulosa cells suitable for next-generation RNA sequencing (RNAseq). I aimed to isolate and analyse the role of granulosa cells to gain insights into their gene expression during primordial follicle activation.

Ovaries are dense, fibrous tissues containing thousands of follicles, each of which tightly hold a single oocyte connected to a number of granulosa cells ranging from 5 to more than 500 depending on the stage of follicle. Ultimately, a modified method of enzymatic digestion was developed using Collagenase II, known for its ability to disrupt follicular structure. To obtain sufficient depth of sequencing during RNA-seq, high quality RNA with an RNA Integrity Number (RIN) ≥8, and total quantity ~50 ng is required. Thus, I took advantage of the initial wave of primordial follicle activation in the mouse ovary that occurs after postnatal day (PND) one to capture mass quantities of granulosa cells from primordial follicles (present in PND1), and from follicles undergoing activation and at the primary follicle stage (which are a dominant follicle stage at PND4). I optimised the protocol to efficiently yield enough cells for RNA-seq replicates, and this was made simpler by only having to use neonatal females from one litter per sample (usually between 2 and 5 mice). The following section comprises of the outcome of methodological optimisation, and contributes to a methodology manuscript reporting on the current temporary culture isolation methodology and an additional method utilising filtration which is presently under review in *Molecular Human Reproduction.*

3.2 – A Protocol for the isolation of mouse neonatal granulosa cells to assess primordial follicle activation

Introduction

The process of primordial follicle activation is the initial committing step in the growth of an ovarian follicle for the eventual goal of ovulation. Through this process a dormant, primordial follicle grows and differentiates into a primary follicle. Primordial follicle activation is a dynamic and tightly controlled process governed by many as yet unknown factors (reviewed in Kallen et al. (2018)). During early ovary development, granulosa cells represent the dominant somatic cell in the ovary. These granulosa cells are responsible for driving primordial follicle activation by facilitating signalling and ensuring follicle survival (Zhang et al., 2014). However, the exact mechanistic function of the granulosa cell in follicle activation, has yet to be investigated in isolation from the oocyte.

There are technical challenges to overcome when seeking to obtain large quantities of granulosa cells for analysis, and these centre on the tight association between granulosa cells and the oocyte, in addition to the lack of knowledge surrounding intrinsic factors distinguishing granulosa cells from primordial and primary follicles. Common methods of tissue analysis like histological sections, or even whole tissue clearing studies (now achieved in ovaries Feng et al. (2017)) observe the cells at a static point in time and always in the context of the follicular structure. Additionally, the isolation of follicles from the ovary, and the use of laser capture microdissection (as in Bonnet et al. (2011); Dolmans et al. (2006); Oktay et al. (1997)) require significant and laborious manual effort into either classifying whole follicles by their diameter using pre-defined criteria (such as Pedersen and Peters (1968)), or by visually identifying each cell of interest to be isolated.

In other heterogeneous cell populations, the isolation of a particular subset of cells is typically performed via Fluorescence-activated Cell Sorting (FACS). However, there are currently no reliable cell surface markers capable of distinguishing the primordial follicle granulosa cells from the primary follicle granulosa cells. Thus, there is a demonstrated need for a method of separating both primordial and primary granulosa cells from the follicle that requires less manual input and to achieve sufficient yield for both standard and advanced laboratory procedures like extraction of protein, DNA, and RNA, immunocytochemistry, discovery proteomics and mass spec, RNA seq. Such a technique should keep cells alive, ideally within minimal experimental steps and using no prior fixation or staining to maintain original cellular expression and other physiological characteristics.

An ideal solution to each of the challenges above is the utilisation of a modified approach to enzymatic dissociation, which is a procedure established in ovarian studies for decades. However, the primary outcomes of historic studies utilising this technique have largely centred on the isolation of whole follicles rather than individual cells (Dolmans et al., 2006), or isolating granulosa cells from mature ovaries (Morbeck et al., 1993). Neither approach distinguishes granulosa cells based on the follicular stage from which they originated. In the mature ovary, granulosa cells have a range of different roles, from the dormant, undifferentiated pregranulosa cell in the primordial follicle, to the steroidogenic, mitotic mural granulosa cell of the antral follicle (Matsuda et al., 2012). When analysing the activation of primordial follicles into primary follicles, the separation of granulosa cells based on follicle type is crucial. Enzymatic dissociation typically relies on the breakdown of the outer structure of the ovary (usually via collagenase, liberase or papain), to release the cells or follicular structures. Collagenase is an efficient enzyme for isolating individual cells in the ovary as it is known to breakdown theca cells and disrupt follicular structure (Newton et al., 1999). Our method is a modified collagenase dissociation featuring the addition of a temporary culture

step, thereby allowing the granulosa cells to adhere to the bottom of the plate while oocytes and debris remain suspended in the media. Herein we present a new method of isolating mass quantities of granulosa cells from primordial, primary, and activating follicles by exploiting the initial wave of primordial follicle activation in the neonatal mouse ovary.

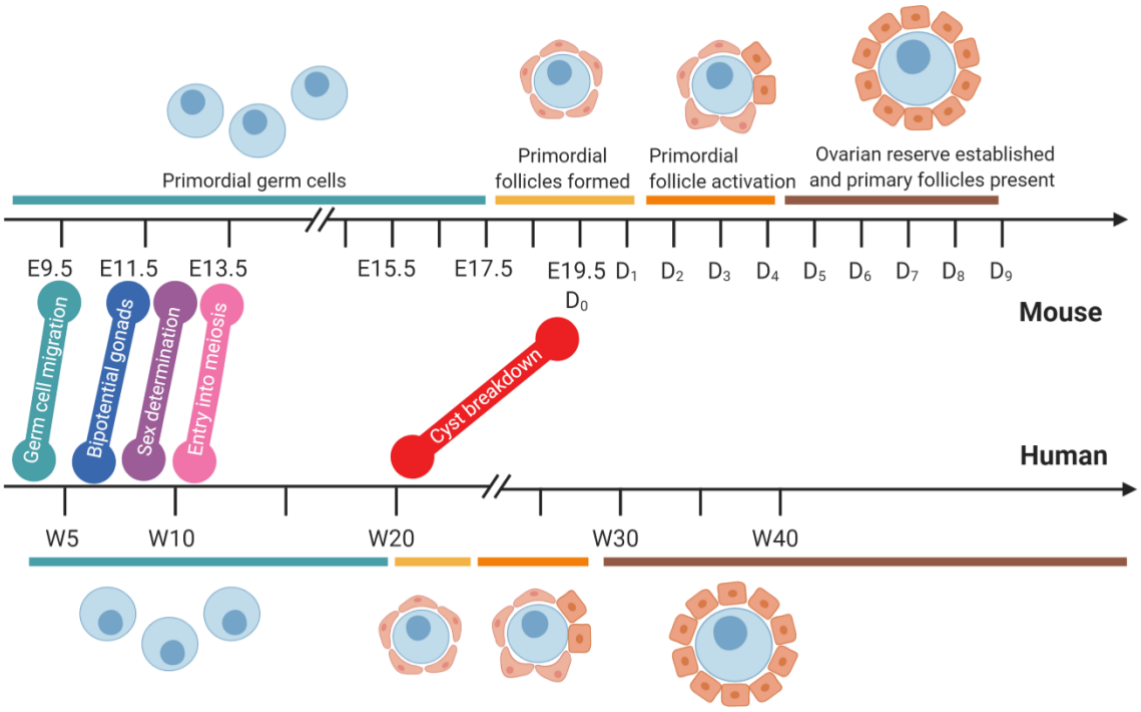

Figure 1: Relative timelines of early folliculogenesis in mice and humans. A timeline of development for mice E=embryonic day, and D=days post birth; and humans with W=weeks post coitus. Schematic representation of major germ cell populations (top and bottom of timeline) and corresponding events in early folliculogenesis (centre between timelines) of both species. Primordial germ cells (teal bar) migrate to the bipotential gonad before sex determination occurs. Following sex determination, germ cells enter meiosis and increase in number, forming cysts. Germ cell cysts, also known as nests, breakdown to form primordial follicles (yellow bar), then the initial wave of primordial follicle activation occurs (orange bar) which sets the ovarian reserve, leaving a proportion of primary follicles (brown bar) present in the ovary. Figure adapted from (Sarraj and Drummond 2012), figure made using Biorender.com.

In the mammalian ovary, there is an established, initial wave of mass primordial follicle activation which drastically depletes the size of the ovarian reserve of primordial follicles (McGee and Hsueh, 2000). The reasons for this activation event are largely unknown, yet follicles activated in the initial wave do contribute to fertility early in reproductive life (Bristol-Gould et al., 2006; Mork et al., 2012; Zheng et al., 2014).

Mice can be used as a model of ovarian development, as the early ovary development timelines are similar, albeit more rapid in the mouse (Sarraj and Drummond, 2012) (Figure 1). In the mouse, primordial follicles develop soon after birth, with up to 89% of oocytes encapsulated within primordial follicles and the rest undergoing germ cell nest breakdown toward the fate of follicle encapsulation or atresia (Pepling and Spradling, 2001). The initial wave of primordial follicle activation causes a large increase in the proportion of primary follicles in the first few days after birth (Bristol-Gould et al., 2006). Then, by postnatal day 4, the ovarian reserve of primordial follicles is markedly reduced and continues on the slow trajectory of depletion throughout reproductive life (Kerr et al., 2013). This protocol utilises collagenase-induced dissociation of ovaries at distinct time points which align to the follicle stage of interest being the dominant follicle type in C57BL/6 mice which are a model of human ovarian follicle development (see Sutherland et al., 2018). We present a protocol for dissociation of postnatal day (PND) 1 ovaries to harvest granulosa cells from primordial follicles, and PND4 ovaries to harvest granulosa cells from activating and primary follicles.

Materials and Methods

Reagents

Antiseptic solution (Perrigo Australia, RIO00802F, used in this protocol)

Ascorbic acid (Sigma, A0278) made up to 5 mg/mL in MilliQ water

Bovine serum albumen (BSA) (Sigma, A7906)

Collagenase type II (sigma, C6885)

Dulbecco's Modified Essential Medium/Ham's F-12 Medium (DMEM/F12) (Sigma, D6421)

DNase I (Roche, 11284932001)

Fetal bovine serum (FBS) (Life Technologies, 10099141)

Hank’s Balanced Salt Solution (HBSS) (Life Technologies, 14025-092)

Hepes solution, 1 M (Thermofisher, SH30237.01)

Insulin-Transferrin-Selenium (ITS -G) (Life Technologies, 41400-045)

Leibovitz medium (L-15) (Life Technologies, 21083-027)

L-Glutamine, 200 mM (Thermofisher, 25030-81)

Penicillin-streptomycin, 10,000 U/mL (Thermofisher, 15140-122)

Trypan blue (Invitrogen, T10282)

Trypsin/Ethylenediaminetetraacetic acid (EDTA) (Sigma, T4049)

Equipment

The following list includes necessary equipment presented with the technical information of the manufacturers and models that were utilised in the collection of this data. The type of equipment is essential, with technical details are merely auxiliary information.

Benchtop centrifuge compatible with 15 mL tubes and capable of 800 x *g* (ELMI SkyLine CM-6MT Swing Rotor Centrifuge)

Biosafety cabinet (Contamination Control Laboratories Biological Safety Cabinet Class II)

Cell counter (manual cell counter used in this protocol)

Cell culture plate, 6-well (Greiner bio-one, Cellstar)

Centrifuge tubes, 15 mL (Greiner Bio-one, 188261)

CO_2 incubator (Thermo Scientific Heracell 150, with 5% CO_2)

Filter, 0.22 μm size (Sarstedt, 83.1826.001)

Haemocytometer

Heated microscope stage

Microcentrifuge (Thermo Scientific Heraeus Pico17)

Microcentrifuge tubes, 1.5 mL (Sarstedt, 72.690.001)

Round bottom polypropylene tube with cap for aeration (BD, 352059)

Serological pipettes, filtered, 10 mL and 25 mL volumes (Sarstedt)

Small petri dish, IVF petri dish (Thermofisher, 150255)

Stereo microscope (Olympus SZ51)

Water bath (ELMI, TW-2.02)

Optional: Cell culture plates, 24 or 48-well

Optional: Glass pipette tips, 20 µL, 200 µL, 1000 µL volumes

Optional: Glass serological pipettes, 1 mL, 10 mL volumes

Optional: Paraformaldehyde diluted to 4% in phospho-buffered saline solution

Solutions

Dissociation working solution: 1 mL of DMEM/F12 in a 1.5 mL microfuge tube heated to 37˚C. 0.02% (2 mg) Collagenase II, and 0.02% (2 mg) DNase I dissolved in solution. To be made fresh each time.

Hank's solution: Under sterile conditions in a 50 mL container 125 mg of BSA, 0. mL 1M Hepes (pH 8.0) was added, and brought to 50 mL with HBSS. Filter sterilised and stored at 4˚C until use.

Ovary culture media: Under sterile conditions, in a 50 mL container, add 22.07 mL DMEM/F12 media, 25 mg BSA, 1 mL penicillin-streptomycin, 1.25 mL FBS, 125 µL ITS-G, and 78 µL. To be stored at 4˚C and heated to 37˚C in a water bath before use.

Protocol

ISOLATION OF GRANULOSA CELLS (DAY 1)

1. Prior to specimen collection, prepare a small petri dish containing L-15 media, supplemented with 1% FBS and allow to heat to 37˚C. This protocol has been optimised for sample size of approximately one litter of C57Bl6 mouse ovaries (typically retrieving between 4 -10 ovaries).

Note: In the instance that a substantially greater number of ovaries are to be dissociated, it is optimal to divide the sample and perform multiple dissociations in parallel than to increase the reaction sizes in a single dish or tube.

2. Add harvested ovaries to the dish (see appendix for ovary dissection details). Transfer the dish to a heated stage (37˚C) under a stereo microscope, remove the ovarian bursa and oviduct keeping only ovary tissue for dissociation.

3. Prepare 1 mL of the dissociation working solution (pre-warmed to 37˚C in a water bath) in a round-bottom tube (with a loose-fitting cap for gas exchange), transferring the tissue from L-15 into the dissociation solution.

4. Incubate at 37˚C in 5% CO_2 for 45-60 minutes depending on the size and degradation level of the tissue, until the tissue has released a majority of its contents. Occasionally some clumps of tissue remain, as fibrous tissue does not completely disintegrate. Pipette the solution every 15 minutes to mechanically facilitate the dissociation of the tissue.

Note: once ovary has started to break down, all pipette tips for subsequent steps should be pre-coated with Hank's solution to prevent cells adhering to pipette walls. Glass pipette tips are an optional addition and when pre-coated in Hank's solution, further reduce cell loss.

5. Transfer, using pre-coated pipette tips, the solution to a microfuge tube and centrifuge for 1,500 x *g* for 3 minutes

6. Remove the supernatant and add 1 mL Trypsin-EDTA. Incubate at 37°C (5% CO_2) for 20 minutes (for tissue sizes <10 mg, incubate for 15 minutes).

7. Inactivate Trypsin by the addition of 100 µL FCS, and mix the solution by gently pipetting. Then centrifuge solution at 1,500 x *g* for 3 minutes.

8. Under sterile conditions in a biosafety cabinet to avoid contamination, remove the supernatant and resuspend in 100 µL of ovary culture media pre-heated to 37°C.

9. Remove an aliquot of cells and dilute 1:1 with Trypan Blue for cell count and viability measurements using haemocytometer or other cell counting method.

Note: cell count on day 1 is a homogenised mix of the assortment of cell types in the ovary and cell count numbers may not necessarily reflect the number of cells obtained on day 2.

When the purpose of the dissociation is to extract protein or RNA, proceed with step 10 and day 2, or see protocol: 'preparation for immunocytochemistry'.

10. Where cell count is ≤3 million in total, prepare a 6-well plate for primary culture by adding 2 mL (volume suited for 6-well plate) of ovary culture media per well, with 10 µL ascorbic acid. Add up to 50,000 cells per cm^2 (or around 500,000 cells per well of a 6-well plate) then place in 37°C incubator (with 5% CO_2) for 18 hours. Upscale reagents as necessary where cell count exceeds 3 million by adding cells to a larger flask or including a second 6-well plate.

GRANULOSA CELL COLLECTION (DAY 2)

1. In a biosafety cabinet and using a serological pipette, remove the media from the plate (granulosa cells adhere to the base of the plate) and dispose of in antiseptic solution for wastage.

Note: avoid disturbing the base or side of the plate as much as possible so as to not disrupt the granulosa cells.

2. Rinse adhered cells with 2 mL HBSS per well, by gently dropping the liquid and swirling the plate for 3 to 5 rotations to thoroughly coat the surface of the wells (while avoiding too much agitation to dislodge cells). Withdraw the wash liquid and discard in antiseptic solution.

Note: for all steps where liquid is being exchanged on the plate of cells, do not let the cells dry out. Proceed through these steps quickly, and cover with the lid to reduce drying when unattended.

3. Pre-treat the cells with a short trypsin digestion by add 1 mL of trypsin-EDTA to each well (again, gently dropping the liquid to avoid disruption) and swirling the plate for 10 to 20 seconds. Then, withdraw and discard the trypsin.

4. Add 1 mL of trypsin to each well of the plate, and incubate at 37°C (with 5% CO_2) for 5 minutes, or until cell projections are removed from the surface of plate (Figure 2).

Note: Structure of granulosa cells resembles cultured fibroblasts (Parvari et al., 2016; Sadowska et al., 2015), with adherent cytoplasmic projections and visible speckled lysosomes (numerous in early follicles, see Ndiaye et al. (2015)).

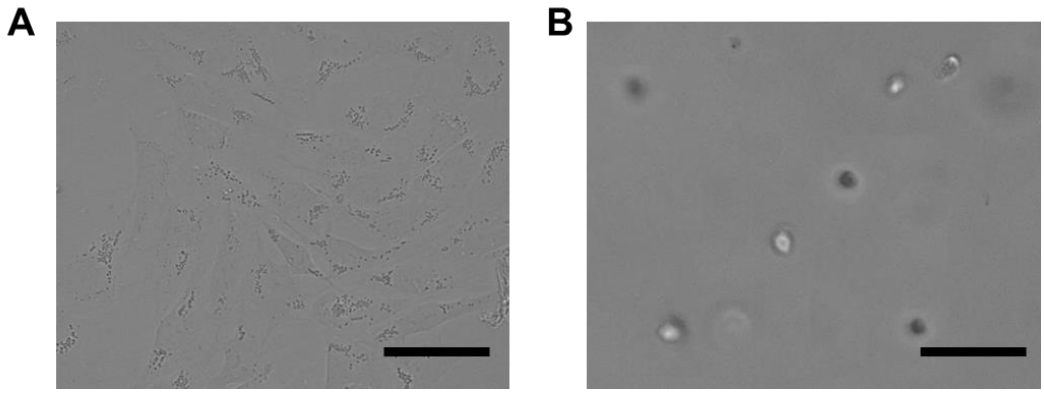

Figure 2: Granulosa cells during isolation visualised via light microscope. (a) Granulosa cells adhering to plate surface following enzymatic dissociation and overnight culture, and (b) granulosa cells after trypsinisation floating in solution with rounded appearance. Images taken at 40 × magnification, scale bar equivalent to 50 µm.

5. Inactivate by adding FBS to the trypsin at a ratio of 1:5 and mix by pipetting.

6. Collect the cells by repeatedly pipetting the trypsin/FBS solution over the surface of the plate to lift any remaining adherent cells and transferring the liquid into a 15 mL centrifuge tube (solution may now be kept at room temperature).

Note: in a 6-well plate, a high proportion of cells adhere along the edge of the wells and the fine tip of a p20 pipette tip can lift these cells with a concentrated ejection of liquid.

7. Wash with 1 mL of HBSS per well, swirling the liquid and pipetting over the surface of the plate, then add the liquid to the 15 mL cell suspension.

Note: Using a microscope on bright field setting, inspect the plate between washes for any remaining cells. An additional wash of 1 mL HBSS can be performed if necessary.

8. Spin the tube containing cells in a benchtop centrifuge for 5 minutes at 800 x *g*.

Note: The final steps may be performed outside of the biosafety cabinet. For final steps it is crucial that pipette tips coming into contact with cells are pre-coated with Hank's solution to avoid cell loss in the plastic tips. Additionally, the pellet may be difficult to see with the naked eye, so ensure the supernatant is carefully removed by pipetting from the opposing side of the tube to the pellet.

9. Discard the supernatant and resuspend in 1 mL of HBSS to wash the cells (transferring to a 1.5 mL tube for efficiency). Spin cells in microfuge for 5 minutes at 1,200 x *g*.

10. Remove the supernatant, and repeat washes by resuspending in HBSS and centrifugation for a total of 3 washes.

11. Discard the supernatant of the final wash and resuspend the cells in a small volume of HBSS for a final cell count as described in day 1 step 9.

12. Centrifuge cells at 1,200 x *g* for 5 minutes and proceed with preparation for either protein or RNA extraction. Alternatively, cell pellets may be stored at -80°C for later use.

PREPARATION FOR IMMUNOCYTOCHEMISTRY

1. Working in a biosafety cabinet, prepare a plate or series of plates (24- or 48-well depending on the number of replicates or cell numbers required) for primary culture by adding an appropriate volume of ovary culture media per well (500 μL for 24-well, and 200 μL for 48-well), with a volume of (5 mg/mL) ascorbic acid totalling 0.5% of the solution. Add up to 50,000 live cells per cm^2, then place in 37°C incubator for 18 hours.

2. Continuing in a biosafety cabinet after incubation, spike the growth media with 4% paraformaldehyde (at a ratio of 1 part 4% paraformaldehyde to 4 parts media) and incubate at room temperature for 2 minutes.

3. Discard liquid into antiseptic solution for wastage and replace with an equivalent volume of 4% paraformaldehyde for 10 minutes at room temperature to fix cells.

Note: for all steps where liquid is being exchanged on the plate of cells, do not let the cells dry out. Proceed through these steps quickly, and cover with the lid to reduce drying when unattended. Additionally, avoid disturbing the base or side of the plate as much as possible so as to not disrupt the granulosa cells.

4. Discard the leftover paraformaldehyde and rinse cells with HBSS to remove excess fixative by gently dropping the liquid and swirling the plate for a few rotations to thoroughly coat the surface of each well. Withdraw the wash liquid and discard in antiseptic solution. Repeat for a second wash.

5. Add 1 mL of HBSS per well (in a 6-well plate), seal the plate with the lid. Can be stored for up to 3 months at 5°C.

Expected results

For an average litter of C57Bl/6 mice (between 4 and 8 ovaries), a sufficient yield of granulosa cells can be accomplished, protocol yields (mean ± SD) $1.36 \pm 0.4 \times 10^6$ total granulosa cells, with additional ovaries increasing the potential cell yield in a weak, positive linear correlation ($r^2 = 0.24$, $p = 0.0131$) as the enzyme approaches saturation point (Figure 3A). We typically achieve 65 ± 11 ng of RNA per 100,000 cells, with no significant differences (N=28, p=0.842) between RNA yield in PND1 and PND4 ovaries (Figure 3B). Thus, for a dissociation using a typical litter size for c57Bl6 mice (between 4 and 8 ovaries), ~1.3×10^6 cells and approximately 845 ng of total RNA can be obtained.

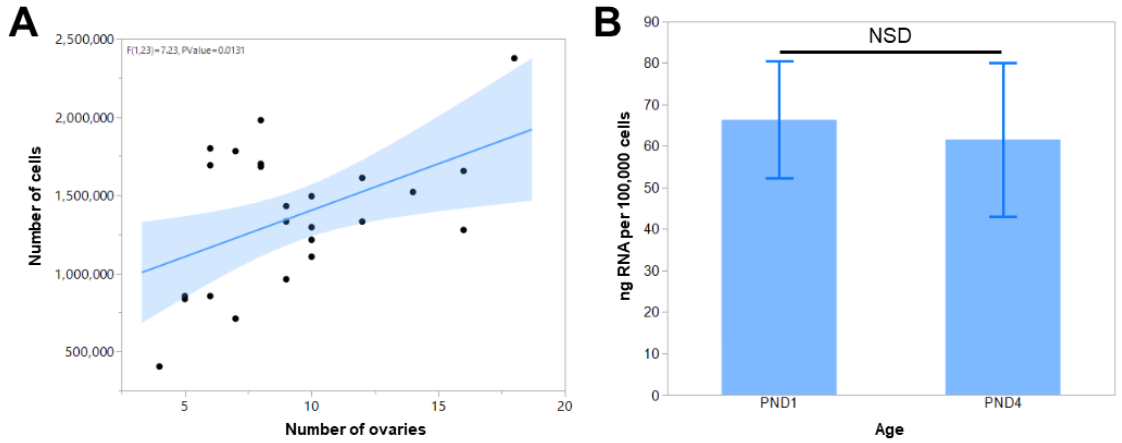

Figure 3: Yield of cells and RNA from granulosa dissociation. (a) granulosa cell yield by number of ovaries dissociated in a single replicate with linear fit (determined via analysis of variance of the slope coefficient, p=0.0131), and (b) RNA yield (ng per 100, 000 cells) by age of ovaries dissociated (compared using student's *t*-test, p=0.8421). Data presented is from 25 dissociation replicates, 11 utilising PND1 ovaries, and 14 replicates of PND4 ovaries. NSD = no significant difference, that is, p≥0.05.

The quality of RNA from dissociated granulosa cells is considerably pure as determined via quality control instrument Agilent 2100 bioanalyzer (see appendix for expanded methods), achieving RIN scores of 9.1 ± 0.24 (supplementary S1), making these samples ideal for next-generation RNA sequencing which typically requires RIN scores > 6 (Kukurba and

Montgomery, 2015). Differences in RNA yield and quality may be due to many factors including, but not limited to: loss of viability of cells obtained after culture, cell numbers, preparation of reagents and buffers, for a troubleshooting guide see Table 1.

Table 1: Troubleshooting guide

Issue	Possible cause(s)	Advice for solution
Ovary tissue not breaking down during enzymatic dissociation step	Enzymatic failure	Ensure reagents stored and used correctly, that solution is completely combined and that the total quantity of tissue is ≤20 mg
	Tissue rigidity	Older ovaries are more fibrous and may require more forceful mechanical agitation when pipetting puncture/tearing with a needle while in the dissociation solution. Additionally, an extra 15-minute incubation in dissociation solution for a total 75 minutes may suffice.
Cells adhering to plate after trypsinisation and wash	Insufficient digestion	Check cells before and after 5-minute incubation with trypsin to ensure they have lifted from the plate. Incubate for a further minute if required. Additionally, tapping plate firmly against a solid surface and pipetting directly onto the plate with force may dislodge any rounded cells remaining on the bottom of the plate
Loss of cell viability after overnight culture	Insufficient nutrients	Ensure ovary culture media is kept at 4ºC when not in use, and always heated to 37ºC before use. Doubling the proportion of FCS in media to 10% may also increase viability.
	Overcrowding on plate	Dilute cell suspension before plating and seed multiple plates, or switch seeding cells in a flask (T25 or T75)
Small/no pellet after collection	Cell loss	Ensure pipette tips and serological pipettes are all coated with Hank's solution before coming into contact with cells. When not in use, store Hank's solution at 4ºC, equilibrate to room temp before use, and do not use following storage exceeding two weeks.

The purity of cell type obtained from performing this dissociation can be determined by immunofluorescent detection of a range of markers indicative of ovarian cell types (see appendix for experimental conditions). Isolated cells stain positively with classical granulosa cell markers GATA4 and FOXL2 (Efimenko et al., 2013; Pisarska et al., 2011) (Figure 4A). Primordial and primary granulosa cell populations were able to be distinguished via

localisation of AMH, which is expressed in growing granulosa cells from the primary to small antral stage (Visser et al., 2006), and thus is expressed in cells dissociated from PND4 ovaries, yet not expressed in cells originating from PND1 ovaries (Figure 4B). It was observed that following overnight culture, there is some loss in viability of the cells as identified by apoptotic cells with condensed nuclei (see Figure 4). Markers for oocytes (GDF9 and DDX4 (Aaltonen et al., 1999; Raz, 2000)) indicated very minimal contamination (Figure 4C) at a proportion of 0.4% (see supplementary S2) compared to granulosa-positive cells (from counts of 300 cells repeated across n=4 biological replicates).

Additionally, there was no expression of theca cell marker, GLI1 (Figure 4D), which is to be expected as theca cells are not observed in the neonatal ovary, but appear once follicles have two or more layers of granulosa cells (Young and McNeilly, 2010), which in the mouse is not until around PND6. While a culture step has the capacity to alter the physiology and gene expression of the cell, RNA transcripts of dissociated cells after culture reveal classic granulosa cell expression compared to very minimal or no detection of other ovarian cell markers (See supplementary S3 and explored later in section 3.4). Thus, the described technique is an efficient way to collect mass quantities of live granulosa cells from primordial, activating and primary follicles for downstream functional analyses without the need for manual cell sorting.

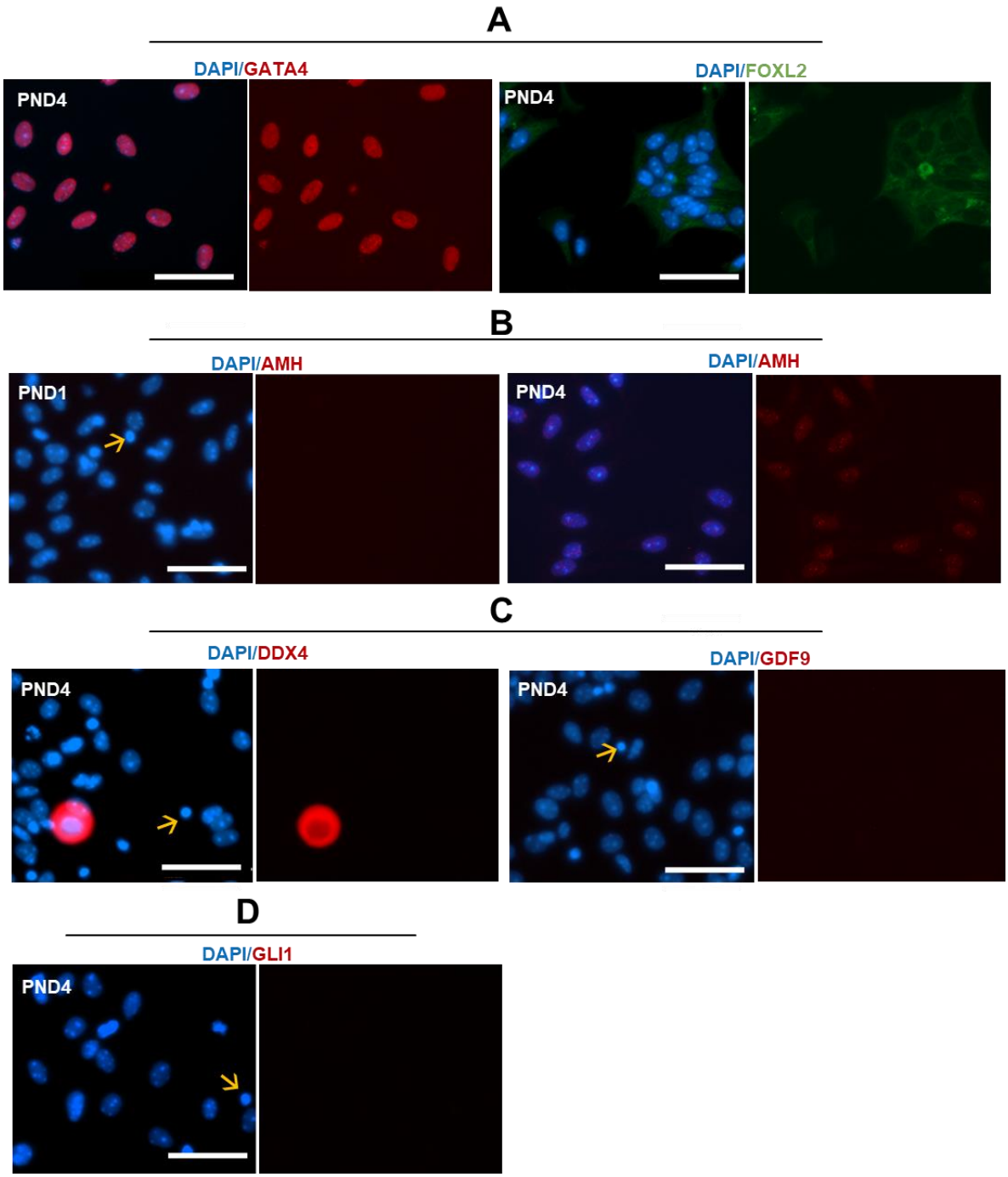

Figure 4: Establishing cell purity using representative markers. Immunofluorescent localisation of ovarian cell markers counterstained with blue nuclear marker (DAPI) (a) granulosa cell markers (GATA4 and FOXL2), (b) mature granulosa cell marker (AMH) in PND1 and PND4 cells, (c) oocyte markers (DDX4 and GDF9), and (d) theca cell marker (GLI1). Arrow indicates apoptotic cells. All experiments performed in PND1 and PND4 n=3 for each group, representative images taken at PND4 stage except where indicated. Images taken at 40 × magnification, scale bar equivalent to 50 µm.

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Appendix

Ethics

C57Bl/6 mice were supplied by the University of Newcastle Animal Services Unit under ethics approval number A-2018-803. All experiments involving the use of animals were conducted in accordance with the Institutes' Animal Care and Ethics Committee guidelines in accordance with Australian NHMRC Guidelines on Ethics in Animal Experimentation.

Animal details

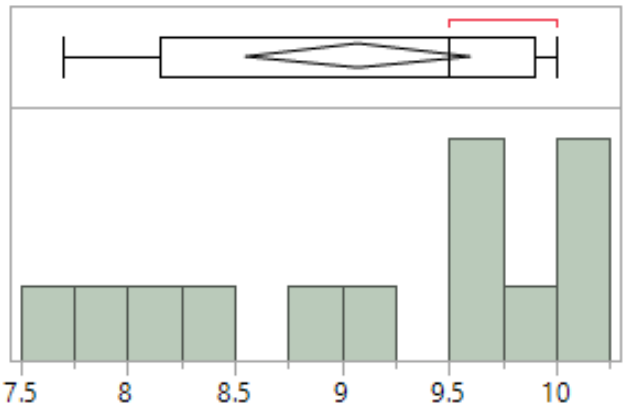
Mice were housed under a controlled lighting regime (16 L: 8 D) at 21–22°C and supplied with food and water *ad libitum*. Neonatal mice (PND1 and PND4) were euthanised by asphyxiation with carbon dioxide, followed by decapitation. Ovaries were collected immediately after euthanasia, and bursa and uterus remnants removed under a dissecting microscope.

Immunocytochemistry

Granulosa cells fixed in 24-well plates (as described in materials and methods) were blocked for non-specific binding by an incubation of 3% BSA, 10% donkey serum in PBS for 1 h at room temperature. Granulosa cells were probed with antibodies specific for GATA4 (ab84593; Abcam), FOXL2 (ab5096; Abcam), AMH (130233; Abcam), DDX4 (ab13840; Abcam), GDF9 (ab93892; Abcam), and GLI1 (ab92611; Abcam) at a dilution of 1:300. Primary antibodies were visualised using either a donkey anti-goat Alexa 555-conjugated secondary antibody (a21432; Invitrogen) or a goat anti-rabbit Alexa 555-conjugated secondary antibody (a21428; Invitrogen) at a dilution of 1:200; DAPI was used as a nuclear counterstain. Slides were imaged using the EVOS FL (AMF4300; Thermofisher).

RNA extraction and quantity

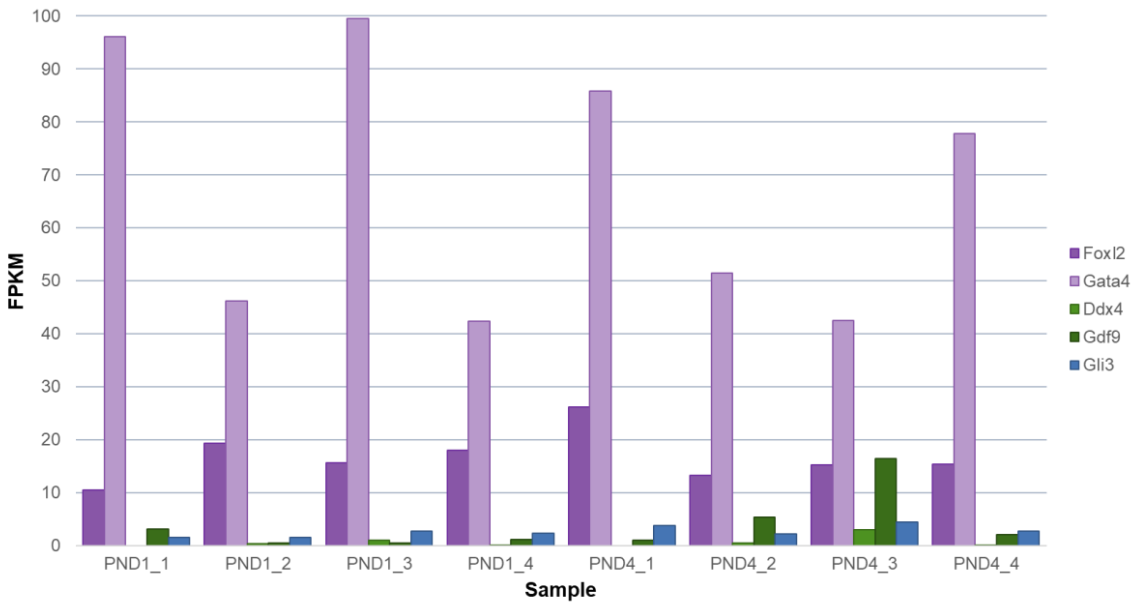
Extractions for granulosa cell RNA were performed using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany; cat no. 74034) as per the manufacturer's instructions. Post-extraction, the synthesis of cDNA was completed using the SuperScript IV VILO system (Invitrogen, Carlsbad, USA; cat no. 11766050). The RNA purity, quality, and concentration were determined using the Agilent Bioanalyser 2100. The samples were prepared for this machine via the Agilent RNA 6000 Nano Assay (5067-1511; Agilent).



S1: Distribution of RIN scores across granulosa cell dissociation replicates

Granulosa cells n(%)	Oocytes n(%)
300 (100)	0 (0)
298 (99.3)	2 (0.7)
299 (99.7)	1 (0.3)
298 (99.3)	2 (0.7)

S2: Granulosa cell counts to determine oocyte contamination



S3: Expression of selected ovarian cell-specific markers. The FPKM (fragments per kilobase of transcript per Million mapped reads) for replicates of isolated cells, with typical cell identity markers. Granulosa cell= purple, Oocyte= green, theca cell= blue. Theca cell markers Ptch1, Gli2, Cyp19a1, Cyp17a1 were not detected.

The following sub-chapter has been formatted as an original research manuscript and submitted to *Biology of Reproduction* journal and is currently undergoing peer reviewed revisions

3.3 – Manuscript overview

Following the successful isolation of granulosa cells, I sought to compare the transcriptome of granulosa cells from primordial follicles to granulosa cells from a mixed population of activating and primary granulosa cells. Within the manuscript below, I first sought to characterise the isolated granulosa cells, expanding on the initial immunolocalisation study from the previous chapter, and through using gene ontology analysis, identify processes commonly occurring in granulosa cells undergoing activation.

The utilisation of next-generation RNA-sequencing (RNAseq), has vastly propelled molecular science, with the opportunity to capture global gene expression of a sample at fixed point in time with high accuracy and repeatability. Herein my aim was to describe the mouse neonatal granulosa cell transcriptome and identify new avenues to investigate in the pursuit of understanding primordial follicle activation. Much of the recent insights into the process of primordial follicle activation comes from new links in previous established pathways, and it is through this method that I successfully identified targets for our downstream studies.

This study provides a valuable platform for future investigation into the potential role of FRZB, POD1, and ZFX particularly, in primordial follicle activation. *Frzb* transcripts were more abundant as granulosa cells were activating, and FRZB protein was identified to interact with WNT3A, which is known to suppress primordial follicle activation through regulating FOXO3A. Thus, we provide preliminary evidence for FRZB as an upstream regulator of primordial follicle activation.

Officially published; Emmalee A Ford, Emily R Frost, Emma L Beckett, Shaun D Roman, Eileen A McLaughlin, Jessie M Sutherland, Transcriptomic profiling of neonatal mouse granulosa cells reveals new insights into primordial follicle activation, Biology of Reproduction, Volume 106, Issue 3, March 2022, Pages 503–514, https://doi.org/10.1093/biolre/ioab193

3.4 – Original research manuscript

Title: Transcriptomic profiling of neonatal mouse granulosa cells reveals new insights into primordial follicle activation

Running Title: Granulosa cell transcriptome during primordial follicle activation

Authors:

Emmalee A Ford [1,2], Emily R Frost[1,2,3], Emma L Beckett[2,4], Shaun D Roman[1,2,5], Eileen A McLaughlin[1,6,7] and Jessie M Sutherland[1,2*†]

Affiliations:

[1] Priority Research Centre for Reproductive Science, Schools of Biomedical Science & Pharmacy and Environmental & Life Sciences, University of Newcastle, Callaghan, New South Wales, Australia, 2308

[2] Hunter Medical Research Institute, New Lambton Heights, New South Wales, Australia, 2305

[3] Stem Cell Biology and Developmental Genetics Laboratory, The Francis Crick Institute, London, United Kingdom, NW1 1AT

[4] School of Environmental & Life Sciences, Faculty of Science, University of Newcastle, Callaghan, New South Wales, Australia, 2308

[5] Priority Research Centre for Drug Development, University of Newcastle, Callaghan, New South Wales, Australia, 2308

[6] School of Science, Western Sydney University, Penrith, Australia, 2750

[7] School of Biological Sciences, Faculty of Science, University of Auckland, Auckland, New Zealand, 1142

*Correspondence: School of Biomedical Science & Pharmacy, the University of Newcastle, Ring Road, Callaghan, NSW 2308. Email: jessie.sutherland@newcastle.edu.au

Keywords: folliculogenesis, granulosa cells, RNAseq, infertility, premature ovarian insufficiency

GEO Accession Number: GSE162927

Abstract

The dormant population of ovarian primordial follicles is determined at birth and serves as the reservoir for future female fertility. Of equal importance to fertility is the rate that primordial follicles activate and enter folliculogenesis. Yet our understanding of the molecular, biochemical, and cellular processes underpinning primordial follicle activation remains limited. The survival of primordial follicles relies on the correct complement and morphology of granulosa cells, which provide signalling factors essential for oocyte and follicular survival. To investigate the contribution of granulosa cells in the primordial-to-primary follicle transition the gene expression profiles of granulosa cells undergoing early differentiation were assessed in a murine model. Ovaries from C57Bl/6 mice were enzymatically dissociated at two time points spanning the initial wave of primordial follicle activation. Post-natal day (PND) 1 ovaries yielded primordial granulosa cells, and PND4 ovaries yielded a mixed population of both primordial and primary granulosa cells. The comparative transcriptome of granulosa cells at these time points was generated via the Illumina NextSeq 500 system which identified 132 significantly differentially expressed transcripts. The qRT-PCR of 8 differentially expressed genes from the dataset significantly validated the RNA sequencing findings, exhibiting consistent expression. This transcriptomic dataset confirmed the expression of factors known to be involved in primordial follicle activation belonging to TGF-B and EIF4E signalling pathways. Following biological network mapping via Ingenuity Pathway Analysis, the functional expression of the protein products of three of the differentially expressed genes,

namely FRZB, POD1 and ZFX, via *in-situ* immunolocalisation in PND4 mouse ovaries was investigated. Finally, evidence is provided that Wnt pathway antagonist, secreted frizzled-related protein 3 (FRZB), interacts with a suppressor of primordial follicle activation WNT3A and may play a role in promoting primordial follicle activation. This study highlights the dynamic changes in gene expression of granulosa cells during primordial follicle activation and provides evidence for a renewed focus into the Wnt signalling pathway's role in primordial follicle activation.

Introduction

Primordial follicle activation is integral to the fertility of sexually reproducing females as it necessary for the follicle development and the committing step to ovulation and subsequent fertilisation. The number of primordial follicles within the ovaries defines the age of menopause in women, and therefore the end of a woman's fertility. Premature ovarian insufficiency (POI) is the premature cessation or absence of ovarian function due to a reduction in the pool of primordial follicles in women before the age of 40 (Shelling, 2010). POI occurs in 1-3% of women, and a common cause of POI is a rapid acceleration in the rate of activation of primordial follicles (Golezar et al., 2019; Haller-Kikkatalo et al., 2015; Nelson, 2009). The process of primordial follicle activation is complex, involving massive parallel molecular, cellular and biochemical events, with limited characterisation (Adhikari and Liu, 2009; Zhang and Liu, 2015). Granulosa cells communicate between neighbouring granulosa cells and intracellularly with the oocyte to coordinate primordial follicle activation (Eppig, 2018), however, the extent of their role in this process is yet to be determined. Importantly, as primordial follicles activate, there must be a sufficient number of granulosa cells supporting the oocyte, and they must all transition correctly to the activated, cuboidal morphological form for successful development (Gougeon and Chainy, 1987; Matsuda et al., 2012). Improving our

understanding of how the rate of primordial follicle activation is controlled is imperative to improving fertility outcomes for women at risk of premature infertility and POI.

Much of the research underpinning our understanding of primordial follicle activation has focused on protein interactions and single gene knockouts (reviewed in (Zhang and Liu, 2015)). Transcriptome studies in primordial follicle activation have been mostly restricted to microarray methodologies or small samples of human data (Hasegawa et al., 2009; Kezele et al., 2005; Kristensen et al., 2015; Yoon et al., 2006), both of which have limitations in identifying unique genes and accounting for sample heterogeneity. For instance, a recent human follicle transcriptome study characterised isolated oocytes and granulosa cells from a range of follicle types, however, these cells originated from only seven patients, six of which had some form of cancer or tumorous growth (Zhang et al., 2018). A recent granulosa cell transcriptome study provided exciting new leads for primordial follicle activation research, yet only samples from three women were collected who were undergoing oophorectomy prior to gonadotoxic treatment of a pathology (Ernst et al., 2018). In each of these studies, the number of cells per sample used for analysis was less than 200.

The use of non-human models overcomes a number of the limitations associated with the study of human samples and is instrumental to our current understanding of primordial follicle activation. (Ford et al., 2020), Animal studies provide important groundwork with advantages such as functional studies via conditional knockouts, drug testing, and robust characterisation through omics technologies. In this study granulosa cells isolated from mouse neonatal ovaries were utilised both before and during the initial wave of primordial follicle activation. These two distinct populations of cells were subjected to RNAseq to compare the gene expression profiles of primordial and activating granulosa cells and identify novel factors contributing to follicle activation. This study presents the first detailed transcriptome comparison of granulosa cells from mouse primordial and activating follicles, we were able to annotate the expression

occurring within granulosa cells and observed a small but significant shift in the transcriptome between the two populations of these cells. From this comprehensive dataset we identified the Wnt pathway inhibitor, secreted frizzle-related protein 3 (FRZB) as significantly increased in expression in activating granulosa cells compared to granulosa cells from primordial follicles and determined an interaction between FZRB and Wnt3A in PND4 granulosa cells. Our findings indicate an important role for Wnt signalling factors in primordial follicle activation.

Methods

Ethics

C57Bl/6 mice were supplied by the University of Newcastle Animal Services Unit under ethics approval number A-2018-803. All experiments involving the use of animals were conducted in accordance with the Institutes' Animal Care and Ethics Committee guidelines.

Tissue and cell collection

Mice were sacrificed at post-natal days one (PND1) and four (PND4). These time points were selected to coincide with immediately prior to the initial wave of primordial follicle activation when the ovary is comprised of only primordial follicles (PND1), and during the initial wave of primordial follicle activation when there is an enriched population of follicles undergoing activation (PND4) (Kerr et al., 2006; Kerr et al., 2013). Follicles that develop during the initial wave of primordial follicle activation do contribute to post-pubertal fertility (McGee and Hsueh, 2000) and thus are used to investigate primordial follicle activation. This approach maximises the quantity and homogeneity of the follicles types of interest retrieved for analysis that would otherwise be confounded by later-stage, gonadotropin-dependent follicles.

Mice were housed under a controlled lighting regime (16 h light: 8 h dark) at 21–22°C and supplied with food and water *ad libitum*. Neonatal mice were euthanised by asphyxiation with carbon dioxide, followed by decapitation. Ovaries were collected immediately after euthanasia and either snap-frozen, fixed in 10% neutral buffered formalin for ≤10 h, or placed into 37°C

Leibovitz's L-15 media supplemented with 1% fetal bovine serum (FBS) for immediate granulosa cell collection. The outer tissue and bursa were removed from the ovary, which was then transferred into Dulbecco Modified Eagle/F-12 Medium (DMEM/F12), containing 0.02% collagenase, and 0.02% DNase I. After incubation for 45-60 minutes at 37°C in 5% CO_2 with gentle pipetting every 15 minutes, cells were retrieved by centrifugation (1,500 *g*, 3'), supernatant removed, and incubated with 0.1% trypsin/EDTA at 37°C in 5% CO_2 for 15-20 minutes.

Cells were recovered by centrifugation (1,500 *g*, 3'), and resuspended in ovary culture media (containing DMEM/F12 with 0.1% bovine serum albumen, 4% penicillin-streptomycin, 5% fetal bovine serum, 0.5% ITS-G and 3% L-Glutamine). An aliquot of cells was removed for cell quantification, and the remaining cells incubated in ovary culture media with 0.5% ascorbic acid 18-24 hours at 37°C for either collection or fixing. When collecting granulosa cells, cells were incubated with 0.1% trypsin/EDTA for five minutes, collected (800 *g*, 15') and washed 3 x 5 minutes at 1,200 *g* in Hanks′ Balanced Salt Solution, counted and stored at -80°C until use. Cell populations were enriched for the cell target of interest, granulosa cells, rather than the other major cell type in neonatal ovaries, the oocytes, as determined by oocyte counts using immunofluorescent targeting. Counts of 300 cells across four biological replicates indicate an average abundance of 0.4% oocytes compared to 99.6% granulosa cells.

RNA extraction and qRT-PCR

Granulosa cell RNA was extracted using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany; cat no. 74034) as per the manufacturer's instructions. Post-extraction, cDNA was synthesised by reverse transcription with the SuperScript IV VILO system (Invitrogen, Carlsbad, USA; cat no. 11766050). Quantitative real-time PCR (qRT-PCR) was performed in triplicate on cDNA with a reaction equivalent to 20 ng of total RNA. Predesigned and validated gene-specific TaqMan Gene Expression Assays (Life Technologies, Carlsbad, USA) were used

for qRT-PCR. Each TaqMan gene expression assay contained gene specific, exon spanning forward and reverse primers for each of the genes of interest (Avpr1a (Mm00444092_m1), Cdkn2b (Mm00483241_m1), Ddr2 (Mm00445615_m1), Fam171a1 (Mm01332727_m1), Frzb (Mm00441378_m1), Ifitm1 (Mm00850040_g1), Rpl19 (Mm02601633_g1), Tcf21 (Mm00448961_m1), Zfx (Mm03053842_s1)) and fluorogenic minor groove binder probes consisting of a target-specific oligonucleotide labelled with a fluorescent dye FAM (6-Carboxyfluorescein) or VIC (2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein), and a non-fluorescent quencher. Data were normalised to the expression of the transcript encoding 60S ribosomal protein L19 (Rpl19). Triplicate expression values of each gene was set relative to the reference gene via the ΔΔCT method (Schmittgen and Livak, 2008), and is presented as the mean ± SEM with statistical analysis determined by unpaired Student's *t*-test. Bonferroni correction for multiple comparison was applied to selected targets.

RNA Sequencing and mapping transcripts

Eight libraries were sequenced on the Illumina NextSeq 2x75bp high output via Auckland Genomics at the University of Auckland. The RNA samples consisted of four replicates each of two groups of mouse granulosa cells (isolated from PND1 and PND4). The bioinformatics team at Auckland Genomics Facility performed the following analyses on the RNA sequencing output. The overall quality of the data was visualised using the program FastqQC (Andrews, 2014). TrimGalore (Lindgreen, 2012), was used to check and remove adapters from the sequences. Sequences with a Phred score less than 30 were trimmed. Reads with a length shorter than 25 base pairs were discarded. Hisat2 (Kim et al., 2015; Pertea et al., 2016) was used to map the cleaned reads to the mouse transcriptome (grcm38). Output sam files from hisat2 were then sorted by genomic position and converted to bam format using samtools sort (Li et al., 2009). To generate expression estimates for each sample, the mapped reads were then assembled into transcripts using StringTie. Differential expression analysis was performed

using the R software package Ballgown (Fu et al., 2018). Low abundance transcripts with a variance less than one across the samples were removed. To view the similarity between the samples, the Pearson correlation and distance between the samples were calculated. Differential gene expression between the two treatments were calculated using FPKM (fragments per kilobase million) values. The data was then sorted by their adjusted p-value (false discovery rate) and absolute log2 fold change values.

In silico *analysis of gene expression*

The analysis of genes expressed within PND1 and PND4 neonatal mouse granulosa cells was undertaken *in silico* using a number of techniques. Briefly, transcript abundance data were assessed via volcano plots to visualise trends associated with differentially accumulating genes in the primordial granulosa cells compared to activating granulosa cells (PND1 versus PND4). The threshold for significant differentially expressed transcripts was defined as having a two-fold change of FPKM in either direction ($\log_2$ fold change ±1), and a significance level of $p \leq 0.05$. Consistency of gene expression among biological replicates for transcripts above the determined threshold was visualised via a heat map of expression (FPKM) for each gene of each replicate normalised to the PND1 average FPKM for the respective gene.

Ingenuity Pathway Analysis (IPA) was used to explore biological networks for differentially expressed genes with significance $p \leq 0.05$. IPA queries a large database of experimental observations between molecules and uses this information to construct biological networks that represent cause-effect relationships between mammalian genes, proteins, and their functions with probability calculations (Krämer et al., 2014). The granulosa cell dataset was interrogated for enrichment of functional pathways using bioinformatic enrichment tools available via the Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.8) (Huang da et al., 2009; Huang et al., 2009). DAVID Gene Ontology (GO) annotation tools were utilised for exploration of biological process (Ashburner et al., 2000) in differentially expressed genes, GO

identifiers identified within the dataset were also subject to enrichment analysis to determine their representation (Mi et al., 2019).

Immunolocalisation

For immunofluorescence of mouse ovaries, tissues were washed with and embedded in Optimal Cutting Temperature (OCT) compound (ProSciTech, IA018), then snap-frozen in dry ice. Blocks were serially sectioned (5 μm thick) with a cryostat (Leica Biosystems) onto Superfrost Plus slides (ThermoFisher; cat no. 4951PLUS4) three ovaries per section for biological triplicate. Before use, slides were dried to room temperature for 5 min and rehydrated in PBS. Heat mediated antigen retrieval was performed by warming slides for 30 min at 65°C in 10 mM sodium citrate buffer (pH 6). To prevent non-specific antibody binding, sections were blocked in phosphate-buffered saline solution with 5% donkey serum and 1% bovine serum albumen for 1.5 h at room temperature. Sections were probed with antibodies in Table 1 (antibody of interest, colocalised with a granulosa cell marker, GATA4 or FOXL2) and incubated overnight at 4°C. After washing, sections were incubated with secondary antibodies Alexa Fluor 488 donkey anti-rabbit IgG (ThermoFisher; cat no. A21206), Alexa Fluor 555 donkey anti-goat IgG (Abcam, cat no. A21432) at a dilution of 1:200 for 1 h at room temperature. Slides were counterstained with 4′-6-Diamidino-2-phenylindole (DAPI) and mounted in anti-fade reagent Mowiol (13% w/v Mowiol4-88, 33% w/v glycerol, 66 mM Tris (pH 8.5), 2.5% w/v 1, 4 diazobcyclo-[2.2.2] octane). Images were taken on an Olympus Fluoview 1000-IX81 confocal microscope (Olympus, Center Valley, USA) under fluorescent optics.

Table 1: Antibody details

Antibody target	Species raised in	Dilution	Company	Catalogue number
FOXL2	Goat	1:100	Abcam	ab5096
FRZB	Goat	1:50	ThermoFisher	PA5-47793
GATA4	Rabbit	1:100	Abcam	ab84593
POD1	Rabbit	1:100	Bios USA	BS-8688R
WNT3A	Rabbit	1:100	Abcam	cat no. ab28472
ZFX	Rabbit	1:200	ThermoFisher	PA5-34376

For immunocytochemistry of fixed granulosa cells, wells were permeabilised in phospho-buffered saline (PBS) with 0.01% Triton-X then blocked for 2 h at room temperature in PBS containing 10% donkey serum and 3% bovine serum albumen. Cells were probed overnight at 4°C with anti-FRZB antibody (ThermoFisher; cat no. PA5-47793), and anti-WNT3A antibody (Abcam, Cambridge, UK, cat no. ab28472), each at a dilution of 1:200. After washing, sections were incubated with secondary antibodies: Alexa Fluor 488 donkey anti-rabbit IgG (ThermoFisher; cat no. A21206), or Alexa Fluor 555 donkey anti-goat IgG (Abcam, cat no. A21432) at a dilution of 1:200 for 1 h at room temperature. Cells were counterstained with 4′-6-Diamidino-2-phenylindole (DAPI) and a layer of PBS was added to prevent drying out. Images were taken on an EVOS FL (AMF4300; Thermofisher).

Duolink proximity ligation assay

Protein interactions were detected via Duolink proximity ligation assay kit (Merck, DUO92105) using an anti-rabbit plus probe and an anti-goat minus probe as according to the manufacturer's instructions. Briefly, cells isolated by dissociation were fixed for immunocytochemistry as above, then blocked with the kit blocking solution for 1 h at room

temperature. Antibodies were applied at a ratio of 1:100 with kit antibody diluent solution and incubated overnight at 4°C. Following this, Duolink probes were added to cells at a ratio of 1:5 with kit diluent solution and incubated for 1 h at 37°C in a humidified chamber and excess was washed in kit wash buffer. Cells were incubated in a humidified chamber heated to 37°C in kit solutions for ligation of probes (30 min), and amplification of the signal (100 min), then cells were counterstained with 4′-6-Diamidino-2-phenylindole (DAPI). The plus and minus probes were bound to the anti-WNT3A antibody, and antibody anti-FRZB respectively. Where the distance between the two bound probes is <40 nm a red signal is generated, which indicates an interaction of proteins of interest (Söderberg et al., 2006).

Protein extraction and immunoblotting

Protein was extracted from granulosa cells using RIPA extraction buffer (150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% SDS, Protease/phosphatase inhibitor cocktail). One million granulosa cells/sample was separated by SDS-PAGE and gels were transferred onto nitrocellulose hybond C-Extra membrane (GE Healthcare, Little Chalfont, UK) then blocked for 2 h at room temperature in 5% skim milk diluted in Tris buffered saline. Immunodetection was conducted using anti-FRZB antibody (ThermoFisher; cat no. PA5-47793) at dilution of 1:2000. Following incubation with a donkey anti-goat HRP-conjugated secondary antibody (Abcam; cat no. ab6885) at a 1:2000 dilution for 1.5 h at room temperature. Labelled antibodies were detected using an Amersham ECL Detection Kit (GE Healthcare UK Limited, Buckinghamshire, UK). GAPDH (G9545, Sigma–Aldrich) was used as a loading control. Densitometry was performed using ImageJ (NIH, Bethesda, USA). The protein expression was normalised to GAPDH and presented as the mean ± SE expression of FRZB protein, relative to GAPDH.

Results

Subtle changes in granulosa cell gene expression during primordial follicle activation

To investigate granulosa cell transcriptional changes associated with primordial follicle activation, RNA-seq analysis was performed on large quantities of granulosa cells isolated from primordial (PND1) ovaries or activating and primary follicles (PND4). A comparison between the PND1 and PND4 date sets did not reveal any transcripts with a false discovery rate ≤ 0.1. Transcripts specific to other ovarian cell types (oocyte and theca cell) were completely absent or were detected at negligible levels compared to granulosa cell specific transcripts *Foxl2* and *Gata4* (See supplementary 1). A total of 11,784 mapped genes were identified from RNA transcripts during bioinformatics processing. There was a total of 131 transcripts differentially expressed ($\log_2$ fold change ± 1; $p \leq 0.05$) between PND1 and PND4 granulosa cells (Figure 1). Comparing the transcripts in the PND1 granulosa cells with those found in the PND4 granulosa cells, 49 RNA transcripts were significantly more abundant whilst 82 transcripts were significantly more abundant in PND4 granulosa cells compared to PND1 (Figure 1); for a complete list of differentially expressed genes, see supplementary 2. This represents a very small (1.1%) change in transcripts from the total number identified between these two fundamental developmental stages.

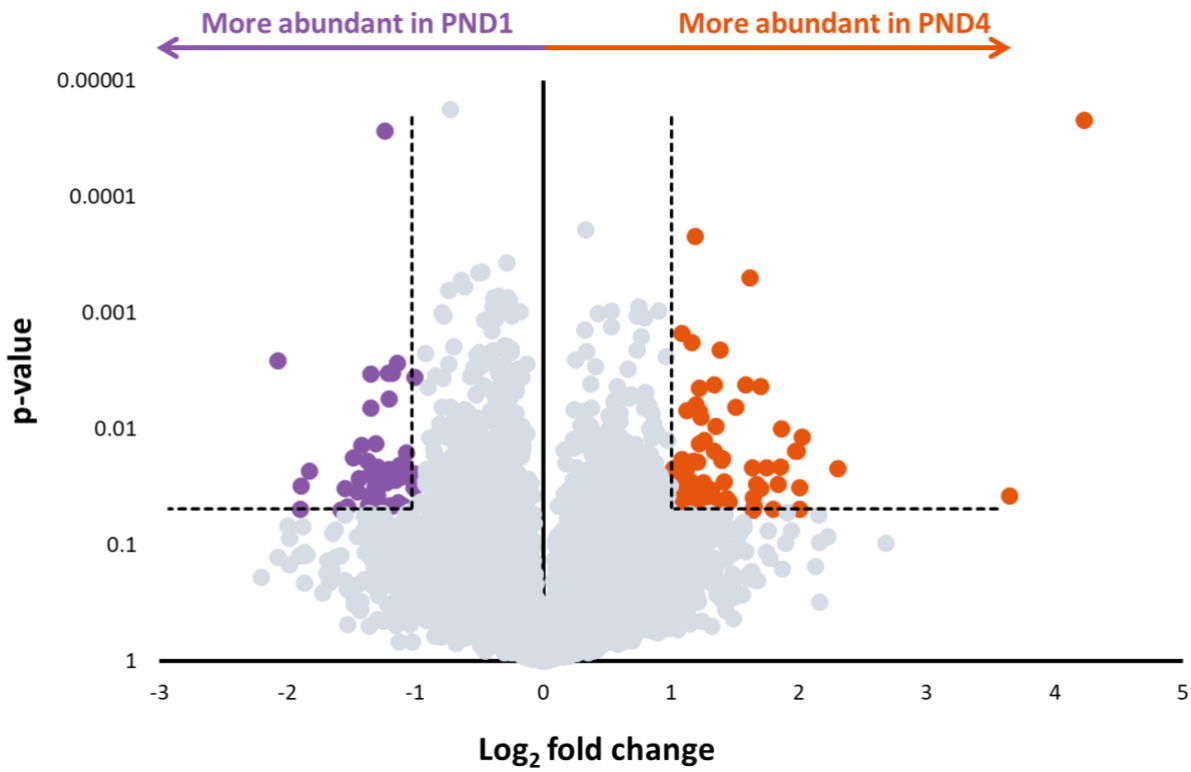


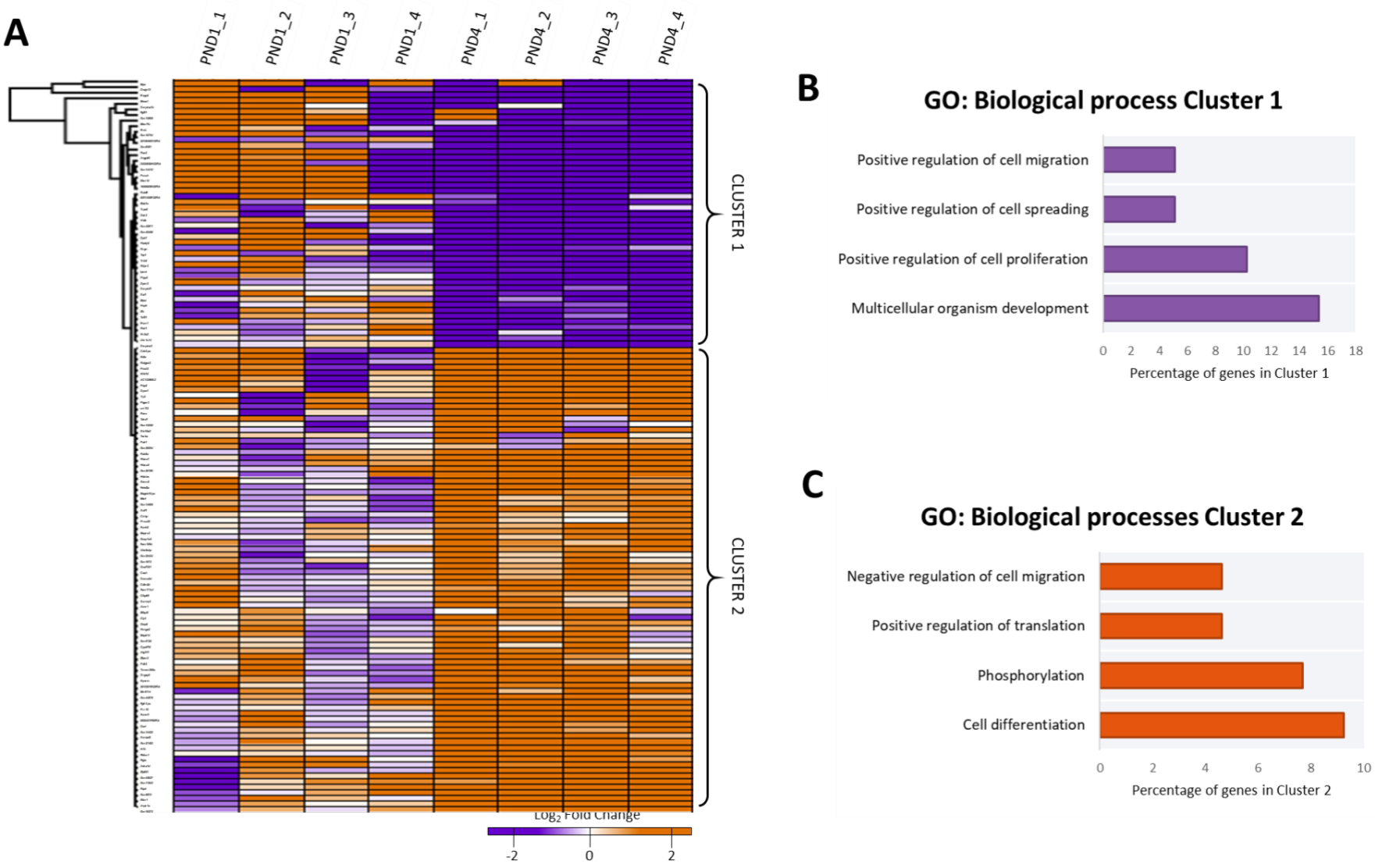
Figure 1: Differential gene expression profiling of neonatal mouse granulosa cells. The volcano plot indicates the differential expression between RNA transcripts from postnatal day 1 (PND1), and postnatal day 4 (PND4) granulosa cells (n=4 replicates from each group). Mapped genes expressed in higher abundance in PND1 granulosa cell RNA transcripts compared to PND4 granulosa cells are coloured purple. Genes expressed in higher abundance in PND4 granulosa cells compared to PND1 are coloured orange. Dashed line indicates the threshold for differential expression; genes with high statistical significance ($p \leq 0.05$), and $\log_2$ fold change of greater than ±1. Points that fall below these thresholds are coloured grey.

A heat map was generaterated to illustrate the expression (normalised to the average FPKM of the PND1 samples) of each gene within the threshold of significantly differentially expressed genes across each of the sample replicates (Figure 2A)., Two clusters of differentially abundant genes are apparent, with Cluster 1 comprising of transcripts that are more abundant in the PND4 granuolsa cells compared to the PND1, and Cluster 2 of transcripts with a decreased abundance in PND4 relative to PND1 granulosa cells. Within Cluster 1, among the top GO annotation of biological processes in terms of the percentage of genes represented within the dataset (Figure 2B) are those associated with development and growth; multicellular organism development (GO:0007275), positive regulation of cell proliferation (GO:0008284), spreading (GO:1900026), and migration (GO:0010634). These processes are indicative of primordial

follicles, known to be regulated by genes associated with development of the ovary (Edson et al., 2009) that establish the ovarian reserve after germ cell nest breakdown (Pepling, 2006). Cluster 2 (Figure 2C) contains notable biological processes: cell differentiation (GO:0030154), phosphorylation (GO:0016310) and positive regulation of translation (GO:0045727). These biological processes signify activation occuring, which is governed by the upregulation of signalling pathways encompassing both the activation and repression of genes and proteins, while granulosa cells differential from their squamous to cuboidal structure (Chen et al., 2020; Zhang and Liu, 2015).

Figure 2: Gene expression clustering of neonatal mouse granulosa cells. (A) a heatmap differential expression within the defined threshold ($p \leq 0.05$, $\log_2$ fold change of $\pm \geq 1$, n=131) depicts the consistency of expression of mapped transcripts identified as having significant between biological replicates. Different genes are represented in different rows, and different replicates in different columns. Expression values (FPKM) are represented as a colour scale from purple for lower expressions to orange for higher expressions and normalised to the average FPKM for the PND1 group. Clusters of differential expression were identified from the heat map and annotated via gene ontology (GO) analysis in DAVID (v6.8). (B) GO information of four biological process categories from Cluster 1 are presented as the percentage of genes from within that cluster belonging to a given category, and similarly (C) the GO information of biological process categories from Cluster 2 for four notable categories with the greatest percentage.

Figure 2


A
PND1_1
PND1_2
PND1_3
PND1_4
PND4_1
PND4_2
PND4_3
PND4_4
CLUSTER 1
CLUSTER 2
Log2 Fold Change
-2
0
2
B
GO: Biological process Cluster 1
Positive regulation of cell migration
Positive regulation of cell spreading
Positive regulation of cell proliferation
Multicellular organism development
0 2 4 6 8 10 12 14 16 18
Percentage of genes in Cluster 1
C
GO: Biological processes Cluster 2
Negative regulation of cell migration
Positive regulation of translation
Phosphorylation
Cell differentiation
0 2 4 6 8 10
Percentage of genes in Cluster 2

Validation of differentially expressed genes

To verify the differential gene expression between primordial granulosa cells and granulosa cells undergoing primordial follicle activation, eight candidate genes were selected for validation of RNAseq data using quantitative qRT-PCR. Genes above the threshold of significance for differential expression were chosen, with four genes decreased abundance in PND1 (primordial) granulosa cells compared to PND4 (activating) granulosa cells (*Frzb, Tcf21, Ddr2, Zfx*), and conversely, four genes exhibiting increased abundance in PND1 granulosa cells relative to PND4 (*Ifitm1, Fam171a1, Cdkn2b, Avpr1a*), see Table 2.

Table 2: Targets selected for validation of RNAseq

Gene Symbol	Absolute fold change	Abundance in PND4 relative to PND1	P-value	Gene name
Frzb	3.56	increased	0.023	Frizzle related protein
Tcf21	2.7	increased	0.014	Transcription factor 21
Ddr2	2.59	increased	0.019	Discoidin domain receptor 2
Zfx	2.5	increased	0.027	Zinc finger protein x-linked
Ifitm1	4.05	decreased	0.012	Interferon-induced transmembrane protein1
Fam171a1	3.95	decreased	0.016	Family with sequence similarity 171 member A1
Cdkn2b	3.91	decreased	0.016	Cyclin dependent kinase inhibitor 2B
Avpr1a	2.99	decreased	0.004	Arginine Vasopressin Receptor 1A

Experiments were performed on at least six pooled biological replicates (*n*= 4-15 animals per sample). The transcript encoding the 60S ribosomal protein L19 (*Rpl19*) was used as an endogenous control in all qRT-PCR analyses and confirmed the differential expression of all eight candidate genes while following a parallel trend to the RNAseq data (Figure 3). Six out of the eight genes validated remained significant ($p \leq 0.05$) following Bonferroni's correction for multiple comparisons. Collectively, these results validate the accuracy our RNA-seq dataset. Importantly this outcome strengthens our justification for the use of this valuable dataset to explore the differential temporal gene expression between mouse neonatal granulosa cells, representative of primordial and activating subtypes.

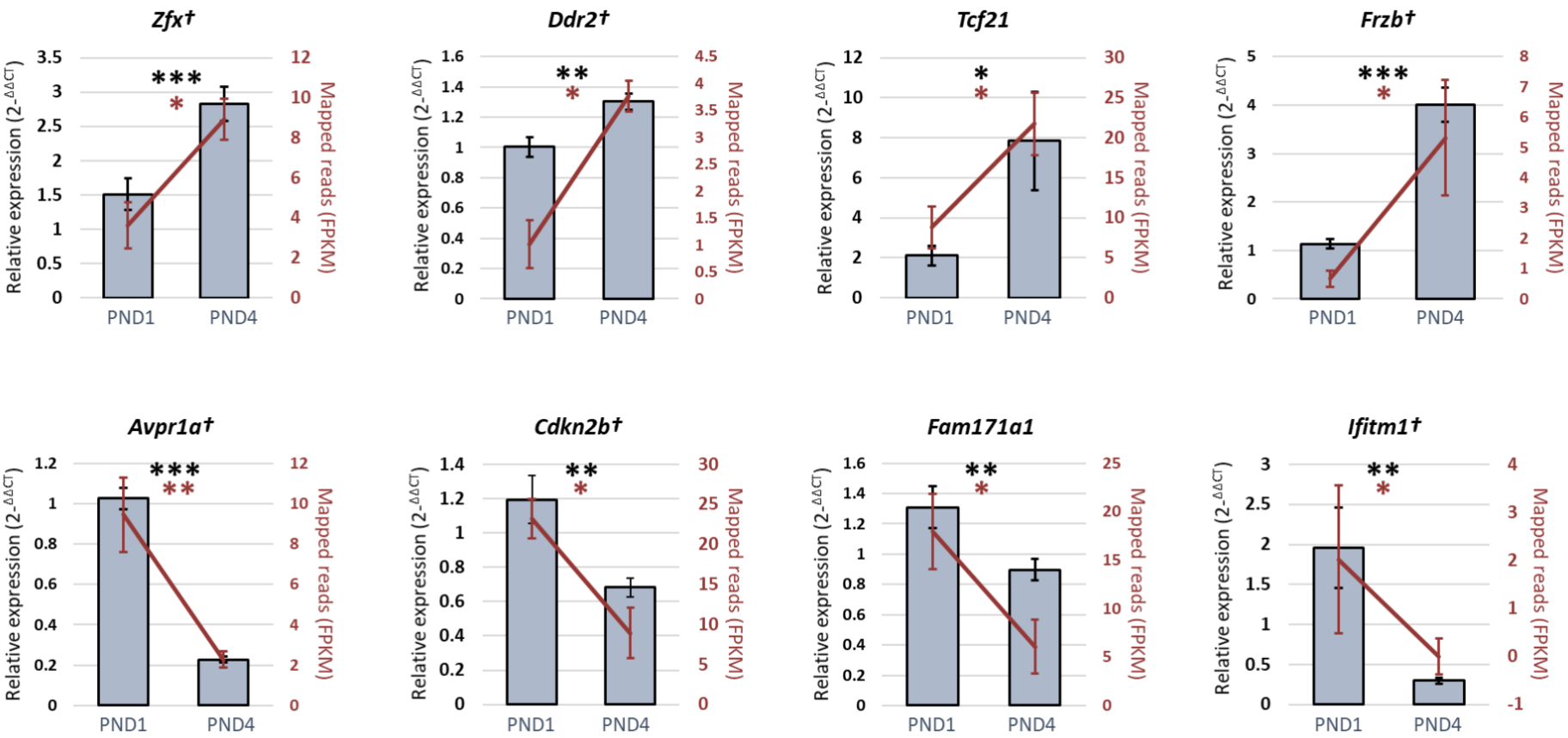


Figure 3: The qRT-PCR validation of differentially expressed genes within neonatal mouse granulosa cells. In verifying the RNA-seq data (represented on the secondary y-axis by the fragment per kilobase of exon per million, FPKM, for each mapped read), eight genes that displayed significantly different levels of expression in PND1 granulosa cells compare to PND4 granulosa cells (see table 2) were selected for orthogonal validation using qRT-PCR. Validation experiments were performed in triplicate using ≥ six distinct pools of biological samples (n= 4-15 animals per sample), statistical significance was investigated via a student's t-test. The 60S ribosomal protein L19 gene was employed as an endogenous control to normalise the expression levels of target genes. 6 out of 8 genes remained significant after Bonferroni correction for multiple comparison and are indicated by †. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Validating protein expression in the mouse ovary

Following gene expression validation, the functional protein expression of notable genes from the RNA-seq dataset was investigated. Through utilising the Ingenuity Pathway Analysis (IPA) network analysis feature, a number of genes from the dataset were identified to encode proteins that interact with molecules demonstrated to be involved in primordial follicle activation. A proportion of the IPA-generated network map is presented in Figure 4 and identifies six molecules from the dataset interacting through binding or regulation and includes two of those validated via qRT-PCR (FRZB, and ZFX). Primordial follicle activation component TGFBR from the dataset was also linked within the network.

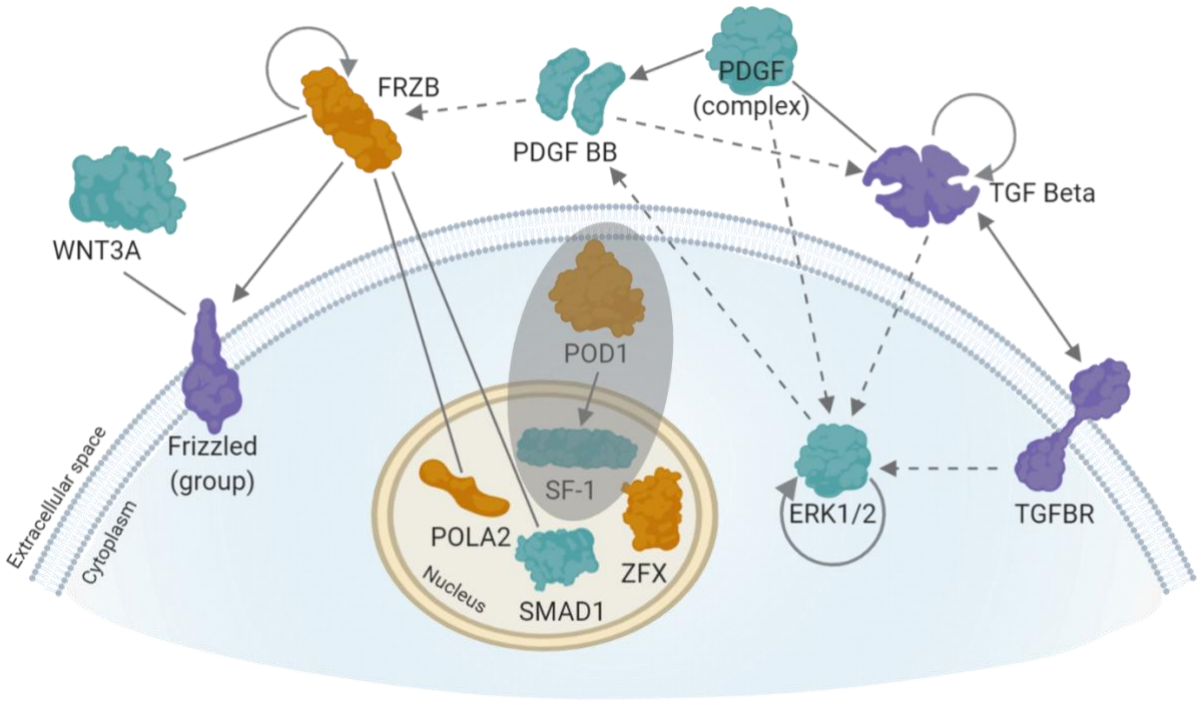


Figure 4: Schematic network diagram of biological network. Pathway analysis (IPA) network explorer was used to generate a biological network of molecules (or groups of molecules) from the dataset, based on their connectivity with other molecules both within the dataset and connected with the literature. A segment of the IPA-generated image was re-created using BioRender.com. Molecules detected within the dataset are colour coded according to their differential expression (orange = increased differential expression in PND1 granulosa cells compared to PND4 granulosa cells, purple = decreased differential expression in PND1 granulosa cells compared to PND4 granulosa cells). purple = decreased differential expression in PND1 granulosa cells compared to PND4 granulosa cells). Teal molecules represent those generated by IPA not present in the dataset. Line between molecules indicates binding, solid arrow indicates direct regulation, dashed arrow indicates indirect regulation, and curved arrow within molecules indicates self-regulation. Shaded circle indicates molecules added to the diagram that were not generated by the IPA network explorer.

To explore a potential relationship with primordial follicle activation, the three molecules from the network that had been validated at the gene expression level were selected for *in situ* immunolocalisation in neonatal mouse ovaries (Figure 5). POD1 did not feature in the IPA-generated network, but was also selected for further investigation, as the expression of its gene (*Tcf21*) increased 3.7-fold ($p<0.05$) in PND4 granulosa cells compared to PND1 granulosa cells as determined by qRT-PCR. ZFX protein was localised to the oocyte cytoplasm of both primordial and primary follicles but was predominantly observed in the extracellular space proximal to the granulosa cells of activating and primary follicles (Figure 5A). There was some expression of POD1 in all granulosa cells, but POD1 protein was more intensely present in the nuclei of cuboidal granulosa cells in primary and activating follicles (Figure 5B). The protein expression of POD1 supported the observed increase in gene expression of *Tcf21* (Figure 3) in the PND4 sample, where primary and activating granulosa cells are the dominant cell population. Primordial follicle oocytes were observed to have punctate expression of POD1. FRZB was expressed in the oocyte cytoplasm of both primordial and primary follicles (Figure 5C) and exhibited intense expression in the cuboidal granulosa cells in activating follicles, with weak expression in all granulosa cells.

The expression and localisation of FRZB was further investigated in neonatal mouse ovaries and granulosa cells to identify possible links to the process of primordial follicle activation considering its 3.5-fold increase ($p<0.001$) in gene expression in PND4 granulosa cells compared to PND1, and its protein localisation in activating granulosa cells (Figure 6). Additionally, FRZB was of interest due to its known interaction with 35% of the molecules within the IPA-generated network (Figure 4), and its canonical role as a Wnt-pathway inhibitor.

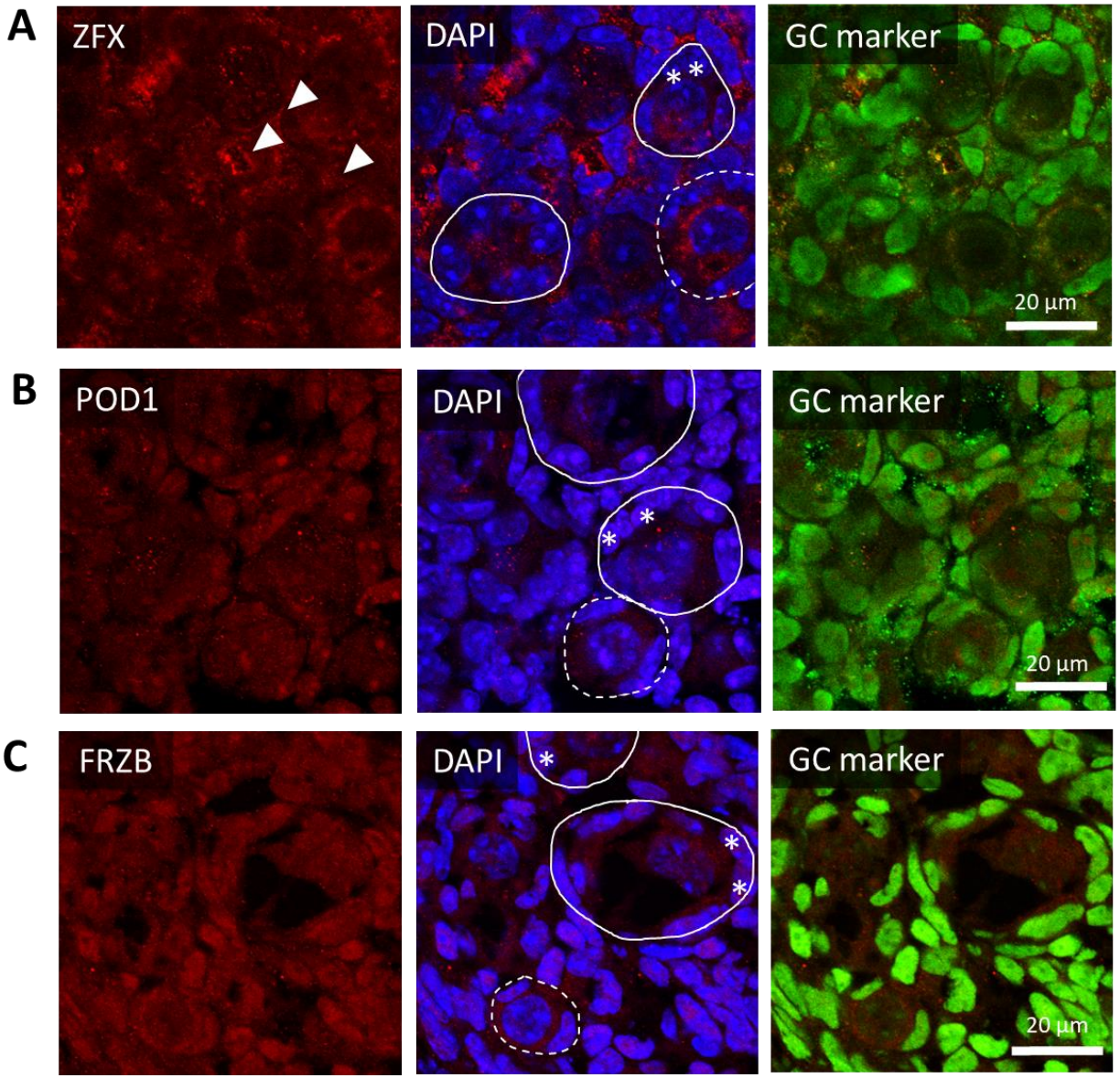


Figure 5: Expression and localisation of proteins of interest within the mouse ovary. The *in situ* immunofluorescent expression of three proteins of interest (A-C) were explored in the neonatal mouse ovaries and were co-localised with a nuclear marker (DAPI, blue), a granulosa cell marker (GATA4 or FOXL2, green), and either (A) ZFX (B) POD1, or (C) FRZB in red. Representative images from PND4 selected as they include populations of primordial, activating and primary follicles. Representative images are indicative of n= 4-6 biological replicates of both PND1 and PND4 performed in triplicate. Images taken at 60 x magnification, scale bars represent 20 µm with dotted circles outlining primordial follicles, solid lines outlining activating or primary follicles. Arrows indicate extracellular staining regions, asterisks indicate activating granulosa cells.

Figure 6

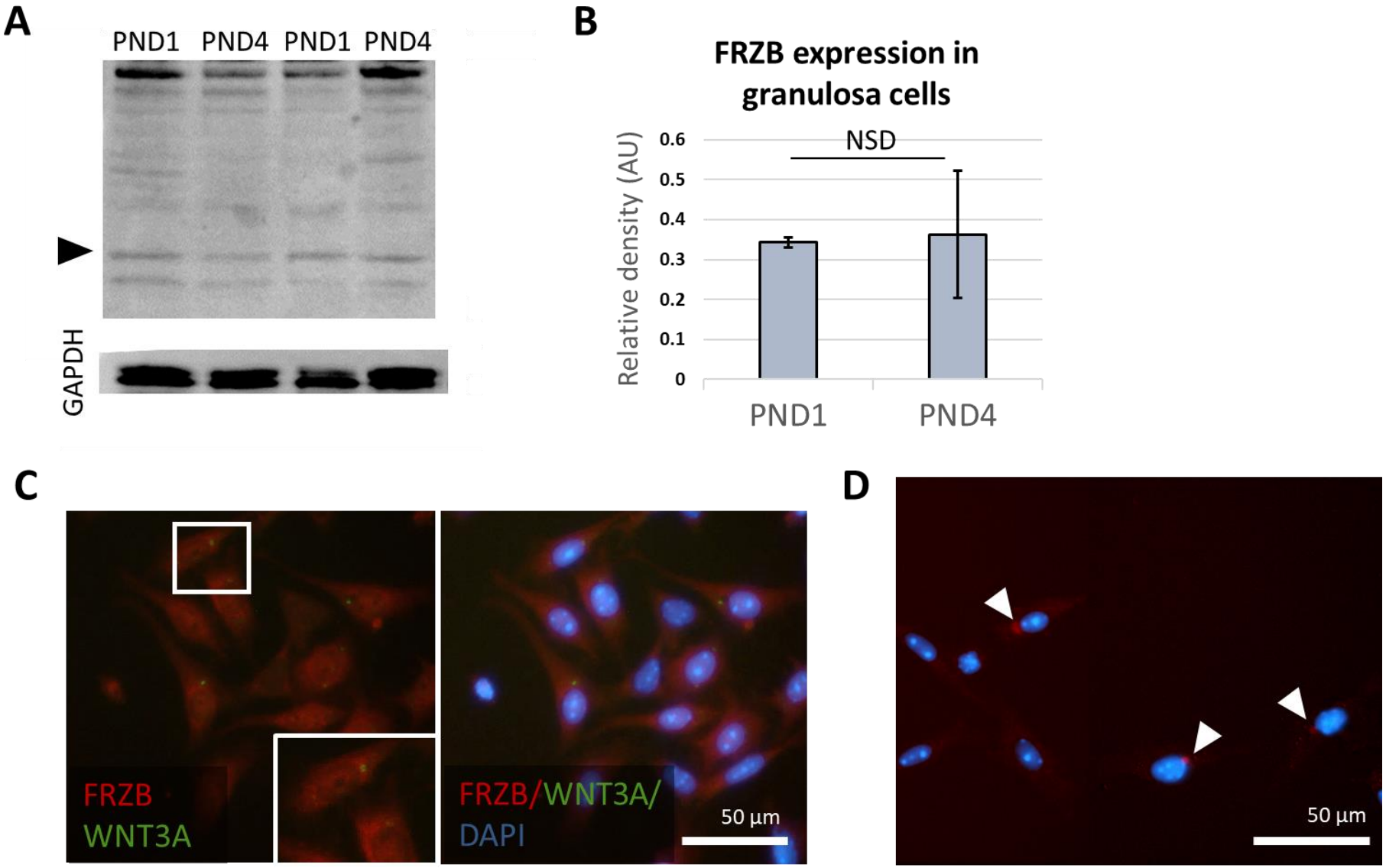

A
PND1 PND4 PND1 PND4
GAPDH
B
FRZB expression in granulosa cells
NSD
Relative density (AU)
0.6
0.5
0.4
0.3
0.2
0.1
0
PND1
PND4
C
FRZB
WNT3A
FRZB/WNT3A/
DAPI
50 µm
D
50 µm

Figure 6: Investigating the role of FRZB in the neonatal mouse ovary. (A) FRZB western blot of granulosa cells from PND1 and PND4 ovaries. Arrow indicates predicted size of FRZB protein (36 kDa). Expression of FRZB was normalised to GAPDH to determine the (B) relative protein expression of FRZB in PND1 and PND4 granulosa cells, via densitometry analysis, expression is in arbitrary units (AU) NSD indicates no significant difference after conducting a student's t-test. Western blots were re-probed to obtain the loading control band, these bands were cropped for presentation to clearly align the band for a given lane. (C) Immunocytochemistry of granulosa cells isolated from a post-natal day (PND) 4 mouse ovary. Frzb (red) co-localised with Wnt pathway activator WNT3A. Insets show zoomed image of boxed area. (D) Duolink proximity ligation assay of granulosa cells isolated from PND4 mouse ovary, red dot indicates proteins are interacting; Representative images are indicative of n=3 biological replicates performed in triplicate. Images taken at 40x magnification

Total expression of FRZB protein was compared in granulosa cells isolated from PND1 and PND4 ovaries and was found to be variable, with no significant difference between the primordial and activating granulosa cells (Figure 6A,B). Next, the expression of FRZB in granulosa cells and to validate its role in the Wnt pathway via WNT3A, a known suppressor of primordial follicle activation (Li et al., 2014) was investigated. WNT3A protein localisation has not previously been reported in neonatal ovaries, only its gene expression (Harwood et al., 2008). Immunocytochemistry was performed on isolated PND4 ovary granulosa cells to determine an association between FRZB and WNT3A. As in the whole ovary, FRZB was detected in both the nuclear and cytoplasmic regions of the isolated PND4 granulosa cells. FRZB co-localised with WNT3A in the granulosa cytoplasm (Figure 6C), the expression of WNT3A was intense and punctate adjacent to the nuclear region of the cell. Duolink proximity ligation assay demonstrated that FRZB and WNT3A are expressed in close proximity and likely interacting in this peri-nuclear region (Figure 6D). Taken together, findings establish the expression of Wnt pathway inhibitor *Frzb* has differential expression in granulosa cells from primordial and activating follicles compared to granulosa cells from primordial follicles. Additionally, that FRZB protein is localised to activating granulosa cells and interacts with WNT3A in these cells.

Discussion

Primordial follicle activation involves the layering of many signalling networks, and a complete picture of this process is essential to our understanding of female fertility (Ford et al., 2020). In granulosa cells, these signalling pathways initiate and control the complex remodelling of cells from a quiescent, flattened shape toward cuboidal structures, and involves the sending and receiving of signals and small molecules from the oocyte (Eppig, 2018; Zhang and Liu, 2015). Studies of global gene expression profiles increase our understanding of primordial follicle activation by identifying departures and extensions to known signalling pathways when they are combined with downstream computational or functional analyses. This study utilised the initial wave of primordial follicle activation in the neonatal mouse ovary to successfully study the granulosa cells during the primordial to primary transition. This study was the first to describe the transcriptome of populations of mouse granulosa cells representing primordial and activating follicles, and we have identified a number of genes differentially expressed in these cells which reflect potential novel interactions contributing to primordial follicle activation in the mouse. Our data highlights the transcription factor, ZFX, as important for ovary development and primordial follicle activation, and introduces FRZB as a new potential regulator in primordial follicle activation.

Overall, the gene expression changes observed were subtle, resembling findings from other transcriptome studies of primordial and primary granulosa cells in humans, primates, pigs and sheep, which also detect ≤1,000 genes when significance thresholds are introduced (Arraztoa et al., 2005; Bonnet et al., 2011; Bonnet et al., 2008; Ernst et al., 2018). The use of proteomic analyses to correlate with transcriptomic data would complement this research, as rodent studies on the proteome of early ovary development (Wang et al., 2009; Xu et al., 2017) have yet to substantially contribute to the understanding of primordial follicle activation. One limitation of the dataset was the false discovery rate above commonly accepted value of 0.1

(Korthauer et al., 2019). False discovery rate predicts the type I error among features deemed significant, so the risk of rejecting a true null is possible but is mitigated by downstream multiple testing correction and biological validation of genes. Thus, in the case of the current dataset, we interpret our data with caution and validated changes biologically, with qRT-PCR experiements mirroring expression changes of the RNAseq.

In the PND4 mouse ovary POD1 protein was expressed in the nuclei of granulosa cells in activating follicles, consistent with previous reports of POD1 expression increasing postnatally in the mouse (Tamura et al., 2001). This study provides further evidence, demonstrating that the expression of *Tcf21* also increases by a factor of 3.7 in granulosa cells from PND4 ovaries, indicative of its activity during primordial follicle activation Tamura et al. (2001) also identified POD1 as a negative regulator of steroidogenic factor-1 (SF-1), an important regulator of granulosa cell regulated ovary development (Pelusi et al., 2008). Further studies focusing on whether there is a link between POD1-induced repression of SF-1 and activation of primordial follicles would be valuable, as the SF-1 gene *Nr5a1* was also detected in the granulosa cells of RNAseq experiment (see GEO accession: GSE162927).

ZFX is a transcription factor previously speculated to be involved in POI as it occurs in the POI-critical region of the X chromosome (Persani et al., 2009), and early research in mice showed a reduced ovarian reserve when *Zfx* was knocked out, but the mechanism by which this occurs is not known (Luoh et al., 1997). The expression of ZFX in the ovaries was notable considering that in other somatic cells, ZFX is associated with promoting cell proliferation through cell cycle control (Harel et al., 2012; Wu et al., 2013), but was not in the nucleus of activating granulosa cells, which are known to proliferate. Our results demonstrate that *Zfx* expression is upregulated in granulosa cells from PND4 mice during primordial follicle activation. Our protein localisation studies indicate that ZFX is predominately expressed in oocyte and extracellular space suggesting the subcellular reorganisation of the transcription

factor. The bidirectional communication between oocytes and granulosa cells is a critical feature of folliculogenesis and follicular survival (El-Hayek et al., 2018; Eppig, 2018). These findings provide a renewed advocacy for investigating the role of ZFX to gain insight to primordial follicle activation and POI in the context of ZFX and oocyte-granulosa cell communications.

An important finding in this study was the 3.5-fold increase in mRNA expression of *Frzb* in PND4 granulosa cells indicative of activating granulosa cells. Importantly, FRZB directly and indirectly has association with or influences cellular function of molecules known to be involved in primordial follicle activation including WNT3A, ERK1/2, and the TGFb superfamily (Adhikari and Liu, 2009; Ding et al., 2013; Li et al., 2014). This study provides compelling evidence for an interaction between FRZB and WTN3A in granulosa cells from PND4 ovaries. This is consistent with previous findings identifying two sites on the FRZB protein for which WNT3A may bind (Bovolenta et al., 2008). WNT3a has been implicated in the maintenance of primordial follicle quiescence through FOXO3a (Li et al., 2014). This study provides novel evidence of FRZB as a potential upstream regulator of primordial follicle activation through its ability to bind to WNT3A in the granulosa cells. A recent human granulosa cell transcriptome study observed a downregulation of Wnt signalling in granulosa cells from primordial follicles compared to primary follicle granulosa cells (Ernst et al., 2018), indicating that WNT3A, and by extension FRZB, may have a conserved role in the human ovary. Taken together with our findings, this emphasises the need for further studies to determine the role of FRZB in primordial follicle activation, including a detailed investigation into a potential FRZB-WNT3A-FOXO3a relationship.

This study has provided a method for the isolation of mouse granulosa cells for the study of primordial follicle activation by taking advantage of the initial wave of primordial follicle activation. The transcriptome of primordial and activating granulosa cells presented in this

study has identified a range of factors previously associated with primordial follicle activation, which signify that future research could be well spent redoubling efforts into extricating the roles of these factors, such as SF-1, ZFX, and WNT3A, and their up- and down-stream effectors. These findings are enhanced by the validation of transcriptome data via qRT-PCR, confirming the dramatic changes in gene expression of granulosa cells during primordial follicle activation. Additionally, novel evidence for Wnt antagonist FRZB as a potential upstream regulator of primordial follicle activation through its interaction with WNT3A in granulosa cells is presented. Understanding the factors which dictate the maintenance of primordial follicle quiescence or their activation into primary follicles will inform our knowledge of early follicle development and may lead to future diagnostic methods and treatment regimens for women with premature ovarian insufficiency.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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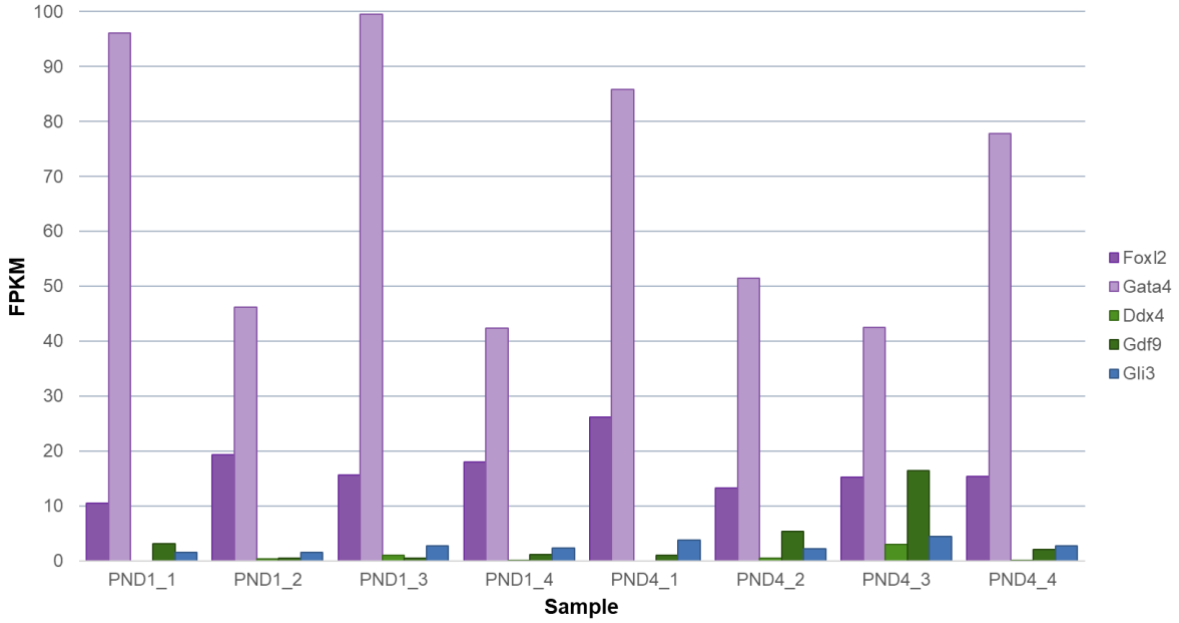
Appendix



S1: Expression of gene markers for ovarian cell types in RNAseq. Expression of selected ovarian cell-specific markers determined via RNA sequencing (FPKM= fragments per kilobase of transcript per Million mapped reads) of collagenase-dissociated cells originating from both postnatal day 1 and postnatal day 4 ovaries (n=4 samples per group). Granulosa cell marker= purple, Oocyte marker= green, theca cell marker= blue. Theca cell markers Ptch1, Gli2, Cyp19a1, Cyp17a1 were not detected. Figure also used in a manuscript submitted to Molecular Human Reproduction journal by the authors.

S2: Gene list of differentially expressed transcripts

Gene name	ID	fold change	log2FC	P-value	Q-value (FDR)
Serpinf1	ENSMUSG00000000753	0.498598	-1.00405	0.003565	0.540645
Nr5a2	ENSMUSG00000026398	0.496597	-1.00985	0.031931	0.742474
Trpc6	ENSMUSG00000031997	0.496568	-1.00994	0.024321	0.742474
Dpm3	ENSMUSG00000042737	0.485143	-1.04352	0.025931	0.742474
Trib3	ENSMUSG00000032715	0.475246	-1.07325	0.01611	0.742474
D630036H23Rik	ENSMUSG00000091007	0.469157	-1.09186	0.021845	0.742474
Gm16794	ENSMUSG00000097777	0.467394	-1.09729	0.020085	0.742474
Gm12898	ENSMUSG00000085626	0.467306	-1.09756	0.046171	0.742474
Heph	ENSMUSG00000031209	0.463058	-1.11074	0.025987	0.742474

Gm45456	ENSMUSG00000109875	0.459393	-1.1222	0.02059	0.742474
Enpp2	ENSMUSG00000022425	0.457628	-1.12775	0.020892	0.742474
Psmb9	ENSMUSG00000096727	0.454631	-1.13723	0.042872	0.742474
Akr1c14	ENSMUSG00000033715	0.453092	-1.14213	0.00273	0.540645
Ipmk	ENSMUSG00000060733	0.449685	-1.15301	0.028064	0.742474
Tap1	ENSMUSG00000037321	0.441438	-1.17972	0.045808	0.742474
Rtel1	ENSMUSG00000038685	0.440493	-1.18281	0.003328	0.540645
Meox1	ENSMUSG00000001493	0.434979	-1.20098	0.046045	0.742474
Gm6981	ENSMUSG00000098076	0.433773	-1.20499	0.022225	0.742474
Nrgn	ENSMUSG00000053310	0.432984	-1.20761	0.04631	0.742474
Dnajc13	ENSMUSG00000032560	0.432652	-1.20872	0.005539	0.633435
Plpp3	ENSMUSG00000028517	0.430474	-1.216	0.003309	0.540645
Mpst	ENSMUSG00000071711	0.427521	-1.22593	0.028851	0.742474
Mterf1b	ENSMUSG00000053178	0.426165	-1.23052	0.047989	0.742474
Pmch	ENSMUSG00000035383	0.424843	-1.235	0.049568	0.742474
Glipr2	ENSMUSG00000028480	0.42462	-1.23576	0.047069	0.742474
Serpine2	ENSMUSG00000026249	0.42278	-1.24202	2.72E-05	0.10671
Rac3	ENSMUSG00000018012	0.408885	-1.29023	0.04503	0.742474
Htatip2	ENSMUSG00000039745	0.406307	-1.29936	0.037179	0.742474
Sat1	ENSMUSG00000025283	0.406093	-1.30012	0.021186	0.742474
5031425E22Rik	ENSMUSG00000073147	0.403757	-1.30844	0.013315	0.742474
Gm45871	ENSMUSG00000110277	0.401909	-1.31506	0.031751	0.742474
Zfx	ENSMUSG00000079509	0.401417	-1.31683	0.026905	0.742474
Dph7	ENSMUSG00000026975	0.393948	-1.34392	0.023829	0.742474
Angptl6	ENSMUSG00000038742	0.392268	-1.35009	0.003368	0.540645
Vldlr	ENSMUSG00000024924	0.392017	-1.35101	0.006631	0.652742
Fgf21	ENSMUSG00000030827	0.390359	-1.35713	0.038036	0.742474
Mpo	ENSMUSG00000009350	0.387606	-1.36734	0.045177	0.742474

Ddr2	ENSMUSG00000026674	0.386124	-1.37286	0.018802	0.742474
Tcf21	ENSMUSG00000045680	0.373125	-1.42227	0.013905	0.742474
Ifitm10	ENSMUSG00000045777	0.368708	-1.43945	0.026674	0.742474
Mob1a	ENSMUSG00000043131	0.365881	-1.45056	0.034762	0.742474
Esm1	ENSMUSG00000042379	0.357213	-1.48514	0.017663	0.742474
Oaz2-ps	ENSMUSG00000083610	0.346448	-1.52929	0.046605	0.742474
Gm14410	ENSMUSG00000078870	0.341374	-1.55057	0.032695	0.742474
Serpina1b	ENSMUSG00000071178	0.333582	-1.58389	0.049979	0.742474
Frzb	ENSMUSG00000027004	0.281013	-1.83129	0.0229	0.742474
1600002K03Rik	ENSMUSG00000035595	0.268707	-1.8959	0.03085	0.742474
Kctd6	ENSMUSG00000021752	0.267849	-1.90051	0.049231	0.742474
2510046G10Rik	ENSMUSG00000066175	0.236852	-2.07794	0.002578	0.540645
Gm16272	ENSMUSG00000086582	18.75232	4.228997	2.19E-05	0.10671
Gm27483	ENSMUSG00000099143	12.51931	3.646084	0.037877	0.742474
Hbb-bs	ENSMUSG00000052305	4.925283	2.300207	0.021914	0.742474
Ifitm1	ENSMUSG00000025491	4.057137	2.020462	0.011826	0.742474
Gm38394	ENSMUSG00000094410	4.017466	2.006286	0.049233	0.742474
Gm1673	ENSMUSG00000070858	4.00333	2.001201	0.032049	0.742474
Fam171a1	ENSMUSG00000050530	3.952833	1.982887	0.015612	0.742474
Cdkn2b	ENSMUSG00000073802	3.912926	1.968248	0.015482	0.742474
Serinc2	ENSMUSG00000023232	3.631806	1.860687	0.009968	0.734057
Hba-a2	ENSMUSG00000069917	3.619256	1.855693	0.021189	0.742474
Hba-a1	ENSMUSG00000069919	3.560032	1.83189	0.030176	0.742474
Gm14453	ENSMUSG00000087442	3.474606	1.796849	0.049236	0.742474
Figla	ENSMUSG00000030001	3.346245	1.742543	0.021647	0.742474
Syce1	ENSMUSG00000025480	3.249891	1.700391	0.004309	0.595397
Sertad3	ENSMUSG00000055200	3.247405	1.699287	0.032695	0.742474
Gm42627	ENSMUSG00000105808	3.176044	1.667231	0.029993	0.742474

Pak1	ENSMUSG00000030774	3.135297	1.648602	0.046185	0.742474
Ptger3	ENSMUSG00000040016	3.114829	1.639153	0.038991	0.742474
Mir8114	ENSMUSG00000099227	3.111674	1.637691	0.049968	0.742474
6820431F20Rik	ENSMUSG00000071796	3.10646	1.635271	0.021645	0.742474
Rorc	ENSMUSG00000028150	3.065783	1.616255	0.000496	0.440157
Avpr1a	ENSMUSG00000020123	2.995055	1.582583	0.004186	0.595397
Srgap3	ENSMUSG00000030257	2.834324	1.503005	0.006485	0.652742
Prss35	ENSMUSG00000033491	2.730224	1.449019	0.04228	0.742474
Cdv3-ps	ENSMUSG00000090389	2.702955	1.434538	0.039633	0.742474
mt-Tl2	ENSMUSG00000064366	2.664992	1.414131	0.044431	0.742474
Clp1	ENSMUSG00000027079	2.664766	1.414009	0.028543	0.742474
Dazl	ENSMUSG00000010592	2.635434	1.398041	0.018116	0.742474
Rab3a	ENSMUSG00000031840	2.630086	1.39511	0.018517	0.742474
Hmga2	ENSMUSG00000056758	2.605195	1.381391	0.04338	0.742474
H19	ENSMUSG00000000031	2.600186	1.378615	0.002081	0.531684
Cirbp	ENSMUSG00000045193	2.573603	1.363789	0.046178	0.742474
Piwil2	ENSMUSG00000033644	2.543889	1.347036	0.009453	0.728807
Nynrin	ENSMUSG00000075592	2.528281	1.338157	0.004132	0.595397
Coch	ENSMUSG00000020953	2.5236	1.335483	0.04033	0.742474
Gm36189	ENSMUSG00000107432	2.519949	1.333394	0.015417	0.742474
Kcne1l	ENSMUSG00000090122	2.493765	1.318325	0.033835	0.742474
Gm11843	ENSMUSG00000082676	2.473795	1.306726	0.046884	0.742474
AC133868.2	ENSMUSG00000116311	2.422303	1.27638	0.033425	0.742474
Yy2	ENSMUSG00000091736	2.404969	1.266018	0.039023	0.742474
Sulf1	ENSMUSG00000016918	2.390539	1.257336	0.012518	0.742474
Mfap3l	ENSMUSG00000031647	2.386414	1.254844	0.029933	0.742474
Gm9725	ENSMUSG00000037982	2.381767	1.252032	0.043352	0.742474
Gucy1a2	ENSMUSG00000041624	2.376282	1.248706	0.029061	0.742474

Mto1	ENSMUSG00000032342	2.375428	1.248187	0.039181	0.742474
Atg101	ENSMUSG00000037204	2.348208	1.23156	0.007938	0.692227
Gm14005	ENSMUSG00000074813	2.331811	1.221451	0.013308	0.742474
Rtp4	ENSMUSG00000033355	2.327732	1.218925	0.007035	0.652742
Prr16	ENSMUSG00000073565	2.326026	1.217868	0.004431	0.600058
Klhl15	ENSMUSG00000043929	2.311394	1.208763	0.049815	0.742474
Slc10a3	ENSMUSG00000032806	2.309374	1.207502	0.032894	0.742474
Sema3d	ENSMUSG00000040254	2.304133	1.204224	0.019383	0.742474
Fahd2a	ENSMUSG00000027371	2.29821	1.20051	0.042931	0.742474
Zfp821	ENSMUSG00000031728	2.290074	1.195394	0.006179	0.652742
Fblim1	ENSMUSG00000006219	2.270401	1.182947	0.000219	0.440157
Adra1d	ENSMUSG00000027335	2.269367	1.18229	0.046915	0.742474
Pdk2	ENSMUSG00000038967	2.25198	1.171194	0.018996	0.742474
Mapk14	ENSMUSG00000053436	2.236465	1.16122	0.049729	0.742474
2610301B20Rik	ENSMUSG00000059482	2.234593	1.160012	0.001784	0.531684
Tdrd1	ENSMUSG00000025081	2.226954	1.155072	0.039419	0.742474
Mapre3	ENSMUSG00000029166	2.221243	1.151367	0.028509	0.742474
Gm44878	ENSMUSG00000108477	2.199116	1.136923	0.028603	0.742474
Acvr1	ENSMUSG00000026836	2.180017	1.12434	0.006944	0.652742
Ralgps2	ENSMUSG00000026594	2.164575	1.114084	0.022489	0.742474
Tor3a	ENSMUSG00000060519	2.16207	1.112413	0.042283	0.742474
Fgfr3-ps	ENSMUSG00000103178	2.129494	1.090511	0.029908	0.742474
Otud4	ENSMUSG00000036990	2.114414	1.080258	0.018345	0.742474
Gm5870	ENSMUSG00000106392	2.113778	1.079824	0.001512	0.508967
Gm29434	ENSMUSG00000100081	2.098615	1.069437	0.023573	0.742474
Dcaf12l1	ENSMUSG00000045284	2.098492	1.069353	0.024107	0.742474
Mageb10-ps	ENSMUSG00000082033	2.094515	1.066616	0.022541	0.742474
Ube2cbp	ENSMUSG00000032415	2.088803	1.062676	0.047533	0.742474

Cyp4f16	ENSMUSG00000048440	2.080276	1.056775	0.036792	0.742474
Pbp2	ENSMUSG00000047104	2.072579	1.051427	0.037048	0.742474
Cfap69	ENSMUSG00000040473	2.070901	1.050259	0.045441	0.742474
Kcnh2	ENSMUSG00000038319	2.068541	1.048614	0.025937	0.742474
Zfpm2	ENSMUSG00000022306	2.039086	1.027923	0.028537	0.742474
Nt5e	ENSMUSG00000032420	2.033172	1.023732	0.04775	0.742474
Stmn2	ENSMUSG00000027500	2.020386	1.014631	0.021876	0.742474
Gm13320	ENSMUSG00000083545	2.014737	1.010592	0.049444	0.742474
Fam189b	ENSMUSG00000032657	2.008167	1.005879	0.023382	0.742474
Tmem255a	ENSMUSG00000036502	2.003457	1.002492	0.023752	0.742474

Chapter 4: A scoping review of the information provided by fertility smartphone applications

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Chapter overview

The use of smartphone applications (apps) designed for women, popularly dubbed 'femtech', has become so prevalent, that it is worth an estimated $820 million in 2019, with one market research forecast projecting an increase to over $3 billion by the end of 2030. Despite the rise of this type of software, which mostly relates to hundreds of apps tracking and observing fertility, pregnancy and the menstrual cycle, overall fertility knowledge has not shown much improvement in studies conducted over the last 5 years. Part of the appeal for some women using femtech is the perception of quality and expertise that some apps claim. This scoping review was conducted to consider the current state of academic literature published on women's reproductive health app content. I evaluated how fertility information is reported across a range of study types and report the accuracy of fertility information within app literature, and the impact this has on the users of apps. Overall, I reason that enhanced and open reporting of app content is vital to academic scrutiny, and apps should strive for content applicable to users from a variety of different racial, educational, and gender backgrounds, who require diverse reproductive needs. Additionally, studies involving apps rarely measure the effect of their information content on ability to inform users, and often in cases where it is reported, it is in a qualitative, self-reported approach.

It is my conclusion that reproductive health apps will continue to be a highly popular resource of observing and measuring women's bodies, and that more rigorous reporting and accuracy of content within apps will further women's fertility knowledge to cater for their present and future reproductive health needs.

Original scoping review

HUMAN FERTILITY
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REVIEW ARTICLE

A scoping review of the information provided by fertility smartphone applications

Emmalee A. Ford[a,b], Alexandra E. Peters[a,b], Shaun. D. Roman[a,b,c], Eileen. A. McLaughlin[a,b,d,e], Emma. L. Beckett[b,f] and Jessie. M. Sutherland[a,b]

[a]Priority Research Centre for Reproductive Science, Schools of Biomedical Science & Pharmacy and Environmental & Life Sciences, University of Newcastle, Callaghan, Australia; [b]Hunter Medical Research Institute, University of Newcastle, New Lambton Heights, Australia; [c]Priority Research Centre for Drug Development, University of Newcastle, Callaghan, Australia; [d]School of Science, Western Sydney University, Penrith, Australia; [e]School of Biological Science, Faculty of Science, University of Auckland, Auckland, New Zealand; [f]School of Environmental & Life Sciences, Faculty of Science, University of Newcastle, Ourimbah, Australia

ABSTRACT

The growth of smartphone application use across areas of female reproductive health has led to increased interest into their functions and benefits. This scoping review aims to determine the nature and extent of the peer-reviewed literature presented on fertility-based apps, to identify the reliability of the information within the apps, and to determine the ability of this information to educate users. A systematic search of six databases was conducted in April 2020, returning a total of 21,158 records. After duplicate removal, title and abstract screening exclusionary steps, 27 records were reviewed and charted. Records covered a variety of reproductive health themes including contraception, sexual health, and family planning, and used a range of methodologies. The accuracy of fertility information within the apps reported in these studies was variable, but overall there was a lack of depth in the coverage of content in apps. It was common for studies in this review to base fertile window algorithms on stringent cycle length and variability requirements, limiting the applicability of information delivered to users. Furthermore, studies from app affiliates often lacked collaborations with researchers, minimising the potential for fertility knowledge improvements integrated across the suite of female reproductive health apps.

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Fertility awareness; education; knowledge; infertility; smartphones; mHealth

Introduction

The pervasiveness of smartphones into our everyday lives, while hard to study on a global scale, can be demonstrated by their ability to influence aspects of our cognitive function including attention, communication, and emotional regulation (Wilmer et al., 2017). There is a vast range of smartphone applications (apps) available to customise smartphone use and function. Certainly, there is a burgeoning market for apps relating to health and wellbeing (also termed mobile health, or mHealth), numbering in the hundreds of thousands (Scott et al., 2020; Viennot et al., 2014). Estimates suggest that mHealth app use in the population is growing rapidly as part of the digital health and 'big data' trends (Lupton, 2014). A 2018 survey of over eight thousand people from seven countries revealed that half of all participants reported downloading an mHealth app in the past, representing an increase of over 30% from the same survey in 2014 (Safavi et al., 2018). In top app stores worldwide (iTunes and Google Play), health apps number well over 300,000, with more than 200 health apps being added daily (Aitken et al., 2017).

Despite the escalating rates of infertility worldwide (Vander Borght & Wyns, 2018) and the subsequent reliance on assisted reproductive technologies (Fitzgerald et al., 2018; United Nations Department of Economic & Social Affairs & Population Division, 2015), women's knowledge about their own fertility remains poor (Pedro et al., 2018). Knowledge about fertility and factors that may prevent infertility (such as sexually transmitted infection screening, smoking cessation programs and family planning to avoid delayed childbearing) is crucial for reducing the incidence of infertility and its burden on the healthcare system (Macaluso et al., 2010).

Women's health and pregnancy apps are incredibly popular, comprising 9% of the apps available in the

CONTACT Jessie. M. Sutherland jessie.sutherland@newcastle.edu.au School of Biomedical Science and Pharmacy, the University of Newcastle, Ring Road, Callaghan, NSW2308, Australia

iTunes marketplace's health category, with women's fertility apps representing 8% of the most downloaded health apps (Safavi et al., 2018). These apps cover a number of functions including, but not limited to, tracking menstrual cycles, recording symptoms, contraception, planning conception, monitoring pregnancy (Gambier-Ross et al., 2018; Hughson et al., 2018). Reproductive health apps are often perceived as helpful by their users, as they provide easy access to information and support where traditional care may be less available (Epstein et al., 2017; Lupton & Pedersen, 2016). It is, therefore, a major concern that the validity of the evidence provided in these apps is frequently unsupported (Lupton & Pedersen, 2016; Starling et al., 2018). However, due to apps being updated, added or removed from the market regularly (Larsen et al., 2016), studies of the reliability of specific apps may quickly become obsolete. Crucially, what remains to be determined is the accuracy of information typically presented in these apps, and what themes of fertility and reproduction they cover. There is mixed evidence on the potential for current apps to improve healthcare service delivery, with success largely dependent on high user engagement, notifications/messaging, and of course, the accuracy of the content within mHealth apps (Free et al., 2013; Kratzke & Cox, 2012). Regardless of the capability of current apps, the potential for mHealth apps to deliver evidence based health information with positive behavioural change outcomes is well established (Hilty & Chan, 2018; Mehdipour & Zerehkafi, 2013).

There have been numerous reviews of women's fertility and/or reproductive health knowledge or literacy (Garcia et al., 2018; Kilfoyle et al., 2016; Musgrave et al., 2019; Pedro et al., 2018) all of which argue for public health interventions to improve women's understanding of fertility before and during their reproductive years. However, there are no reviews reporting fertility and reproductive health apps' ability to change attitudes and knowledge, only critical analyses on the themes and attitudes presented to women (Lupton, 2014, 2015), or of the research of these types of apps more broadly (Earle et al., 2020). The importance of compiling the evidence on the information provided to women through female reproductive health apps is necessary for determining the viability of apps as a resource for public health promotion and education. Herein, we aim to use a scoping review to consider the peer-reviewed literature of fertility-based reproductive health apps to systematically examine the information provided to women through these apps. Specifically, this review includes fertility/reproductive health content that are reported in both studies of apps and in intervention studies involving apps. Finally, we will determine how fertility information within apps is reported in peer reviewed literature, and, whether the relationship between this information and consequent knowledge of app users has been evaluated.

Materials and methods

This systematic scoping review adheres to the PRISMA extension for scoping reviews (Tricco et al., 2018), no protocol is registered.

Study criteria

To be eligible for inclusion in this review, a study had to report on information available via fertility apps. Fertility apps were defined as containing content relating to the theme of fertility and may include the menstrual cycle, ovulation, preconception care, lifestyle behaviours, contraception and reproductive health. Studies were included regardless of whether they involved human participants to measure learning outcomes of the apps. Studies could include researcher-developed apps (with or without pre and post-testing of knowledge, behaviour change or other metrics included in app research), reviews of apps in the marketplace targeted to women and/or for the female reproductive system (gender non-conforming/non-binary inclusive), and observational/descriptive studies that outline information contained with women's fertility apps. The authors acknowledge that not all people with a female reproductive system or assigned female at birth identify as 'women'. Studies reporting on apps developed for use by health professionals (i.e. diagnostic, requiring medical equipment, specialist knowledge required) or in languages other than English were excluded. Systematic/scoping and narrative reviews, case studies, editorials, conference abstracts, and grey literature (non-academic publishing) were excluded. However, some of these resources were used to contextualise the rationale and discussion in this study.

Search strategy

Six databases (Medline, Scopus, The Cochrane Library, CINAHL, Web of Science, and embase) were searched using relevant search terms (see Table 1 for the details of the MEDLINE search as an example) for human studies in English dated between January 2007 and April 2020 (last search 22 April 2020). Search terms

Table 1. Search strategy in MEDLINE.

#	Searches	Results
1	((fertilit* or period or contracept* or pregnan* or sex* or reproduct* or menstrual or ovulat* or infertilit*) adj3 (app or application* or smartphone* or smart phone* or iphone* or mobile phone* or cell phone*)).mp.	3501
2	Animals/ not humans/	4,657,796
3	1 not 2	2675
4	Limit 3 to yr="2007 -Current"	1595

and operators were modified according to the database requirements. The year of the development of modern touchscreen smartphones, 2007, was chosen as the lower limit (Zapata et al., 2015). The references section of key studies was manually examined by reviewers to determine any additional sources that met the criteria for inclusion in the review.

Study selection

Duplicates were removed, and the remaining titles screened by an independent reviewer (EAF), using the previously defined inclusion criteria. The titles were screened for inclusion, foremost for references to mobile applications. Additionally, the title screening process sought to include records that contained references to sexual and reproductive health, fertility and preconception care. Following the separation of studies into included and excluded lists, a second independent reviewer (AEP) examined the lists to ensure correct classification had occurred. Final study inclusion involved three actions: (i) the screening of abstracts for inclusion based on pre-defined criteria (see study criteria section above); (ii) the scanning of references within key studies to add any relevant studies not identified in the database search; and (iii) the obtaining of full texts for summary and synthesis. Discrepancies were reviewed and resolved by a third independent reviewer (JMS).

Data charting

Firstly data were extracted by summarising each record and taking note of the type of study, participant information, aims, key findings and identifying any references to fertility information and a statement of how the paper relates to the research objectives. Next, data was charted in a form independently by two reviewers (EAF and AEP) and included information in Table 2. Any disagreements between reviewers were resolved by a third independent reviewer (JMS). Extracted data included country, study design, primary aim, participant information, a summary of the fertility information in the study, if the information was evidence based, and if the record measured the effect of app information on the education or knowledge of participants. The purpose of this scoping review was to identify the current state of research on fertility information in fertility-based reproductive health apps and its effect on app users, and thus the quality of records in this review was not assessed as is typical in scoping reviews (Tricco et al., 2018).

Results

Search results

Searching of six databases returned 21,518 records (see Figure 1 for overview). Following the removal of duplicates, 16,458 records remained, removal of records published before 2007 returned a total of 14,370 studies. At the conclusion of title screening, 299 records remained.

After abstract screening, 242 records were excluded as not fitting the inclusion criteria defined above, resulting in 53 records progressing to full text summary and synthesis. Two abstracts/full texts were unable to be acquired and were subsequently excluded, and one additional record obtained from a reference search of key studies was added to the inclusion list. Upon screening of full texts, 26 studies were excluded for not addressing the criteria, with reasons including: (i) text-messaging or web-browser services rather than downloadable apps; (ii) studies reporting user data collected from apps without reporting predictions or information received on the user interface; and (iii) containing themes related to fertility without any explicit references to fertility. One study protocol was included in the records list, where information contained within the app could be determined (van Dijk et al., 2017), several other study protocols were excluded where the app was yet to be developed, or no description of contents were given (Nuwamanya et al., 2018; Simmons et al., 2017). Following full text examination of the retained abstracts, a final list of 27 records were included for review.

Study designs

The most frequent type of study design in the reviewed records was evaluation studies (29.6% of records), followed by prospective and descriptive studies (each 18.5%) (Table 2). There were three retrospective studies, one of which was two-armed with

Table 2. Summary of records included.

Authors (year)	Country	Study design	Primary aim	Participants: information recorded	Summary of fertility information	Evidence based fertility information?	Measured effect on knowledge?
Berglund Scherwitzl et al. (2016)	Sweden	Retrospective	Determine effectiveness of app 'Natural Cycles' as contraceptive method	4054 participants aged 18–45. Collected BMI, menstrual cycle data, previous contraceptives and number of children	Reported only that users receive a daily prediction of fertile or not fertile based on their input factors. Other information provided in-app is not disclosed	Y*	N
Berglund Scherwitzl et al. (2015)	Sweden	Retrospective	Effectiveness of app 'Natural Cycles' to accurately detect ovulation	317 participants aged 18–39 years. Collected BBT, menstrual cycle data, BMI, medication and smoking status	Reported only that users receive a daily prediction of fertile or not fertile based on their input factors. Other information provided in-app is not disclosed	Y*	N
Bull et al. (2019)	Sweden	Prospective observational	Evaluate contraceptive effectiveness of app 'Natural Cycles' for women who previously used hormonal birth control	16,331 participants, aged 18–45. Collected menstrual cycle data, previous contraception information, pregnancy, daily information (sexual activity, symptoms)	Daily predictions of fertile or not fertile, and ovulation. Other information available to users contained within the app is not reported	Y*	N
Eschler et al. (2019)	USA	Two-armed; descriptive, evaluation study	Create a definition of menstrual literacy evaluate the menstrual literacy of menstrual tracking apps	For definitions, used 8 popular and publicly available websites. Evaluated 17 apps	12 of apps featured fertility-related features, but across the apps the information was mainly limited to fertile days and did not support menstrual literacy	N	N
Faust et al. (2019)	USA	Observational	Compare self-reported fertility data from an app 'Ovia Fertility' to traditionally obtained fertility data	98,902 participants aged 18–45. Collected demographics (race, previous children), daily information (menstrual cycle data, symptoms, sexual activity, fertility indicators of cervical fluid, LH test)	Definitions of how the fertile window and ovulation is determined in app. Users receive fertility rating.	Y*	N
Freis et al. (2018)	Germany	Evaluation study	Scored apps' ability to predict conception using novel ranking system	12 apps	Only reporting app information relating to conception: cervical fluid thickness, ovulation and fertile window.	Y* – most apps relied on expected menstrual cycle length and gave incorrect information	N
Haile et al. (2018)	Egypt, Ghana, Jordan, India, Kenya, Nigeria, Rwanda	Evaluation	Assess women's' interests and purposes using a fertility app 'CycleBeads'	356,520 participants aged 18–39. Collected relationship status, location, menstrual cycle data, previous contraceptive, app intentions and satisfaction	Users receive fertile/not fertile prediction. Qualitative assessment did not cover content	N/A how prediction delivered not reported	N

(*continued*)

Table 2. Continued.

Authors (year)	Country	Study design	Primary aim	Participants: information recorded	Summary of fertility information	Evidence based fertility information?	Measured effect on knowledge?
Hamper (2020)	UK	Qualitative study	Identify women's' motives and perceptions of fertility apps	15 participants aged 30–44, 27 interviews.	Information in apps is generically described contextualised through second-hand via interviewees.	N/A	Yes, most women self-report gains in fertility knowledge
Jennings et al. (2019)	USA	Prospective observational study	Evaluate effectiveness of 'Dot' app as a contraceptive	718 participants aged 18–39 years. Collected demographics (race and relationship status), menstrual cycle data, and sexual activity	Fertile window of menstrual cycle	Y*	N
Jennings et al. (2019)	USA	Prospective study	Evaluate effectiveness of 'Dot' app as a contraceptive	718 women aged 18–35. Collected demographics (race and relationship status), menstrual cycle data, and sexual activity	Fertile window of menstrual cycle and barrier contraceptive methods for fertile days	Y*	N
Johnson et al. (2018)	UK	Evaluation study	Measure effectiveness of 73 apps for predicting ovulation by comparison to biometric data of app users and traditional methods	949 participants aged 18–50. Collected BMI, previous pregnancies, length of time trying to conceive, daily urine, menstrual cycle data	Fertile window, ovulation day but apps could predict ovulation with 21% accuracy	N/A how prediction delivered not reported	N
Johnson et al. (2020)	UK	Randomised control trial	Evaluates effectiveness of app-connected 'ClearBlue' ovulation detector	844 participants aged 18–40 years ($n = 382$ in test group). Collected urine samples for pregnancy and ovulation testing, menstrual cycle data, and sexual activity	Provided fertile and ovulation days. No other information users received was reported	Y*	N
Kalke et al. (2018)	USA	Descriptive study	Identify the availability of sexual health education apps and determine quality of evidence-based apps	679 apps addressing sexuality and sexual health, 15 of those were evidence based and content was analysed	80% of the 15 evidence-based apps had fertility information but most did not incorporate health behaviour techniques or interactivity principles	Y	N
Lee and Kim (2019)	Korea	Randomised control trial	Determine if new method of app selection based on user needs improves health-related factors	72 participants aged 'in their 20 s and 30s'. Collected occupation, PMS and dysmenorrhoea information, previous app use, and app information: menstrual	Fertile window and ovulation.	N/A how prediction delivered not reported	Yes, 90.3% of users reported apps helpful for period cycles, 24.5% reported apps helpful for PMS patterns,

(*continued*)

Table 2. Continued.

Authors (year)	Country	Study design	Primary aim	Participants: information recorded	Summary of fertility information	Evidence based fertility information?	Measured effect on knowledge?
				cycle data, sexual activity, daily symptoms			1.9% reported helpful for dysmenorrhoea/PMS
Levy and Romo-Aviles (2019)	Spain and Austria	Qualitative study	Analysing women's' practices and experiences with menstrual cycle tracking apps	Interviewed 26 participants aged 18–40 years. Collected demographics (education, race, location, sexuality), self-reported digital media literacy	Users reported and describe their use of apps for contraception, conception, and self-exploration reasons (linked to fertility)	N/A apps used by participants not reported	Yes, several participants report educational benefit and increased awareness from tracking menstruation and symptoms
Li et al. (2016)	USA	Retrospective and prospective study	Evaluate effectiveness of app 'Dot' to predict fertility for contraception and conception	Two retrospective cohorts sourced from a study based in North Carolina ($n = 68$), the Early Pregnancy Study ($n = 221$), and prospective WHO trial ($n = 706$). Collected age, race, location, menstrual cycle data, sexual activity, cervical fluid.	Reported fertile window and ovulation days	Y*	N
Mangone et al. (2016)	USA	Evaluation study	Identify, describe and evaluate apps used to prevent pregnancy	218 apps	5.5% scored 15 out of 21 for best advice about pregnancy prevention. Fertility tracking apps incorporated the fewest best practices for pregnancy prevention	Y	N
Mesheriakova and Tebb (2017)	USA	Evaluation study	Improve sexual health knowledge and contraceptive use in adolescents using app 'Health-E-You'	120 participants aged 12–18 years. Collected race, sexual activity, contraceptive use and intentions, knowledge of contraceptives	Effects of contraceptives on long term fertility is mentioned	Y	Yes, increased knowledge scores by 21%
Moglia et al. (2016)	USA	Evaluation study	Evaluate accuracy and functionality of menstrual cycle tracking apps	Identified 108 cycle tracking apps, but analysed 20 apps deemed accurate	Of the 20 apps examined, 55% contained fertility information. 15% of apps have information useful for 'fertility medications'	Y*	N

(*continued*)

Table 2. Continued.

Authors (year)	Country	Study design	Primary aim	Participants: information recorded	Summary of fertility information	Evidence based fertility information?	Measured effect on knowledge?
Scherwitzl et al. (2017)	Sweden and 36 other countries (not identified)	Prospective observational study	Evaluate efficacy of app 'NaturalCycles' as contraception	22,875 participants. Collected BBT, menstrual cycle data, sexual activity, LH test, daily symptoms	Daily predictions of fertile or not fertile, and ovulation. Other information available to users contained within the app is not reported	Y*	N
Setton et al. (2016)	USA	Descriptive study	Evaluate validity of websites and apps to standard 28-day cycle	20 apps	3 apps accurately reported fertile window duration, no other app information reported	Y*	N
Shopov et al. (2019)	Bulgaria	Use case analysis	Presents a use-case for a multi-interface app for patients and healthcare workers with a BBT device for ovulation detection	Not tested	Provides information about fertility and information. Shares between patient and healthcare worker but specific details not reported	Y*	N
Sohda et al. (2017)	Japan	Evaluation study	Optimising self-reported health tracking data in app 'Luna Luna' for ovulation prediction	7043 participants aged 20–45 years. Collected menstrual cycle data, ovulation via LH test	Reported only on correct ovulation prediction of different methods. No other information within app is reported	Y*	N
Sridhar et al. (2015)	USA	Randomised control trial	Compare contraceptive information app 'Plan A Birth Control' (test) to contraceptive counselling with healthcare worker (control)	120 participants aged 18–45 years. Collected education, relationship status, pregnancy history, knowledge of contraceptives	Provides contraceptive selection based on fertility/ reproductive goals	Y	Yes, no difference in knowledge of those who received app or counselling
Staric et al. (2019)	Italy	Descriptive study	Analysis of menstrual cycle tracking apps	6 apps selected by highest rating by users	Reported whether apps contained fertile window and ovulation predictions, and health information. However, this information was not evaluated for accuracy	N/A content described not evaluated	N
Starling et al. (2018)	USA	Pilot study	Determined preferences of apps and user knowledge in people who used apps or intended to use apps for preventing pregnancy	1000 participants aged 18–39. Collected demographics (race, relationship status, employment status, income), app preferences and fertility knowledge	App content reported second hand through participants: ovulation prediction, fertile window	N/A	Yes, participants who had received fertility-based awareness education knew more about fertility
Vanya et al. (2017)	Hungary	Descriptive study	Overview of novel app 'Infertility Handling'	8 people's cycles tested in app. No information of demographics and what data was collected	Reports fertile window and ovulation predictions	N/A how prediction delivered not reported	N

*Information has limitations in cycle length for fertile days calculations.

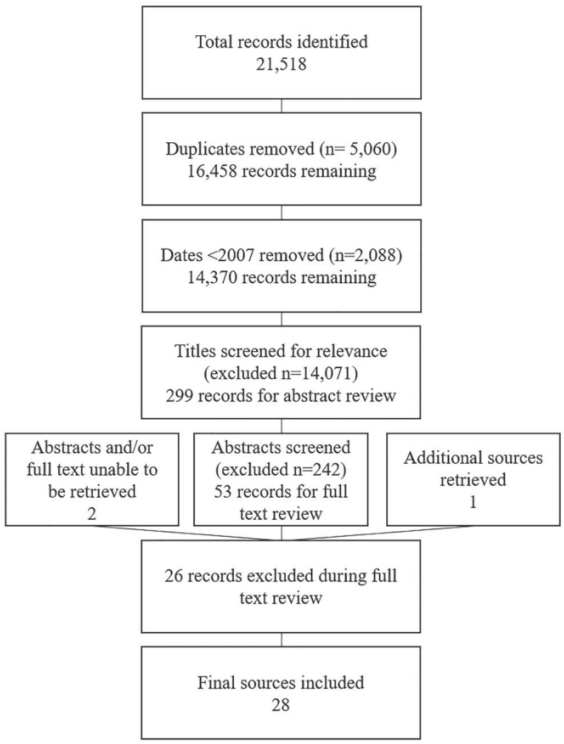

Figure 1. Flow diagram of the search strategy for including in the scoping review.

both retrospective and prospective components (Li et al., 2016), three randomised control trials, two qualitative studies, one use-case study and one pilot study. A third of all records contained authors that were affiliates/developers from an app company (where the app was not developed by an academic institution for research purposes).

The most common type of fertility apps were those for preventing unplanned pregnancy (33.3% records in this review), yet fertility information can be conveyed in other types of apps. The second most common type of apps providing information on fertility were those designed for conception and cycle tracking (each with 14.8% of records). Notably, for some studies reporting on a single app (rather than an evaluation of a suite of apps), the function was multipurpose and enabled the user to indicate their reproductive goals to receive either pregnancy prevention, contraception or cycle tracking information (Haile et al., 2018; Li et al., 2016; Setton et al., 2016). This flexibility in functionality is evident in an app evaluation study by Moglia et al. (2016) which excluded 'fertility tracking' apps in their review of apps for menstrual cycle tracking. Nevertheless, their search returned apps containing fertility information, illustrating the level of interconnectedness within women's reproductive health information.

Of the 17 records which evaluated the effectiveness of an app directly via its ability to accurately predict the fertile period, one study measured the effectiveness of the app compared to a control group receiving counsel with a healthcare professional (Sridhar et al., 2015). The study highlights the intention of the developers to use this app as an alternative to traditional care by pre-emptively discussing the content with patients prior to their appointment, rather than as an extension of learning or continuing personal development. This style of 'waiting room app' was also observed in two other studies in this review (Mesheriakova & Tebb, 2017; Shopov et al., 2019), whose purpose was to inform patients prior to consultations with healthcare professionals.

Accuracy of information provided in apps

The majority of fertility information reported in apps focussed on delivering a fertile window or ovulation prediction (63%) (Table 2). Many of the studies that relied on evaluating predictions and parameters within apps did not report on other information within the app, and did not reference where this information would be available outside of downloading the app firsthand, or laboriously providing an extrapolation of all app content and coding in an open source repository. Critical to receiving accurate information regarding achieving or avoiding pregnancies during the fertile window is the tracking of sexual intercourse. Fourteen of the records mention the ability for users to self-report intercourse. Yet, only records in this review relating to the apps 'Natural Cycles' and 'Dot' specify that their perfect and typical use calculations exclude cycles with missing intercourse data from their algortithms.

Fourteen records (51.9%; Table 2) reported evidenced-based information limited to a defined menstrual cycle range. Typically this was between 20 and 40 days, with some apps extending this to up to 60 days (Faust et al., 2019). Disconcertingly, it was reported that some apps strictly adhered to a 28-day menstrual cycle (Setton et al., 2016). In five of the 14 records, the algorithm or methods used to define cycle phases for predicting fertile days were made available which is a valuable addition for independent review. However, there remains some concern for those studies which sought validation for an app as a contraceptive method that recruited participants from

the pool of current users of the app who fit the strict cycle length and variability criteria for algorithm evaluation. This implies a cohort of current app users who do not fit within the apps' predictability criteria that may be receiving invalidated or incorrect personal fertility information. We would expect that these apps would have disclaimers warning of app limitations, but this was not reported in any records.

Other types of fertility information identified by this scoping review relate to contraceptive advice (Mangone et al., 2016; Mesheriakova & Tebb, 2017; Sridhar et al., 2015), quantifying the quality of fertility information in apps (Johnson et al., 2018; Kalke et al., 2018; Mangone et al., 2016; Moglia et al., 2016), and descriptions of the types of content without depth of examination or reporting (Haile et al., 2018; Hamper, 2020; Lee & Kim, 2019; Shopov et al., 2019; Staric et al., 2019; Vanya et al., 2017). Fertility information for contraceptive advice apps, while limited in scope to the short-term consequences of long acting, reversible contraceptives (LARCs) on fertility (Mesheriakova & Tebb, 2017; Sridhar et al., 2015), was accurate and instructive.

For studies that quantified information within apps (by a systematic selection and review of apps), the accuracy of information was more variable. Johnson et al. (2018) compared apps to participant biometric indicators of fertility and identified that out of 73 fertility apps, 24.7% did not provide an ovulation day prediction, 8.2% didn't provide a fertile window, and the apps that did predict ovulation, could only do so with 21% accuracy. A study of evidence-based sexual educational apps identified 12 apps with qualified information relating to fertility, but the details of this content was not reported (Kalke et al., 2018). In an evaluation of apps for preventing pregnancy (Mangone et al., 2016) fertility tracking apps were among the category which had the least representations of best practices for pregnancy prevention. Another evaluation study of women's reproductive health apps focussed only on menstrual cycle tracking apps identified as accurate (determined by their ability to accurately predict a 23-day cycle after at least 3 prior cycles input) (Moglia et al., 2016). This limitation to a 23 day-cycle for verifying accuracy makes an inference that an app able to predict a 'short' cycle is reliable, potentially overlooking those with longer or variable cycles. Of these 20 'accurate' apps, 55% contained fertility information, and 15% had information useful for 'fertility medication'. However, the specific content within these categories of information was not elaborated on, only the presence of this information within an app was quantified. Additionally, this study emphasises the range of apps containing information without expert input; as 65% of the 20 apps evaluated had medical disclaimers yet only 5% contained either professional involvement or cited literature (Moglia et al., 2016).

Finally, the accuracy of apps' fertility information in a number of records was difficult to ascertain due to the limited descriptions: mainly centred around qualitative statements unable to be verified, such as 'excellent in providing awareness of the menstrual cycle', 'receive useful advice concerning their health' (Staric et al., 2019; Vanya et al., 2017) or via accounts of app content by participants (Hamper, 2020; Levy & Romo-Aviles, 2019). Simplified descriptions were also present in records where the focus of the study was to evaluate the functions of apps rather than to evaluate information, as in Lee and Kim (2019) which provided a report of capabilities of two apps in reference to people with premenstrual syndrome (PMS) and/or dysmenorrhoea, or the market-testing of the uptake of a family planning app (Haile et al., 2018).

Overall, the depth of fertility information within apps does not seem to be adequately reported in peer-reviewed literature, which has consequences for the independent review and interpretation of content. The need for integration and cohesion of reproductive health and fertility information is ever present, as apps should be able to cater for specific needs and goals or be clear about the intended audience.

Impact/reception on users

Despite the limited reporting of information in peer reviewed studies of women's reproductive health apps, the users of these apps, which number in the hundreds of thousands, are nonetheless being exposed to the content within these apps. Indeed, the effectiveness and value of fertility apps may be determined indirectly through the qualitative analysis of app users as identified in this review. Women consistently report the value of learning about their bodies, often without any prior desire to seek care or education (Hamper, 2020; Lee & Kim, 2019; Levy & Romo-Aviles, 2019). This reiterates the importance of integrating validated women's reproductive health content in topics within apps, as it enables the opportunity for exposure to pertinent topics of reproductive health and wellbeing that the user may deem as irrelevant, or have overlooked altogether.

Six studies (22%) quantified the differences in knowledge of their participants. For researcher-

developed apps by the University of California, the use of apps for contraceptive information were able to improve knowledge about contraceptives by 21% compared to those who didn't use the app (Mesheriakova & Tebb, 2017), and in their waiting-room app, the group that received advice from a healthcare worker had no differences in the level of knowledge as app users (Starling et al., 2018). However, the proportion of information specifically relating to fertility within these apps was limited. Ultimately when apps are developed in conjunction with experts, and elements of behaviour theory and digital learning are considered, then there is potential for substantial improvements in fertility knowledge to occur.

Gender, race, education, and their intersections are important considerations that were underreported in the records returned by the scoping review. Twelve studies (44%) were from the USA, which has a predominantly (76%) white population (U.S. Census Bureau, 2019), and race is reported in only seven (26%) records. Additionally, participants assigned female at birth, but not identifying as female were reported in only one study (Levy & Romo-Aviles, 2019), with all other human studies assuming cis-gender. The gendered content of apps in evaluation studies is discussed in Eschler et al. (2019), noting that of the 17 apps evaluated, all conflated sex with gender. For other sociodemographics; education, income or occupational information was only obtained in three records. It is crucial to consider how the participant selection and reporting limits the applicability of the records examined in this scoping review.

Discussion

This scoping review sought to summarise the peer-reviewed evidence of information about fertility provided to the public through fertility-based female reproductive health apps, and to determine if this information is able to educate app users effectively on these topics. Reproductive health apps are embraced by the public for many reasons ranging from data tracking and informing health, to preventing pregnancy and family planning (Levy & Romo-Aviles, 2019). It is essential that their current impact on decision-making and knowledge be understood, as well as the opportunity they present to become a trusted, reliable source of information and advice.

By undertaking this review, it was found that a majority of the peer-reviewed information within apps is limited to identifying the fertile window of the menstrual cycle, and that very few studies explore the influence of app content on the fertility knowledge of participants. Unsurprisingly, fertile window predictions were a common feature of apps within the studies in this review, as they can be used for both the prevention and planning of pregnancies depending on the intentions of the user, thus making them valuable commodities. However, much of the reporting on these predictions in apps measured the effectiveness of the app as a fertility-awareness based contraceptive method, and it is unclear if multi-function capabilities were available in the apps described in those studies.

This study raised a number of concerns regarding the recruitment of participants for studies which sought to measure the efficiacy of an application as a contraceptive or conception aid tool. For studies where the primary app function was as a contraceptive, there were often limits placed on participant inclusion to be within a defined cycle length and variability. These restrictions are problematic as only approximately 13% of women adhere to a 28 day menstrual cycle. Indeed, 65% of cycles range between 25 and 30 days in length, and a further 7–8.5% of women experience cycles over 35 days in length (Bull et al., 2019b; Grieger & Norman, 2020). In studies where the focus was conception, participants were recruited only if they had 'just started' trying to conceive (Faust et al., 2019), or had been trying for <6 months (or if aged over 35 years, <3 months) (Johnson et al., 2020). A potential bias is thus created, limiting the both participation and applicability of findings to people outside of this range. Further questions arise regarding the justification of exclusions of conception history for an app arguably designed for people trying to conceive over a range of time. A recent study evaluating apps' ability to increase fecundability included participants trying to conceive without exclusion limits on the duration (range: <3 months to >6 months) without finding increased fecundability in app use when adjusting for the length of time trying to conceive as a covariate (Stanford et al., 2020). Together these suggest that while excluding populations which may potentially confound efficacy or success in app functions, the applicability suffers as a consequence, and efforts should be made to identify potential users whose cycle or conception history behaviours have not been tested in the app.

Fertile window predictions were the sole report of fertility information reported in the peer-reviewed literature of fertility apps. This does not necessarily mean there is no other fertility information contained within these apps, as evaluation reviews of apps and

qualitative studies in this review report the contrary (Eschler et al., 2019; Mesheriakova & Tebb, 2017), but rather that the peer-reviewed studies on these types of apps do not provide an accessible option for academic review of content. If apps are to be used as decision making health informatics tools, then they must be able to accommodate for a variety of needs across a number of interrelated subjects, and should be reported openly for interpretation within academic literature.

This review demonstrated that app evaluation studies were more useful for understanding the types of information provided to app users and the accuracy of the content than studies directly reporting on a function or retrospective result. Evaluation studies are conducted by independent researchers and often explore the user interface and thus have the resources to adequately determine the accuracy of information first-hand. However, their reporting is only in reference to the specific aims of the study, which are not always able to extensively cover the breadth of information within apps and thus limit our understanding of content within apps. This reiterates the need for app developers to deposit their coding, or extrapolate their content, to an open access repository for independent academic review. This recommendation has additional advantages for healthcare professionals, who are known to 'prescribe' reproductive health apps to their patients for use (Lunde et al., 2017), to be informed about the most suitable apps. There is evidence indicating an informative future, where app developers and researchers collaborated in the analysis of large datasets obtained through apps, to study the changing parameters of menstrual cycles and fertility in the modern age (Bull et al., 2019a; Bull et al., 2019b; Faust et al., 2019; Grieger & Norman, 2020).

Sociodemographic factors of race and educational level were not consistently reported in the studies in this review, even fewer studies reported on gender diversity of participants. This creates concerns about the participant diversity included in these studies, and how applicable their results are for different demographic groups. The variability of users' race, education and gender may affect the way app information is received, as it may impact the perceived relevance, accessibility and retention (Flanders et al., 2017; Karlsson, 2019; Kressbach, 2019; McDaniel, 1996; St. John, 1982). Future app developers should consult with potential consumers when creating content, as this has been shown to improve reception and uptake of information (Akinfaderin-Agarau et al., 2012; Brayboy et al., 2017; Gold et al., 2010).

The nature of a scoping review imposes a number of limitations that may impact the application of this research. The search results were limited to the English language, which may account for the loss of potentially relevant studies. Additionally, the nature of the database search was framed around potential app purposes and functions with the intention of identifying fertility information within, and due to the range of female reproductive health apps which may contain fertility information. As such, there may be some fertility information in apps for other purposes that were not identified. However, it is important to note that app stores enable multi-language capabilities for published apps, and indeed a number of studies in this review were conducted in non-English speaking countries, and made available in English and other languages. The rigorous search strategy in this review was designed to cover a breadth of reproductive health topics and many records were held for abstract review to ensure that fertility information was captured as much as possible.

Female reproductive health apps have the ability to fulfil a range of reproductive health needs, but they currently do so with severe limitations when it comes to the menstrual cycle variability, and they do not adequately succeed in multi-functional capabilities. It is recommended that future apps consider the diverse and changing needs of their users, and cater a wider variety of purposes (contraception, trying to conceive, measuring and observing changes in response to medications or reproductive illnesses, etc), for a variety of people (race, sexuality, and gender). Future app studies should also explore the influences of culture on the effective communication of information in apps. In Africa, as sexual health studies have shown promise with navigating cultural sensitivities around disclosing sexual information through text-messaging style apps and services (Nyanchoka, 2011; Rokicki & Fink, 2017; Shelus et al., 2017). Furthermore, the scope of information available via the user interface is a major barrier to independent review for accuracy, and it is recommended that through open source sharing of app contents, developers would be held accountable to their consumers, healthcare professionals and researchers. A final conclusion from this review was the lack of studies that determine the effectiveness of an app's contents to translate to knowledge or behaviour change in participants. There is evidence that women who use apps have differing knowledge of fertility than non-app using counterparts (Ford et al., 2020), and future iterations of apps would benefit from including factual information about factors

influencing fertility with an accompanying evaluation. This would strengthen apps as tools for acquiring knowledge and measuring/recording reproductive health. Despite the limitations of studies in this review, apps are continually identified as a useful tool to women for understanding their bodies and family planning decision making, and as such their popularity will only continue to grow. Therefore, continued efforts towards rigorous reporting and accuracy will achieve large strides in public health promotion for fertility and reproductive health.

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ORCID

Emmalee A. Ford http://orcid.org/0000-0003-0371-2399

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Chapter 5: The association between reproductive health smartphone apps and fertility knowledge in Australian women

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Chapter overview

As established in chapter 4, some currently available women's reproductive smartphone apps contain evidence-based information. However, there are concerns with the range of women this information may apply to, and whether the information translates to improvements in knowledge. As such, I sought to investigate fertility knowledge in the context of the use of apps relating to female reproductive health to determine if there was a knowledge difference between those who did or did not use women's reproductive health apps.

In this study, I surveyed a large cohort of women in Australia to examine their general fertility knowledge, and also gathered information about respondents' use of reproductive health mobile applications, and the preferred utilities within these apps. The popularity of menstrual cycle tracking apps as identified in this study presents a viable opportunity to target pertinent reproductive health information to these women.

Overall, this study provides preliminary evidence for an association between fertility knowledge and reproductive health app use. This significant finding presents a valuable platform for future attempts to target women of reproductive age for information about fertility. This research also is valuable in replicating similar levels of inadequate fertility knowledge among the general population. Smartphone applications may be an avenue for the dissemination of fertility information, and we present a novel opportunity for public health interventions using this method.

Original research article

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The association between reproductive health smartphone applications and fertility knowledge of Australian women

Emmalee A. Ford[1,2*], Shaun D. Roman[1,2,3], Eileen A. McLaughlin[1,4,5], Emma L. Beckett[2,6†] and Jessie M. Sutherland[1,2†]

Abstract

Background: Previous studies have identified that women living in developed countries have insufficient knowledge of factors which may be contributing to the increasingly high global infertility rates such as maternal age and assisted reproductive technologies. There is a large market of reproductive health smartphone applications, yet little is known about the advantages these apps may confer to users in regards to reproductive health knowledge.

Methods: An anonymous, online survey of women living in Australia aged 18 and above was open March–June 2018, until ≥200 responses were acquired for statistical power. Respondents answered questions regarding knowledge about general fertility and related factors (age, cyclic fertility, smoking, obesity, miscarriage rate, and success of assisted reproductive technologies). Fertility knowledge was compared in respondents who did or did not use apps relating to female reproductive health. Additionally the functions preferred in reproductive health apps was described by app using respondents. Sociodemographic information was also collected, and relevant data within the dataset was subject to multivariable modelling for the outcome of the fertility knowledge questions.

Results: Of the 673 respondents that completed the survey, 43.09% reported using mobile phone applications relating to female reproductive health. On average, respondents answered only three of the six fertility knowledge questions correctly. App using respondents were more likely to score better on one question, related to fertility during the menstrual cycle ($p < 0.001$). App users most commonly reported using the menstrual tracking function in apps (82.4%), which may account for the increased knowledge of cyclic fertility.

Conclusions: This data provides preliminary evidence toward the usefulness of smartphone applications as a medium for providing information about fertility to women. A limited understanding of one's own fertility was demonstrated despite being essential for the decision-making of women throughout their reproductive years.

Keywords: Female infertility, Education, mHealth, Fertility awareness, Menstrual cycle

* Correspondence: emmalee.ford@uon.edu.au
†Emma L. Beckett and Jessie M. Sutherland contributed equally to this work.
[1]Priority Research Centre for Reproductive Science, Schools of Biomedical Science & Pharmacy and Environmental & Life Sciences, University of Newcastle, Ring Road, Callaghan, New South Wales 2308, Australia
[2]Hunter Medical Research Institute, New Lambton Heights, New South Wales 2305, Australia
Full list of author information is available at the end of the article

Background

Human infertility is recognised as a global public health issue by the World Health Organisation, [1, 2]. Importantly, multiple modifiable lifestyle factors, including smoking and obesity, are known to be detrimental to fertility [3, 4] and the success of reproductive treatment outcomes [5, 6]. However, the age-related fertility decline remains the single most limiting factor in reproductive success [7, 8], with advanced maternal age (> 35 years) associated with an increased risk of infertility, miscarriage, fetal abnormalities, and stillbirth [9–11]. Despite these considerable health risks almost one-quarter of all women giving birth in Australia in 2016 were > 35 years of age [12]. Moreover, within Australian and New Zealand, for 61% of all couples accessing assisted reproductive treatment cycles in 2016, the female partner seeking treatment was > 35 years of age [12].

Although a well-established concept, the consequences of advanced maternal age on fertility and pregnancy may remain poorly understood among the general public. A series of recent international studies have demonstrated that women of reproductive age from Western and European societies consistently underestimate the impacts of maternal age on fertility [13–18]. Additionally, there is a common misconception within Western societies that assisted reproductive treatments can effectively compensate for age-related infertility [19]. These studies support the notion that insufficient knowledge of these factors may be contributing to the number of people struggling with infertility. This data highlights a requisite need for further public education on the consequences of advanced maternal age on fertility and pregnancy for women during their reproductive years.

The United Nations Educational, Scientific and Cultural Organisation recommends that sexual health education include fertility; however this is not currently mandated within many national curricula, including Australia and Britian [20–22]. Despite this, there is an abundance of publicly available information regarding fertility and reproductive health, with many people preferencing the internet to access this content [23, 24]. However, studies demonstrate that people accessing health-related information online are reluctant to go beyond the first page of search engine inquiries, their evaluative skills are limited, and indicators of credibility are often missed (reviewed in [25]). The development of strategies for optimising accessibility and visibility of fertility information is a valuable avenue to improve knowledge.

The global expansion in mobile education products [26] presents the ideal platform for mobile health (mHealth) smartphone applications (apps) to address this knowledge gap. Smartphones are arguably the most accessible form of mixed-modal communication today, with higher online growth than personal computers [27]. In Australia, 84% of all adults, and 99% of 18–29 year-olds possess a smartphone [28]. Demand for mHealth services is demonstrated by the millions of annual downloads across the hundreds of thousands of health and lifestyle apps available [29]. Women's reproductive health apps account for 7% of all health-related apps [30, 31]. Despite containing a myriad of accessible functions including menstrual cycle tracking, pregnancy planning, and contraception, it remains unknown how these functions influence the fertility knowledge of users. Harnessing these technologies to disemminate reliable and accessible information requires improved understanding of the relationship between their use and the acquisition of relevant knowledge.

Regarding the reliability of reproductive health apps, studies report that only 20% are of good quality [30, 32]. It remains unclear as to whether currently available apps confer understanding, with previous studies of fertility knowledge in women lacking any reference to apps [17, 18, 23, 33–36]. The aim of this study was to determine the differences between fertility knowledge, based on the use of female reproductive health apps, via an anonymous online survey of Australian women. It was hypothesised that women who utilised reproductive health apps would perform better in general fertility knowledge questions Additionally, for women using apps, determining the reproductive health functions employed was a secondary aim. A better understanding of these relationships may reveal new opportunities for the integration of public health interventions within reproductive health apps as a strategy to improve fertility knowledge.

Methods

Study participants

This study was approved by the Human Research Ethics Administration at the University of Newcastle; protocol number H-2018-0014. Participation in the survey was limited to women (and for inclusivity, to those not identifying as women, but assigned female at birth), living in Australia, aged 18 and over. Exlcusion from participation was restricted to people with male-assigned reproductive systems, aged under 18 years, or not living within Australia. The use of "female reproductive health" or simply "reproductive health" in this paper refers to aspects surrounding general functions of the female reproductive system including menstruation, conception, and pregnancy.

Survey design

An anonymous, online fertility knowledge survey was created to capture the usage habits of those with reproductive health smartphone applications (apps), and what relationships exist between differences in knowledge or

function of these tools (see supplementary file 1). All respondents provided mandatory information regarding sociodemographic factors including age, education level, income, postcode, relationship status, and pregnancy history. Respondents also completed six, multiple-choice general fertility knowledge questions (supplementary figure 1). These questions covered; fertility during the menstrual cycle, age-related fertility, impact of lifestyle factors (obesity, cigarette smoking) on fertility, the frequency of miscarriage in Australia, and the success rates of assisted reproductive technologies in Australia. These questions were chosen to capture a range of factors relating to aspects of fertility and education including personal management (cyclic fertility and age), lifestyle factors (smoking, obesity), and common misconceptions (miscarriage rate and IVF success). Similar questions have been included in other general fertility knowledge surveys [17, 18, 37, 38] and thus serve the additional purpose of contextualising the results of this study. For respondents using reproductive health apps, the functions and utility preferences of the apps were collected. Reproductive health apps were defined to respondents as "applications that have to do with the female reproductive system and may include features like: menstruation tracking/calendar, pregnancy, or contraception/birth control."

The survey was implemented using the host website eSurvey Creator (enuvo GmbH, Zurich).

Data collection

The survey was open in March – June 2018, and advertised on social media (Twitter, Facebook, Instagram), and at the University of Newcastle's Callaghan, Ourimbah and New Space campuses.

The responses to fertility knowledge multiple-choice questions were coded into a binary of being correct or incorrect. Area data was provided in the survey as the respondents' postcodes and was re-coded according to the Accessibility/Remoteness Index of Australia (ARIA), which defines remoteness as accessibility based on road distances [39]. In this study, locations with ARIA service centre score 'A' were re-coded as "city", score 'B' as "inner regional", and score 'C' as "outer regional". Annual household income data was provided as a multiple choice of different income brackets. In this study, income data were presented alongside government definitions of low, middle and high income households according to equivalised disposable household income estimates within given quintiles of the population [40]. These estimates are adjusted by equivalence factors to standardise them for variations in household size and composition, while taking into account the economies of scale that arise from the sharing of dwellings [40].

App users were asked about function and utility preferences for their reproductive health apps in a multiple response style question, with an additional free-text response option. Thus, within a given category of this question, the number of responses refers to the proportion of all app using respondents selecting the given category, with the free text option re-coded as "other".

Statistical analysis

It was determined prior to launch that the survey required a minimum of 200 responses to allow the detection of a quantitative difference mean response of experimental (app users) and control subjects (non-app users) with probability (power) 0.8 at 0.05 significance level, assuming equal response rates between users and non-users of the apps in question. Data from the survey were entered in JMP version 13 (SAS Institute, Inc.).

Frequencies and proportions were used to describe the range of categorical responses, and comparisons of proportions between app users and non-app users were made by chi-square likelihood ratio tests, with effect sizes reported using the r-squared statistic. $P < 0.05$ was considered to be statistically significant. Where the proportions of a sociodemographic category differed significantly between app users and non-app users, the category was then used as a predictor in multivariable models for the outcome of the fertility knowledge questions.

Nominal logistic regression was used for multivariable models. The chi-square statistic for the whole model was reported, with adjustments for the following variables: age, app use, whether the respondent had conceived in the past, and if they were currently trying to conceive. Effect likelihood ratio tests, odds ratios, and 95% confidence intervals were reported for predictors.

Results

Sociodemographic and reproductive history of respondents

At the end of the survey period a total of 759 respondents had accessed the online survey, with 673 respondents completing all mandatory items allowing their inclusion in data analyses. A majority of the respondents were of reproductive age (between 18 and 36 years of age), making up 83.8% of all respondents (Table 1). Those who had completed a tertiary degree (or above) comprised 43.3% of all respondents (Table 1). Of the 227 respondents who had conceived, 78.4% (178) had given birth. The current use of smartphone apps relating to reproductive health was reported by 43.1% (290) of respondents. App users were as a group younger than non-app users (Table 1; $\chi^2 = 24.5$, $p < 0.001$). A larger proportion of app users lived outside of metro areas, in inner or outer regional areas (27.9% vs 18.3%; $\chi^2 = 8.8$,

Table 1 Sociodemographic and reproductive history of respondents sorted by reproductive health application usage status

	All respondents (*n* = 673)	App-users (*n* = 290)	Non-app users (*n* = 383)	X^2 (*P*-value)[a]
Age category	*n* (%)	*n* (%)	*n* (%)	
18–24	285 (42.4)	130 (44.9)	155 (40.5)	24.540 (< 0.0001[c])
25–30	161 (23.9)	71 (24.5)	90 (23.5)	
31–36	118 (17.5)	59 (20.3)	59 (15.4)	
37–42	46 (6.8)	20 (6.9)	26 (6.8)	
43+	63 (9.4)	10 (3.5)	53 (13.9)	
Location by ARIA category				
City	522 (77.6)	209 (72.1)	313 (81.7)	8.769 (0.0125[c])
Inner regional	138 (20.5	74 (25.5)	64 (16.7)	
Outer	13 (1.9)	7 (2.4)	6 (1.6)	
Education attained				
< Year 12 (or equivalent)	25 (3.7)	10 (3.5)	15 (3.9)	5.261 (0.2615)
Year 12 (or equivalent)	157 (23.3)	80 (27.6)	77 (20.1)	
Technical diploma	109 (16.2)	43 (12.9)	66 (17.2)	
Bachelor's degree	256 (38)	106 (36.5)	150 (39.2)	
Postgraduate	126 (18.7)	51 (17.6)	75 (19.6)	
Annual household income				
< $20,000	78 (11.6)	34 (11.7)	44 (11.5)	11.511 (0.0738)
$20,000 - $34,999: low income[b]	74 (11)	32 (11)	42 (10.9)	
$35,000 - $49,999: mid income[b]	67 (10)	25 (8.6)	42 (10.9)	
$50,000 - $74,999	109 (16.2)	41 (14.1)	68 (17.8)	
$75,000 - $99,000	98 (14.6)	53 (18.3)	45 (11.8)	
$100,000 - $149,999: high income[b]	141 (21)	68 (23.5)	73 (19)	
$150,000 +	106 (15.8)	37 (12.8)	69 (18)	
Relationship status				
Married/de facto	358 (53.1)	158 (54.5)	200 (52.2)	1.7 (0.6368)
In a relationship, living apart	133 (19.8)	55 (19)	78 (20.4)	
Not in a relationship	170 (25.3)	70 (24.1)	100 (26.1)	
Other/not disclosed	12 (1.8)	7 (2.4)	5 (1.3)	
Have a close friend/relative who has experienced fertility issues				
Yes	381 (56.6)	156 (53.8)	225 (58.8)	1.647 (0.1993)
No	292 (43.4)	134 (46.2)	158 (41.3)	
Currently trying to conceive				
Yes	30 (4.4)	24 (8.3)	6 (1.6)	18.052 (0.0001[c])
No	639 (95)	264 (91)	375 (97.9)	
Not disclosed	4 (0.6)	2 (0.7)	2 (0.5)	
Have conceived before				
Yes	227 (33.7)	114 (39.3)	113 (29.5)	7.073 (0.0078[c])
No	446 (66.3)	176 (60.7)	270 (70.5)	
Have given birth				
Yes	178 (26.5)	87 (30)	91 (23.7)	3.287 (0.0698)
No	495 (73.5)	203 (70)	292 (76.3)	

[a]Indicates that statistical data derived from comparison between app users and non-app users
[b]Reference to Australian Bureau of Statistics definitions of household wealth [40]
[c]Indicates statistical significance

$p = 0.013$). Additionally, it was more likely that app users were trying to conceive (8.3% vs 1.6; $\chi^2 = 18.1$, $p < 0.001$), and were more likely to have previously conceived compared to non-app users (39.3% vs 29.55%; $\chi^2 = 7.1$, $p = 0.008$).

General fertility knowledge of respondents

The cumulative scores for the six multiple-choice fertility knowledge questions are shown in Fig. 1. The data follows a Poisson distribution (χ^2 goodness of fit = 423.1, $p = 1$). The mean score was 3.03 correct answers, with a standard deviation of 1.38. The modal score was three correct answers, (27.8% of respondents; 95% CI: 24.4–31.2). Surprisingly 3.1% of respondents did not answer any questions correctly, with 31.5% getting only 2 or fewer questions correct. Only 2.8% of respondents answered all six questions correctly.

A clear majority of app users tracked their cycles (91.4%, or 265 out of 290 app users), with the next most selected function "plan a pregnancy" accounting for only 19%, or 55 responses (see Fig. 2). Fifteen respondents utilised the free-text tool enabled for 'other' to identify that birth control reminders were a primary function they used reproductive health apps. A further 9 items within the 'other' category were identified by respondents who use reproductive health apps for monitoring cycle-related symptoms (i.e. headaches, bloating, and acne).

Comparisons between reproductive health app and non-app users

The proportion of correct responses for each category are shown in Table 2. App users were more likely than non-app users to correctly identify the most fertile time in the menstrual cycle (15.7% $\chi^2 = 16.7$, $p < 0.001$). These results remained significant in the fully adjusted model ($\chi^2 = 44.4$, $p < 0.001$). The parameters responsible for this effect were app use ($\chi^2 = 16.4$, $p < 0.001$) and age ($\chi^2 = 10.1$, $p = 0.039$). For this question, app users were 1.9 times more likely to answer correctly (95% CI: 1.4–2.8, $p < 0.001$) than non-app users.

For the question regarding miscarriage rate in Australia, there were no significant differences in the correct responses of app users compared to non-app users ($\chi^2 = 3.22$, $p = 0.07$). However, the adjusted model for this question was significantly different ($\chi^2 = 44.4$, $p < 0.001$), with the effects primarily driven by whether the respondent was trying to conceive ($\chi^2 = 24.1$, $p < 0.001$), whether they had conceived before ($\chi^2 = 14.7$, $p < 0.001$), and age ($\chi^2 = 11.7$, $p = 0.02$). The same adjustments were applied to each subsequent fertility knowledge question, yet there were no significant differences. For the remainder of the questions, there were no statistically significant differences between app users and non-app users.

Discussion

There is an increasingly large volume of users accessing the range of health apps for female reproductive health. Yet these types of apps have previously only been studied in small samples with a focus on reviewing the information within apps [30, 32, 41, 42], or the design and testing of novel apps [43–47]. Presently, the association between knowledge about female reproductive health and the use of reproductive health-themed apps has yet

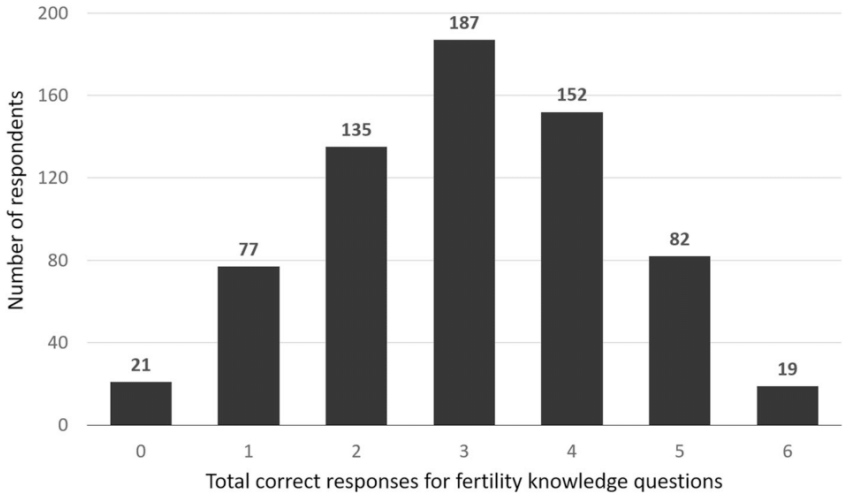

Fig. 1 Total score for fertility knowledge questions. In the fertility knowledge survey, respondents were asked six general knowledge fertility questions (Supplementary figure 1) with multiple choice responses, and the total score of correct responses for each respondent across the questions is represented in the graph ($n = 673$)

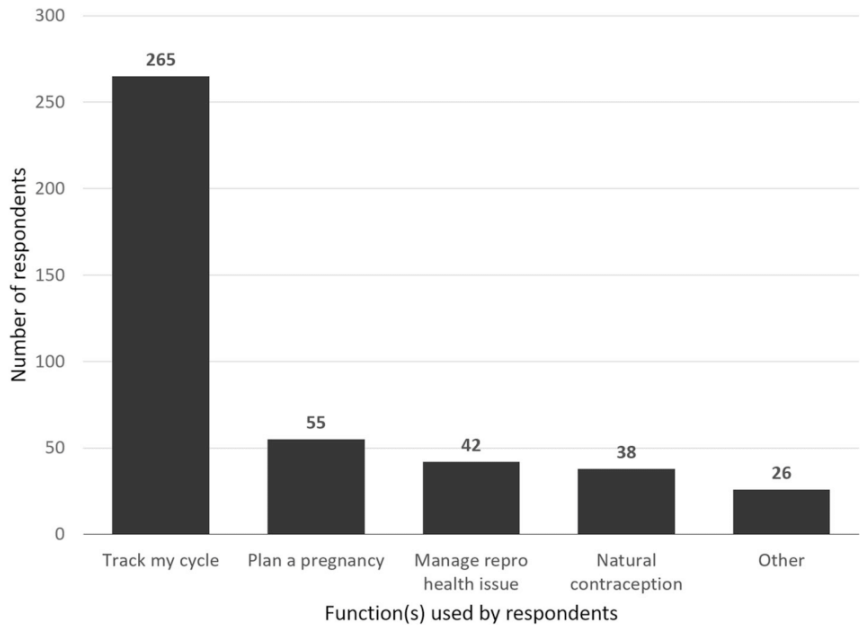

Fig. 2 Application user functions and utility preferences. Respondents who use reproductive health applications were instructed to select all that apply from a list of common utilities and functions available in reproductive health smartphones, with a free text option to add other options. Additional functions that respondents commonly identified in 'other' included reminders to take birth control [15], and monitoring cycle-related symptoms [9]. Data is presented as a count of the respondents that selected the corresponding option ($n = 290$)

to be investigated. This study is the first to address the fertility knowledge gap by exploring the relationship between app use and knowledge within a considerably large cohort of Australian women. This study identified a novel association between app use and knowledge of fertility during the menstrual cycle. This association provides compelling preliminary evidence that may be used to assess the viability of apps as a medium to promote public health messages and address the gap in fertility knowledge. This study also found that the most popular function within reproductive health apps was menstrual cycle tracking, making this type of app an ideal opportunity for public health intervention.

A number of demographic variables were different between the app using and non-app using groups in this study. App users were more likely to be younger, aged 18–24, which is expected, as the adoption of smartphones is much greater in this generation [48]. In a 2017 national mobile consumer survey, 95% of 18–34 year olds reported smartphone ownership, compared to older Australians (85% in 45–54 year olds, and this further decreases with advancing age) [48]. A greater proportion

Table 2 Proportions of correct responses by question

Question	All respondents correct (%)	App users correct (%)	Non-app users correct (%)	*P*-value[a]	*P*-value for adjusted model[c]
When is the most fertile time in the menstrual cycle?	58.8	64.8	49.1	< 0.0001[b]	< 0.0001[b]
At what age does a woman's fertility begin to decline?	46.2	42.8	45.2	0.5326	0.1220
How much does cigarette smoking negatively impact fertility?	61.9	57.6	60.3	0.4762	0.7137
How much does obesity negatively impact female fertility?	65.5	60	64.5	0.2336	0.1885
On average, how frequently do you think miscarriages occur in Australia?	50.7	53.4	45.4	0.0726	< 0.0001[b]
On average, how often do you think IVF achieves a live birth in Australia?	34.5	31.4	34.2	0.4398	0.2025

[a]indicates that statistical data derived from comparison between app users and non-app users
[b]Indicates statistical significance
[c]model is adjusted for the following predictors: app use, age, location, whether respondent was trying to conceive, and whether they had conceived before

of app users lived in regional centres (defined as fewer than 250,000 people), which was an unexpected finding, as regional and rural internet accessibility has traditionally been a challenge for people living away from city centres [49]. However, there is a renewed research focus in the equal distribution of care into rural and region locations in Australia via smartphone technology [50–52], and our findings have positive implications for rural mobile health interventions of the future. Finally, app users were more likely to be trying to conceive, or already had conceived compared to non-app users. This may be attributed to the app functions used by respondents (such as fertility tracking and pregnancy planning), fitting with previous studies which have demonstrated that women who are struggling to conceive often actively try and improve their knowledge through multiple sources [24, 34, 53, 54].

Overall the fertility knowledge of respondents in this survey, regardless of app use, was mediocre, which may suggest that women are lacking education in some fundamental aspects of fertility and reproductive health. This is consistent with the literature surrounding women's understanding of fertility related to age of fertility decline, cyclic fertility, and lifestyle factors that can influence fertility [13, 15–18, 23, 33, 34, 55]. Misunderstanding aspects relating to fertility may risk women's future plans of parenthood. In particular, the decision to delay childbearing without an understanding of the negative impact of advanced maternal age on reproductive success [56]. This can ultimately burden the health system, with Australian government-funded health care claims surpassing $200 million for assisted reproductive treatments in 2010 alone [57]. The burden is predicted to rise as the use of assisted reproductive technologies is increasing, while live birth rates as a result of these technologies remains staggeringly low at only 18.1% [12]. Almost two thirds of all respondents overestimated the success rate of IVF. This creates an additional burden as the consequence of delaying conception is not as easily rescued as respondents estimate. Importantly, infertility can have mental health impacts for patients [58, 59], thus the current study only adds to the evidence that more targeted and further reaching public health interventions are required to bridge the fertility knowledge gap. Increasing knowledge about general aspects of fertility and pregnancy may have additional benefits beyond successful conception; it may help adjust women's expectations and hence their experiences. For instance, amongst women who had experienced a miscarriage, those with more education about miscarriage beforehand were better able to cope with the event when it occurred [60].

The potential response bias occurring due to the large proportion of women in this survey cohort who used menstrual cycle tracking as a function in their reproductive health apps may be a limitation of the present study. The knowledge differences among women who used apps for different reasons was not able to be examined in great depth in this study, thus a larger sample of diverse reproductive health app functions is required in future research. This would enable the link between app function and knowledge improvements to be studied more conclusively. Increasing the specificity of response options available for the 'track my cycle' selection in further surveys also may shed more light on this aspect in future research. It is important to note that the proportion of women in the app using cohort that had completed a tertiary degree (or above) is 24% higher than the overall proportion of Australian women with the same qualification at an estimated 30.1%, which needs to be considered in interpretation [61]. In the survey, 24 app using respondents self-identified that they were trying to conceive, yet 55 app using respondents were reportedly using a 'plan a pregnancy' app. There are a range of pregnancy-related apps available to accommodate for many stages throughout pregnancy (planning to conceive, trying to conceive, predicting gestation monitoring pregnancy, etc) [42], and respondents may have selected this to cover this broad range of applications. Apart from the free-text option, 'plan a pregnancy' was the only selectable option that mentioned pregnancy. Indeed, increasing the specificity of the ranges of reproductive health apps used, or creating further defining questions in futures studies may assist in interpreting any associations between fertility and knowledge.

Prior research has shown that app users of health and medical-type apps [62, 63], and female reproductive health apps [64, 65] often self-report during research interviews the usefulness and 'benefits' they obtain from their app use. However, previous research has not quantitatively studied whether these 'benefits' are associated with knowledge about the content in question. This is important to consider in order to evaluate the usefulness of these apps as tools for education, to identify the strengths of current apps, and to highlight the challenges for future apps. In this study, it was observed that those who used reproductive health apps were more likely to be knowledgeable of the most fertile time in the menstrual cycle ($p < 0.0001$ in adjusted model). This may be due to the large proportion of app using respondents that utilised menstrual cycle tracking functions. Despite the popularity of menstrual cycle tracking in this study, its use may not necessarily be linked to fertility. A recent study found that women identified many reasons for tracking menstruation using apps, and of the five priorities, only two could be directly connected to providing knowledge about fertility (becoming pregnant, inform conversations with healthcare providers) [64], further

description of the purposes for each type of reproductive health app would serve to examine this link more closely. Nevertheless, the fact that 91.4% of women in this study tracked their menstrual cycles using reproductive health apps, provides an excellent intervention opportunity for education regarding cyclic fertility.

While the marketplace of menstrual cycle tracking and other reproductive health apps is fraught with inaccuracies and misinformation [30, 66], there are a small number that meet adequate standards, with a US study finding 18% of the reviewed menstrual cycle tracking apps were accurate [30], and a recent study validating some components of a fertility app [67]. Additionally, around 90% of women have a 'normal' cycle frequency (between 24 and 38 days) [68], thus apps that may have flaws in their content relating to those with irregular cycles, may still be beneficial to a majority of users with no cycle irregularity Further research into education gained from apps could involve testing the knowledge of a sample of women before and after prolonged periods using an accurate menstrual tracking application purposed for fertility awareness. Additionally, further analyses linking the quality of apps used by respondents to their demonstration of fertility knowledge obtained from the survey would enable the link between app use and knowledge to be understood in greater detail.

Although not associated directly with app use, an additional and important finding from this study was the observation that women with a history of conception ($p < 0.001$) or who were actively seeing to get pregnant ($p < 0.001$) were more aware of the miscarriage rate ($p < 0.001$ in adjusted model). This is consistent with findings that the general population often underestimate the prevalence of miscarriage [37], and couples who have experienced miscarriage reported that they were not previously aware of the frequency in which it occurs in the people around them [69]. As miscarriage is not often discussed amongst the general population, it is likely that lived experience was responsible for the significant differences in response rates to this question in women who had conceived in the past or that were trying to conceive.

Conclusions

This study has provided preliminary evidence for an association between fertility knowledge and reproductive health app use. This will be able to inform public health strategies aimed at increasing awareness of fertility throughout a woman's reproductive years. Fertility knowledge and awareness are important, not only for family planning, but for young people to have realistic expectations and make lifestyle choices accordingly. The association between knowledge and app use in this study while significant, had a small effect size, and covered only one aspect of fertility. This may be a reflection of the disadvantages in the apps themselves as discussed above, or a reflection of the limited knowledge of the general population. In both cases, the distribution of fertility information to women is imperative, and should be delivered in a way that is highly accessible, and likely to be received by women. Smartphone applications may be one such avenue for the dissemination of this information, and this study presents a novel opportunity for public health interventions using this method. Regardless of the sources developing them, mobile phone applications play an increasingly important role in the provision of personal health information [41], and this study provides an insight into how smartphone applications may be able to educate women about their own fertility.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12905-020-00912-y.

Additional file 1.

Additional file 2: Figure S1. Description of data: Response to fertility knowledge questions by app use status. Each of the questions in the fertility knowledge quiz is displayed with the responses as shown to participants (Δ indicates the correct answer). Black bars represent the number of non-app using respondents ($n = 383$) that selected a given response, and grey bars indicate app users ($n = 290$).

Abbreviations

Apps: Smartphone applications; ARIA: Accessibility/Remoteness Index of Australia; IVF: In vitro fertilisation; mHealth: Mobile health; US: United States

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Author's contributions

EAF drafted the manuscript, and contributed to the design of the study. EAF also completed the acquisition and analysis of data for the work. SDR revised the manuscript critically for important intellectual content, and provided assistance in the interpretation of data for the work. EAM revised the manuscript critically for important intellectual content. ELB Contributed to the conception and design of the study, and assisted with interpretation of data for the work. ELB also contributed to the final approval of the version to be published. JMS was responsible for initial conception of the study, contributed to study design and interpretation of data. JMS additionally revised the manuscript critically for important intellectual content and was responsible for final approval of the version to be published. All authors have read and approved the final version of the manuscript.

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Availability of data and materials
The datasets generated and/or analysed during the current study are not publicly available due to the likelihood that they will be used in future novel analyses, but are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The online survey from which this data originated obtained written evidence of consent. The survey contained an information statement document that when read, declares informed, recorded consent via a short statement outlining that by submitting the completed survey, the participant consents to their data being stored and used. This study was approved by the Human Research Ethics Administration at the University of Newcastle; protocol number H-2018-0014.

Consent for publication
Not applicable.

Competing interests
The authors declare they have no competing interests.

Author details
[1]Priority Research Centre for Reproductive Science, Schools of Biomedical Science & Pharmacy and Environmental & Life Sciences, University of Newcastle, Ring Road, Callaghan, New South Wales 2308, Australia. [2]Hunter Medical Research Institute, New Lambton Heights, New South Wales 2305, Australia. [3]Priority Research Centre for Drug Development, University of Newcastle, Callaghan, New South Wales 2308, Australia. [4]School of Science, Western Sydney University, Penrith, NSW 2751, Australia. [5]School of Biological Sciences, Faculty of Science, University of Auckland, Auckland 1142, New Zealand. [6]School of Environmental & Life Sciences, Faculty of Science, University of Newcastle, Callaghan, New South Wales 2308, Australia.

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Publisher's Note

Chapter 6: Final discussion

6.1 – Preamble

The studies within this thesis apply a multidisciplinary approach to improving fertility outcomes. This was achieved through the molecular investigation of gene expression in mammalian granulosa cells undergoing primordial follicle activation, and the potential for reproductive health smartphone apps to influence knowledge about fertility. Two themes emerge from the findings in this thesis; the subtle gene expression changes in granulosa cells during primordial follicle activation reignites the need for rigorous pursuit of delineating the evidence formerly identified, and that reproductive health smartphone apps provide an opportunity for bridging the fertility knowledge gap to improve fertility outcomes. A novel model was applied to isolate granulosa cells, before and during the initial wave of primordial follicle activation. The genes expressed in these cells during the process of primordial follicle activation were identified, and the analyses signify the complexity and subtlety of the transition of these cells. Through a systematic appraisal of peer-reviewed evidence on smartphones and the collection of self-reported data of app use in Australian women, the unique opportunity to educate people about fertility using reproductive health apps was identified.

The prevalence of infertility is increasing globally due to a range of factors that differ according to region (reviewed in Petraglia et al. (2013)). In Australia, maternal age is a major contributor to infertility, evidenced by the average age of mothers giving birth at 30.6 years of age in 2017 or the 45% rise in assisted reproductive technology cycles from 2008 to 2018 (Newman et al., 2020; Wang et al., 2010; AIHW, 2019). Intrinsic to the delay in childbearing years is a lack of understanding about fertility (García et al., 2018), which also contributes to misconceptions about the detrimental impact of lifestyle factors on fertility (Pedro et al., 2018), and may result in a resistance to seeking medical advice for reproductive health issues (Bunting and Boivin,

2007; Kilfoyle et al., 2016). Women with premature ovarian insufficiency (POI) may be diagnosed upon the presentation of menopausal symptoms and therefore struggle to respond to typical fertility preservation techniques (ESHRE, 2015). Beyond genetic, autoimmune and iatrogenic induction of POI which only account for between 30 and 50% of cases (Chapman et al., 2015), little is known about the factors that bring about the rapid loss of primordial follicles from the ovarian reserve. Treatment directed at primordial follicle activation may be helpful for fertility in women diagnosed with POI but in this setting follicle loss may have progressed to such an extent that there are insufficient follicles remaining. Treatment strategies will be of greatest use in women identified as poor ovarian responders undergoing ART in addition to those women at risk of POI secondary to genetic abnormalities or chemotherapy. Additionally, further research directed at identification of biomarkers for accelerated primordial follicle activation will assist with identification of women at risk of POI to facilitate timely introduction of fertility planning or preservation strategies.

Clinical findings and current research have shaped the trajectory of this thesis in the years it was developed (spanning 2017 to 2020). As fertility preservation strategies for POI patients has resulted in live births through the *in vitro* activation of cryopreserved tissue (reviewed in Dolmans et al., 2019), the need for fundamental research underlying the causes of excessive activation of primordial follicles has remained ever present. The publishing of transcriptome findings on human samples (Ernst et al., 2018; Zhang et al., 2018) after transcriptomics data for this thesis was being analysed illustrate how timely next generation sequencing projects are for building substantial knowledge of primordial follicle activation and served as a reference point for similarities between humans and the mouse model. These findings allowed the scope of the thesis to gain new clinical relevance through a renewed focus on pathways understudied in primordial follicle activation that, with further study have the potential to lead to new preservation or early diagnostics for people at risk of early fertility loss through accelerated

primordial follicle activation. Additionally, the interest in women's reproductive health smartphone apps has been building, so too has research in this field. A consequence of the findings in Chapter 5 were to critically review the apps used by participants and those available in Australia to determine their potential to influence fertility knowledge. However, proceedings from the Fertility Society of Australia conference in 2019 identified a systematic review of Australian women's health apps (Costa & Yazdani, unpublished). This led to the development of a scoping review which has provided recommendations for the future of app use, which was emphasised by new research reviewing the fertile window information in popular women's health apps in the USA (Ali et al., 2020). Together, this thesis has outlined the clinical potential women's health apps have as both a health resource in Australia and as a platform to provide public health messaging about fertility and reproductive health.

6.2 – Modelling primordial follicle activation in neonatal mice

The mechanism of primordial follicle activation remains somewhat elusive in mammalian systems. Much of the research has been conducted in rodents and livestock, with little evidence from these model systems validated in human samples (Adhikari and Liu, 2009; Grive, 2020). Human oocytes, whole follicles, or ovary sections are much less accessible than animal equivalents – and the results in human research are not always analogous to findings from animal systems (discussed in Chapter 2, Ford et al. (2020a)). However, the utilisation of animal models, particularly mice, are fundamental for a systematic approach to studying the process of primordial follicle activation. A broad perspective is becoming increasingly relevant, with primordial follicle activation studies identifying confounding influences on activation outside of the follicle unit, such as functional redundancy (Tarnawa et al., 2013), physical/structural effects (Nagamatsu et al., 2019), and gene ablation studies causing off-target effects and/or

embryonic lethality where pathways have multiple roles in an organisms' tissues (Yu et al., 2016).

The work in this thesis utilised a systems approach in understanding primordial follicle activation; comparing the transcriptome of an intrinsic, yet often overlooked contribution of the granulosa cells. Granulosa cells are integral to oocyte maturation, follicle growth and metabolism through the initiation of signalling and transfer of materials through gap junctions (El-Hayek et al., 2018; Eppig, 2018; Zhang et al., 2014). Approaches in gene expression characterisation in younger stage follicles have previously been at the whole-follicle level (Dharma et al., 2009; Ernst et al., 2018; Hasegawa et al., 2009; Herrera et al., 2005; Kezele et al., 2005; Yoon et al., 2006), with fewer studies on the individual components; oocytes (Arraztoa et al., 2005; Ernst et al., 2017; Grøndahl et al., 2013; Pan et al., 2005) and granulosa cells (Bonnet et al., 2011; Bonnet et al., 2008; Ernst et al., 2018). These studies, though very valuable, require further functional validation to determine the relationship of these gene expression changes to primordial follicle activation.

The transcriptome study in this thesis provided the first comprehensive comparison of the gene expression changes that occur in granulosa cells during primordial follicle activation in the mouse. To achieve such an analysis, it was necessary to develop a novel method of retrieving mass quantities of granulosa cells specific to the follicle stages of interest. By exploiting the initial wave of primordial follicle activation that occurs just after birth in mice (Bristol-Gould et al., 2006), the enzymatic dissociation method presented in this thesis (detailed protocol provided in Chapter 3.2) overcomes the technical challenges of sorting early-stage granulosa cells based on as yet unknown cell markers, or manually with time-consuming equipment. Importantly, biological processes identified via gene ontology analysis (cell differentiation, transcription, translation), and detected genes (*Tgfbr1, Eif4b*) in the transcriptome of these

dissociated cells validated the appropriateness of this technique as a model to study primordial follicle activation (Edson et al., 2009; Ernst et al., 2017; Yang et al., 2013). The publication and dissemination (protocol under review in *Molecular Human Reproduction* journal) of this novel method of dissociating granulosa cells from follicles undergoing primordial follicle activation will support future attempts to elucidate this process.

The transcriptome study in this thesis (Chapter 3.4) served to draw attention to the range of genes or proteins that have been indirectly linked to primordial follicle activation or POI, yet mechanistically remain unstudied in that regard. Aberrant or absent ZFX genes are found in patients with POI and, as a consequence, ZFX has long been associated with this condition (França et al., 2020; Klenov and Cooper, 2016) but its mechanism remains unstudied beyond a knockout mouse study decades old (Luoh et al., 1997). The detection of *Frzb* in the activating follicles provided by this thesis demonstrated the potential for an additional upstream regulator of primordial follicle activation operating via the Wnt signalling pathway. FRZB was observed to interact with WNT3A, a protein linked to the maintenance of primordial follicle dormancy via FOXO3a through an unknown mechanism (Li et al., 2014). In human oocytes undergoing the primordial to primary transition, Wnt signalling pathway genes were significantly altered and suggest a conserved role during primordial follicle activation (Ernst et al., 2017). The Wnt signalling pathway has a multitude of roles in the ovary throughout folliculogenesis (Hernandez Gifford, 2015; Suzuki et al., 2015), with new evidence implicating Wnt signalling in differentiation of granulosa cells from squamous to cuboidal (Habara et al., 2020). The evidence presented in this thesis only adds to need for understanding the role of Wnt signalling in primordial follicle activation, which remains critically underdeveloped.

The detection and exploration of *Zfx* and *Frzb* as a finding of the transcriptome study highlights the necessity to explore how factors may influence the balance between dormancy and

activation of primordial follicles, and how the crosstalk between oocytes and granulosa cells drives this project. The delineation of pathways interacting during primordial follicle activation will eventuate in providing clinics with the correct complement of inhibitory or growth factors to preserve fertility in patients at threat of early fertility loss, such as those with POI or undergoing chemotherapy. One example is the research trajectory of Hippo signalling, first identified to influence primordial follicle activation in mice through established PI3K-Akt signalling, to its eventual use in patients to induce follicle growth *in vitro* (reviewed in Hsueh and Kawamura (2020).

Future research focused on other 'omics' technologies would benefit greatly from harnessing the method of ovary dissociation presented in this thesis and make large contributions to our understanding of primordial follicle activation. Future experiments based upon the work in thesis should be focused on refining the role of Frzb in primordial follicle activation by knockout, or *in vitro* inhibition of Frzb in mouse ovary culture from postnatal day 1 to postnatal day 4 as studied in Chapter 3. Additionally, proteomic analysis during primordial follicle activation using granulosa cells obtained from the enzymatic dissociation method will yield further insights into correlating gene expression and protein abundance neonatal granulosa cells. The use of proteomic analysis is still relatively novel and focused on follicle formation and development in rodents and livestock (Ferrazza et al., 2017; Fu et al., 2016; Wang et al., 2009; Xu et al., 2017), rather than primordial follicle activation specifically. Furthermore, our knowledge that a number of interacting and overlapping signalling pathways operate simultaneously to promote primordial follicle formation warrants the use of phosphoproteomics to specifically explore protein signalling through detecting posttranslational phosphorylation (usually signifying activation of a protein to perform biological functions), currently only used in ovaries in the context of cancer (Francavilla et al., 2017; Hu et al., 2020).

6.3 – An opportunity for smartphones apps to provide fertility knowledge

The expanding market of smartphone applications targeted to women's reproductive health, also called 'femtech' reflects the huge popularity these apps have among users. Investments in femtech increased by almost 7-fold in the 6 years over 2012-2018 to a collective sum of $392 million USD (Lu, 2019). There have been several studies conducted on the efficacy of these apps (reviewed in Earle et al. (2020)), but due to the high rates of turnover and in-app updates, there is difficulty in maintaining a consensus for a given apps' validity. However, the evidence-based claims made by many femtech apps are used as a marketing tool, and are seen as valuable in a persons' decision to use an app (Gambier-Ross et al., 2018; Grenfell et al., 2020; Hamper, 2020). Indeed, even doctors recommend the use of femtech apps for their patients (Comstock, 2014; Ford et al., 2020b). As women are using femtech apps for personal and medical decision-making based on claims of scientific evidence, it is therefore important to understand how femtech apps and their contents are studied in such a dynamic area of research.

A scoping review was utilised (Chapter 4, accepted for publication in *Human Fertility*) to consider the current state of academic literature published on female fertility app content, and mapped recommendations for future research. It was discovered that peer-reviewed studies of apps were commonly limited to evaluation studies where multiple apps are compared in response to a specific research question, or validating accuracy of a given app as a contraceptive via predictions of fertile window. These findings uncovered very little evidence of the fertility information contained within these apps which was further complicated by the concealment of algorithms; a factor also encountered in a recent app evaluation study (Ali et al., 2020). It is critical to consider the types of studies conducted on apps as the narrow research focus has implications for the applicability of the research to diverse users with variable reproductive characteristics and with differing reproductive needs. This thesis has contributed to the

discourse for app developers, researchers, doctors and patients on the design and implementation of femtech app research; highlighting the broad needs of users in regards to education, functionality, race, ethnicity, gender and sexuality.

The scoping review provides substantial evidence that although fertility femtech apps have been the feature of many peer-reviewed studies, there is insufficient examination of the information within them that is provided to users. The extent of information about reproductive health within femtech apps is thus hard to discern, but some studies observe that women self-report these apps as useful and add to their reproductive health knowledge (Hamper, 2020; Lee and Kim, 2019; Levy and Romo-Avilés, 2019; Starling et al., 2018), with fewer studies able to quantitatively measure knowledge of femtech app users (Mesheriakova and Tebb, 2017; Sridhar et al., 2015). This thesis has provided additional evidence about the educative capacity of femtech apps through a survey study which examined the fertility knowledge of women to compare the knowledge of those who use apps against those who do not (Chapter 5, Ford et al. (2020b)). This study also explored app usage preferences and found that among app users, an overwhelming majority used apps which had a menstrual cycle tracking function.

A critical finding of the study in Chapter 5 was an association between the use of reproductive health smartphone applications and knowledge of the most fertile time in the menstrual cycle. Participants that used a reproductive health app were 1.9 times more likely to correctly identify the most fertile time in the cycle than those who didn't use an app, with statistical modelling indicating app use status as significantly able to predict a correct response. It is likely that the abundance of app using participants who use an app featuring a menstrual cycle tracking function (82.4%) can explain this increased knowledge. Most femtech apps which track the menstrual cycle are used for contraception or conception timing (Chapter 4; Earle et al. (2020)), and thus, provide the user a monthly fertile prediction. Indeed, even less precise apps that

operate on primitive algorithms with limited data input typically estimate the fertile time as the middle of a cycle, or 2 weeks before the next cycle (Ali et al., 2020), returning a correct answer in the survey study. The outcomes of the survey study in Chapter 5 provide compelling evidence that femtech apps are popular within the community (43% uptake in the cohort), especially in people of reproductive age, and that these apps can provide some beneficial information about fertility. Additionally, femtech apps may be utilised also to increase awareness regarding risk factors associated with an earlier age of menopause or POI (Mishra et al., 2019).

The work performed in this thesis provides the justification for public health intervention in the reproductive health application market. Women identify learning about themselves as a motivator for continued use of apps (Levy and Romo-Avilés, 2019), but there are severe limitations in the capacity for currently available apps to do this in a way that is broadly applicable to those with diverse cycle lengths, and with different reproductive needs. There are also issues with the privacy and accessibility of currently available apps as identified by both users and experts (Lupton, 2015; Shipp and Blasco, 2020). Subsequent studies should trial the effectiveness of researcher-developed, evidence-based apps to determine if knowledge is received and retained with prolonged use/daily monitoring behaviours of users. The studies within this thesis demonstrate that apps have the potential to change the fertility knowledge of their users. Thus, we advocate for the development of freely available reproductive health apps with content that is transparently available for independent peer review, providing multi-functionality for users at different stages in their reproductive journey, and with reliable educational content. Establishing how the participants obtain information based on the availability of this information, this thesis has made recommendations from which meaningful and relevant content can be conveyed to the public, and foster a greater interest in reproductive health throughout a person's lifetime.

6.4 – Concluding remarks

The diversified approach to improving fertility outcomes adopted for this thesis have collectively provided new insights to both granulosa cell-driven primordial follicle activation, and the fertility knowledge content provided by smartphone applications. Taken together, the findings from this thesis provide a meaningful platform for which we may empower women to have a realistic knowledge of, and ability to manage their own fertility and reproductive health. This may be through self-education via reproductive health smartphone applications, or through enhanced fertility preservation options for women with premature ovarian insufficiency.

By establishing a novel dissociation protocol to capture mass quantities of primordial and activating granulosa cells, the transcriptome study assessed the initial wave of primordial follicle activation. The findings of factors previously linked to POI or upstream in pathways involved in follicle activation emphasises the importance of a strong research foundation in animal models to completely delineate the pathways, genes, and proteins operating during this process. This research highlights the complexities within the process of primordial follicle activation, and the availability of this dataset in the gene expression omnibus (Barrett et al., 2010) will ensure its legacy as an enduring instrument in future interpretations of primordial follicle activation research.

Providing fertility education to the public has the potential to reduce the number of people struggling with infertility directly by timing conception (as in Stanford et al. (2020)), or indirectly by making people aware of the factors able to influence fertility. The work in the latter part of this thesis have collectively identified the strengths and weaknesses of using reproductive health smartphone applications to reduce the fertility knowledge gap. The accessible nature and high uptake of these applications make them an ideal instrument to

provide public health messaging about factors influencing fertility, and the recommendations outlined in this thesis will be used to lobby smartphone developers and policy makers. Overall, this thesis has supplied valuable evidence for the use of smartphone applications to improve fertility knowledge of the public, and has contributed to a deeper understanding of primordial follicle activation for the eventual goal of improved diagnostics and treatment of premature ovarian insufficiency.

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