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Dysregulation of granulosal bone morphogenetic protein receptor 1B density is associated with reduced ovarian reserve and the age-related decline in human fertility

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ABSTRACT

Reproductive ageing is linked to the depletion of ovarian primordial follicles, which causes an irreversible change to ovarian cellular function and the capacity to reproduce. The current study aimed to profile the expression of bone morphogenetic protein receptor, (BMPR1B) in 53 IVF patients exhibiting different degrees of primordial follicle depletion. The granulosa cell receptor density was measured in 403 follicles via flow cytometry. A decline in BMPR1B density occurred at the time of dominant follicle selection and during the terminal stage of folliculogenesis in the 23–30 y good ovarian reserve patients. The 40+ y poor ovarian reserve patients experienced a reversal of this pattern. The results demonstrate an association between age-induced depletion of the ovarian reserve and BMPR1B receptor density at the two critical time points of dominant follicle selection and pre-ovulatory follicle maturation. Dysregulation of BMP receptor signalling may inhibit the normal steroidogenic differentiation required for maturation in older patients.

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1. Introduction

Reproductive ageing is linked to the declining capacity to regenerate cells and tissues, causing irreversible changes to ovarian cellular dynamics and ultimately reducing the capacity to reproduce. As the average age of fertility-challenged patients climbs towards 40 years (y), there is an urgency to characterise the cellular changes that occur in the ovary with time. The response of the ovaries to cyclic recruitment of primordial follicles forms the basis of the clinical documentation of the antral follicle count (AFC) (Almog et al., 2011). The AFC and age are highly correlated to histologically determined ovarian primordial reserve (Hansen et al., 2011; van Rooij et al., 2005). As the primordial follicle reserve declines, the endocrine, paracrine, and autocrine regulation adapts to a changing environment. It is this changing landscape that requires further investigation to provide an alternative treatment to preserve the primordial follicles, and to adjust the cellular regulation to achieve oocyte competence and improve fertility rates in older patients.

Earlier research has highlighted the potential role of bone morphogenetic protein (BMP) signalling in regulating ovulation rate in sheep (Campbell et al., 2006; Galloway et al., 2000; Juengel et al., 2011), and has led us to further investigate the molecular regulation of folliculogenesis by the BMPs (Regan et al., 2015; Ruoss et al., 2009). During a natural cycle, small antral follicles with sufficient granulosal follicle-stimulating hormone receptor (FSHR) expression are recruited in response to the intercycle rise in FSH, and one of these is subsequently selected to become the dominant follicle. Follicles with reduced FSHR and luteinising hormone receptor (LHR) become less responsive as the dependence from pituitary FSH stimulation shifts to LH, and circulating FSH concentrations decline (Lapolt et al., 1990; Xu et al., 1995; Zeleznik et al., 1974). These subordinate follicles are destined for atresia. The

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selected dominant follicle is the one with greatest gonadotrophin responsiveness, and is dependent on the acquisition of FSHR-induced LHR by granulosa cells; and this follicle continues growing to the ovulatory stage.

Previous research has shown that, at the time of declining FSH levels, a reduction in BMP6 (Erickson and Shimasaki, 2003), BMP15 (Feary et al., 2007) and the type 1 TGFβ superfamily receptor BMPR1B (Feary et al., 2007; Regan et al., 2015), occurs after dominant follicle selection. Once selected, follicle growth and cellular proliferation continues, leading to an increased oestrogen and inhibin production by the granulosa cells. Together, these hormones suppress pituitary FSH output further, ensuring the demise of subordinate follicles. When the threshold for oestrogen's positive feedback action on the hypothalamus-pituitary axis is met, preparation for the ovulation phase begins with a cessation of cell proliferation, and early luteinisation changes taking place. During cellular and steroidogenic differentiation, activin (Young et al., 2012), insulin-like peptide 3 (INSL3) (Anand-Ivell et al., 2013), anti-mullerian hormone (AMH) (Andersen et al., 2010; Ogura Nose et al., 2012; Weenen et al., 2004) and gonadotrophin surge attenuating factor (GnSAF) activity (Martinez et al., 2002) declines, from dominant follicle selection to the termination of folliculogenesis at ovulation.

The functional role of BMPR1B receptor in follicle development has received considerable attention in recent years following the discovery that hyper-prolific sheep with the Booroola (FecB) phenotype have a naturally occurring mutation in the kinase domain of BMPR1B that perturbs antral follicle development and ovulation rate (Souza et al., 2001; Mulsant et al., 2001). The BMP ligands, 2, 4, 6, 7, and 15 form a receptor-ligand complex with the type 1 TGFβ receptor BMPR1B, and recruit the type 2 TGFβ receptor BMPR2. The complex initiates phosphorylation of the intracellular substrate molecules, which are the receptor-regulated Smads. The Smad forms a complex with a common mediator, Smad 4, and translocates to the nucleus where transcription of BMP-responsive genes takes place. Smad signalling is modulated by repressor and activator molecules in the nucleus, cytoplasm, and in the extracellular matrix. Alternatively, BMPs activate the non-Smad pathway mitogen-activated protein kinase (MAPK) such as extracellular signal-regulated kinase (ERK 1/2) or Ark (Inagaki et al., 2009; Ryan et al., 2008).

In the ovary, granulosa cell signalling induced by the gonadotrophins, FSH and LH, activate the FSHR or LHR and stimulate cAMP-PKA, which increases the CYP19A1 aromatase to facilitate oestrogen synthesis. Progesterone synthesis is inhibited by the suppression of steroidogenic regulatory protein (StAR) (Abdo et al., 2008; Pierre et al., 2004; Tajima et al., 2003; Val et al., 2003), which is essential for progesterone synthesis in the granulosa cell (Moore et al., 2001). Alternatively, or in addition, BMPs inhibit ERK 1/2 signalling, which provides inhibitory control over the balance of progesterone and oestrogen (Miyoshi et al., 2007; Nakamura et al., 2012; Ogura Nose et al., 2012).

Given the particular focus of interest on BMPR1B in ovarian function, the current study aimed to comprehensively profile the expression of granulosal BMPR1B in a range of patients, of different ages and stages of ovarian primordial follicle depletion, who were receiving treatment for infertility. Previous reports documenting ovarian BMPR1B expression have evaluated expression at the mRNA level in pooled follicles from different size classes (Chen et al., 2009; Estienne et al., 2015). However, mRNA expression does not necessarily reflect expression of translated functional BMPR1B protein on the cell surface. In contrast, in this study we collected an average of ~8000 granulosa cells from each individual follicle over a comprehensive range of follicle diameters from 4 mm to 27 mm. Immunofluorescent labelling and flow cytometry were then used to measure the granulosa cell surface-expressed mature receptor protein density for the BMPR1B receptor.

2. Materials and methods

2.1. Patients

A total of 401 follicles were collected from 53 patients undergoing standard fertility treatment previously reported in accordance with the PIVET Medical Centre Algorithm, and are presented in Table 1 (Yovich et al., 2012). Follicles were collected irrespective of previous aetiology, but limited to exclude unusual medical conditions, hormonal dysfunction, and polycystic ovarian syndrome; patients were aged between 23 and 45 y.

2.2. Human IVF: ovarian stimulation, follicular fluid and oocyte

Patient treatment consisted of two types of GnRH-LH suppression in conjunction with rFSH, from cycle day 2 for ~10 days (Puregon or Gonal F). A GnRH antagonist treatment (Cetrotide) (0.25 μg/day) was administered from day seven until ovulation induction. Alternatively, a GnRH flare agonist treatment (Lucrin) (0.25 μg/day) was administered in conjunction with rFSH on day 2. Ovulation was triggered with either 10 000 IU hCG derived from a urinary preparation (Pregnyl) or a pituitary derived analogue to LH (Ovidrel). Oocyte retrieval was scheduled for 36 h post-trigger, by transvaginal oocyte aspiration (Yovich and Stanger, 2010).

2.3. Antral follicle count

Patients received rFSH based on the patient's profile of age and AFC, to predict the rFSH dose required to stimulate multiple preovulatory follicles (Yovich et al., 2012). The dose of rFSH was then adjusted to the patient's ovarian response to stimulation. Considerable overlap in rFSH dose was present between age groups, which allowed for a rFSH dose comparison between different ovarian reserve patient groups of the same age. Ovarian reserve was measured indirectly by the antral follicle count (AFC) (Hansen et al., 2011). AFC was defined as the number of follicles between 2 and 10 mm in size that are present on day 2–5 of a cycle. Determination of AFC was ascertained by transvaginal ultrasound and patients were divided into groups accordingly: Group A+ = 30–39; group A = 20–29; group B = 13–19; group C = 9–12, group D = 5–8; group E = ≤ 4. The groups were established based on ovarian response to gonadotrophin hormone stimulation during IVF cycles. (Yovich et al., 2012).

2.4. Collection of granulosa cells

The diameter of the follicle was calculated using ultrasonography before the clinical aspiration of individual follicles. The first aspiration was collected without flush medium into a test tube, and handed to the embryologist to locate the oocyte and attached cumulus cells if present. Further flushing of the follicle (Quinn's Advantage with Hepes, Sage Media, Pasadena, California) at ~1.24–1.72 MPa removed the loosely attached layers of granulosa cells. Once the oocyte was located and removed, the clinician proceeded to the next follicle and repeated the process. The follicular fluid and flush was then layered onto a ficoll density gradient (555485; BD Biosciences, Perth, Australia) and centrifuged to isolate the granulosa cells.

2.5. Immunolabelling of granulosa cells

Aliquots of suspended granulosa cells (1×10^6 cells in 100 μl)

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