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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.

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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Table 1
Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

Age year	IVF patient	Total follicle	AFC	Major group	Number of follicles collected per group							
					Sub group	#	Sub group	#	Sub group	#	Sub group	#
21–30	8	95	20–40	A+ &A	A+	31	A	64	C	6		
31–34	11	86	13–29	A & B	A	60	B	26	C	17		
35–39	16	102	9–19	B & C	B	50	C	16	D	30	E	6
40–45	18	118	3–8	D & E	D	59	E	19	B	34		

Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2 and 10 mm on day 2–5 of a cycle: A+ = 30–39 follicles; A = 20–29; B = 13–19; C = 9–12, D = 5–8; E = ≤4.

were immunolabelled using a double-indirect method as previously described (Abir et al., 2008; Cai et al., 2007; Gao et al., 2007). The cells were incubated separately with an optimised concentration of 4 μg/ml affinity purified polyclonal antibody to goat BMPR1B (sc-5679), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), for 25 min at 5 °C; washed with PBS and then incubated with a second antibody, donkey anti-goat conjugated to the flurochrome Alexa 488 (Al-Samerria and Almahbobi, 2014). The cells were washed again with PBS and centrifuged at 300 g at 5 °C for 5 min. In addition, these antibodies have been used previously in human studies (Abir et al., 2008; Haÿ et al., 2004), including flow cytometry analyses (Gao et al., 2007; Regan et al., 2015; Whiteman et al., 1991).

The routinely used monoclonal antibody against CD45 was added to BMPR1B antibody-containing tubes to enable the subtraction of leukocyte common antigen-positive cells (~3%) not removed during isolation of the granulosa cells using the ficoll density gradient. Unstained samples or the substitution of primary antibody with pre-immune goat IgG (Fig. 1A) (Millennium Science, Surrey Hills, Victoria Australia) at the same concentration of the primary antibody served as a negative control for auto-fluorescence; and a blocking peptide for BMPR1B also confirmed binding specificity (Fig. 1B), (sc-5679P; Millennium Science, Surrey Hills, Victoria Australia) and as previously published (Abir et al., 2008; Al-Samerria and Almahbobi, 2014; Haÿ et al., 2004; Regan et al., 2015; Weall et al., 2015).

In the current study, the 'normal' goat IgG and unstained control cells emitted an average MFI that was very similar for each individual follicle but different between follicles and patients; therefore, to optimise accuracy, the auto-fluorescence and the nonspecific binding determined by the unstained control for each follicle, was subtracted from each individual follicle. The data were analysed using FlowJo software (Tree Star Inc., Oregon, USA).

2.6. Fluorescent microscopy

Re-suspended 10 μl aliquots of BMPR1B immunolabelled, live granulosa cells were placed on slides and visualized using an Olympus DP 70 camera fitted to a Olympus BX-51 upright fluorescent microscope with a 40× UPlan N 0.4 N.A. objective; (Olympus Imaging Australia, Macquarie Park, Australia), (Fig. 1B). The granulosa cell slides were allowed to air dry to reduce movement during digital capture, which would account for the more clumpy appearance compared to the more typical single granulosa cells analysed by flow cytometry. Fluorescent microscopy revealed a positive staining of the cell membrane-bound BMPR1B, as an intermittent, bright, ring-like pattern around the cells. All control samples showed negative staining. Granulosa cells ranged from 8 μm to 25 μm, with the average being 15 μm.

2.7. Flow cytometry

Selective gating of the whole sample to identify a pure granulosa cell population was achieved by graphing forward scatter to remove doublets (FSC-H verses FSC-A) (Regan et al., 2015). Then Alexa Fluor 488 fluorescent intensity was plotted against Allophycocyanin (APC) intensity to identify and subtract the cells positive for the leukocyte common antigen antibody CD45, which emits in the APC spectrum (Fig. 2A). Auto-fluorescence and nonspecific binding were identified by the unstained sample control BMPR1B expression, and subtracted from the measurement (Fig. 2B).

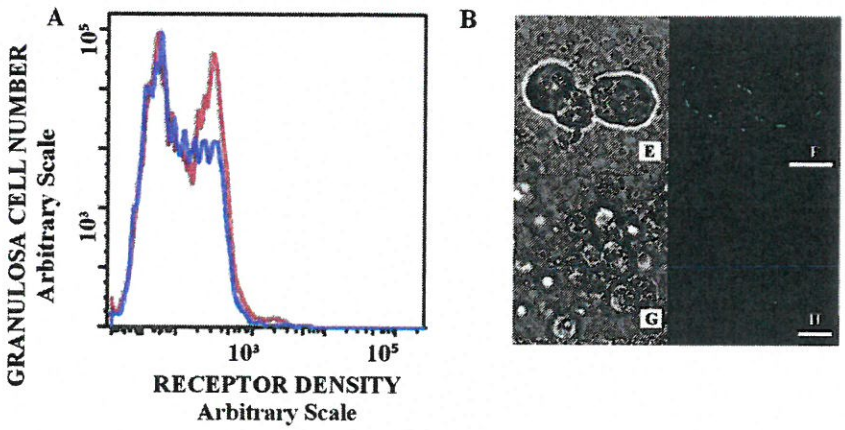

Fig. 1. Validation of immunofluorescent labelling. A. Unstained control (blue) compared to IgG Isotope control (red) for nonspecific binding and auto-fluorescence. B. Live human granulosa luteal cells with positive fluorescence for BMPR1B (EF), and negative blocking agent for BMPR1B (GH). Bar 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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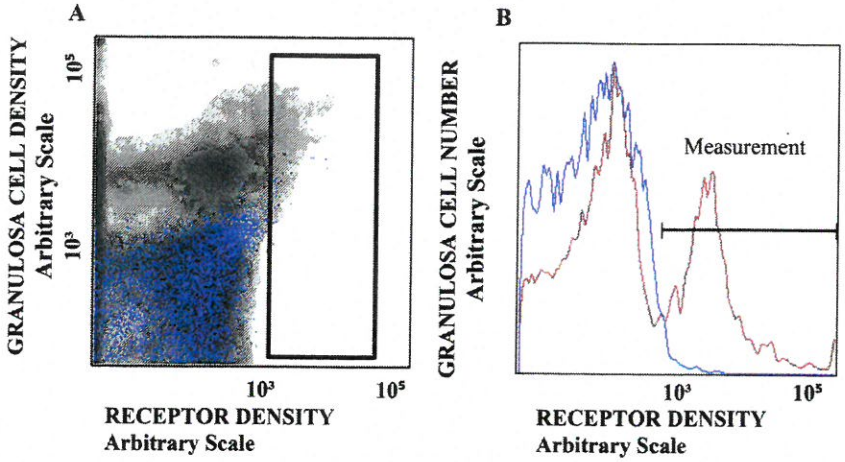

Fig. 2. Validation of gating to measure average receptor density in flow cytometry. A. Unstained control granulosa cells, represented as blue dots (auto-fluorescence) and immunostained granulosa cells (grey). A rectangle subtraction gate for the leukocyte common antigen CD45 positive cells. B. Subtraction of nonspecific binding and auto-fluorescence at 10^3; mean granulosa cell fluorescent intensity (MFI) measurement. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.8. Statistics

Mean fluorescent intensity (MFI) was obtained using ~8000 granulosa cells per individual follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size using GraphPad Prism 6. Values in graphs are means ± S.E.M., and differences were considered significant if $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, and $^{****}p < 0.001$. The letter, such as 'a', signifies a statistical difference to the matching letter, and an attached asterisk (a*) indicates the significance level for that follicle size category.

2.9. Human ethics

Informed consent was obtained from patients undergoing standard fertility treatment at PIVET fertility clinic Perth, Australia. Approval by the Human Research Ethics Committee of Curtin University of Technology and KEMH Women and Newborn Health Service ethics committee (WNHS) was obtained for this study.

3. Results

3.1. Follicle development and ovarian reserve

Relative to older (40+ y) patients, the level of granulosal BMPR1B expression was lower in the 23–30 y IVF patients in combined AFC groups A+ & A, and showed a biphasic receptor density pattern (Fig. 3). The biphasic pattern of receptor density consisted of an initial decrease in BMPR1B in follicles from 8 mm to 10 mm ($p = 0.0201$), followed by an up-regulation in the follicles to 16 mm ($p = 0.0084$), which was further followed by a significant decline in follicles to the terminal-end of folliculogenesis of 24–26 mm ($p = 0.0301$, Fig. 3). In marked contrast, the BMPR1B density increased with follicular size ($p = 0.0044$) in a monophasic reversed profile in the 40+ y group (Fig. 3). The level of receptor density in the small antral follicles of 8 mm was greater in the young patients than the older patients ($p = 0.0405$).

3.2. Dysregulation of BMPR1B receptor density young patients with poor ovarian reserve

In the youngest age group 23–30 y, the majority of the patients had an AFC within groups A+ & A (Fig. 4). There was no significant difference between the A+ group and the A group. In contrast, the follicles from the C group patients with low AFC had increased BMPR1B expression compared to the similar size follicles in the A+ & A group ($p < 0.05$ to $p < 0.001$, Fig. 4), similar to the over expression profile of the 40+ y E AFC patients. Young patients with a very poor ovarian reserve (group C) for their age (0.1% of women under 30 y) do not have many follicles available for collection as they approach premature ovarian failure (Shelling, 2010). The rFSH dose administered ranged from 87 IU to 150 IU in the A+ & A group, and was 190 IU in the C group.

3.3. Association between AFC and BMPR1B receptor density in older (31–34 y) patients

The high level of BMPR1B density was not observed in the 31–34 age group compared to the youngest age group in the 8 mm follicles. Rather an increase in receptor density in the larger follicles from patients with an ovarian reserve of B and C ($p = 0.0076$). Within the 31–34 y age group, the decline in ovarian reserve was associated with a loss of receptor density of BMPR1B in the granulosa cells from a peak in the 19 mm follicles in the A group to a significantly lower value in the B and C groups ($p = 0.002$, Fig. 5A). The rFSH dose given ranged from 83.5 to 266 IU, and when the AFC group comparison was restricted to those patients who received a comparable rFSH dose (200–266 IU), a similar BMPR1B receptor density profile was observed in the B and C group patients (Fig. 5B).

3.4. BMPR1B receptor density in older patients with declining AFC

The 35–39 y combined B & C group demonstrated a significant increase in BMPR1B receptor density in the 10 mm follicles, which significantly reduced much later compared to the youngest age group, when the follicle reached 16 mm ($p = 0.007$). With a further decline of the ovarian reserve (group D & E) the receptor density in the smaller follicles was reduced followed by a steady increase with increasing follicular size similar to the oldest patients monophasic profile, ($p = 0.0372$), Fig. 6A). The rFSH dose given ranged from 83.5

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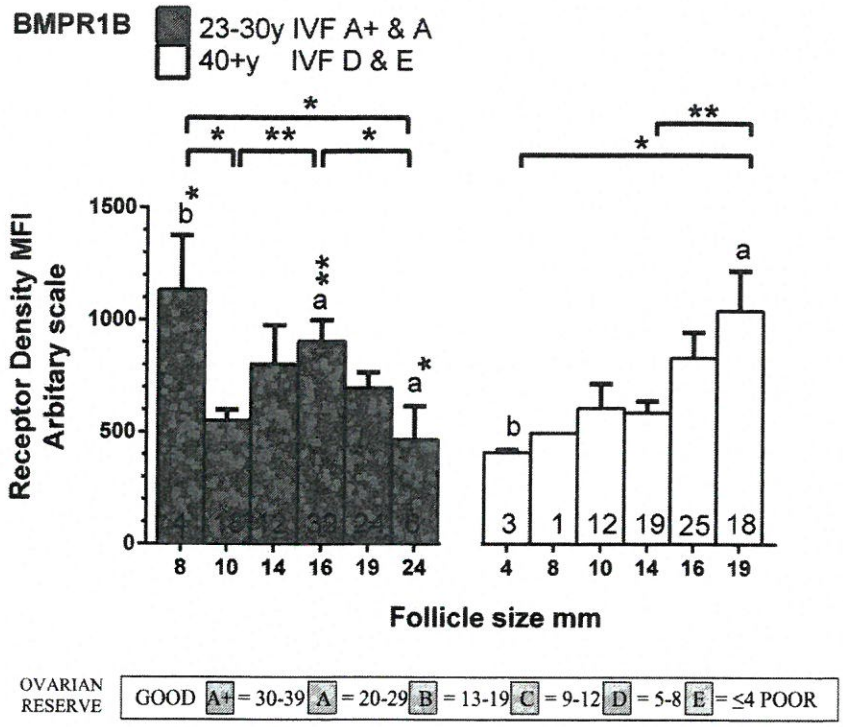

Fig. 3. Granulosal BMPR1B density from follicles of different sizes collected from young and older IVF patients compared to an unstimulated natural healthy cycle. Granulosal BMPR1B protein density and follicle size profile of, 23–30 y patients in a stimulated, IVF cycle with an AFC of A+ & A, (grey bar). Patients, 40+ y stimulated IVF cycle with an AFC of D & E, (white bar). IVF patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values in graphs are means ± S.E.M., and differences were considered significant if *p < 0.05 and **p < 0.01. The letter, 'a' signifies a statistical difference to the matching letter with an attached asterisk(s) (a*, a**). The number within the column represents the number of follicles analysed for that group.

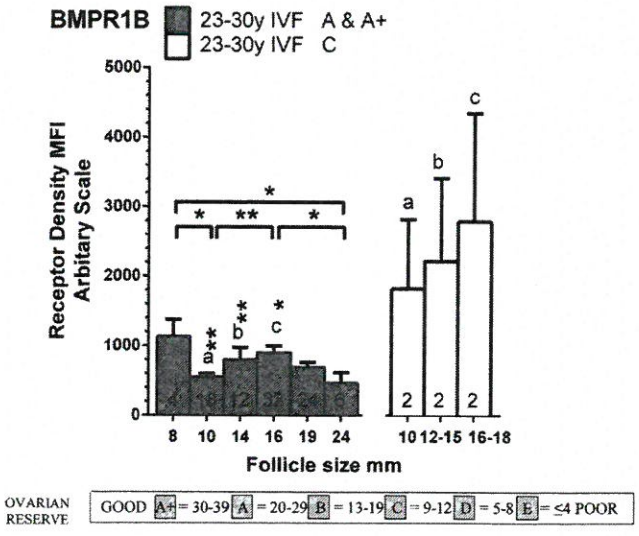

Fig. 4. Granulosal BMPR1B density and ovarian reserve depletion in 23–30 year-old patients. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2 and 10 mm on day 2–5 of a cycle. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means ± S.E.M., and differences were considered significant if *p < 0.05. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level. The number within the column represents the number of follicles analysed for that group.

to 600 IU, and when the AFC group comparison was restricted to those patients who received a similar FSH dose (190–241 IU), a similar BMPR1B receptor density profile was observed (Fig. 6B).

The 40+ y patients (40–45 year-old) ranged in AFC from group B to group E (Fig. 7A). The group B & C patients combined demonstrate a higher BMPR1B receptor density in the small follicles, followed by significant down-regulation of receptors as follicle size increased ($p = 0.0176$). With a decline in the ovarian reserve to group D, the receptor density in the smaller follicles was reduced compared to group B ($p = 0.0059$). With a further decline of ovarian reserve from D to group E, the receptor density significantly increased (19 mm follicles, $p = 0.03$); and within the E group, increased with follicle size (10 mm–22 mm, $p = 0.0058$, Fig. 7A). This was similar to the ageing effect observed in the youngest group C patients (Fig. 4). The rFSH dose given ranged from 300 to 600 IU, and when the AFC group comparison was restricted to those patients who received an identical FSH dose (600 IU), a very similar BMPR1B receptor density profile was observed (Fig. 7B).

3.5. *BMPR1B receptor density independent of patient age and ovarian ageing*

The depletion of the ovarian reserve appears to be different at different ages and may reflect the ageing process, rather than a clear cut rise or fall in density (Fig. 8). When the data are pooled the re-grouping condenses the data, and the effect of the sequential loss of ovarian reserve is lost.

The extended graphs at a given age demonstrates the continual process of ovarian ageing and the regression of receptor density; followed by the eventual low density in the smaller antral follicles,

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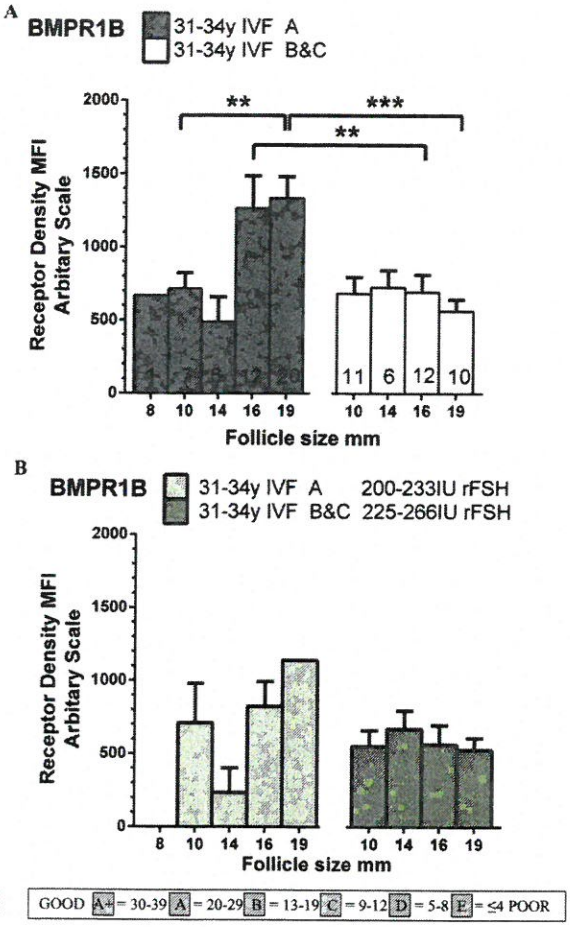

Fig. 5. Granulosal BMPR1B density and ovarian reserve depletion in 31–34 year-old patients. A. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2 and 10 mm on day 2–5 of a cycle. B. As above, patients were grouped according to AFC but only those who received an equivalent rFSH dose were included in the analysis. Data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means ± S.E.M., and differences were considered significant if $^*p < 0.05$. The letter, such as 'a' signifies a statistical difference to the matching letter with an attached asterisk (a*) which indicates the significance level (i.e. 'a' is s.d. to all a*). The number within the column represents the number of follicles analysed for that group.

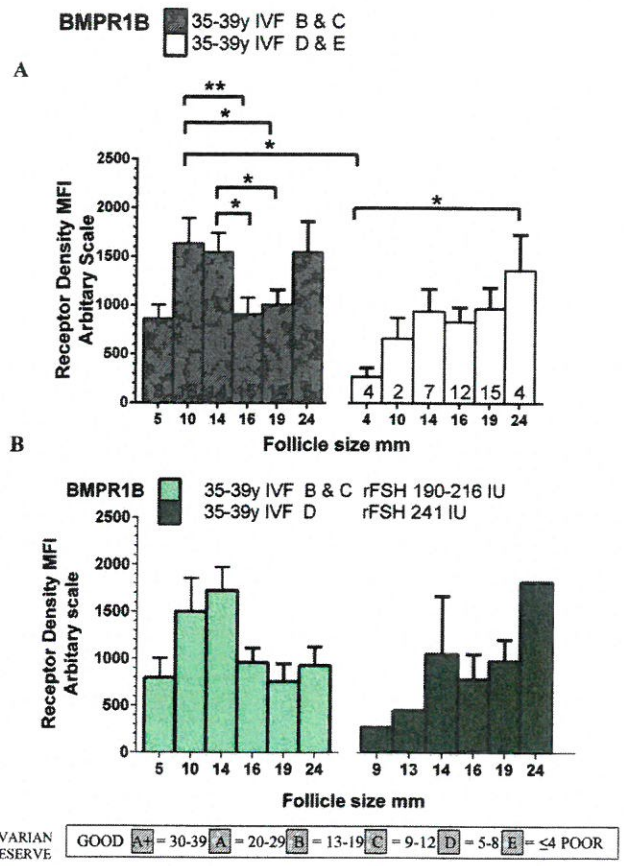

Fig. 6. Granulosal BMPR1B density and ovarian reserve depletion in 35–39 year-old patients. A. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2 and 10 mm on day 2–5 of a cycle. B. As above, patients were grouped according to AFC but only those who received s similar rFSH dose (190–241 IU) were included in the analysis. Data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means ± S.E.M., and differences were considered significant if $^*p < 0.05$. The number within the column represents the number of follicles analysed for that group.

and by a reversal of expression, without down-regulation of the receptor in the largest pre-ovulatory follicles (Fig. 4–7).

4. Discussion

A continuous process of activation of primordial follicles in the ovary leads to the inevitable depletion of the ovarian reserve in women (Almog et al., 2011). The gradual decline in ovarian reserve can be indirectly measured by the number of small antral follicles at the beginning of a cycle, which is termed the AFC (Hansen et al., 2011). The response of the ovary to exogenous gonadotrophins used to treat infertility declines with age, which is strongly correlated to the ovarian reserve (Hansen et al., 2011). Patients with a poor ovarian reserve are treated with increasing doses of rFSH in an attempt to increase the number of small antral follicles with sufficient FSHR to develop into pre-ovulatory follicles.

In sheep carrying the Booroola (FecB) mutation, follicle development was perturbed and ovulation rate increased as a consequence of a point mutation in the BMPR1B gene (Regan et al., 2015). Therefore, the potential role of BMPR1B within the context of follicle development, ovarian ageing, and fertility in humans is of considerable interest. In the present study, the density of expression of mature cell surface protein for BMPR1B was measured by flow cytometric analysis. We found that a reduction in the number of growing follicles was linked to the sequential disruption of BMPR1B density on the surface of granulosa cells.

Ovarian reserve depletes sequentially with age in a slow continuous process. The results in this study show a gradual degradation in the density of receptors, which is perpetuated through the different age groups. The change observed may appear difficult to interpret; however, within each age group, there is evidence of over-expression followed by a weakness in expression, a lack of down-regulation, and eventually, reduced receptor density, ultimately leading to increased levels of BMPR1B in the largest follicles of the oldest, poorest ovarian patient groups.

An important finding of this study was that a decline in granulosal BMPR1B receptor density occurs in follicles of a size that would correspond to the time of cyclic dominant follicle selection (small follicles), and again in the largest follicles from the best prognosis IVF patients, aged 23–30 y (ovarian reserve group A). In

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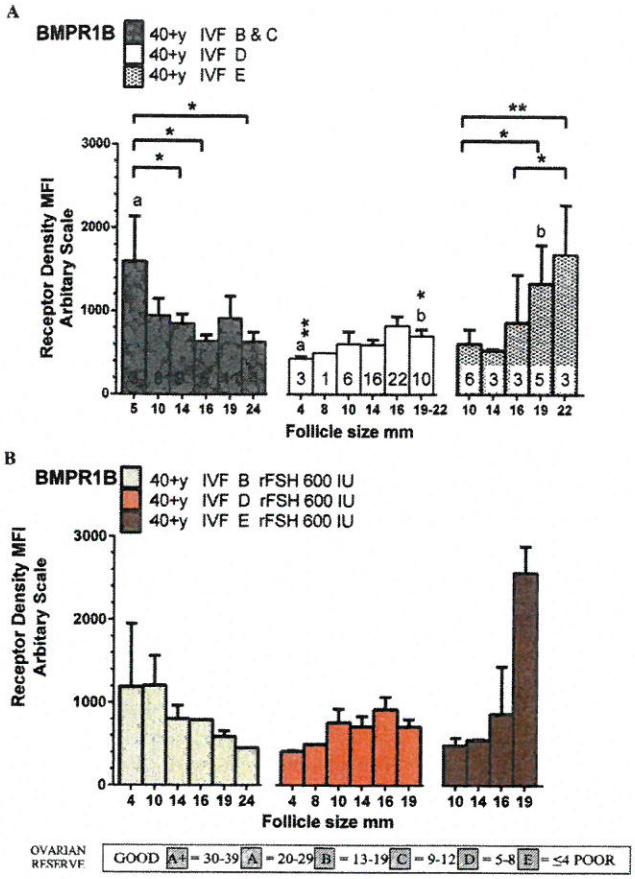

Fig. 7. Granulosal BMPR1B density and ovarian reserve depletion in 40+ year-old patients. A. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2 and 10 mm on day 2–5 of a cycle. B. As above, patients were grouped according to AFC but only those who received an equivalent rFSH dose (600 IU) were included in the analysis. Data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means ± S.E.M., and differences were considered significant if $^{*}p < 0.05$ and $^{**}p < 0.01$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level (i.e. 'a' is s.d. to all a*). The number within the column represents the number of follicles analysed for that group.

comparison, the older, 40+ y, poor ovarian reserve patients (ovarian reserve group D & E) exhibited a reversal of this pattern (Fig. 3).

A decrease in small antral follicle number has been associated with a rise in luteal and start-of-cycle FSH and LH with a corresponding decrease in inhibin B, AMH, and IGF 1 (Klein et al., 2000; Pal et al., 2010). The increase in FSH and LH has been shown to accelerate the early growth of small follicles, followed by reduced growth rates of the pre-ovulatory follicles in older patients. Other ovarian age related changes were associated with an increase in mitochondrial deletions in granulosa cells (Seifer et al., 2002) and an increase in the number of chromosomal errors (Handyside et al., 2012). It is, therefore, speculated that enhanced BMP signalling arising from elevated pre-ovulatory BMPR1B levels would inhibit the normal steroidogenic differentiation required for maturation of the follicle in older patients with reduced ovarian reserve.

4.1. BMPR1B down-regulation and dominant follicle selection

The biphasic down-regulation of the density of the TGFβ superfamily type I receptor, BMPR1B, during folliculogenesis in a stimulated cycle was similar to the pattern of down-regulation observed in natural cycle young adult sheep (Regan et al., 2015). During dominant follicle selection in sheep, and at an equivalent size in gonadotrophin stimulated humans, granulosal expression of BMPR1B was reduced, followed by a sequential increase with follicle size (Fig. 3). The similarity between the sheep in natural cycles and the human IVF model suggests that rFSH has minimal impact on receptor expression levels and on the timing of dominant follicle selection. The addition of gonadotrophin in the form of rFSH masks the normal physiological pituitary drop in FSH, allowing a prolonged recruitment phase that enables multiple follicles to grow (Rice et al., 2007). The timing of recruitment and dominant follicle selection should therefore be comparable to a normal unstimulated IVF cycle.

In other studies, granulosa cell expression of BMPR1B has been shown to increase with follicle size (Chen et al., 2009; Estienne et al., 2015), which is consistent with our findings. However, the pre-ovulatory, leading dominant follicle in sheep was pooled with smaller follicles in these studies, which would effectively mask the down-regulation (Regan et al., 2015). The down-regulation of granulosa cell BMPR1B expression in the present study was

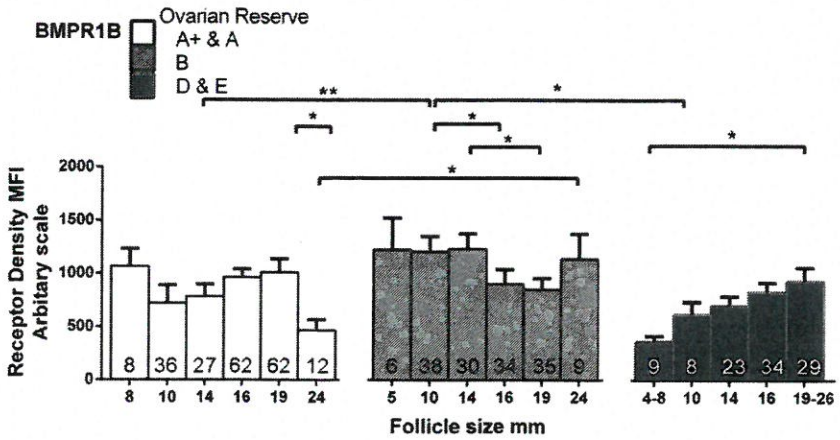

Fig. 8. Granulosal BMPR1B density and ovarian reserve depletion independent of age. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2 and 10 mm on day 2–5 of a cycle. Data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means ± S.E.M., and differences were considered significant if $^{*}p < 0.05$ and $^{**}p < 0.01$. The number within the column represents the number of follicles analysed for that group.

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consistent with findings for sheep dominant follicles compared to the subordinate follicles reported in another recent study (Gasperin et al., 2014). The interrelationship between FSH and BMP regulation has been previously reported (Miyoshi et al., 2006; Shi et al., 2009, Shi et al., 2010), and the decline in pituitary FSH secretion initiating the dominant follicle selection process would, therefore, appear to be temporally related to the decline in BMPR1B expression on the granulosa cell surface.

As a function of age, the quantity and quality of oocytes produced is reduced in older women, and disruptions to follicular growth and hormone profiles is increased (Santoro et al., 2003; Stanger and Yovich, 1985; Yovich and Stanger, 2010). Increasing levels of gonadotrophin treatment are used to extend the dominant recruitment phase of antral follicles that results in an increased quantity of maturing follicles. However, the low levels of receptor expression in the small antral follicles of older patients with reduced ovarian reserve suggest a possible cause of poor quality follicles and oocytes typical of older patients. Administration of BMP6 to old mice during superovulation increased the quantity and quality of oocytes collected (Park et al., 2012). Whereas in humans, the pregnancy rate was increased during IVF when *BMP15* mRNA in the cumulus cells surrounding the oocyte was higher (Li et al., 2014). Moreover, high levels of *BMP15* mRNA in the follicular fluid has also been associated with oocyte quality (Wu et al., 2007).

4.2. BMPR1B down-regulation and the maturation of pre-ovulatory follicles

The degenerative ageing of granulosal BMPR1B density is highlighted by the observation that 40+ y patients (groups B & C) with a favourable ovarian reserve for age exhibit a pattern of declining receptor density with follicle size, whereas the pattern in the 40+ y patients (groups D & E) with reduced ovarian reserve is reversed (Fig. 7). Similar ovarian ageing was found in the 35–39 y B & C group with the same steady increasing density in the reduced ovarian reserve D & E group (Fig. 6). Younger Patients with a C antral follicle count are not common because they are approaching premature ovarian failure (Shelling, 2010). Evidence of ovarian ageing was seen in the youngest patients with a severely reduced for age ovarian reserve (AFC group C), where the receptor density was increased substantially (Fig. 4). The over-expression of BMPR1B was also present in the oldest patients with severe ovarian depletion (AFC E; Fig. 7A). In the 31–34 y patient group with an A ovarian reserve, the receptor density increased only in the largest follicles followed by a general decrease in the poorer ovarian reserve patients for that age group (Fig. 5A). The level of receptor expression in the small follicles appears unchanged but the lack of down-regulation in the larger follicles is evident and may negatively be associated with the maturation process. As age increases, and the ovarian reserve declines, fewer follicles are stimulated; hence, the extra-large follicles are rare as the follicles mature at a smaller size (Santoro et al., 2003). In the younger patients of 31–34 y, it would be expected that a 24+ mm follicle would be common; however, none were analysed. The reversal of receptor density compared to the younger patients provides evidence of an early fundamental shift in granulosal BMPR1B receptor density with ovarian ageing. Sustained high levels of BMPR1B in pre-ovulatory follicles would promote oestrogen synthesis and inhibit progesterone synthesis, which could potentially suppress maturation of the follicle (Otsuka, 2010; Shimasaki et al., 1999).

4.3. Could the apparent effect of ovarian ageing on BMPR1B receptor density be due to different degrees of rFSH stimulation in the treatment cycle?

Patients with declining ovarian reserves are prescribed greater doses of rFSH and this could potentially confound the interpretation of the present observation that ovarian ageing affects granulosal BMPR1B receptor density. However, when 'like-with-like' comparisons were made, with only those patients prescribed similar doses of rFSH included in the analyses, the effect of ovarian ageing on receptor expression persisted. The changes observed in BMPR1B density are therefore, unlikely to be attributable to the degree of rFSH stimulation that the patient received during a treatment cycle, at least within patients of a similar chronological age. In support of this, unpublished findings from one of our laboratories (PGK) have indicated that treatment of cultured bovine granulosa cells with FSH promotes a marked increase in CYP19A1 mRNA expression and oestrogen secretion but has no effect on BMPR1A, BMPR1B or BMPR2 mRNA expression (C Glister and PG Knight, unpublished observations).

4.4. Conclusion

Taken together, the results demonstrate the disrupting effect that ageing-induced depletion of the ovarian reserve has on granulosal BMPR1B receptor density in antral follicles. Age-induced depletion is associated with a loss of the biphasic down-regulation of granulosal BMPR1B density during follicle development. Grouping the AFC subgroups regardless of age does not show the demise of receptor density as women age and the ovarian reserve is depleted (Sup graph). Both age and ovarian reserve are factors in declining fertility and the quality of the oocyte. The findings extend previous research by the comprehensive nature of the range of follicle sizes and age groups studied, together with measurement of the translated mature, BMPR1B protein as opposed to measurement of receptor expression at the mRNA level (Ascoli et al., 2002). Further work is needed to confirm the identity of the locally-produced TGFβ family ligand(s) (BMP2, BMP4, BMP6, BMP 7, and BMP15) whose signalling may either promote, or be impacted by this change in BMPR1B receptor density on the granulosa cell surface, and also to explore the consequences of altered signalling on oocyte quality.

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Authors' roles

S.L.P.R. performed the experiments, analysed and interpreted the data, and wrote the manuscript. All authors contributed to the study design, manuscript revision, and final approval.

Conflicts of interest

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

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