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

Sheena L.P. Regan[a]*, Phil G. Knight[b], John Yovich[c], Jim Stanger[c], Yee Leung[d] , Frank Arfuso[a], Arun Dharmarajan[a], Ghanim Almahbobi[a]

[a]School of Biomedical Sciences, Stem Cell and Cancer Biology Laboratory, Curtin Health Innovation Research Institute, Curtin University, Perth, Australia. [b]School of Biological Sciences, Hopkins Building, University of Reading, Whiteknights, Reading RG6 6UB, UK. [c] PIVET Medical Centre, Perth, Australia. [d] Western Australian Gynaecologic Cancer Service, King Edward Memorial Hospital for Women, Perth, Western Australia.

* Sheena LP Regan,
School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, GPO Box U1987, Perth, WA 6845, Australia
Email: sheenaregan@aapt.net.au

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 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. One patient, out of three patients undergoing risk reduction removal of the uterus and ovaries, was selected to represent an unstimulated natural healthy cycle prior to the LH surge, and was recruited from King Edward Memorial Hospital (KEMH).

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

Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 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.

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		
40	1	Natural Cycle		Healthy	D	2						

OVARIAN RESERVE GOOD [A+] = 30-39 [A] = 20-29 [B] = 13-19 [C] = 9-12 [D] = 5-8 [E] = ≤4 POOR

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 hours 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 pre-ovulatory 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 2-10 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. Natural healthy unstimulated cycle collection

The natural cycle patients scheduled for risk reduction removal of the reproductive organs was timed to coincide with day 12 of the menstrual cycle (Table 1). Before removal, ultrasound confirmation of the size of the follicles and the number of follicles present was made. After removal of the uterus and ovaries, the whole follicle was excised and transported to the laboratory. The collection of follicular fluid, isolation of the granulosa cells, and the analysis was performed as described above and below.

2.6. Immunolabelling of granulosa cells

Aliquots of suspended granulosa cells ($1x10^6$ cells in 100 µl) 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. 2014)

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).

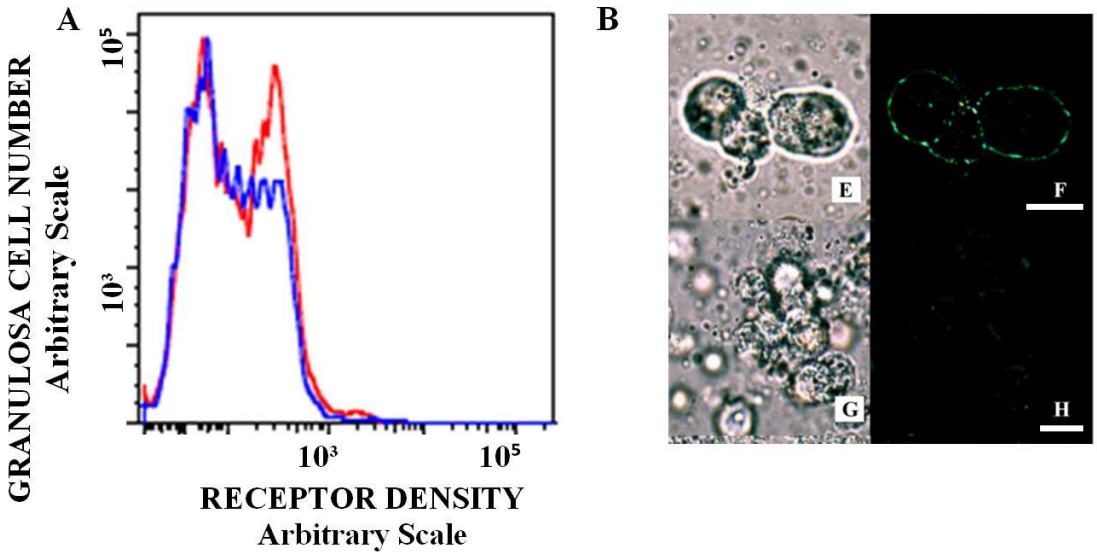


Fig. 1 Validation of immunofluorescent labelling.

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.

2.7. Fluorescent microscopy

Re-suspended 10µl 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 40x 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.

2.8. 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).

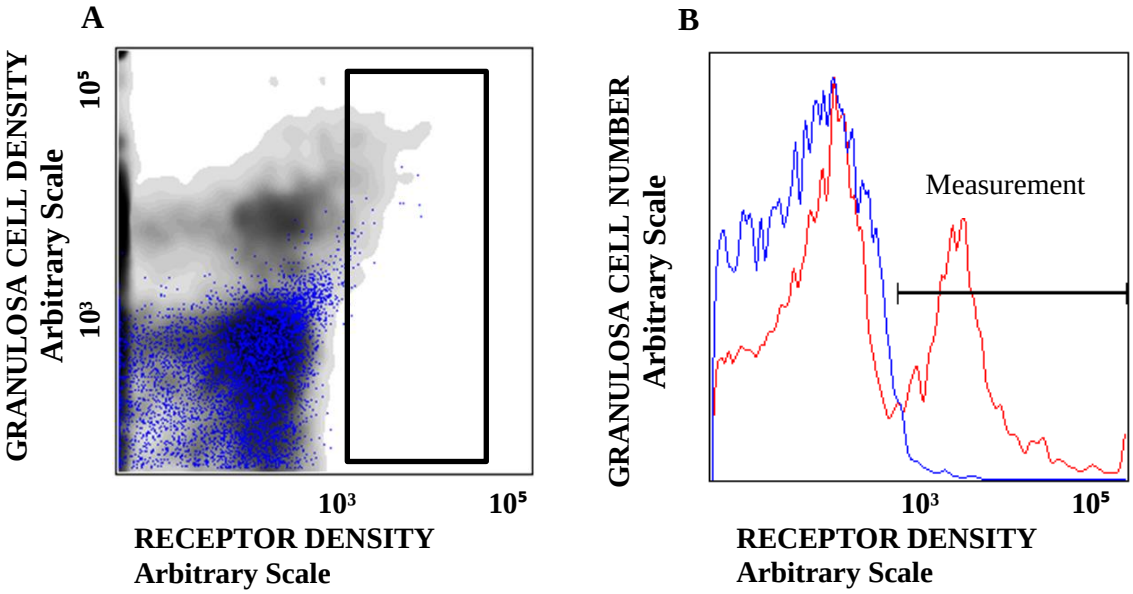


Fig. 2 Validation of gating to measure average receptor density in flow cytometry.

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.

227

2.9. 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.

2.10. Human Ethics

Informed consent was obtained from 57 patients undergoing standard fertility treatment at PIVET fertility clinic Perth, Australia, and three patients undergoing risk reduction removal of the uterus and ovaries which were recruited from King Edward Memorial Hospital (KEMH) 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.

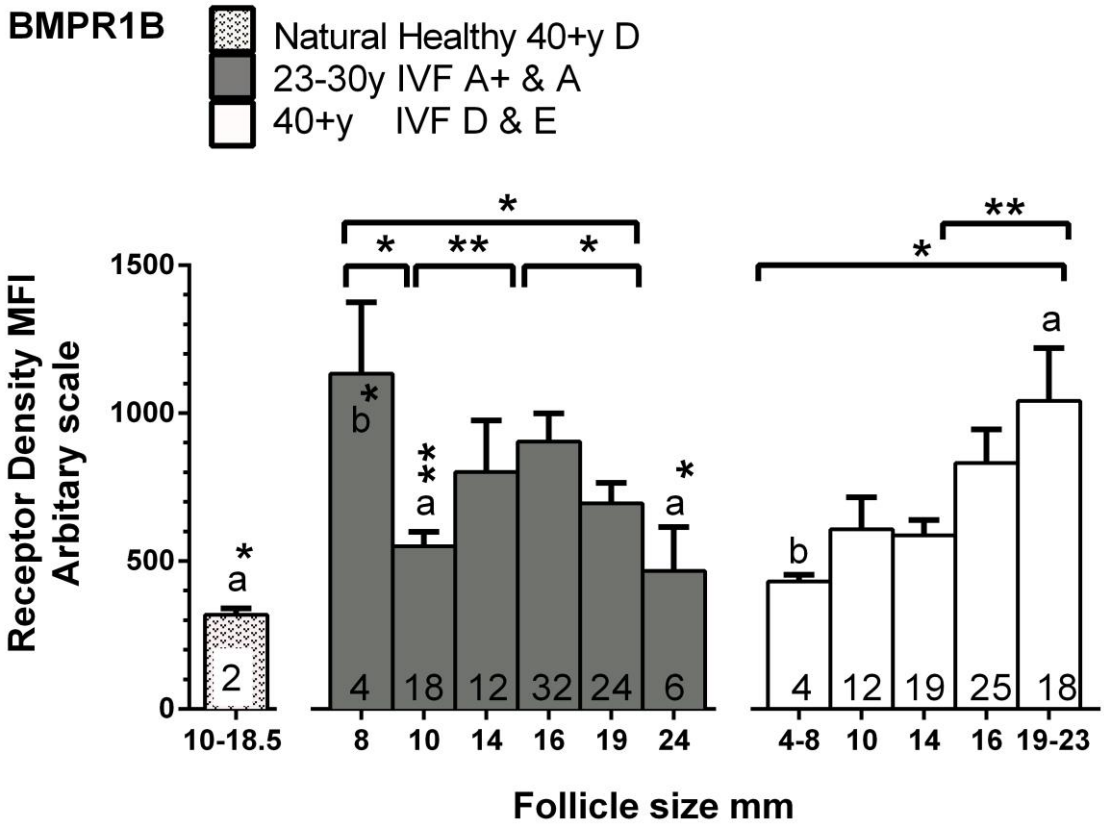
3. Results

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

253 small antral follicles of 8 mm was greater in the young patients than the older patients
254 ($p<0.0405$).

255 In a natural healthy cycle, the granulosa cells were collected from a healthy 40+ y with a
256 group D AFC. When combined, the two follicles of 10 mm and 18.5 mm had a significantly
257 lower density of BMPR1B compared to the largest follicles of the 40+ y IVF patients with a
258 group D & E AFC (Fig. 3). The receptor level in the natural cycle was not significantly
259 different to the level recored in the younger patients and provides a baseline comparison.



OVARIAN RESERVE | GOOD | A+ = 30-39 | A = 20-29 | B = 13-19 | C = 9-12 | D = 5-8 | E = ≤4 POOR

260 with the limitation of reduced interpretation.

261
262 **Fig. 3** Granulosal BMPR1B density from follicles of different sizes collected from young
263 and older IVF patients compared to an unstimulated natural healthy cycle.
264
265 Granulosal BMPR1B protein density and follicle size profile of a natural healthy
266 unstimulated patient of 41y with an AFC of D, before the LH surge (patterned bar). Patients,
267 23-30 y 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.

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 profile of the 40+y E AFC patients. Young patients with a very poor ovarian reserve (group C) for their age do not typically have many follicles available for collection. The rFSH dose administered ranged from 87 IU to 150 IU in the A+ & A group, and was 190 IU in the C group.

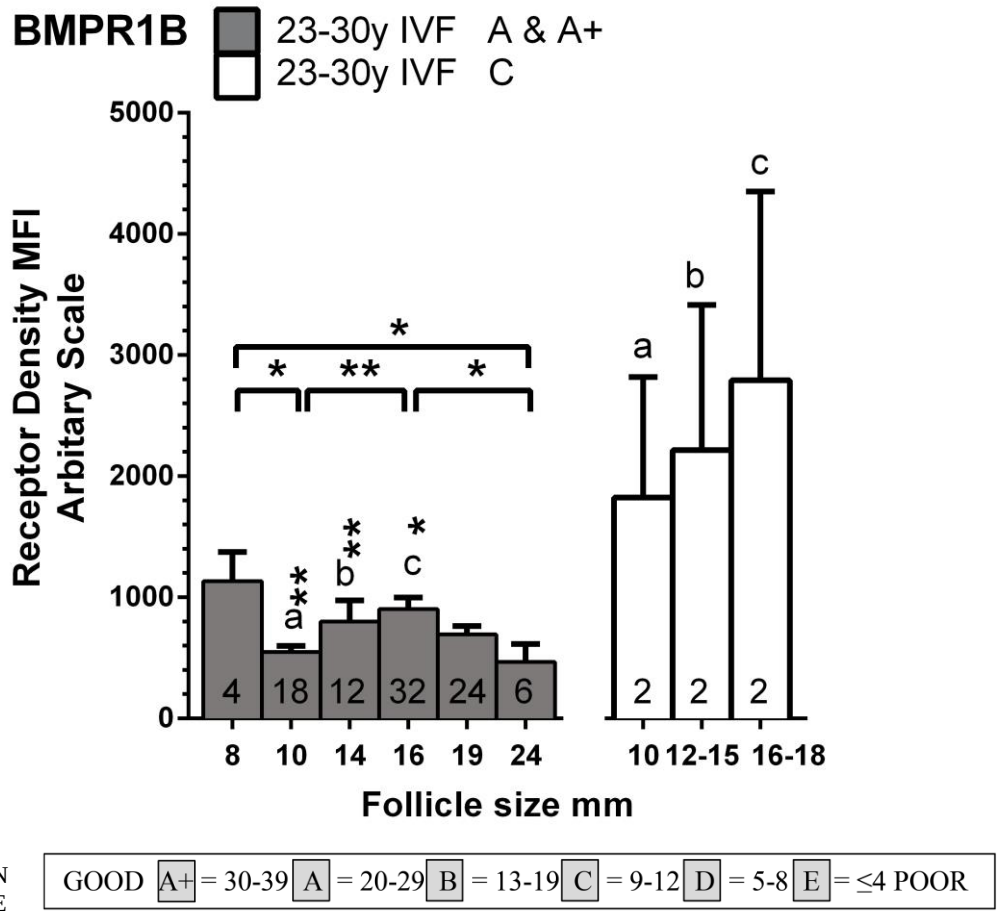


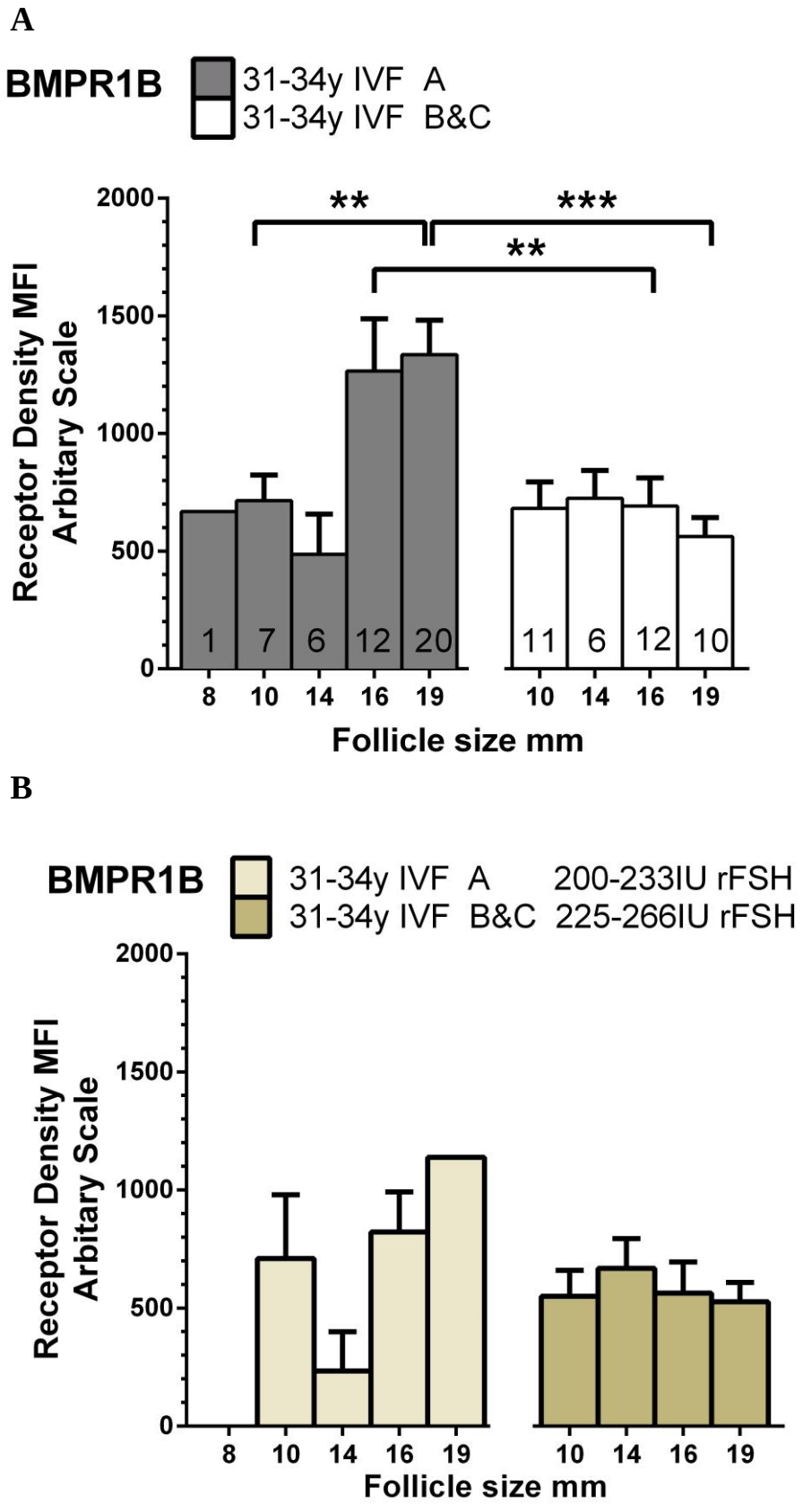
Fig. 4 Granulosal BMPR1B density and ovarian reserve depletion in 23-30 year-old patients.

Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 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.

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

In 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 83.5-266 IU, and when the AFC group comparison was restricted to those patients who received a comparable rFSH dose (200-233 IU), a similar BMPR1B receptor density profile was observed in the B and C group patients. However, in group A, the profile was different in the 16-19 mm follicles, which showed reduced receptor density (Fig. 5B).



OVARIAN RESERVE	GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

309 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 2-10 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 (ie. 'a' is s.d. to all a*). The number within the column represents the number of follicles analysed for that group.

3.5. BMPR1B receptor density in older patients with declining AFC

The 35-39 y combined B & C group demonstrated a significant reduction in BMPR1B receptor density in the 10 mm to 16 mm follicles (p=0.007), similar to the youngest age group. 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=0.037), Fig. 6A). The rFSH dose given ranged from 83.5-600 IU, and when the AFC group comparison was restricted to those patients who received a similar FSH dose (190-241 IU), a very similar BMPR1B receptor density profile was observed (Fig. 6B).

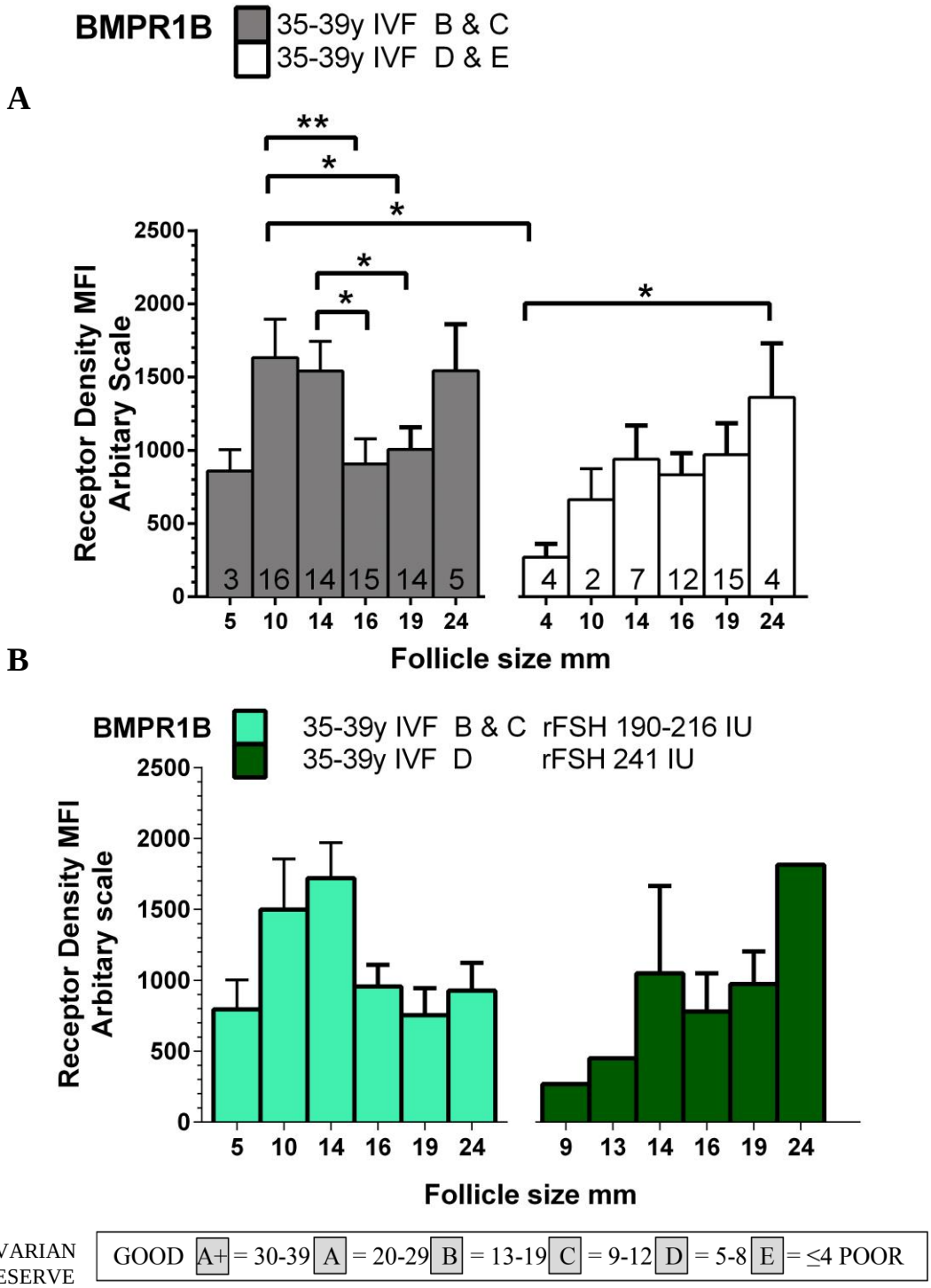


Fig. 6 Granulosal BMPR1B density and ovarian reserve depletion in 35-39 year-old patients.

A. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 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.

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 to 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 300-600 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).

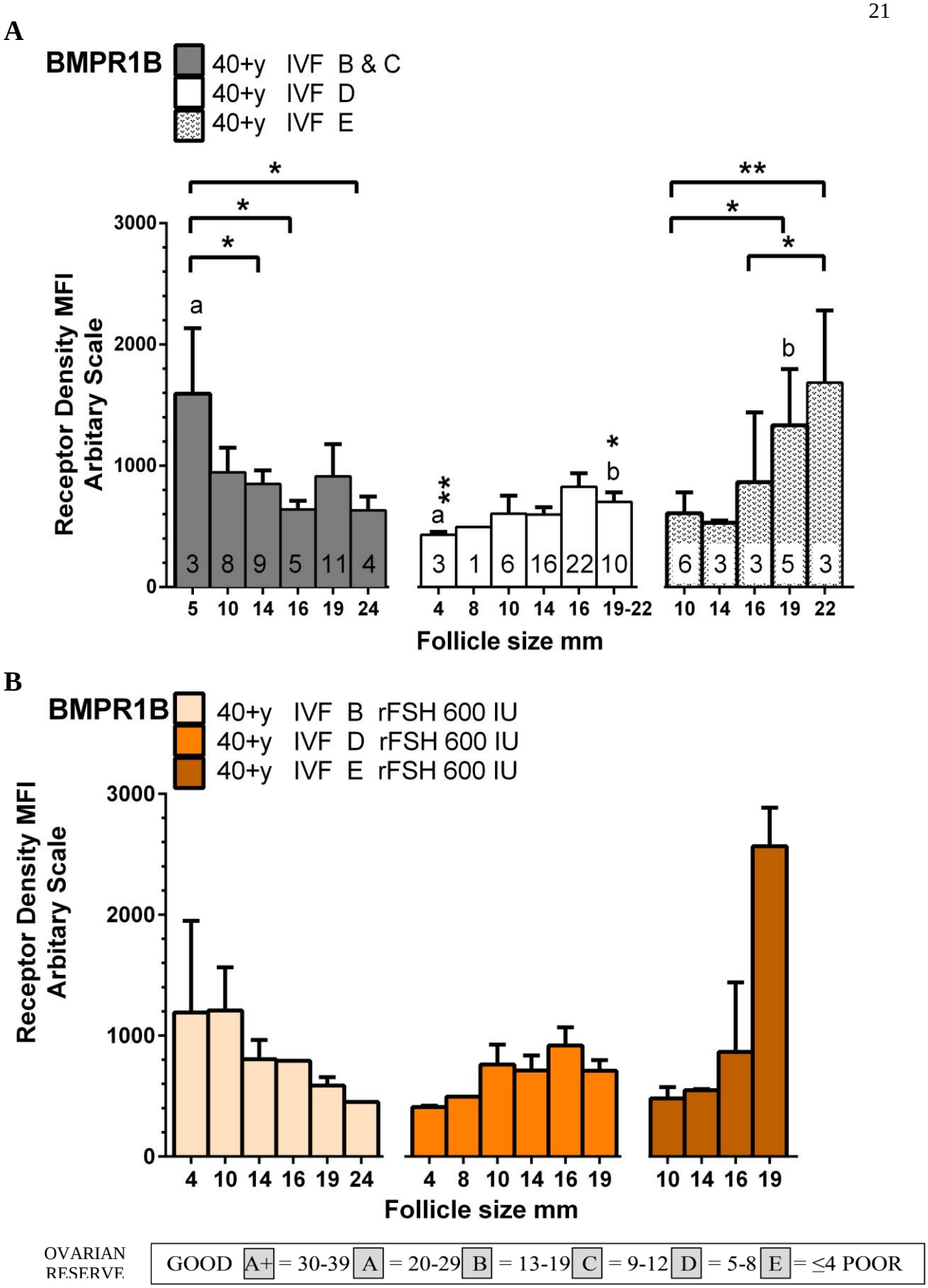


Fig. 7 Granulosal BMPR1B density and ovarian reserve depletion in 40+ year-old patients.

A. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 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 (600IU) 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 (ie. 'a' is s.d. to all a*). The number within the column represents the number of follicles analysed for that group.

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, and again in the largest follicles from the best prognosis IVF patients, aged 23-30 y. In comparison, the older, 40+ y, poor ovarian reserve patients exhibited a reversal of this pattern (Fig. 3). The emergence of the dominant follicle in a natural cycle and the multiple 'dominant' follicle cohort of an IVF stimulated cycle occur at the same follicular size (Baerwald, et al. 2003, Baerwald, et al. 2009). The growth rate of ovulatory follicles from a major wave was also not significantly different in an IVF cycle (Baerwald, et al. 2009). The rFSH only extends the window of recruitment that promotes multiple dominant follicles (Baird 1987, Fauser and Van Heusden 1997). Therefore, it is possible to compare the receptor density to the physiological process indicated by the size of the follicle, such as dominant follicle selection. 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 AFC. 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).

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 was similar to our previous finding in unstimulated 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 process 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 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. 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. Oocytes surrounded by cumulus cells with greater levels of *BMP15* mRNA were shown to have an increased

pregnancy rate after IVF (Li, et al. 2014), and reduced apoptosis (Hussein, et al. 2005). Moreover, an association between high levels of BMP15 in the follicular fluid and oocyte quality has been reported (Li, et al. 2014, 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). In addition, 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 in the largest follicles followed by a general decrease in the poorer ovarian reserve patients for that age group (Fig. 5A). As age increases, and the ovarian reserve declines, fewer follicles are stimulated; hence, the extra-large follicles are rare. 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 provides evidence of a fundamental shift in granulosal BMPR1B receptor density with ovarian ageing. High levels of BMPR1B in pre-ovulatory follicles would promote oestrogen synthesis and inhibit progesterone synthesis, which could potentially supress 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 E2 secretion but has no effect on BMPR1A, BMPR1B or BMPR2 mRNA expression (C Glister and PG Knight, unpublished observations).

4.4. Ovarian gonadotrophin surge attenuating factor (GnSAF) and BMPR1B receptor density during folliculogenesis: is there a link?

The BMPs have been described as inhibitors of the LH surge and luteinisation (Otsuka 2010, Shimasaki, et al. 1999). GnSAF is an uncharacterised follicular-derived factor purported to reduce GnRH-induced pituitary LH secretion (Dimitraki, et al. 2014). An inverse relationship between GnSAF activity and follicle size has been reported (Fowler, et al. 2001). Furthermore, the age-related decline in ovarian reserve was associated with reduced GnSAF activity during follicle development (Martinez, et al. 2002). Could this be linked to the somewhat similar effect of age on granulosal BMPR1B receptor density shown in the present study? In the current study the observed changes in granulosal BMPR1B density in the young and older patients mimic the changes in GnSAF activity described by Martinez et al. (2002). Martinez et al. (2002) concluded that the GnSAF bioactivity prevented the premature onset of the LH surge, which bears comparison with the proposed role of BMPs as an inhibitor of luteinisation as evidenced by others (Otsuka 2010, Shimasaki, et al. 1999). In this context, it should be mentioned that both BMP ligands and receptors are expressed at the anterior pituitary gland level and BMP signalling has been implicated in the regulation of gonadotrophin production (Nicol et al 2008). Thus, it is tempting to speculate that ovarian GnSAF bioactivity, as yet uncharacterised, might actually

be attributed to BMPs synthesized and secreted by ovarian follicles. Clearly, further detailed experiments would be required to evaluate the tenability of this suggestion.

4.5. 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. 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.

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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.

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Conflict 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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