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Amphiregulin cooperates with bone morphogenetic protein 15 to increase bovine oocyte developmental competence: effects on gap junction-mediated metabolite supply

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Abstract

This study assessed the participation of amphiregulin (AREG) and bone morphogenetic protein 15 (BMP15) during maturation of bovine cumulus oocyte complexes (COCs) on cumulus cell function and their impact on subsequent embryo development. AREG treatment of COCs enhanced blastocyst formation and quality only when in the presence of BMP15. Expression of hyaluronan synthase 2 was enhanced by follicle stimulating hormone (FSH) but not by AREG, which was reflected in the level of cumulus expansion. Although both FSH and AREG stimulated glycolysis, AREG-treated COCs had higher glucose consumption, lactate production and ratio of lactate production to glucose uptake. Autofluorescence levels in oocytes, indicative of NAD(P)H and FAD^{++}, were increased with combined AREG and BMP15 treatment of COCs. In contrast, these treatments did not alter autoflouresence levels when cumulus cells were removed from oocytes, even in the presence of other COCs, suggesting oocyte-cumulus gap-junctional communication (GJC) is required. FSH contributed to maintaining GJC for an extended period of time. Remarkably, BMP15 was equally effective at maintaining GJC even in the presence of AREG. Hence, AREG stimulation of COC glycolysis and BMP15 preservation of GJC may facilitate efficient transfer of metabolites from cumulus cells to the oocyte thereby enhancing oocyte developmental competence. These results have implications for improving in vitro oocyte maturation systems.

Introduction

Oocytes from *in vitro* maturation (IVM) have reduced developmental competence in clinical settings. To improve IVM outcomes and hence the clinical update of the procedure, understanding of the mechanisms regulating oocyte developmental competence and how these are impacted by IVM is imperative.

Complex cellular processes occur during oocyte maturation within the ovarian follicular environment that determine the oocyte's developmental competence, defined as the capacity of the oocyte to support fertilization and early embryo development (Gilchrist and Thompson, 2007). Significantly, this underpins fetal and neonatal development, postnatal health and prevention of programmable diseases (Gilchrist and Thompson, 2007, Mtango *et al.* , 2008). Oocyte maturation is coordinated by follicular cells, including granulosa and cumulus cells. These respond to stimulation by gonadotrophins (luteinizing hormone (LH) and follicle stimulating hormone (FSH)), which in turn interact with a complex array of local growth factors. How these signaling processes within the somatic cell compartment of the ovarian follicle endow the oocyte with developmental competence is a subject of intensive research, as these processes set the life course of healthy development and provides opportunities for clinical interventions to treat infertility.

Oocyte maturation occurs during the period between the LH surge and ovulation. LH, via the cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent pathway, induces a rapid and transient expression of epidermal growth factor (EGF)-like factors amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC), which are ligands for the EGF receptor (EGFR) as well as other members of the ErbB family in both mural granulosa and cumulus cells, thereby inducing maturation of the cumulus-oocyte complex (COC) (Hsieh *et al.* , 2007, Park *et al.* , 2004, Shimada *et al.* , 2006). EGF-like factors are also produced by cumulus cells upon stimulation with FSH in vitro, where

1 they trigger resumption of meiosis (Downs and Chen, 2008). During the maturation of
2 COCs, extracellular signal-regulated kinases 1 and 2 (ERK1/2), phosphoinositide 3-
3 kinase (PI3K)/Akt and p38 mitogen-activated protein kinase (p38 MAPK) have been
4 identified as downstream effectors of EGFR signaling in cumulus cells following ligand
5 binding (Prochazka *et al.* , 2012, Shimada *et al.*, 2006, Yamashita *et al.* , 2007).
6 Furthermore, EGF-like factors stimulate gene expression in cumulus cells that enable
7 the production of hyaluronic acid and its organization within the extracellular matrix.
8 Together, these promote modification and expansion of COCs which is essential for
9 ovulation, but not for oocyte developmental competence.

10 Oocyte-secreted factors (OSFs) such as bone morphogenetic protein 15
11 (BMP15) (Laitinen *et al.* , 1998) and growth differentiation factor 9 (GDF9) (Dong *et al.* ,
12 1996), which are members of the transforming growth factor beta (TGF-β) superfamily,
13 are required for correct differentiation of cumulus cells which is crucial for normal oocyte
14 maturation (Eppig, 2001, Gilchrist *et al.* , 2006). OSFs regulate proliferation (Li *et al.* ,
15 2000), gene expression (Paradis *et al.* , 2010, Regassa *et al.* , 2011), luteinization (Eppig
16 *et al.* , 1997), apoptosis (Hussein *et al.* , 2005) and expansion of the extra-cellular matrix
17 (Buccione *et al.* , 1990) within cumulus cells. In the mouse, GDF9 and BMP15 regulate
18 EGFR expression in cumulus cells (Su *et al.* , 2010). Furthermore, in granulosa and
19 cumulus cells, GDF9 requires the activation of EGFR-ERK1/2 signaling (Sasseville *et*
20 *al.* , 2010), suggesting that oocyte paracrine signals work cooperatively with EGFR
21 signaling to regulate the function of follicular cells in the somatic compartment of the
22 follicle.

23 Previous studies have indicated that FSH, AREG and EREG enhance oocyte
24 developmental competence (Demeestere *et al.* , 2012, Prochazka *et al.* , 2011, Richani
25 *et al.* , 2013). Interestingly, COCs cultured with EGF-like factors have higher
26 developmental competence compared to those cultured with gonadotrophins such as

1 FSH, LH and equine chorionic gonadotrophin (Prochazka *et al.*, 2011, Richani *et al.*, 2 2013).

3 *Bmp15* and/or *Gdf9* null mice exhibit reduced fertility, at least in part due to 4 impaired functions of granulosa cells and lower developmental competence of oocytes 5 (Su *et al.* , 2004). In addition, we have demonstrated that supplementation of 6 recombinant BMP15 or GDF9 during *in vitro* maturation of COCs significantly improves 7 oocyte developmental competence, as assessed by increased blastocyst and fetal yield 8 and blastocyst cell number (Hussein *et al.* , 2006, Yeo *et al.* , 2008). This may be related 9 to the observation that, in the mouse at least, IVM oocytes are deficient in processed 10 BMP15, relative to *in vivo* matured oocytes (unpublished data)

11 Gonadotrophins and OSFs also regulate some of the metabolic processes that 12 occur within follicular cells to support oocyte maturation. FSH stimulates glucose uptake 13 by PI3K-mediated translocation of glucose transporter 4 protein to the membrane of 14 granulosa cells (Roberts *et al.* , 2004). Endogenous OSFs control amino acid uptake 15 (Eppig *et al.* , 2005), cholesterol biosynthesis (Su *et al.* , 2008) and glycolysis in cumulus 16 cells (Sugiura *et al.* , 2007). These processes are important for metabolite supply to the 17 oocyte, which may be mediated via cumulus-oocyte gap junctions that are regulated by 18 FSH (Sommersberg *et al.* , 2000) as well as by local paracrine signals.

19 In the present study, our aim was to investigate the mechanisms underpinning 20 how EGF-like factors and OSFs act together to regulate bovine oocyte developmental 21 competence. First, we examined the effect of AREG in the presence of native OSFs and 22 the recombinant OSFs, human GDF9 and human BMP15, on oocyte meiotic maturation, 23 *in vitro* development and blastocyst cell number. Our results indicate that AREG 24 cooperates with BMP15 to enhance oocyte developmental competence. To gain further 25 understanding of the improved oocyte developmental competence, we examined the 26 effect of AREG and BMP15 on cumulus cell expansion; cumulus cell expression of

transcripts encoding such as hyaluronic acid 2 (*HAS2*), tumor necrosis factor alpha-induced protein 6 (*TNFAIP6*), pentraxin 3 (*PTX3*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*); COC metabolism such as glycolysis and levels of autofluorescence within the oocyte, indicative of the metabolic coenzymes reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and oxidized flavin adenine dinucleotide (FAD^{++}). The effect of AREG and BMP15 on gap-junctional communication (GJC) between cumulus cells and the oocyte was assessed.

Materials and Methods

Chemicals and culture media

Unless specified, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Oocyte in vitro maturation (IVM) medium was bicarbonate buffered TCM 199 (B-199, ICN Biochemicals, Irvine, CA, USA) supplemented with 4 mg/ml of fatty-acid free bovine serum albumin (FAF-BSA; ICP biological; Auckland, New Zealand), 0.5 mM pyruvate and kanamycin sulphate (0.1 mg/ml). Wash medium was VitroWash (IVF Vet Solutions, Adelaide, SA Australia) plus 4 mg/ml of FAF-BSA. *In vitro* fertilization (IVF) medium was VitroFert (IVF Vet Solutions) plus 4 mg/ml FAF-BAS. Embryo cleavage and blastocyst media were VitroCleave (IVF Vet Solutions) plus 4 mg/ml FAF-BSA and VitroBlast (IVF Vet Solutions) plus 4 mg/ml FAF-BSA, respectively.

Oocyte collection and *in vitro* maturation

Collection and IVM of bovine COCs was performed as previously described (Sutton-McDowall *et al.* , 2012). Bovine ovaries from a slaughterhouse were transferred to the laboratory, and then washed and stored in physiological saline at 38.5°C. COCs were aspirated from small follicles (2-6 mm in diameter) using a 10-ml syringe equipped with an 18-gauge needle and collected in HEPES-buffered tissue culture medium 199

(HEPES-buffered TCM199; ICP biological) supplemented with 4 mg/ml of FAF-BSA. Compact COCs with greater than 3 layers of cumulus cells and a homogeneous ooplasm were washed twice with HEPES-TCM 199. COCs were cultured in IVM medium without ligand addition (control), with 0.1 IU/ml FSH (Puregon; Organon, Oss, The Netherlands) (FSH), or with 100 ng/ml recombinant human amphiregulin (AREG; R&D systems, Minneapolis, MN, USA) in the presence or absence of DOs (0.5 oocyte/μl), 100 ng/ml of human GDF9 or 100 ng/ml of human BMP15. GDF9 and BMP15 were produced in our laboratory as concentrated preparations of the pro- and mature-region complexes, produced in 293T cells, as previously described (Pulkki *et al.* , 2012, Pulkki *et al.* , 2011). Ten COCs were incubated in 100 μl of IVM medium, covered with paraffin oil in 35-mm Petri dishes at 38.5°C for 22 h in a humidified atmosphere with 6% CO_2 in air. About 20 COCs were used per treatment per replicate.

Assessment of meiotic status

Following IVM for 22 h, COCs were mechanically denuded by pipetting and then fixed using 4% paraformaldehyde for 1 h at 4 °C. Fixed oocytes were incubated in a permeating solution (0.5% triton X-100 and 0.5% sodium citrate) for 15-30 min, followed by incubation in 3 μM 4′,6-diamidino-2-phenylindole solution in the dark for 15 minutes. Oocytes were washed in phosphate buffered saline (PBS) containing 1 mg/ml BSA and then mounted on a slide with glycerol. Chromosome configurations were assessed using a Nikon Eclipse TE2000-E microscope (330–380 nm excitation wavelength; Nikon, Melville, NY, USA).

***In vitro* embryo production**

In vitro embryo production was performed as previously reported (Hussein *et al.*, 2006). Briefly, following IVM, COCs were washed once with wash media and once with

1 IVF medium. Frozen sperm samples (0.5 ml) were thawed at 37°C for 30 s and then
2 centrifuged in a discontinuous Percoll (GE Healthcare, Piscataway, NJ USA) gradient
3 (45%:90%) at 271 × *g* for 25 min. The pellet was then resuspended in wash medium and
4 centrifuged at 56 × *g* for 5 min. Spermatozoa were resuspended in IVF medium, and 20
5 μl of this suspension was added to 380 μl IVF medium containing COCs to yield a final
6 concentration of 1 × 10^6 sperm/ml. After 20 h of culture (day 1), putative zygotes were
7 mechanically denuded from cumulus cells and spermatozoa by gently pipetting using a
8 glass pipette. A total of 5 zygotes were placed in a droplet of 20 μl of cleavage medium
9 and cultured at 38.5°C in 6% CO_2, 7% O_2, nitrogen balance. On day 5 of culture, embryos
10 were transferred into blastocyst medium. The rates of cleavage and blastocyst formation
11 were calculated on day 2 and days 7 and 8, respectively.
12

13 **Differential staining of blastocyst inner cell mass and trophectoderm cells**

14 The allocation of cells within blastocysts was assessed by differential staining of
15 ICM and TE cells as described previously (Hardy *et al.* , 1989). Briefly, the zona-pellucida
16 was removed using 0.5% pronase at 37°C. Embryos were washed in wash medium
17 minus protein and cultured in 10% 2,4,6-trinitrobenzene sulfonic acid for 10 min at 4°C
18 in the dark. Embryos were then incubated in anti-2,4-dinitrophenol (1:10) for 10 min at
19 37°C , followed by a 10-min incubation in complement (2 μg/ml of propidium
20 iodine:guinea pig serum 1:1). Embryos were then fixed in ethanol containing 25 μg/ml
21 Hoechst 33342. Fixed and stained embryos were mounted in 100% glycerol, and the
22 number of ICM and TE cells was assessed under an epifluoresence microscope
23 (excitation = 340-380 nm and emission = 440-480 nm). ICM and TE nuclei appear blue
24 and pink, respectively.
25

26 **COCs expansion assay**

1 Cumulus expansion was assessed according to the scoring system of
2 Vanderhyden *et al.* (1990). Briefly, a score of 0 indicates no detectable response; +1
3 indicates minimum response, with cells in the peripheral 2 layers beginning to expand;
4 +2 indicates expansion extending inwards to several layers; + 3 indicates expansion of
5 all layers of cumulus except the corona radiata cells; and +4 indicates expansion of the
6 entire cumulus including corona radiata cells. Cumulus expansion was examined at 3, 6,
7 12 and 22 h of IVM.
8
9 **RNA extraction and real-time RT-PCR analysis**
10 *HAS2*, *TNFIPA6*, *PTX3* and *PTGS2* genes were analyzed by real-time PCR as
11 previously described (Gilchrist and Ritter, 2011). Ribosomal protein L19 (*RPL19)* was
12 used as the endogenous control, as this was found to be stably expressed regardless of
13 the experimental treatments. COCs cultured for 3, 6, 12 and 22 h, were mechanically
14 stripped of their cumulus cells by vigorous pipetting using a 200 μl pipette. Cumulus cells
15 from 30 COCs were lysed in 300 μl of RTL buffer containing 10 μl/ml of 2-
16 mercaptoethanol and stored at -80°C until RNA extraction. Total RNA was extracted from
17 each sample using the RNeasy Micro Kit (QIAGEN, Valencia, CA) according to the
18 manufacturer's instructions and quantified using a NanoDrop spectrophotometer
19 (Thermo Fisher Scientific, Scoresby, VIC, Australia). Residual genomic DNA was
20 removed by digesting with recombinant RNase-free DNase I (QIAGEN). RNA (200 ng)
21 was reverse transcribed with random primers (Invitrogen) using Super-Script III
22 (Invitrogen Australia Pty Limited, Mount Waverley, Australia). Quantitative real-time PCR
23 analysis was performed on a Corbett Rotor-Gene 6000 (QIAGEN) in a 20-μl reaction
24 volume containing 3 μl cDNA, 2.5 μl each of forward and reverse primers (Table 1), that
25 were designed from bovine sequences, 2 μl nuclease-free water and 10 μl SYBR Green
26 PCR Master Mix (Applied Biosystems, Foster City, CA, USA). All PCR reactions were

1 carried out in duplicate. Universal thermal cycling parameters (initial step of 2 min at
2 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C) were
3 used to quantify the expression of all genes. At the end of the real-time PCR analysis,
4 melting curve analysis was carried out on the real-time cycler to check the specificity of
5 the reaction. A standard curve was generated for the gene of interest and for the
6 endogenous control gene (RPL19) in every PCR run by using a serial 5-fold dilution of
7 the amplified cDNA derived from cumulus cells. Final quantitative analysis of each gene
8 was performed using the standard curve method and results were reported as a relative
9 to the housekeeping gene *RTL19*.
10
11 **Glucose and lactate metabolism**
12 Glucose uptake and lactate production by COCs were determined by measuring
13 the concentration of each substrate in the spent media as previously described (Sutton-
14 McDowall *et al.*, 2012). After 22 h of culture, spent media samples were snap frozen and
15 stored at −80°C. Glucose and lactate concentrations in 50 μl of spent media were
16 determined using a Hitachi 912 chemical analyzer (F. Hoffmann-La Roche Ltd; Basal,
17 Switzerland) and 10 media samples (10 COCs per 100 μl) were measured for each
18 treatment. Glucose uptake and lactate production were expressed as pmol COC^{-1} h^{-1}.
19
20 **Redox state**
21 Redox state in the oocyte was determined following measurement of
22 autofluorescence emissions that relate to the concentration of NAD(P)H and FAD^{++},
23 using methodologies previously described and validated (Dumollard *et al.* , 2004). After
24 22 h of culture, COCs were denuded by gentle pipetting using a 200 μl pipette. Oocytes
25 were washed twice in wash medium and transferred into 5 μl of wash medium overlaid
26 with mineral oil in glass bottom confocal dishes (Cell E&G; Houston, TX, USA). Laser

1 intensity for autofluorescence detection (excitation: 405 nm, emission: 420–520 nm) and
2 green (excitation: 473 nm, emission: 490–590 nm) was set at 50% maximal power and
3 detection sensitivity was 75% (blue) and 65% (green), respectively. Images were
4 captured at 40× magnification, 521 × 521 pixel image size and 8× speed/quality by using
5 an Olympus Fluoview FV10i confocal microscope (Olympus; Tokyo, Japan). Microscope
6 and image settings remained constant throughout. Fluorescence intensity was measured
7 with FV10-ASW 3.0 software (Olympus). The intensity was normalized to the mean value
8 of the background excluding oocytes in each photograph. Autofluorescence was also
9 assessed in oocytes which were matured in groups of 10 denuded oocytes (DOs) or 5
10 DOs co-cultured with 5 COCs.

11

12 **Gap junctional communication assay**

13 The degree of gap junction communication in COCs was assessed using lucifer
14 yellow (LY) dye microinjected into the ooplasm, as previously described (Luciano *et al.* ,
15 2004). After 0, 3, 6 and 12 h of IVM, 3% LY in 5mM lithium chloride was microinjected
16 into the oocyte. The spread of dye into the surrounding cumulus cells was assessed with
17 the confocal microscope within 15 min of injection. As a positive control, the coupling of
18 COCs was assessed immediately after collection, and as a negative control, COCs were
19 cultured in IVM medium for 12 h supplemented with the gap-junction inhibitor,
20 carbenoxolone (CBX, 100 μM) and assessed at 12 h. Individual COCs were scored as
21 +2 when the dye was completely transferred to the entire cumulus mass, +1 when the
22 dye was transferred to limited number of cumulus cell layers just beyond the corona
23 radiata, and 0 when the dye was transferred to only the corona radiata cells or was not
24 transferred to any cumulus cells at all (Fig. 6A). The average score of 10 COCs in each
25 treatment group was calculated to generate the GJC coupling index.

26

1 **Statistical analysis**

2 Data were analyzed using analysis of variance (ANOVA) followed by Tukey-
3 Kramer test or Student *t*-test. All percentage data were arcsine transformed prior to
4 ANOVA. For all data, $P < 0.05$ was considered significant. All analyses were conducted
5 using StatView (SAS Institute Inc, NC, USA).

6

7 **Results**

8 **Amphiregulin enhances oocyte developmental competence in the presence of**
9 **exogenous oocyte-secreted factors**

10 The effect of AREG and FSH during the COC maturation phase in vitro on oocyte
11 meiotic progression and subsequent embryo development were examined, firstly alone
12 and then in the presence of native oocyte-secreted factors (denuded oocytes; DOs),
13 recombinant GDF9 and BMP15 in their pro-mature form. Regardless of the presence or
14 absence of native or recombinant oocyte-secreted factors, AREG and FSH
15 supplementation did not affect the resumption and completion of oocyte nuclear
16 maturation (Fig. S1).

17 Post fertilization, there were no significant differences in embryo cleavage rates
18 or the proportion of pre-compaction embryos across all treatments ($P > 0.05$; Fig. 1). In
19 the absence of native or recombinant oocyte-secreted factors (Fig. 1A), FSH increased
20 total blastocyst development 1.6-fold on day 8 (on time development) compared to the
21 control, whereas AREG did not influence total blastocyst development on either day 7 or
22 8. However, treatment of oocytes during maturation with AREG and with either DOs or
23 BMP15 further increased total blastocyst development on both days 7 and 8, compared
24 to the control group ($P < 0.05$; Figs. 1B and 1D). This additive effect of oocyte-secreted
25 factor and AREG enhancing pre-implantation embryo development was not observed
26 when GDF9 was added during oocyte maturation (Fig. 1C).

1 The FSH and AREG treatment groups had significantly higher rates of
2 hatching/hatched blastocysts compared to the control group, in absence of oocyte-
3 secreted factors (Fig. 1A).
4 Cell allocation to the inner cell mass cell lineage (ICM; fetal progenitor) or
5 trophectoderm (TE; placenta progenitor cells) within blastocysts is an indicator of embryo
6 health and predicts post-implantation embryo and fetal developmental potential
7 (Sugimura *et al.* , 2012). In the absence of oocyte-secreted factors, there was no
8 difference in the number of ICM and TE cells between the various experimental groups
9 ($P > 0.05$; Fig. 2A). In contrast, the number of total and ICM cells in the subsequent
10 blastocysts was significantly increased in the groups supplemented with AREG and DOs
11 (Fig. 2B) or BMP15 (Fig. 2D) during the oocyte maturation phase, but not in the FSH
12 group. As with embryo development, supplementation with GDF9 ± FSH ± AREG had
13 no influence on embryo quality as assessed by ICM, TE and total cell numbers within
14 blastocysts (Fig. 2C).
15

16 **Regulation of cumulus cell expansion by amphiregulin, FSH and BMP15**

17 Cumulus cell production of extracellular matrix proteins is a hallmark of the
18 ovulatory cascade, and expression of some of the related genes are amongst the most
19 reliable markers of oocyte developmental competence (Adriaenssens *et al.* , 2010,
20 Wathlet *et al.* , 2011). We measured cumulus expansion and expression of relevant
21 genes within cumulus cells of COCs treated with either FSH or AREG, with and without
22 BMP15 (Fig. 3 and 4). As expected, cumulus expansion was observed from 12 h of
23 oocyte maturation in both FSH- and AREG-treated groups (Fig. 3, A). Overall, BMP15
24 did not promote morphological cumulus expansion or expression of any matrix gene, nor
25 affect the capacity of FSH or AREG to do so (Fig. 4, B-E). The level of expansion in
26 AREG treated COCs was 80% of the FSH group at 22 h ($P < 0.05$), and was not improved

1 by the addition of BMP15 (Fig. 4A). FSH stimulated higher *HAS2* expression than AREG,
2 with maximum expression observed at 6 and 12 h of oocyte maturation (Fig. 3B).
3 Consistent with the morphological measures of cumulus cell expansion, AREG did not
4 alter cumulus cell *HAS2* gene expression at any stage during COC maturation (Fig. 3B).
5 *TNFAIP6* expression within cumulus cells of the FSH-treated group was higher at 3 h
6 than in the AREG treated group ($P < 0.05$; Fig. 3C). Furthermore, cumulus cells of FSH-
7 treated COCs at 22 h had higher *TNFAIP6* expression compared with control, and
8 although this response was not achieved with AREG alone it was with AREG combined
9 with BMP15 (Fig. 4C). The expression level of *PTX3* was significantly higher following 3
10 h of oocyte maturation in the FSH stimulated group, but otherwise no difference was
11 observed at any other time point (Figs. 3D). In both FSH and AREG stimulated groups,
12 *PTGS2* expression increased from 12 to 22 h (Fig. 3E). Expression level of *PTGS2* at 22
13 h did not differ with FSH or AREG stimulation (Fig. 4E). These results demonstrate that
14 the slightly diminished cumulus cell expansion observed within the AREG-stimulated
15 COCs correlated with less expression of extracellular matrix related genes, most notably
16 an absence of change in *HAS2* expression.
17
18 **Amphiregulin stimulates cumulus-oocyte complex glycolysis**
19 Within the COC, glucose is mostly metabolized to lactate via glycolysis for energy
20 production and this primarily occurs within the cumulus cell compartment of the complex
21 (Sutton-McDowall *et al.* , 2010). Glucose uptake, lactate production and lactate:glucose
22 ratio were measured in COCs cultured with FSH or AREG in the presence or absence of
23 BMP15 (Fig. 5). Theoretically, under conditions that enable complete glycolysis, two
24 molecules of lactate are produced from one glucose molecule, hence the lactate:glucose
25 ratio can indicate the proportion of glucose metabolized via glycolysis, as opposed to
26 other metabolic fates of glucose. Overall, AREG stimulated COC glycolysis to a greater

1 extent than FSH, as evidenced by higher levels of glucose consumption and lactate
2 production (Figs. 5, A and B). BMP15 had no appreciable effect on COC glycolysis,
3 although the combination of BMP15 and AREG stimulated more glucose consumption
4 than those treated with FSH ($P < 0.05$; Fig. 5A). The ratio of lactate production to glucose
5 uptake was significantly higher in AREG-treated COCs than in FSH-treated COCs, and
6 the lactate:glucose ratio was further increased with the addition of BMP15 ($P < 0.05$; Fig.
7 5C). These results indicate that AREG stimulation of oocyte maturation results in a
8 higher glycolytic activity compared with FSH-stimulation.
9

10 **NAD(P)H and FAD^{++} in oocytes is increased by the combination of AREG and**
11 **BMP15 and requires cumulus cell-oocyte contact**

12 Monitoring oocyte autofluorescence of NAD(P)H and FAD^{++} provides information
13 about oocyte metabolism and cellular REDOX state (Dumollard *et al.* , 2007). When
14 oocytes were treated as intact COCs with the combination of BMP15 plus AREG, intra-
15 oocyte NAD(P)H and FAD^{++} levels were significantly increased, compared to the control
16 group without BMP15 (COCs; Fig. 6, A1 and B1). No other combination of ligands altered
17 oocyte NAD(P)H and FAD^{++} levels. No significant difference was found amongst
18 experimental groups in the FAD^{++}:NAD(P)H ratio (REDOX ratio; Fig. 6, C1). No
19 difference was observed in oocyte NAD(P)H and FAD^{++} levels or the REDOX ratio ($P >$
20 0.05) between treatments when oocytes were denuded of their cumulus cells prior to
21 culture (DOs; Fig. 6, A2-C2). Similarly, these measures were unchanged in denuded
22 oocytes that were exposed from the start of culture to intact COCs (DOs+COCs; Fig. 6,
23 A3-C3), suggesting that a metabolic difference in COCs caused by the ligand treatments
24 could not be transmitted by paracrine means to neighboring denuded oocyte in culture.
25 These results suggest that the elevated levels of intra-oocyte NAD(P)H and FAD^{++},
26 caused by incubation in media supplemented with AREG and BMP15, requires the

coupling of oocytes to cumulus cells, and is not the result of a direct action of the ligands on oocytes or paracrine secretions by cumulus cells to oocytes.

BMP15 sustains cumulus-oocyte gap-junctional communication

Because metabolic coenzymes levels were increased in oocytes within intact COCs following treatment with AREG and BMP15, but not within denuded oocytes (Fig. 6), we next investigated the role of AREG and BMP15 in intercellular coupling of cumulus cells with oocytes by injecting lucifer yellow dye into the ooplasm (Fig. 7). Diffusion of the dye from the oocyte into cumulus cells is a standard measure of oocyte-CC GJC (Wert and Larsen, 1989). Prior to maturation (Fig. 7A), most COCs had open gap junctions (69.1% ± 3.2%) or at least partly open gap junctions (26.3% ± 5.4%), and few COCs had completely closed junctions (5.2% ± 3.4%). The coupling index was 1.74 ± 0. 05 at this time point. The coupling index at 3 h and 6 h was significantly higher in groups treated with FSH and FSH+AREG than in the control and those treated with AREG (Fig. 7B). Levels of coupling in FSH and FSH+AREG groups remained steady until 3 h of oocyte maturation, unlike in the control and AREG groups, indicating that AREG could not maintain oocyte-CC GJC to levels maintained by FSH. No significant differences were observed among the experimental groups at 12 h, nor with COCs treated with the gap junction inhibitor carbenoxolone (CBX), indicating that oocyte-CC GJC in all groups was completely closed at 12 h of maturation. In the presence of BMP15 for 3 h, coupling indexes in the control and AREG groups were not different from that of the FSH group (Fig. 7C) and were similar to levels at 0 h. By 6 h of oocyte maturation, oocyte-CC coupling was sustained by FSH or by BMP15 alone or in combination with FSH or AREG. Taken together these results indicate that BMP15 has the capacity to sustain oocyte-CC GJC during oocyte maturation.

1 **Discussion**

2 The means by which oocytes acquire and maintain developmental competence
3 is an extraordinarily complex, yet a fundamentally important biological process, as it sets
4 the developmental life course for the ensuing embryo, fetus and offspring. During oocyte
5 maturation in vivo and in reproductive medicine, where immature oocytes are removed
6 from ovarian follicles and cultured as intact COCs prior to fertilization, hormones and
7 growth factors control the fundamental processes of oocyte maturation (Gilchrist and
8 Ritter, 2011). Previous studies in various animal models have shown that COCs cultured
9 with either EGF-like factors (such as AREG and EREG (Prochazka *et al.*, 2011, Richani
10 *et al.*, 2013)) or with OSFs (Hussein *et al.*, 2006) have increased oocyte developmental
11 competence. This is consistent with emerging critical roles of OSFs in regulating cumulus
12 cell differentiation (Gilchrist *et al.* , 2008) and EGF-like factors mediating the endocrine
13 ovulatory signal in the ovarian follicle (Conti *et al.* , 2006). However, our understanding
14 of how EGF-like factors and OSFs may work together to regulate oocyte competence is
15 limited. Here we demonstrate that AREG works cooperatively with BMP15 to enhance
16 oocyte developmental competence, possibly by increasing the metabolite supply from
17 the cumulus cells to the oocyte through extended gap-junction coupling.

18 As summarized in Table 2, in the absence of any exogenous OSFs, both FSH
19 and AREG during the oocyte maturation phase improved the hatching capacity of
20 blastocysts. However, blastocyst formation could only be enhanced by FSH and not by
21 AREG. In contrast, in the presence of either native OSFs (co-cultured DOs) or BMP15,
22 AREG-treated COCs showed enhanced preimplantation development to the blastocyst
23 stage, with increased blastocyst ICM numbers, compared with FSH treated COCs. This
24 suggests that AREG works cooperatively with exogenous OSFs such as BMP15 to
25 enhance oocyte developmental competence and via a different mode of action to that of
26 FSH. In addition, these results have important practical implications for the treatment of

1 human infertility and for advanced domestic animal breeding, as they suggest that the
2 addition of either native or recombinant OSFs are more efficacious in AREG-stimulated
3 oocyte maturation, than in the current universal practice of FSH-stimulated oocyte
4 maturation.

5 Numerous studies have now shown that native or recombinant OSFs, when
6 added to IVM, enhance mammalian oocyte developmental competence. This suggests
7 that the secretion or availability of resident OSFs from within the IVM COC is aberrant or
8 deficient in some manner. In support of this, we have recently found that IVM mouse
9 COCs, but not *in vivo* matured COCs, lack the processed form of BMP15 (Gilchrist et al;
10 unpublished data), and that recombinant mouse GDF9 (in pro-mature complex form)
11 improves mouse oocyte competence in the presence of FSH (Yeo *et al.*, 2008). Unlike
12 most members of the TGFβ superfamily, there are marked species-specific differences
13 in the expression and bioactivity of GDF9 and BMP15 ligands. For example, the pro-
14 mature complex of human GDF9, but not mouse GDF9, is naturally latent and requires
15 dissociation of the pro-mature complex for biological activity (Mottershead *et al.* , 2008,
16 Simpson *et al.* , 2012). In the current study, we used human GDF9 and BMP15 proteins,
17 as the bovine growth factors are not yet available and the human proteins are most
18 relevant to future human applications. Moreover, as the species-specific expression and
19 activity of these proteins is related to, or potentially determines, natural mammalian
20 ovulation rate (Crawford and McNatty, 2012, McNatty *et al.* , 2005), human (*c.f.* murine)
21 variants of GDF9 and BMP15 proteins are currently most relevant for bovine studies, as
22 both species tend to be mono-ovular. Human GDF9 in the pro-mature form had little
23 effect on oocyte competence (current study). This suggests that neither FSH- or AREG-
24 stimulated COCs are capable of activating latent human GDF9.

25 Cumulus cell expansion, which plays a key role in normal fertilization (Fulop *et*
26 *al.* , 2003), involves the production and stabilization of an extracellular matrix between

cumulus cells surrounding the oocyte. An increase in the expression of the matrix genes *HAS2* (Fulop *et al.* , 1997), *TNFAIP6* (Fulop *et al.*, 2003), *PTX3* (Salustri *et al.* , 2004) and *PTGS2* (Davis *et al.* , 1999) genes, which are stimulated by gonadotrophins or EGF-like factors, is associated with normal cumulus expansion. In the present study, we observed that stimulation of COCs with AREG did not lead to an increase in the expression of *HAS2* and *TNFIPA6* as compared to those stimulated with FSH. In particular, *HAS2* responded poorly to AREG-stimulation. Previous studies in pigs have also shown that cumulus cells of COCs treated with EGF-like factors, such as AREG, had lower expression levels of cumulus expansion related genes compared to FSH (Prochazka *et al.*, 2011). Although both FSH and EGF-like factors activate MAPK1/3, PI3K/AKT and MAPK14 pathways via EGFR, EGF-like factors do not mimic all signals elicited by FSH in culture. EGF-like factors do not activate cAMP-PKA and do not stimulate progesterone production, as does FSH (Prochazka *et al.*, 2011). This could be one of the reasons for the diminished cumulus expansion and lower expression levels of cumulus expansion-related genes in AREG-treated COCs seen in the present study. However, a recent study suggested that a PKA-independent pathway is involved in the regulation of *HAS2* expression in cumulus cells (Prochazka *et al.*, 2012). PKA is not the only transmitter of cAMP signals in mammalian cells (Gerdin and Eiden, 2007). PKA-independent cAMP signaling has been recently reported in rat granulosa cells via Epac-Rit (Wayne *et al.* , 2007). Thus, PKA-independent signaling bypassed in AREG stimulation may be associated with the reduced *HAS2* expression.

OSFs activate SMAD2/3 and/or SMAD1/5/8 signaling in cumulus cells; an event that is essential for cumulus expansion in the mouse (Buccione *et al.*, 1990, Dragovic *et al.* , 2007). These events differ markedly in most other species where cumulus expansion is not critically depending on the presence of OSFs (Gilchrist and Ritter, 2011, Ralph *et al.* , 1995). Consistent with this, in the present study, BMP15 did not increase cumulus

1 expansion of COCs in the absence or presence of FSH or AREG, nor did it alter FSH- or
2 AREG-stimulated expression of genes associated with expansion.

3 During the ovulatory cascade, cumulus expansion requires glucose, which is
4 converted to UDP-*N*-acetyl glucosamine via the hexosamine biosynthesis pathway, and
5 is necessary for hyaluronic acid production by HAS2 (Rilla *et al.* , 2013). Likewise,
6 glucose metabolism via glycolysis in cumulus cells allows for ATP generation. Just as
7 importantly, cumulus cell glycolysis generates metabolites such as pyruvate and lactate
8 that can then be utilized by oocytes, which have a poor capacity to metabolize glucose
9 (Donahue and Stern, 1968, Sutton-McDowall *et al.*, 2010). Thus cumulus cell glycolysis
10 and glucose consumption have fundamental roles in providing energy and substrates for
11 oocyte metabolism that is necessary for subsequent post-fertilisation development. In
12 the present study, both FSH and AREG stimulated glucose uptake and lactate production
13 by COCs, regardless of BMP15 addition. However, glycolysis was stimulated to a greater
14 extent in AREG-treated COCs and furthermore they exhibited a higher ratio of lactate
15 production to glucose consumed compared to the FSH-treated group. This suggests that
16 AREG may stimulate a higher proportion of glucose down the glycolytic pathway, and
17 thereby possibly a reduction of glucose metabolism via the hexosamine biosynthesis
18 pathway, a known "energy sensing" pathway. Over-stimulation of the hexosamine
19 biosynthetic pathway adversely affects oocyte developmental competence in cattle and
20 pigs (Sutton-McDowall *et al.* , 2006) and in mice, over-stimulation promotes adverse
21 long-term programming effects on fetal development (Schelbach *et al.* , 2010, Schelbach
22 *et al.* , 2013). It is likely that AREG reduces glucose flux through the hexosamine pathway,
23 consistent with the failure of AREG to stimulate *HAS2* expression and reduced cumulus
24 expansion, thus providing more ATP production from glycolysis within cumulus cells,
25 compared to COCs treated with FSH. We propose this is one mechanism by which
26 AREG improves oocyte developmental competence.

1 Pyruvate, lactate and glucose, that enter the oocyte from cumulus cells via 2 glucose catabolism, are metabolised and can provide coenzymes such as NAD(P)H in 3 the cytosol as well as in the mitochondria (Li *et al.* , 2011). Activation of the Kreb cycle 4 by uptake of pyruvate in the mitochondria provides more NADH and leads to the 5 reduction of $FADH_2$ by oxidative phosphorylation via the electron transport chain, which 6 is necessary for ATP production. On the other hand, NADPH from the pentose phosphate 7 pathway reduces oxidized glutathione, a reaction important for maintaining antioxidant 8 defense in the oocyte (de Matos *et al.* , 1997). Thus, changing the supply of energy 9 substrates entering the oocyte from the cumulus cells should be reflected in levels of 10 NAD(P)H and FAD^{++}, (Dumollard *et al.*, 2007). Although AREG increased glucose uptake 11 and glycolysis in COCs, the intensity of NAD(P)H and FAD^{++} and the REDOX ratio in 12 oocytes were similar to those observed in the control. However, in the presence of 13 BMP15, their levels were increased by AREG stimulation. This increase in the levels of 14 NAD(P)H and FAD^{++} autofluorescence was not observed in denuded oocytes or denuded 15 oocytes co-cultured with COCs upon stimulation with AREG and BMP15. This suggests 16 that AREG increases metabolic activity in the oocyte cooperatively with BMP15 via 17 oocyte-cumulus cell coupling and that these growth factors do not have this effect directly 18 on the oocyte or via paracrine actions from cumulus cells.

19 Oocyte-cumulus cell gap-junction coupling is vitally important for oocyte growth 20 and is a key component of oocyte developmental competence (Gilchrist *et al.* , 2004). 21 Intercellular communication in COCs can be regulated by dynamic changes in 22 connexin43 (Cx43) and connexin37 (Cx37)-mediated gap-junction communication (GJC) 23 (Sutovsky *et al.* , 1993, Veitch *et al.* , 2004). Cyclic AMP generated in cumulus cells 24 stimulated by gonadotrophins or forskolin enhances the synthesis of Cx43 and GJC 25 (Sasseville *et al.* , 2009, Thomas *et al.* , 2004). Furthermore, it is known that FSH 26 positively regulates Cx43-mediated GJC activity through phosphorylation of specific sites

1 via a PKA-depend pathway (Granot and Dekel, 1994, Yogo *et al.* , 2006, Yogo *et al.* ,
2 2002). In contrast to FSH, AREG is not thought to activate cAMP/PKA in cumulus cells
3 (Prochazka *et al.*, 2011). During the ovulatory cascade, LH-induced expression of AREG
4 and EREG activates cumulus cell EGF receptors which in turn leads to closure of
5 cumulus-oocyte gap junctions via a MAPK-dependent mechanism (Norris *et al.* , 2008,
6 Norris *et al.* , 2010). These reports are consistent with the present study where we found
7 that FSH but not AREG maintained cumulus-oocyte GJC. Perhaps surprisingly, BMP15
8 prevented the normal closure of cumulus-oocyte GJC that occurs from 0 to 3 h of oocyte
9 maturation and levels were still elevated by 6 h. This represents a previously undescribed
10 role for BMP15 in oocyte biology. BMP15 even maintained GJC in the presence AREG,
11 which typically is associated with closure of cumulus-oocyte gap junctions (Norris *et al.*,
12 2010). The intracellular mechanism by which BMP15 maintains cumulus-oocyte GJC will
13 be an important issue for future studies. Nonetheless, in the current study, BMP15-
14 enhanced GJC may be an important means by which these oocytes achieve elevated
15 levels of cumulus-derived metabolites that are vital for subsequent embryo development.
16 In conclusion, the sophisticated communication axes and supply of metabolic
17 substrates between the somatic cells and the germ cell of the COC are central to the
18 oocyte's capacity to support subsequent pre-implantation embryo development. Glucose
19 uptake in cumulus cells stimulated by AREG may be utilized for the production of energy
20 via glycolysis rather than being utilized for extracellular matrix and cumulus cell
21 expansion. BMP15 maintains cumulus-oocyte GJC, possibly thereby allowing glycolytic
22 metabolites to be more efficiently transferred to the oocyte from cumulus cells, facilitating
23 oocyte energy production (Fig. 8). This may be the mechanism by which AREG in
24 combination with BMP15 improves oocyte developmental competence, leading to
25 improved embryo development. This knowledge has significant implications for
26 improving the developmental competence of oocytes derived from IVM for human clinical

1 applications.

2

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10
11

12 **Table I**. Sequences of primers for real-time PCR

13

Gene name	GenBank accession number	Primer sequences forward (F) and reverse (R)	Amplicon size (bp)
HAS2	NM_174079.2	F: 5'-GGATCTCCTTCCTCAGCAGTGT-3'	106
		R: 5'-ATTCCCAGAGGTCCGCTAATG-3'	
TNFAIP6	NM_001007813.2	F: 5'-TGAAAGATGGGATGCATATTGC-3'	101
		R: 5'-CATTTGGGAAGCCTGGAGATT-3'	
PTX3	NM_001076259.2	F: 5'-CATGTATGTGAATTTGGACAACGA-3'	101
		R: 5'-GCTTGTCCCACTCGGAGTTC-3'	
PTGS2	NM_174445.2	F: 5'-CTTAAACAAGAGCATCCAGAATGG-3'	106
		R: 5'-GCTGTACGTAGTCTTCAATCACAATCT-3'	
RPL19	NM_001040516.1	F: 5'-TGAGGCCCGCAGGTCTAAG-3'	101
		R: 5'-CTTCCTCCTTGGACAGAGTCTTG-3'	

14

Table II. Summary of the effects of treatment of oocytes during the maturation phase with FSH, growth factors and native oocyte-secreted factors, on oocyte maturation and subsequent embryo development and embryo quality.

Exogenous OSFs	Ligands	MII	Cleaved	<4 cell	D7BL	D8BL	Hing/Hed	Number of cells in blastocyst		
								Total	ICM	TE
None	FSH	-	-	-	-	↑	↑	-	-	-
	AREG	-	-	-	-	-	↑	-	-	-
DOs	FSH	-	-	-	-	↑	↑	-	-	-
	AREG	-	-	-	↑	↑	↑	↑	↑	-
GDF9	FSH	-	-	-	-	-	↑	-	-	-
	AREG	-	-	-	-	-	-	-	-	-
BMP15	FSH	-	-	-	-	↑	↑	-	-	-
	AREG	-	-	-	↑	↑	↑	↑	↑	-

1 ↑ and -, indicate a significant increase and no change to control, respectively; MII, % of oocytes to reache metaphase II; cleaved and <4
2 cell, % of cleaved and <4 cell embryos, respectively; D7BL and D8BL, % of blastocysts at day 7 and 8 of cleaved embryos, respectively;
3 Hing/Hed, % of hatching/hatched blastocysts of cleaved embryos.

Figure legends

Fig. 1. Effect of FSH and AREG in the presence of oocyte-secreted factors during oocyte maturation on subsequent oocyte developmental competence. COCs were cultured without ligand (control) or with FSH or AREG supplemented medium, with either no oocyte-secreted factors (**A**); native oocyte-secreted factors (DOs; **B**); GDF9 (**C**); or BMP15 (**D**). Effects of treatments on oocyte development competence were examined by measuring the following points of embryo development: 2 cell cleavage (Cleaved), > 4 cell embryos (>4 cell) at day 2, embryos developed to blastocysts at days 7 (D7BL) and 8 (D8BL), and those that reached the hatching or hatched blastocyst stage (Hing/Hed) by day 8. D7BL, D8BL and Hing/Hed are expressed as percentages of cleaved embryos. All values are represented as mean ± s.e.m of 7 replicate experiments. Asterisks indicate significant differences at $P < 0.05$.

Fig. 2. Effect of FSH and AREG in the presence of oocyte-secreted factors during oocyte maturation on blastocyst cell number at day 8. COCs were cultured without ligand (control) or with FSH or AREG supplemented medium, with either no oocyte-secreted factors (**A**); native oocyte-secreted factors (DOs; **B**); GDF9 (**C**); or BMP15 (**D**). Effects of treatments during oocyte maturation on subsequent blastocyst quality were examined by counting the number of total, ICM and TE cells in blastocysts at day 8. All values are represented as mean ± s.e.m of 7 replicate experiments. Asterisks on the bars indicate significant differences at $P < 0.05$.

Fig. 3. Kinetics of cumulus cell expansion and its related mRNA gene expression in cumulus cells treated with FSH or AREG. (**A**) Cumulus cell expansion index (CEI) in COCs cultured with FSH or AREG was examined at 3, 6, 12 and 22 h of oocyte

maturation. Values represented as mean ± s.e.m of 4 replicates. Asterisks indicate significant difference compared with AREG ($P < 0.05$; Student *t*-test). Expression of *HAS2* (**B**), *TNFIPA6* (**C**), *PTX3* (**D**) and *PTGS2* (**E**) in cumulus cells of COCs cultured in FSH or in AREG determined at various time points. FSH-treatment at 3 h was assigned a value of one and all other points are expressed relative to this point. Values are represented as mean ± s.e.m of 4 replicates. Asterisks indicate significant difference compared with AREG ($P < 0.05$; Student *t*-test).

Fig. 4. Cumulus cell expansion and its related mRNA gene expression in cumulus cells treated with FSH or AREG in the presence or absence of BMP15. (**A**) CEI in COCs cultured with no ligand (control), FSH or AREG in the presence or absence of BMP15 was examined at 22 h of oocyte maturation. Values are represented as mean ± s.e.m of 4 replicates. Different letters indicate significant differences at $P < 0.05$. Expression of *HAS2* (**B**), *TNFIPA6* (**C**), *PTX3* (**D**) and *PTGS2* (**E**) in cumulus cells of COCs cultured with no ligand (control), FSH or AREG in the presence or absence of BMP15 were examined at 22 h of oocyte maturation. Control group in the absence of BMP15 was assigned a value of one and all other points are expressed relative to this point. Values are represented as mean ± s.e.m of 4 replicates. Different letters indicate significant differences at $P < 0.05$.

Fig. 5. Glucose metabolism of COCs cultured with FSH or AREG in the presence or absence of BMP15. Glucose uptake (**A**), lactate production (**B**) and the ratio of lactate production to glucose uptake (**C**) were calculated from COCs cultured with no ligand (control), FSH or AREG in the presence or absence of BMP15 for 22 h of oocyte maturation. Values are represented as mean ± s.e.m of 5 replicates. Different letters indicate significant differences at $P < 0.05$.

1

2 **Fig. 6.** Regulation of oocyte NAD(P)H, FAD^{++} and redox ratio by ligands and cumulus-
3 oocyte contact. Intensity of oocyte autofluorescence of NAD(P)H (**A1-A3**) and FAD^{++}
4 (**B1-B3**) and the redox ratio (**C1-C3**) in oocytes that were cultured 0-22h as intact COCs
5 (COCs; **A1-C1**), or cultured 0-22h as denuded oocytes (DOs; **A2-C2**), or cultured 0-22h
6 as denuded oocytes in co-culture with COCs (DOs+COCs; **A3-C3**). All cultures were
7 treated with no ligand (control), FSH or AREG, in the presence or absence of BMP15.
8 Within each graph, the control group in the absence of BMP15 was assigned a value of
9 one and all other means are expressed relative to this value. Values are represented as
10 mean ± s.e.m of 5 replicates. Different letters indicate significant differences at $P < 0.05$.

11

12 **Fig. 7.** Regulation of oocyte-CC gap-junctional communication of COCs by FSH and
13 AREG in the presence or absence of BMP15. (**A**) LY diffusion in COCs at 0 h of oocyte
14 maturation. Fluorescence and bright field images of open (+2), partially open (+1), and
15 closed (0) GJC. (**B**) LY diffusion in COCs cultured with no ligand (control), FSH, AREG
16 or FSH+AREG was examined at 3, 6, and 12 of oocyte maturation in vitro. LY diffusion
17 of COCs at 0 h and COCs cultured with CBX at 12 h were measured as positive (black)
18 and negative controls (white). (**C**) LY diffusion in COCs cultured with no ligand (control),
19 FSH or AREG in the presence or absence of BMP15, was examined at 3 h or 6 h of
20 oocyte maturation in vitro. Dotted line indicates mean value at 0 h of IVM. Values are
21 represented as mean ± s.e.m of 5 replicates. Different letters reflect significant
22 differences within a time point at $P < 0.05$. Asterisks indicate significant differences
23 compared to the GJC index at 0 h ($P < 0.05$; Student *t*-test).

24

25 **Fig. 8.** Hypothesized mechanism by which AREG and BMP15 enhance oocyte
26 developmental competence and ensures subsequent embryo development. *HAS2* and

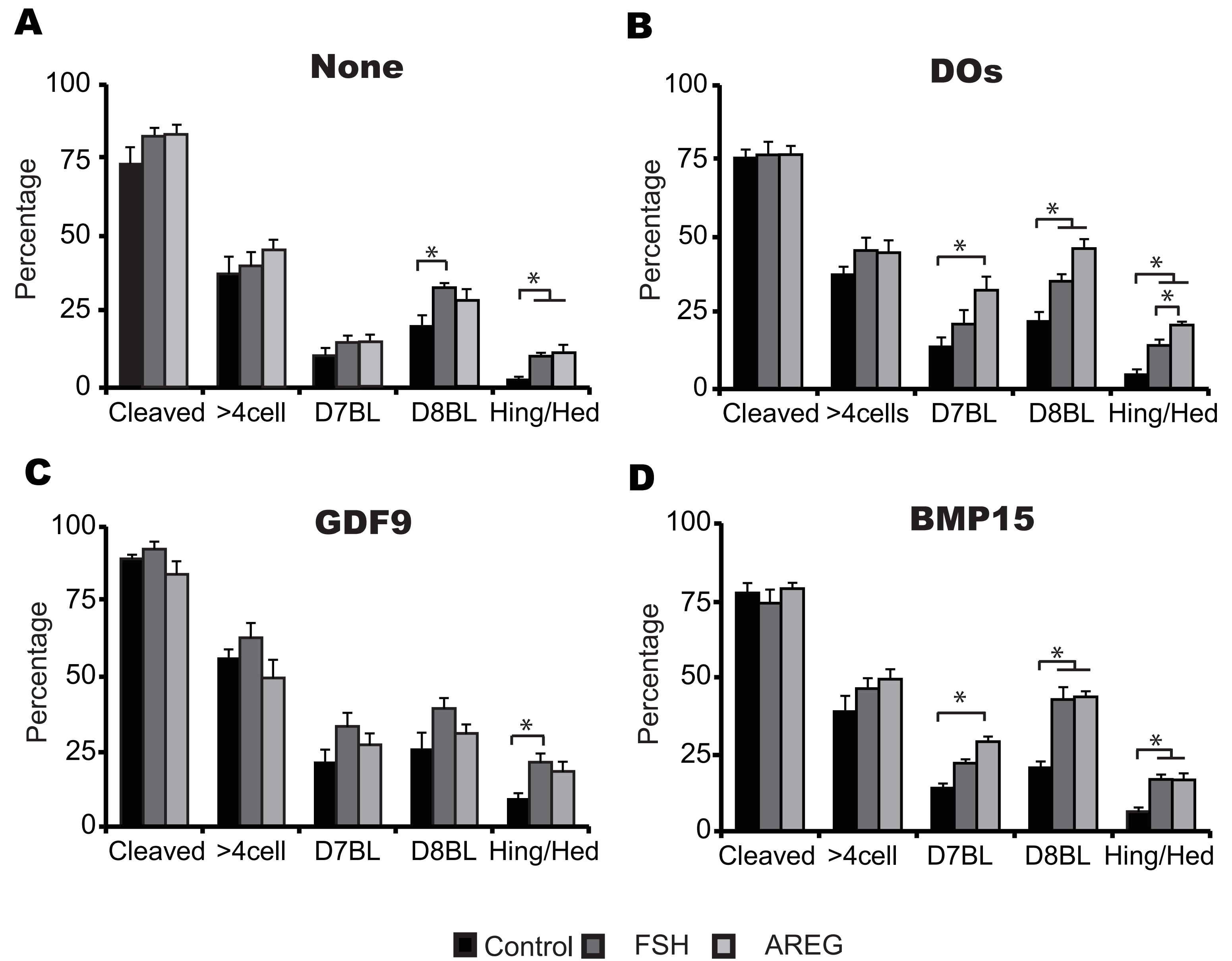
TNFIPA6 are efficiently stimulated by FSH (**A**) but not by AREG (**B**). AREG with BMP15 increase *TNFIPA6* expression but not *HAS2* expression (**D**). AREG enhances cumulus cell glucose metabolism via glycolysis (**B**) compared to FSH (**A**) but nonetheless leads to somewhat diminished cumulus expansion. Intra-oocyte oxidative phosphorylation is crucial for oocyte developmental competence and the oocyte is dependent on cumulus cells for supply of substrates from metabolized glucose via glycolysis. Accordingly, intra-oocyte NAD(P)H and FAD^{++} levels are increased by the combination of AREG and BMP15 but only when cumulus cells and oocytes are physically coupled, which is sustained by BMP15 (**D**). Thus AREG and BMP15 cooperate to regulate oocyte metabolism by enhancing glycolysis and CC-oocyte gap-junctional communication, which increases oocyte developmental competence and the subsequent development and quality of embryos.

1 **Supplemental figure legends**

2

3 **Fig. S1** Effect of FSH and AREG in the presence of OSFs on meiotic resumption. Bovine
4 COCs were cultured in without ligand (control) and in FSH or AREG supplemented
5 medium with no OSFs (**A**); DOs (**B**); GDF9 (**C**); and BMP15 (**D**). We examined the
6 following: rates of oocytes arrested at the germinal vesicle stage (GV) and GVBD to
7 telophase I (GVBD) and the percentage of cells that had reached metaphase II (MII) at
8 22 h of IVM. All values are represented as mean ± s.e.m of 7 replicates.

Fig. 1. *Sugimura et.*



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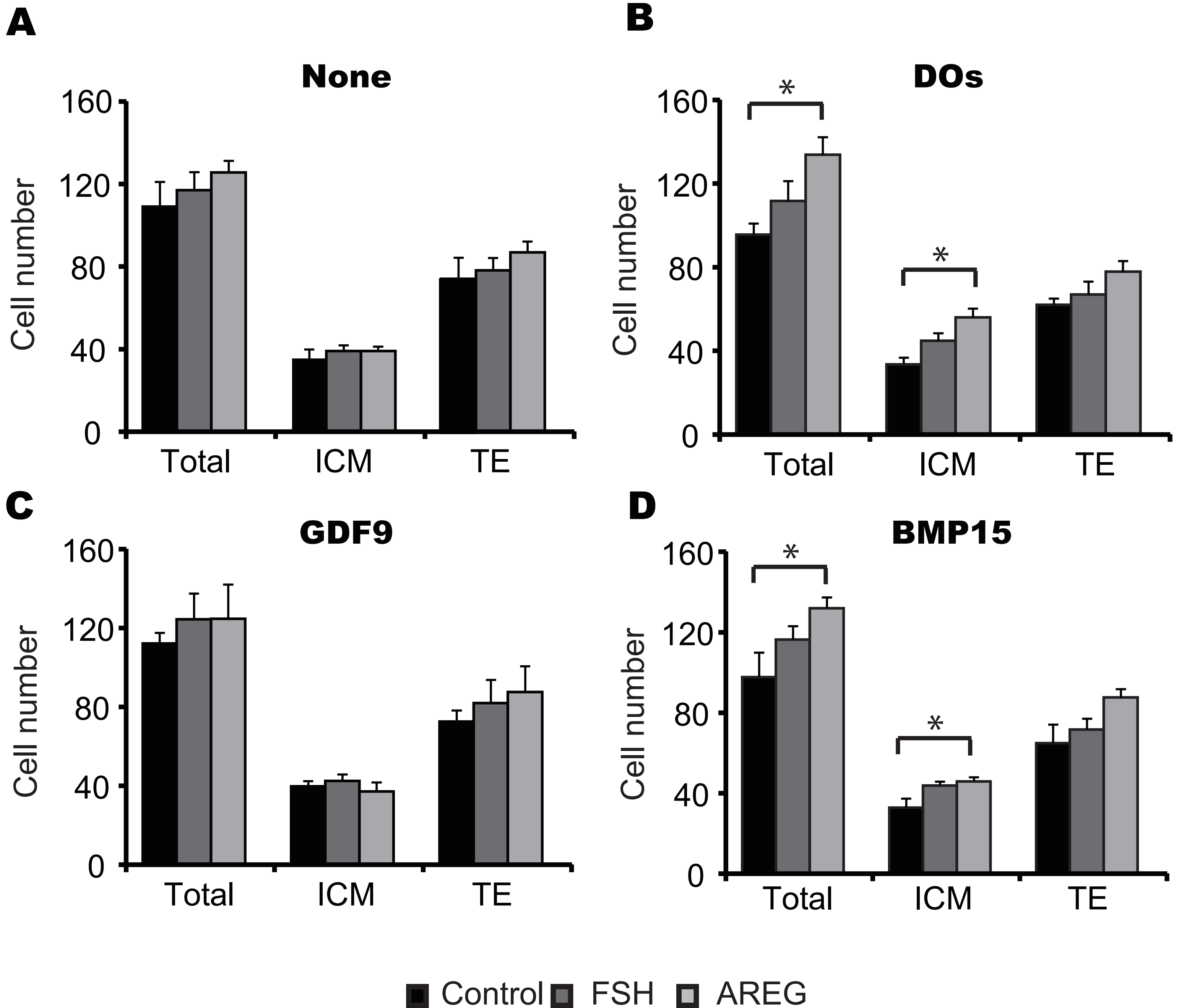

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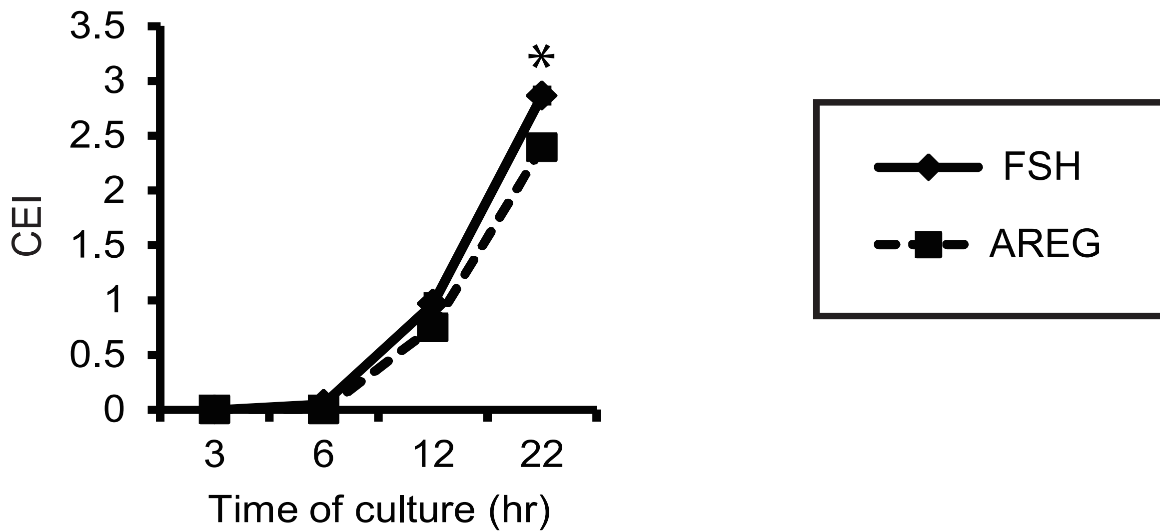

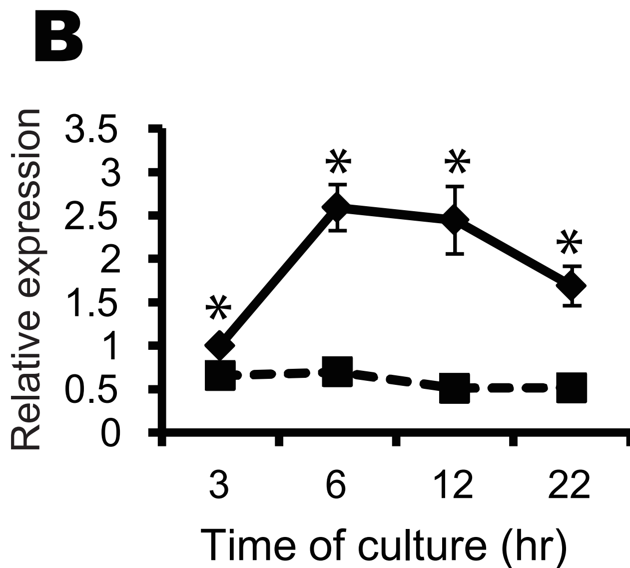

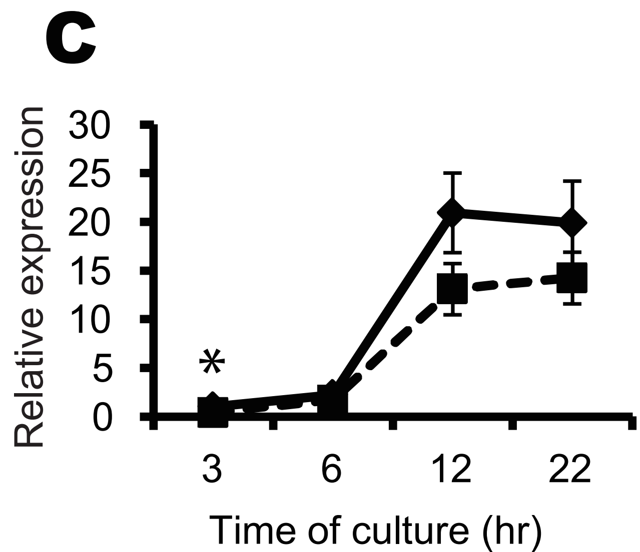

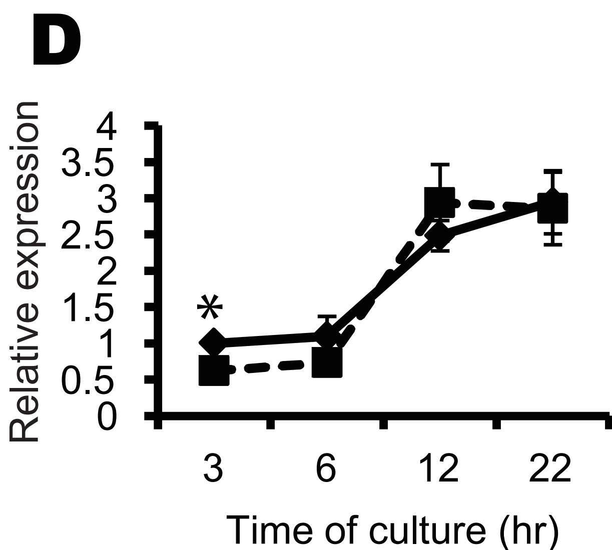

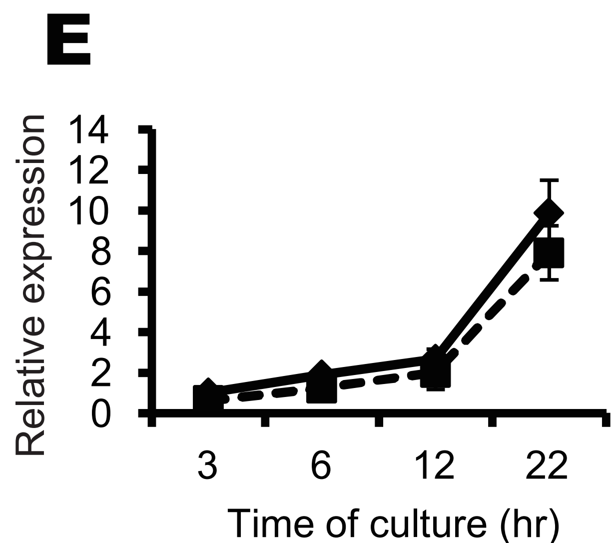

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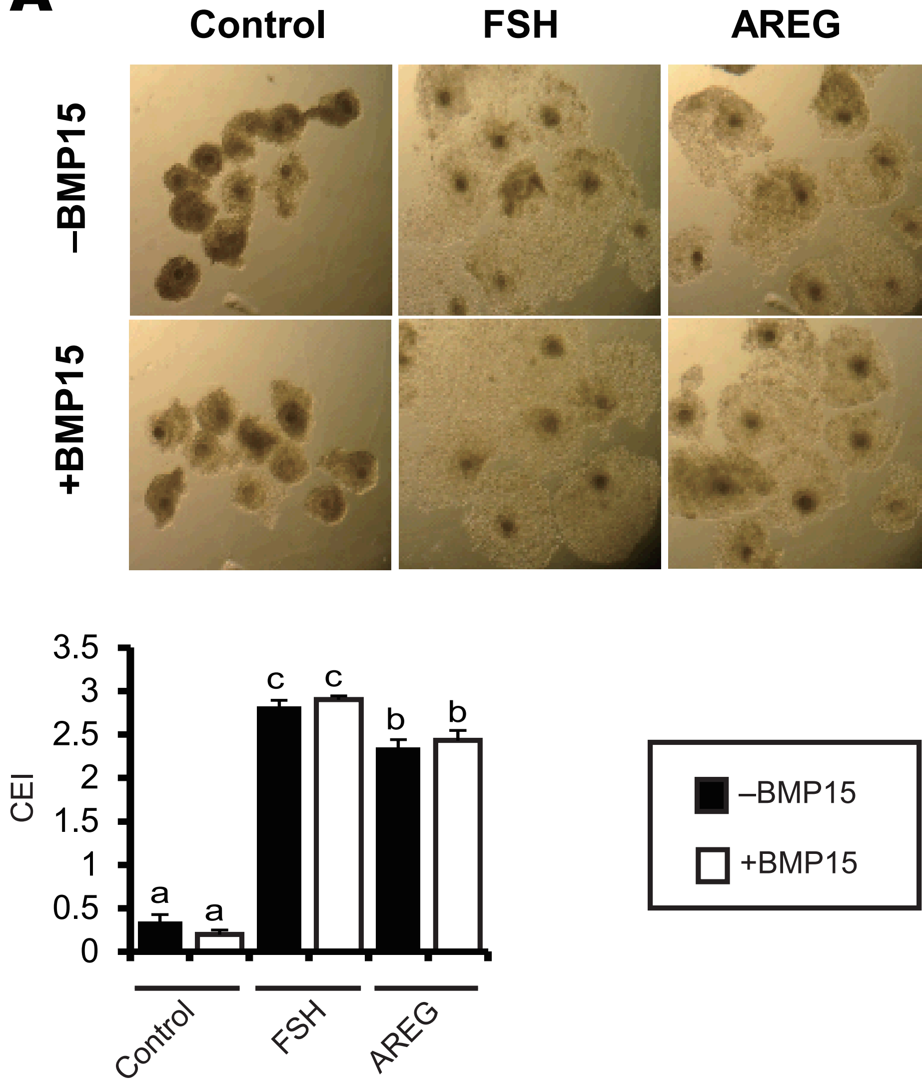

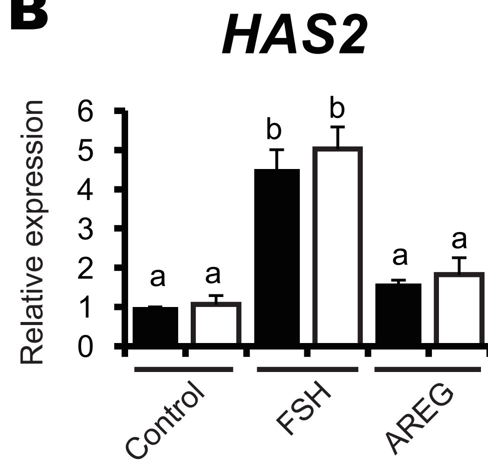

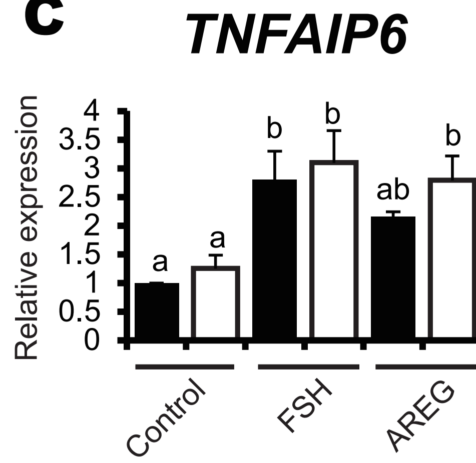

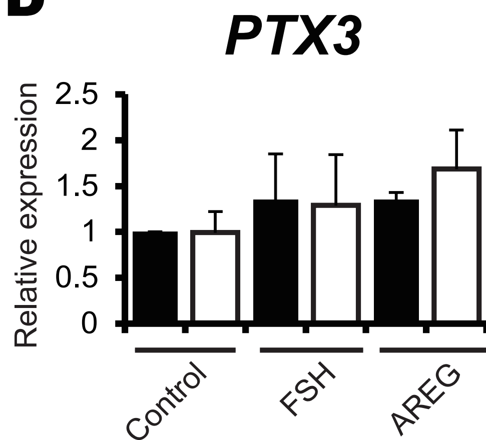

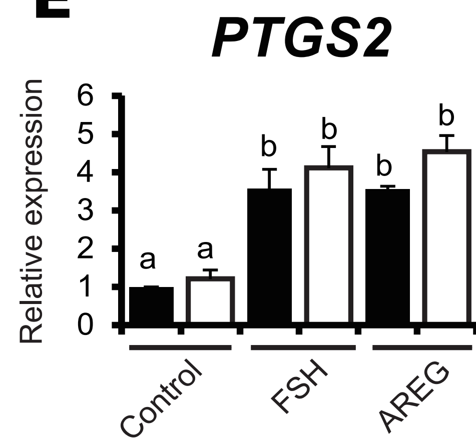

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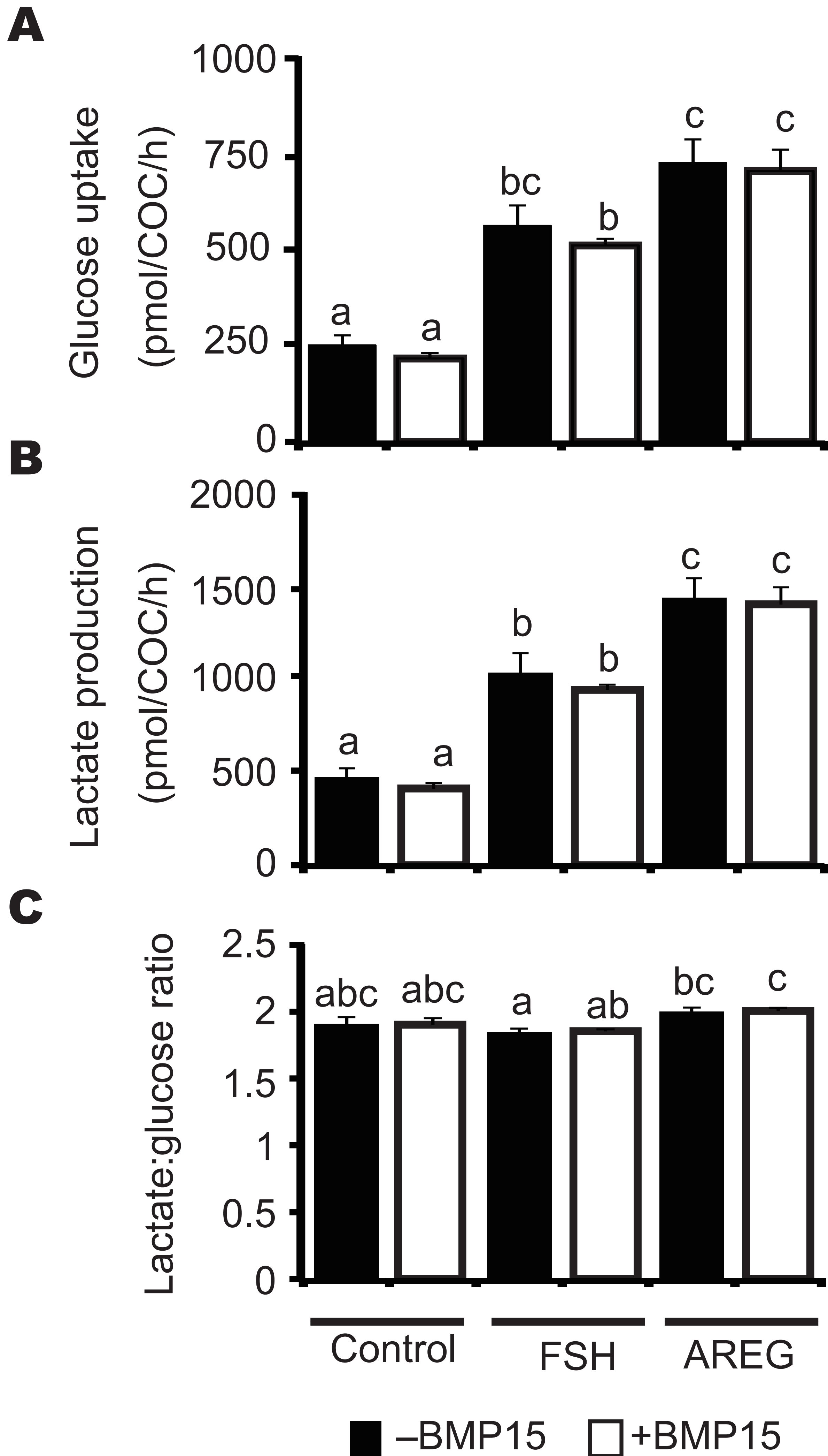

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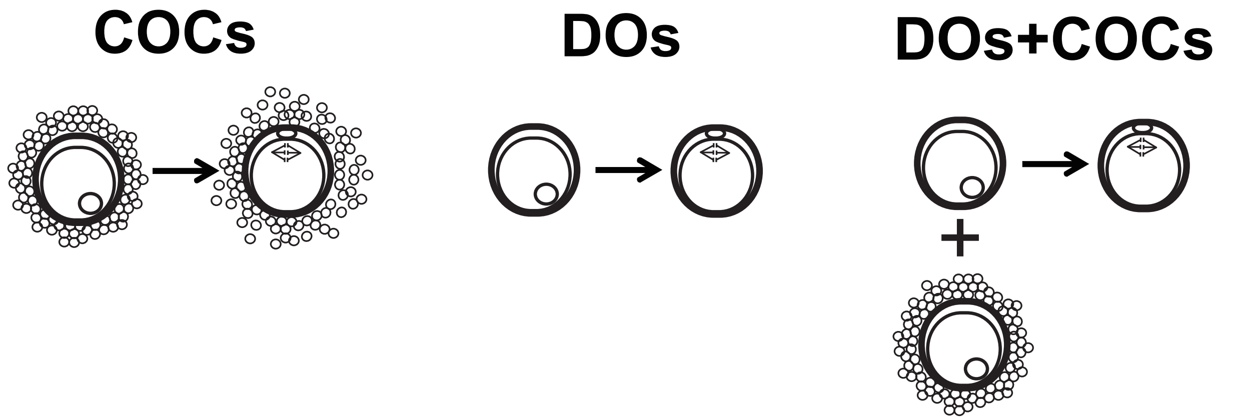

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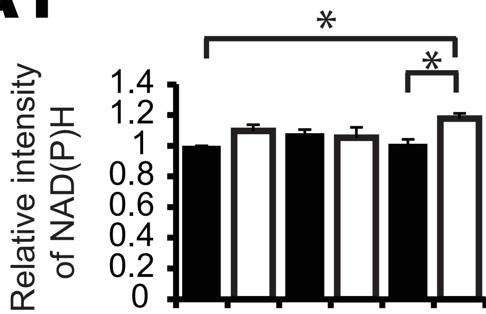

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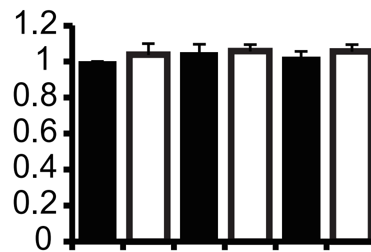

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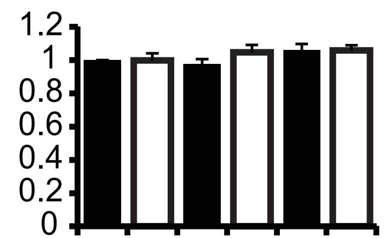

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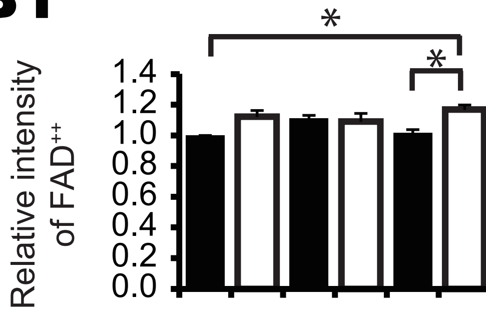

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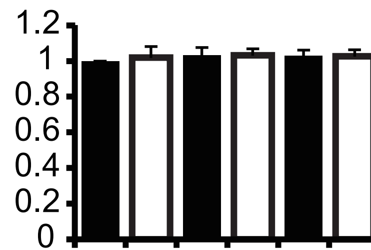

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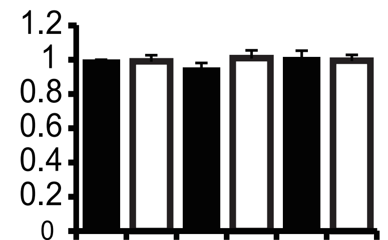

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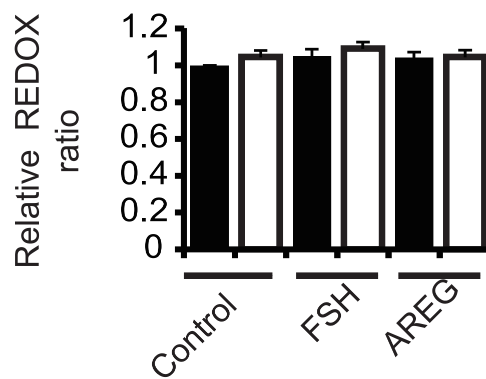

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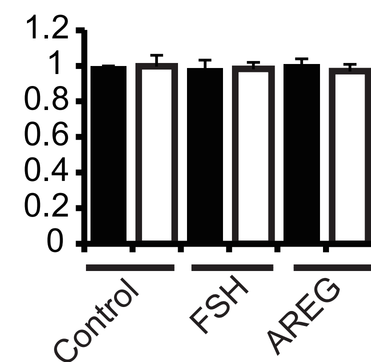

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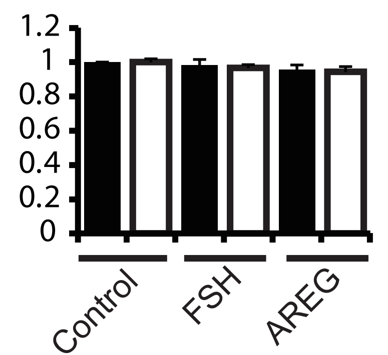
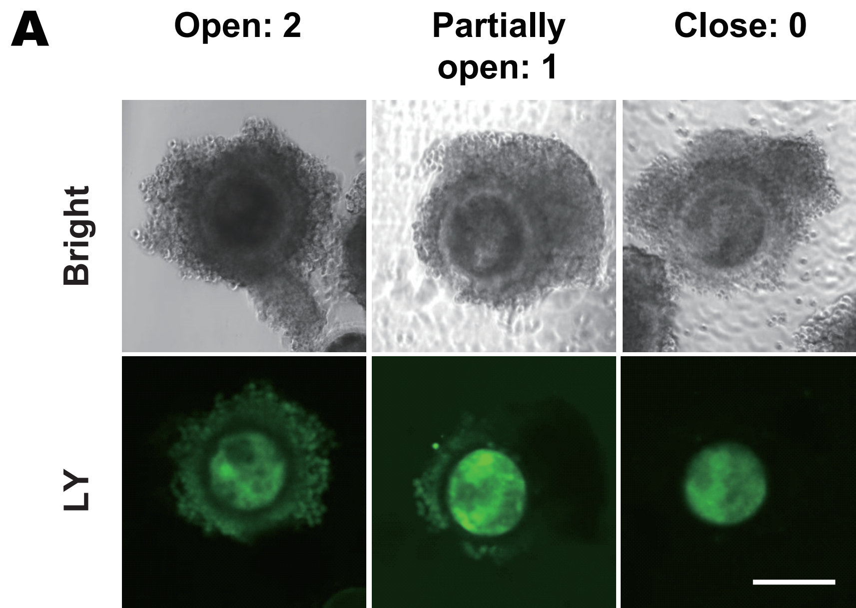
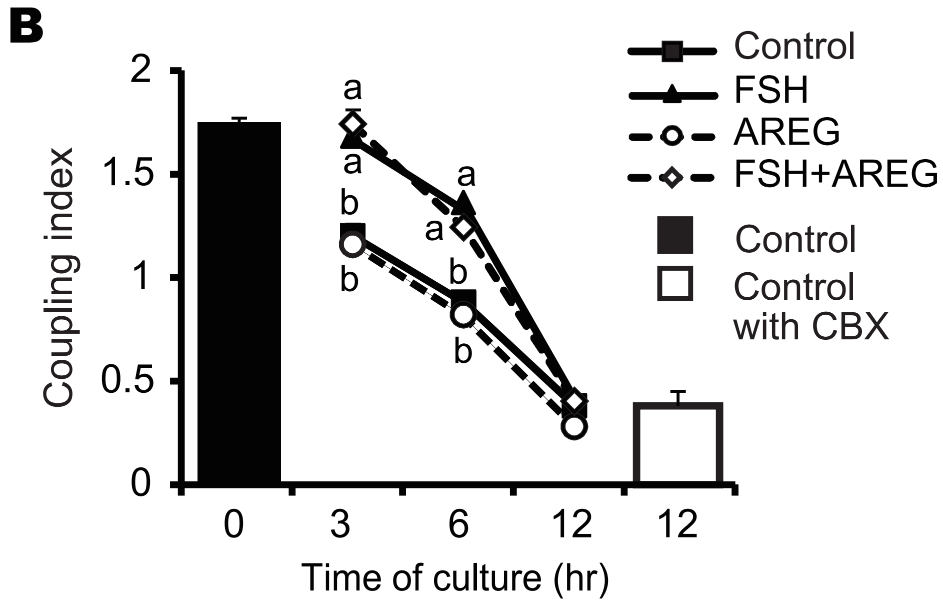
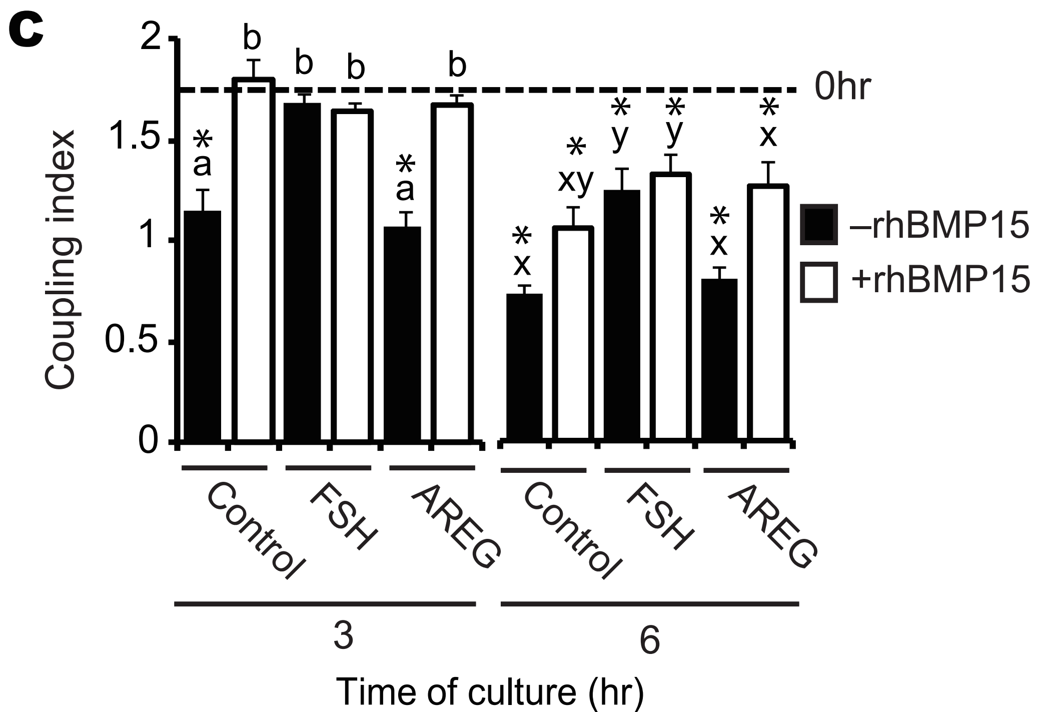


–BMP15 +BMP15

Fig. 7. *Sugimura et.*







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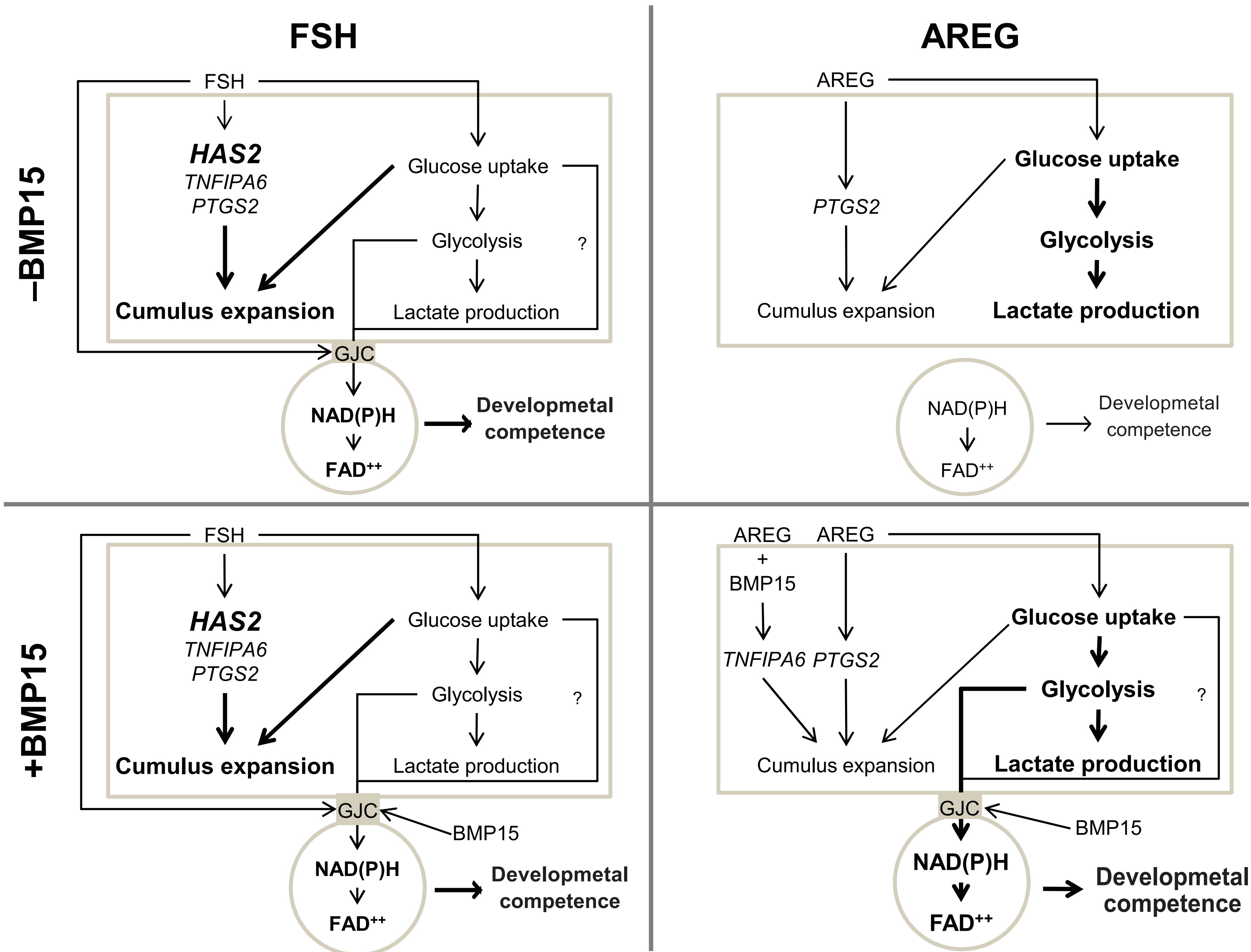

Fig. S1. *Sugimura et.*