Aldosterone enhances progesterone biosynthesis regulated by bone morphogenetic protein in rat granulosa cells.

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Abbreviations:
ActRII, activin type-II receptor; Aldo, aldosterone; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPRII, BMP type-II receptor; 11β-HSD, 11β-hydroxysteroid dehydrogenase; FSH, follicle-stimulating hormone; FSHR, FSH receptor; GPER, G-protein estrogen receptor; MR, mineralocorticoid receptor; PCOS, polycystic ovary syndrome; P450arom, P450 aromatase; P450scc, P450 steroid side-chain cleavage enzyme; RAS, renin-angiotensin system; Sp, spironolactone; and StAR, steroidogenic acute regulatory protein.

Introduction

Aldosterone (Aldo) is a major mineralocorticoid hormone that is synthesized and secreted by the zona glomerulosa of the adrenal cortex [1]. Aldo mainly acts on the distal tubules and collecting ducts of the kidney via the mineralocorticoid receptor (MR), leading to a homeostasis of sodium and fluid balance in the body. Recently, Aldo has been shown to be involved in cardiovascular and metabolic disorders as a proinflammatory hormone [1]. In a clinical situation, systemic Aldo levels can be elevated in a variety of diseases including primary aldosteronism due to adrenal hyperfunction and secondary hyperaldosteronism caused by congestive heart failure, renal failure and liver cirrhosis.

Maintenance of the ovarian renin-angiotensin system (RAS) has been recognized to be important for normal follicular function. High levels of plasma Aldo have been reported in patients with polycystic ovary syndrome (PCOS), which causes infertility, hyperandrogenism and insulin resistance in women, suggesting that an excess of Aldo is involved in the pathogenesis of PCOS [2, 3]. It has also been reported that obese women with PCOS had high total renin levels compared to the levels of angiotensin-converting enzyme activity and Aldo and

that the high total renin levels are related to the development of insulin resistance in PCOS patients [4].

Evidence indicating extra-adrenal mineralocorticoid production including production in the ovary has been accumulating [5-7]. In ovarian tissues, the existence of functional MR has been demonstrated in various mammals [8-10]. A potent inhibitory action of the MR antagonist spironolactone in blocking gonadotropin-induced steroidogenesis was also shown, implying that endogenous mineralocorticoids may play a key role in initiating or maintaining periovulatory progesterone synthesis [5]. Hence, these results indicate that the ovary is constantly exposed to circulating as well as local mineralocorticoids. It has been shown that Aldo is involved in the induction of bovine oocyte maturation [9]. These findings further indicate that mineralocorticoids act not only as endocrine factors but also as autocrine/paracrine factors to regulate ovarian physiology. Despite these observations, there have been only a few studies in which the effects of mineralocorticoids on ovarian reproductive functions were examined.

In the present study, we utilized rat primary granulosa cells to elucidate the functional impact of Aldo in the ovarian steroidogenesis regulated by follicle-

stimulating hormone (FSH) and ovarian growth factors. Functional interactions among FSH and various ovarian factors including activins/inhibins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) have been shown to be indispensable for follicle growth and maturation [11, 12], in which the BMP system regulates FSH receptor (FSHR) activity in granulosa cells, leading to suppression of progesterone synthesis and luteinization [13-15]. The present study uncovered a unique activity of Aldo in progesterone enhancement and its interaction with the BMP system in granulosa cells.

Materials and Methods

Reagents and supplies

Medium 199 and McCoy's 5A were purchased from Thermo Fisher Scientific (Waltham, MA). Recombinant protein of human BMP-6 was purchased from R&D Systems Inc. (Minneapolis, MN). Aldosterone (Aldo), diethylstilbestrol (DES), spironolactone (Sp), ovine FSH, 4-androstene-3,17-dione and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO).

Preparation of rat primary granulosa cells

Silastic capsules each containing 10 mg of DES were subcutaneously implanted in 22-day-old female Sprague-Dawley rats (Charles River, Wilmington, MA). After exposure to DES for three days, the ovaries were removed and punctured using syringe needles of 27 gauge. A cell suspension of Medium 199 containing granulosa cells and oocytes was passed through nylon meshes with pore sizes of 100 and 40 μm (BD Falcon, Bedford, MA). Granulosa cells were isolated by the filtering process, which allowed granulosa cells but not oocytes to pass through, as we previously reported [16-18]. The isolated granulosa cells were subsequently cultured in McCoy's 5A medium containing penicillin-streptomycin without serum. Based on the results of our previous *in vitro* experiments [19, 20], Aldo concentrations ranging from 1 to 300 nM were selected, and 100 nM of Aldo was found to exert significant effects. The concentrations of BMPs (100 ng/ml) and FSH (10 ng/ml) were selected according to our previous data obtained under the same experimental conditions [16-18]. The animal protocol was approved by Okayama University Institutional Animal Care and Use Committee (OKU-2018704).

Assays for ovarian steroids and cAMP

Granulosa cell culture was performed in 96-well plates with serum-free McCoy's 5A medium (1 × 10^5 viable cells in 200 μl) containing 100 nM of 4-androstene-3,17-dione as a substrate for aromatase. The indicated concentrations of Aldo were added to the culture media either alone or in combination with FSH (10 ng/ml) and Sp (100 nM), and the cells were cultured for 48 h. Architect estradiol and progesterone kits for enzyme-linked immunosorbent assay (ELISA; Cayman Chemical Co., Ann Arbor, MI) were used for measuring the concentrations of ovarian steroids in the culture media. The steroid contents were within undetectable ranges in the cell-free medium: progesterone < 10 pg/ml and estradiol < 15 pg/ml. Synthesis of cellular cAMP was evaluated in granulosa cells cultured with the phosphodiesterase inhibitor IBMX (0.1 mM) in 96-well plates (1 × 10^5 viable cells in 200 μl) for 48 h. The extracellular cAMP concentrations in the culture media were measured by an ELISA after acetylation with an assay sensitivity of 0.039 nM (Enzo Life Sciences, Inc., Farmingdale, NY).

RNA extraction from granulosa cells and real-time PCR

Granulosa cells were cultured in 12-well plates with serum-free McCoy's 5A medium (5×10^5 viable cells in 1 ml). The cells were treated with FSH (10 ng/ml) and BMP-6 (100 ng/ml) either alone or in combination with Aldo (100 nM) and Sp (100 nM). Total cellular RNA was extracted using TRI Reagent® (Cosmo Bio Co. Ltd., Tokyo) after treatments for 24 to 48 h, and then the amount of RNA was quantified using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific). Primer pairs for genes related to steroidogenesis including *Star* (also known as steroidogenic acute regulatory protein, StAR), *Cyp11a* (steroid side-chain cleavage enzyme, P450scc), *Hsd3b* (3β-hydroxysteroid dehydrogenase, 3βHSD), and *Cyp19* (aromatase, P450arom), for genes related to the BMP system including *Acvr1* (activin receptor-like kinase, ALK-2), *Bmp6*, *Bmpr1a* (ALK-3), *Bmpr1b* (ALK-6), *Bmpr2* (BMP type-II receptor, BMPRII), *Acvr2a* (activin type-II receptor, ActRIIA), *Acvr2b* (activin type-II receptor, ActRIIB), *Smad6*, *Smad7*, and *Id1* (inhibitor of DNA binding 1: the BMP target gene), for *Mr* (mineralocorticoid receptor), and for a housekeeping gene, *Rpl19* (ribosomal protein L19, RPL19) were selected [16-19]. The primer pairs were chosen from different exons of the corresponding genes in order to distinguish the target PCR products from chromosome DNA contaminants. The extracted RNA (1 μg) was

reverse transcribed using ReverTra Ace® (TOYOBO Co., Ltd. Osaka) with a random hexamer. Real-time PCR was performed using the LightCycler® 96 system (Roche Diagnostic Co., Tokyo) under the optimized annealing conditions and amplification efficiency. The Δthreshold cycle (Ct) method was used to determine the relative mRNA expression. The ΔCt values were calculated by subtracting the Ct value of *Rpl19* mRNA from that of the target mRNA. The target mRNA levels were expressed as $2^{-(\Delta Ct)}$ and the results were expressed as mRNA ratios of the target to *Rpl19*.

Western blot analysis

Granulosa cells were cultured in serum-free McCoy's 5A medium in a 24-well plate (2.5×10^5 viable cells in 500 μl). After preculture either alone or with Aldo (100 nM) for 24 h, the cells were treated with BMP-6 (100 ng/ml) for 60 min. In another experiment, granulosa cells were treated with Aldo (100 nM) and/or BMP-6 (100 ng/ml) in a serum-free medium for 48 h. After the treatments, cells were solubilized in RIPA lysis buffer (50-100 μl; Upstate Biotechnology, Lake Placid, NY) containing 2% SDS and 4% β-mercaptoethanol. The cell lysates were subjected to SDS-PAGE/immunoblotting analysis using antibodies against

phopho-Smad1/5/9 (pSmad1/5/9), total-Smad1 (tSmad1; Cell Signaling Technology, Inc., Beverly, MA), Smad6 (Novus Biologicals, LLC, Centennial, CO), MR (Santa Cruz Biotechnology, Inc., Dallas, TX), and actin (Sigma-Aldrich Co. Ltd.). The C-DiGit® Blot Scanner System (LI-COR Biosciences, Lincoln, NE) was used for analyzing the integrated signal density of each band. Ratios of the target signal intensities were standardized by the corresponding actin intensities.

Statistics

All of the data are shown as means ± SEM from at least three individual experiments with triplicated samples. ANOVA and subsequently Fisher's protected least significant difference (PLSD) test or Tukey-Kramer's post hoc test and unpaired *t*-test were used for statistical analyses (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). *P* values < 0.05 were accepted as significant changes.

Results

We first investigated the effects of Aldo on ovarian steroidogenesis induced by FSH using rat primary granulosa cells. Treatment with Aldo (100 nM) alone had no significant effect on the basal levels of estradiol (**Fig. 1A**) and progesterone production for 48-h culture (**Fig. 1B**). Of interest, treatment with Aldo (100 nM) significantly increased progesterone production induced by FSH (10 ng/ml) (**Fig. 1B**). On the contrary, Aldo (100 nM) had no significant effect on FSH-induced estrogen production by granulosa cells (**Fig. 1A**).

To clarify the mechanism by which Aldo increases FSH (10 ng/ml)-induced steroidogenesis, the change in cAMP synthesis by granulosa cells was determined by ELISA. FSH-induced cAMP levels were increased by treatment with Aldo (100 nM) for 48 h, whereas the basal cAMP synthesis was not changed by Aldo treatment (**Fig. 1C**). As shown in the effects of Aldo on steroid biosynthesis, Aldo treatment (100 nM) did not alter the basal expression levels of steroidogenic factor and enzyme mRNAs, whereas it was revealed that Aldo increased mRNA levels of progesterogenic enzymes including *Star* and *Cyp11a* stimulated by FSH (10 ng/m) (**Fig. 1D**). The mRNA levels of FSH (10 ng/ml)-induced *Hsd3b* and *Cyp19* were not affected by treatment with Aldo (100 nM)

(**Fig. 1E**). Co-treatment with Sp (100 nM) reversed Aldo-induced enhancement of FSH-induced progesterone production (**Fig. 1F**) and *Star* mRNA expression but not *Cyp11a* mRNA expression (**Fig. 1G**), indicating that the effects of Aldo were, at least in part, involved in an MR-mediated genomic effect.

Since the effects of Aldo on FSH-induced steroidogenesis were shown more clearly in the progesterone levels than in estrogen levels, the action of the BMP system that specifically suppresses FSH-induced progesterone synthesis in granulosa cells [13] was evaluated. To clarify the effects of Aldo on BMP-receptor signaling, phosphorylation of Smad1/5/9 and transcription of *Id1* induced by BMP-6 were examined in the presence of Aldo in granulosa cells. BMP-6 treatment (100 ng/ml) for 1 h stimulated phosphorylation of Smad1/5/9, although Aldo (100 nM) alone had no effect on phosphorylation of Smads (**Fig. 2A**). Importantly, co-treatment with Aldo (100 nM) significantly suppressed the phosphorylation of Smad1/5/9 stimulated by BMP-6 (100 ng/ml) (**Fig. 2A**). Similar to the effects on Smad phosphorylation, the transcription of *Id1* elicited by BMP-6 (100 ng/ml) was suppressed by co-treatment with Aldo (100 nM) for 24 h (**Fig. 2B**). On the other hand, endogenous BMP-6 mRNA expression was found to be upregulated by treatment with Aldo (100 nM) (**Fig. 2C**).

Next, to investigate the mechanism by which Aldo suppressed BMP-receptor signaling, the expression levels of BMP-receptor components were assessed by real-time PCR. Aldo treatment (100 nM) for 24 h significantly increased the mRNA level of *Smad6*, but not that of *Smad7*, in the presence of BMP-6 (100 ng/ml) (**Fig. 2D**), though treatment with Aldo (100 nM) alone had no effect on the mRNA expression of inhibitory *Smad6 and Smad7* (**Fig. 2D**). Also, Aldo treatment (100 nM) for 48 h significantly increased the protein level of Smad6 in the presence of BMP-6 (100 ng/ml) (**Fig. 2E**), suggesting that Aldo suppressed BMP-receptor signaling by upregulating inhibitory Smad6 in granulosa cells.

Furthermore, treatment with Aldo (100 nM) did not affect the mRNA levels of BMP receptors, including type-I receptors such as *Acvr1*, *Bmpr1a* and *Bmpr1b* and the type-II receptor *Acvr2a*, while Aldo treatment significantly increased the expression of the type-II receptor *Bmpr2* but decreased the expression of the type-II receptor *Acvr2b* in granulosa cells (**Fig. 3A**). On the other hand, treatment with BMP-6 (100 pg/ml) significantly suppressed the MR expression at the levels of mRNA (**Fig. 3B**) as well as protein (**Fig. 3C**), suggesting the

presence of a feedback system between the BMP and Aldo systems in granulosa cells.

Discussion

In the present study, we revealed that Aldo treatment increased FSH-induced progesterone, but not estradiol, biosynthesis by rat granulosa cells. Furthermore, Aldo treatment attenuated BMP-6-induced Smad1/5/9 pathway activation by upregulating inhibitory Smad6 expression. Moreover, BMP-6 downregulated MR expression in granulosa cells, suggesting a functional link between BMP and MR signaling as a negative feedback. Considering that BMP ligands act as specific suppressors of progesterone production as luteinizing inhibitors [13-15], it is likely that Aldo is involved in the inhibition of endogenous BMP activity in granulosa cells, leading to enhanced progesterone production by growing follicles (**Fig. 4**).

The ovarian BMP system comprised of BMP ligands and receptors is expressed in a cell-specific manner in ovarian follicles [13]. The ovarian BMP system plays critical roles in the maintenance of female fertility in mammals [12-15]. A common activity of BMP ligands was found to be regulation of the sensitivity of FSHRs on granulosa cells during the process of folliculogenesis in addition to the ligand-dependent unique actions of BMPs [21-23]. In ovarian tissues from PCOS patients, BMP-6 expression was revealed to be

predominantly enhanced in granulosa cells compared with its expression in normal tissues [24-26]. Upregulated BMP-6 in granulosa cells may indicate some disruption of follicular development as shown in PCOS ovaries. In the present study, the expression levels of BMP type-II receptors, including BMPRII and ActRIIB, and endogenous BMP-6 were found to be modulated by Aldo treatment, suggesting a functional link between MR signaling and the BMP system in granulosa cells.

The possible involvement of Aldo in various gynecological disorders characterized by inflammation, hypertension and increased cardiovascular and metabolic risks has been reported [1]. In the present study, relatively high concentrations of Aldo were used to assess the impact of Aldo on steroidogenic activities by granulosa cells, based on the findings that high concentrations of Aldo with its precursors such as corticosterone ranging from 1 to 500 nM were present in human ovarian follicular fluid [7]. Recent studies have indicated local synthesis and action of mineralocorticoids in the ovary. It has been reported that granulosa and thecal layers expressed CYP21A2 and MR, whereas CYP11B1 was expressed at very low or undetectable levels [27], and Aldo was detected in various stages of developing follicles, indicating possible endocrine and

autocrine-paracrine roles of mineralocorticoids in the ovary. Considering preliminary evidence of follicular Aldo synthesis and MR expression in oocytes [7], Aldo may play a key role in folliculogenesis and oocyte maturation as an ovarian RAS.

On the other hand, it was shown that MR is expressed in rat granuloma cells and that gonadotropin stimulation downregulates MR expression [9]. It was also revealed that treatment with Sp reversed the enhanced effects of Aldo on FSH-induced progesterone production and StAR expression, suggesting that a genomic MR action is functionally involved in the effects of Aldo on steroidogenesis by granulosa cells. Of interest, it was shown that MR expression levels were not different among the phases of early diestrus, late diestrus, estrus, and post-ovulation in the rat ovary and that MR protein expression was the highest in corporal lutea cells, moderately intense in oocytes and granulosa cells, and slightly detected in theca cells [8]. In the present study, MR expression in granulosa cells was suppressed by treatment with BMP-6, while endogenous BMP-6 expression was increased by treatment with Aldo, suggesting the presence of a functional link between BMP and MR actions. It was also shown in previous studies that the total renin level was significantly

higher in plasma from PCOS women than in plasma from controls [28] and that renin, Aldo, and androgen levels and insulin sensitivity indices were decreased after treatment with metformin [28], suggesting the clinical significance of ovarian RAS in the pathophysiology of PCOS.

Androgen and the androgen receptor, which are expressed in various stages of developing follicles, have been shown to play a key role in the pathophysiology of PCOS [29]. An excess of androgen is clinically associated with metabolic syndrome and insulin resistance in women [30]. In our previous study, it was revealed that androgen in the presence of insulin-like growth factor-I promoted FSH-induced progesterone production by rat granulosa cells [16]. The androgen effects that enhanced progesterone synthesis were similar to the present findings regarding the Aldo effects, both of which are commonly linked to counteracting the endogenous BMP activity, leading to a progesterogenic property of granulosa cells.

Collectively, the results of this study revealed that a high concentration of Aldo specifically enhances FSH-induced progesterone production by inhibiting BMP signaling in granulosa cells (**Fig. 4**). It was also found that the actions of Aldo were, at least in part, involved in an MR-mediated genomic effect. However,

further approaches are necessary to clarify the significance of the non-genomic action of MR and the involvement of its translocation, since Aldo can exert non-genomic MR signaling such as that by G-protein estrogen receptors (GPERs) [31]. Besides, roles of isoforms 1 and 2 of 11β-hydroxysteroid dehydrogenase (11β-HSD) expressed in granulosa cells may also be linked to ovarian function and fertility [32]. The present findings regarding the effect of Aldo in granulosa cells may reflect the changes in steroidogenic capacity shown in hyperaldosteronism as seen in PCOS.

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Figure Legends:

Fig. 1. Effects of aldosterone (Aldo) on FSH-induced steroidogenesis by rat granulosa cells. **A**, **B**) Granulosa cells were cultured with Aldo either alone or in combination with FSH in a serum-free condition for 48 h. The concentrations of estradiol (**A**) and progesterone (**B**) in the medium were evaluated by ELISA and then expressed as fold changes. **C**) Cells were cultured with Aldo either alone or in combination with FSH in a serum-free medium containing IBMX for 48 h. The cAMP levels in the medium were examined by ELISA. **D**, **E**) Cellular RNA was extracted from granulosa cells treated with FSH and Aldo for 48 h. *Star*, *Cyp11a*, *Hsd3b* (**D**) and *Cyp19* (**E**) mRNA levels were determined by real-time PCR. The target gene mRNA levels were standardized by *Rpl19* levels and expressed as fold changes. **F**) Granulosa cells were cultured with FSH either alone or in combination with Aldo and spironolactone (Sp). After 48-h culture, the level of progesterone in the medium were determined by ELISA, and the level of progesterone was expressed as fold change. **G**) Cellular RNA was extracted from granulosa cells treated with FSH, Aldo and Sp for 48 h, and *Star* and *Cyp11a* mRNA levels were determined by real-time PCR. Results in all panels are shown as means ± SEM of data from at least three individual experiments with

triplicated samples. The results were analyzed by ANOVA. Values with different superscript letters show significant difference at $P < 0.05$. *$P < 0.05$ between the indicated group; and n.s, not significant.

Fig. 2. Effects of Aldo on BMP signaling in rat granulosa cells. **A**) After 24-h preculture with Aldo, granulosa cells were stimulated with BMP-6 for 60 min. The cells were lysed and subjected to Western blot analysis using anti-pSmad1/5/9, anti-Smad1 and anti-actin antibodies. **B**) Cellular RNA was extracted from granulosa cells treated with BMP-6 and Aldo for 24 h, and *Id1* mRNA levels were determined by real-time PCR. The target gene mRNA levels were standardized by *Rpl19* levels and expressed as fold changes. **C**) Cellular RNA was extracted from granulosa cells treated with Aldo for 48 h, and *Bmp6* mRNA levels were determined by real-time PCR. **D**) RNA was extracted from granulosa cells treated with Aldo in the absence of BMP-6 for 48 h and in the presence of BMP-6 for 24 h, and *Smad6* and *Smad7* mRNA levels were examined by real-time PCR. **E**) Cells were cultured in a serum-free condition with Aldo in the presence of BMP-6 for 48 h. The cells were lysed and subjected to Western blot analysis using anti-Smad6 and anti-actin antibodies. The signal

intensities of pSmad1/5/9 and Smad6 were standardized by actin signal intensities in each sample and then expressed as fold changes. Results in all panels are shown as means ± SEM of data from at least three individual experiments with triplicated samples. The results were analyzed by ANOVA (**A**, **B**) and the unpaired *t*-test (**C**-**E**). Values with different superscript letters show significant difference at $P < 0.05$; and $^{*}P < 0.05$ vs. control group.

Fig. 3. Mutual effects of Aldo and BMP-6 on the expression of MR and BMP receptors in rat granulosa cells. **A**) Cellular RNA was extracted from granulosa cells treated with Aldo for 48 h, and mRNA levels of BMP type-I (*Acvr1*, *Bmpr1a* and *Bmpr1b*) and type-II (*Bmpr2*, *Acvr2a* and *Acvr2b*) receptors were determined by real-time PCR. The target gene mRNA levels were standardized by *Rpl19* levels and expressed as fold changes. **B**) Cellular RNA was extracted from granulosa cells treated with BMP-6 for 24 h, and mRNA levels of *Mr* were determined by real-time PCR. **C**) Cells were cultured in a serum-free condition with BMP-6 for 48 h. The cells were lysed and subjected to Western blot analysis using anti-MR and anti-actin antibodies. The signal intensities of MR were standardized by actin signal intensities in each sample and then expressed

as fold changes. Results in all panels are shown as means ± SEM of data from at least three individual experiments with triplicated samples. The results were analyzed by the unpaired *t*-test. $^{**}P < 0.01$ vs. control group.

Fig. 4. The mechanism by which Aldo regulates progesterone biosynthesis in granulosa cells. Aldo increases FSH-induced progesterone, but not estradiol, production by enhancing mRNA expression of progesterogenic enzymes including *StAR* and *P450scc* induced by FSH in granulosa cells. BMP-6 suppresses FSH-induced cAMP synthesis by granulosa cells [15, 21]. Aldo suppresses BMP-6-induced Smad1/5/9 phosphorylation and *Id1* transcription by upregulating inhibitory Smad6. BMP-6 downregulates the expression of MR on granulosa cells, while Aldo modulates endogenous BMP-6 and BMP type-II receptor expression, suggesting the possibility of feedback actions between the BMP and Aldo systems in granulosa cells. Dotted lines show the feedback function. BMPRs: BMP receptors, FSHR: FSH receptor, and Sp: spironolactone.

Figure 1

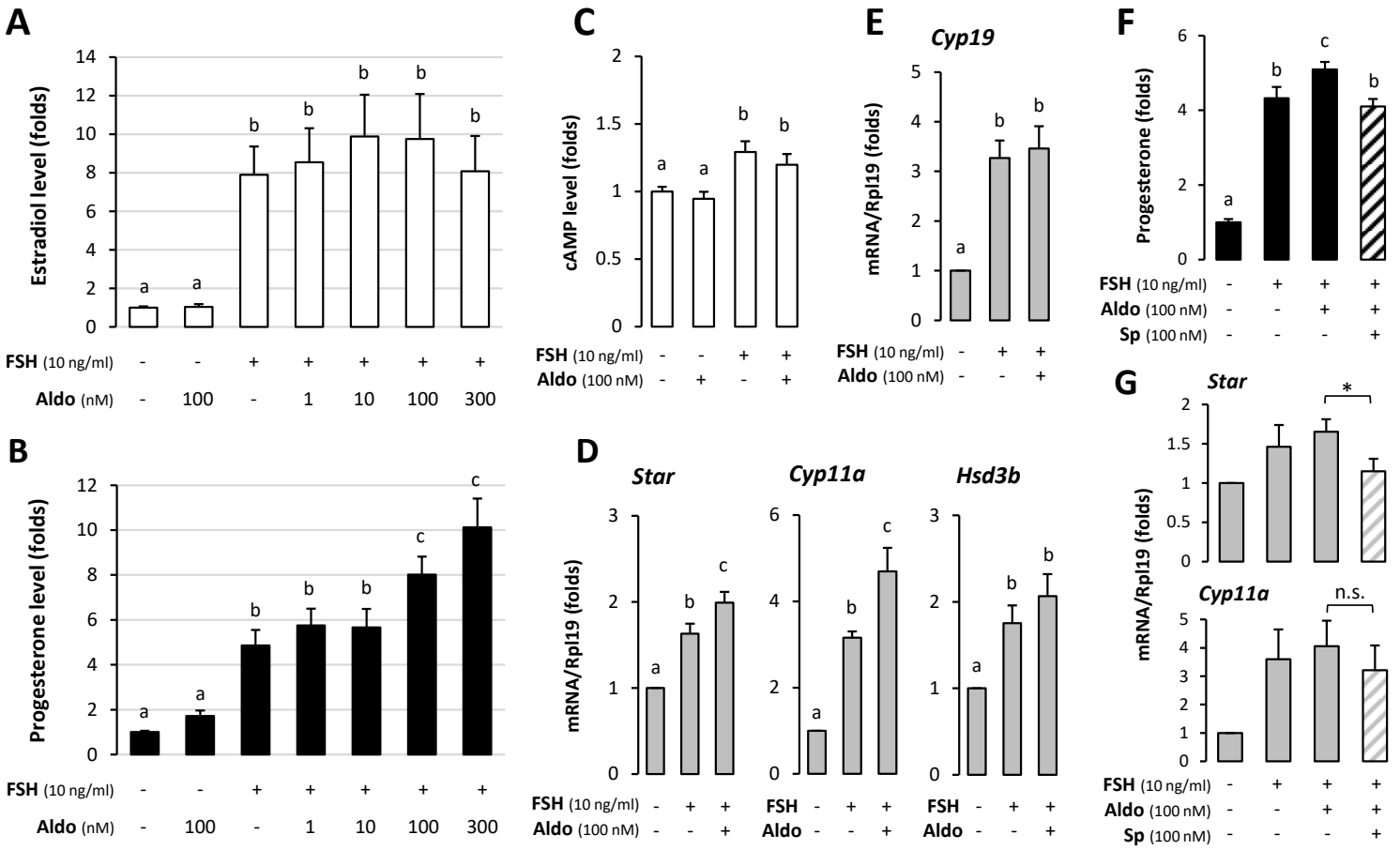

Figure 2

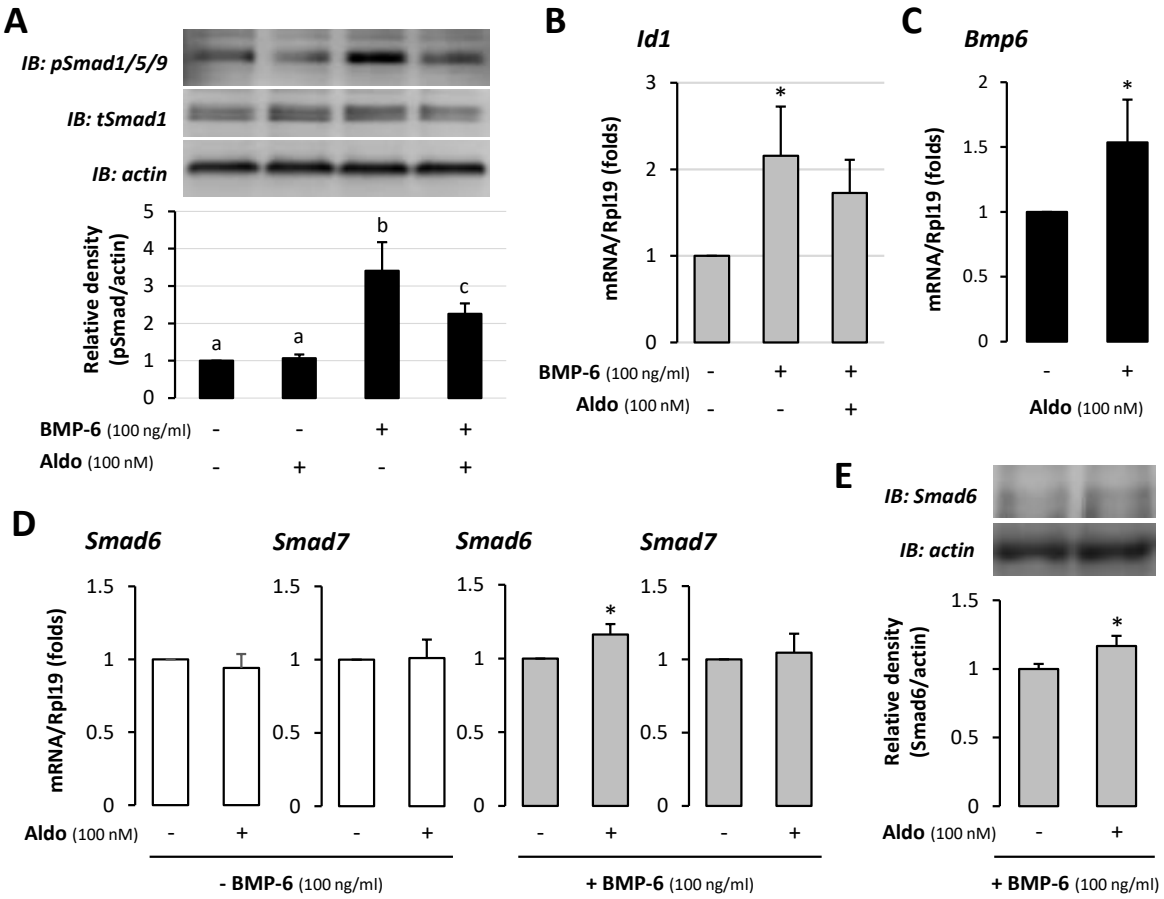

Figure 3

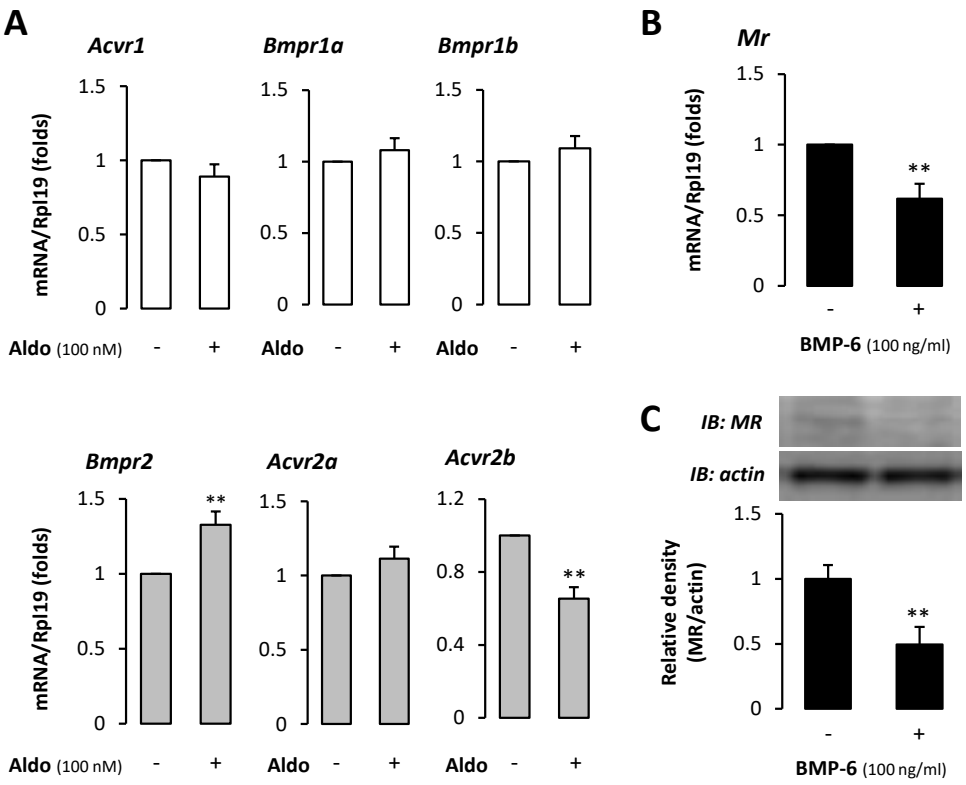

Figure 4