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Abnormal Follicle Development in GGPPS Conditional Knockout Mice

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Abstract: GGPPS is a member of prenyl transferase family catalyzing the synthesis of GGPP (Geranylgeranyl diphosphate) from Farnesyl diphosphate (FPP) and Isopentenyl diphosphate (IPP). Using cre/loxp system, researchers got oocyte specific knockout ggpps mice. Mutation of ggpps resulted in abnormality of follicular development, granulosa cell premature luteinization, theca cell hypertrophy and multi-oocyte follicles. Interaction of oocyte and granulosa cell was also changed. Oocyte-specific knockout of ggpps mice was acquired. Follicles grew abnormally in ggpps mutation mice. Interaction of oocyte and granulosa cell was also changed.

Key words: ggpps, conditional knockout, follicular development, multi-oocyte follicles, IPP

INTRODUCTION

The *GGPPS* (Geranylgeranyl diphosphate Synthase, also known as GGPS1) gene has a length of 10.97 kb, contains four exons and is located on chromosome 13 in mice (Kainou *et al*., 1999). The length of the GGPPS transcript is 2387 bp which encodes a protein of 300 amino acids. GGPPS is a member of prenyl transferase family catalyzing the synthesis of GGPP (Geranylgeranyl diphosphate) from Farnesyl diphosphate (FPP) and Isopentenyl diphosphate (IPP). FPP is not only the intermediate product of cholesterol synthesis (Grunler *et al*., 1994) but also the precursor of other isoprenoid compounds such as GGPP, isoprenoid, dolichol and ubiquinone (Coenzyme Q) synthesis in organisms. Farnesyl pyrophosphate can also be used as a source of farnesyl groups for protein post-translational modifications. Such modified proteins have a highly hydrophobic isoprene units and can be anchored in the cell membrane (Takai *et al*., 2001). Currently, there are approximately 300 different proteins modified by isoprenylation. These modified proteins are involved in various functions including, cell growth, differentiation, cytoskeleton formation and vesicle transport.

Researchers wanted to investigate whether GGPPS has an effect on follicular development and reproduction. In order to address this, researchers used a positive and negative dual screening strategy where researchers inserted loxp sites upstream of exon 3 and downstream of exon 4 and neo floxed FRT adjacent to loxp site. After mating with ZP3 Cre mice (De Vries *et al*., 2000; Sun *et al*., 2008; Hu *et al*., 2012) in which ZP3 (Zona Pellucida glycoprotein 3) promoter drives Cre expression, ZP3 creggpps loxp/loxp mice were produced.

MATERIALS AND METHODS

Generation of ZP3$^{cre/-}$ ggpps $^{loxp/loxp}$ conditional knockout mice: A positive and negative screening strategy was used where a loxp site was inserted upstream of exon 3 and downstream of exon 4 and neo was inserted adjacent to the second loxp site as positive marker. TK (Thymidine Kinase) was used as negative selection marker in the vector terminal. Electroporation of ES (Embryonic Stem) cells with the prepared vector was performed using conditions of 600 V and 30 μF. Blastocyst injection and embryo transfer were performed as described (Nagy, 2003).

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Identification of ggpps$^{-/-}$ mice: Mouse genomic DNA was extracted using an extraction kit (DNA isolation kit for cells and tissues, ROCHE, Germany) following the manufacturer's instructions. Mice were genotyped from tail genomic DNA using PCR primers 5'-AATTGTGT GTGGTAGGGGTA-3';5'-AACTTGCTTCAGAACTGAGC-3'. ggpps$^{loxp/loxp}$ mice were bred to ZP3$^{cre/-}$ mice to generate ZP3$^{cre/-}$ ggpps$^{loxp/loxp}$ mice. The preparation of transgenic mice expressing Cre recombinase in oocytes under the control of the ZP3 promoter was described earlier (De Vries *et al*., 2000).

The primers for the Cre transgene were sense 5'-ATGTCCAAT TTACTGACCG-3' and antisense 5'-CGCCGC ATAACCAGTGAAAC-3'.

Animals and treatment: All mice used were C57BL/6J line and from Medical College of Nanjing University. Mice were produced and raised at 22~25°C under artificial light (14 h light, 10 h dark) in a controlled greenhouse with free access to water and food. Mice were maintained and dealt with according to the Guide for the Care and Use of Laboratory Animals. Ovaries from 10, 14 and 22 days, 28-30, 35-38 days and 8-10 weeks old healthy female mice were used for serial paraffin sections. The 8 weeks old mice were used for long-term mating experiments (4 consecutive months) and 22-24 days old mice were used for PMSG (Pregnant Mare Serum Gonadotropin) treatment and superovulation experiments. The 8 weeks old male mice were used for *in vitro* fertilization.

Histological analysis of ovaries: Mice from different ages were sacrificed based on experimental needs. Ovaries were separated under a dissecting microscope and immediately immersed in Bouin's fluid (for sections) or 4% neutral formalin (for TUNEL) for 18-24 h. Ovaries were weighed directly after separation as appropriate. After dehydration, ovaries were embedded in paraffin and 6 μm serial sections prepared and stained with HE (Hematoxylin and eosin staining). For electron microscopy of gap junctions, fresh ovaries from 4 weeks old mice were cut into 1 mm^3 size and put into ice cold 2.5% glutaraldehyde promptly, sectioning, examining and analysis was done following reference (Weakley, 1966; Makabe *et al*., 2006).

Statistical analysis: Statistical analysis was carried out using SPSS Software. Data were analyzed using One-Way ANOVA or Independent-Samples t-test. Each test was repeated at least three times. The results are described as the mean±standard error (mean±SEM). The $p<0.05$ was accepted as being statistically significant.

RESULTS

Generation of ggpps conditional knockout mice: A targeting construct encompassing exons 2-4 of the *ggpps* gene was generated with LoxP sites flanking exons 3 and 4 since exons 3 and 4 contain the majority of the coding sequence of the *ggpps* gene. The targeting construct also contained a neomycin resistance cassette flanked by FLP/FRT (Flippase (FLP) recombinase/FLP recombinase target) recombination sites (Fig. 1). ZP3^{Cre} mice have normal expression levels of Cre under the control of ZP3 promoter in mouse oocytes (De Vries *et al*., 2000). When ggpps floxed mice are mated with ZP3^{Cre} mice, four

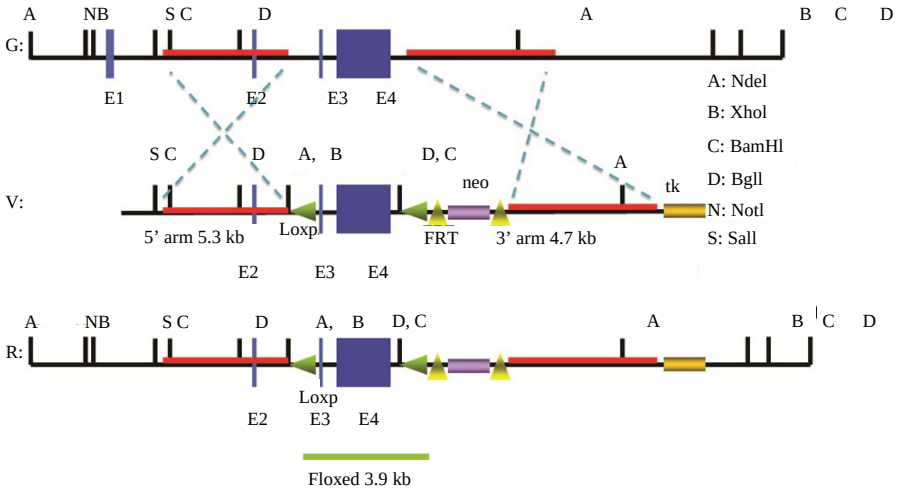

Fig. 1: Schematic representation of the ggpps gene-targeting strategy. E1, E2, E3, E4: exons1-4; G = Genome sequence; V = Vector sequence; R = sequence after recombination; neo = neo gene; tk = thymine kinase gene. The distance between twe loxp site is 3.9 kb. A, B, C, D, N, S is enzyme site respectively. A: NdeI; B: XhoI; C: BamHI; D: BglI; N: NotI; S: SalI

genotypes are expected in the offspring: Cre+/-, Cre+/-loxp+/-, Cre+/- loxp+/+, loxp+/+. Cre+/-loxp+/ and Cre+/-loxp+/+ are the oocyte specific knockout mice *ggpps* genes as determined by PCR on genomic DNA.

Follicles developed asymmetrically and blood vessels growth faster in homozygous ggpps$^{-/-}$ mice at 2 weeks old: Analysis of paraffin serial sections of mouse ovaries at 14 days after birth, developed at the secondary follicle stage, showed that some of the follicles in knockout homozygous mice developed asymmetrically (Fig. 2a) and oocytes usually lay at the edge of follicles rather than at a central location (Fig. 2b).

In paraffin serial sections researchers observed that blood vessels in the ovarian cortex of homozygous mice were larger than in heterozygotes or Wild Type (WT) at 2 weeks (Fig. 2c) and 3 weeks of age after birth. At 3 weeks of age the diameter of blood vessels in the ovarian medulla appeared similar with the control group (heterozygote or WT) but vessels of homozygous mice filled with blood cells (Fig. 3).

Follicles in homozygous mice developed luteal-like configuration and mofs (multi-oocyte follicles) after pmsg treatment: To determine whether hormone treatment has an effect on homozygous ggpps$^{-/-}$ mice, 3 weeks old mice were injected with PMSG. After treatment for 36 and 46 h, follicles in homozygous mice developed more corpus luteal-like tissue with oocytes trapped inside than did heterozygotes (Fig. 4). This indicates that the corpus luteal-like tissue is not formed after ovulation. The average number of luteinized follicles in knockout homozygotes was 21 whereas in heterozygotes it was 11.3 (Fig. 5). However, the early secondary follicles after PMSG treatment exhibited normal morphology suggesting that FSH (the main active ingredient in PMSG) may rescue the degenerated follicles in homozygotes before follicle cavity formation.

Another interesting finding was a Multi-Oocyte Follicles (MOFs) phenomenon. MOFs means two or more oocytes in a follicle. Figure 6a shows four oocytes in one follicle in a knockout homozygote mouse after PMSG treatment. In serial sections, researchers found that this follicle contains nine oocytes in total. The same phenomenon was also found in untreated 3 weeks old homozygous mice.

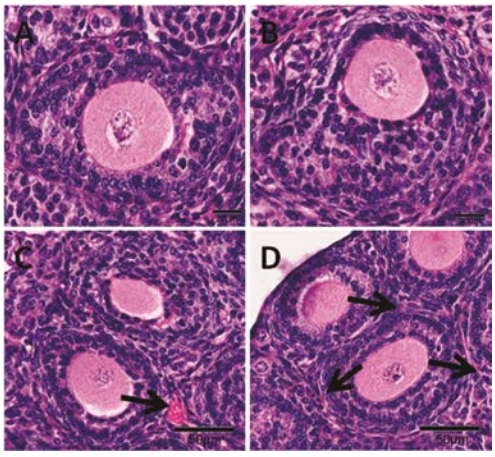

Fig. 2: Follicle asymmetric growth and blood vessel growth in ggps$^{-/-}$ mouse of 2 weeks old. A, B) Granulosa cells in follicle of 2 weeks ggps$^{-/-}$ mouse grow asymmetrically. C) Blood vessel in the ovary of ggps$^{-/-}$ mouse. D) Blood capillary in the ovary of ggpps$^{+/-}$ mouse

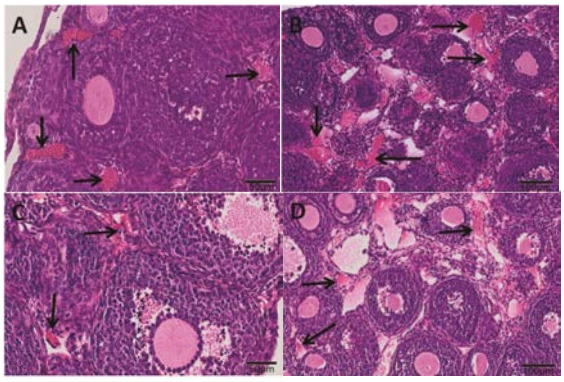

Fig. 3: Blood vessel development in the ovary of mouse of 3 weeks old. In the cortical ovary (A, C) of ggpps$^{-/-}$ mouse, blood vessel development was earlier than in ggpps$^{+/-}$ mouse. The blood vessel in the medulla ovary (B, D) was filled with red blood cells. Arrows show blood vessel. A, B) ggpps$^{-/-}$; C, D) ggpps$^{+/-}$

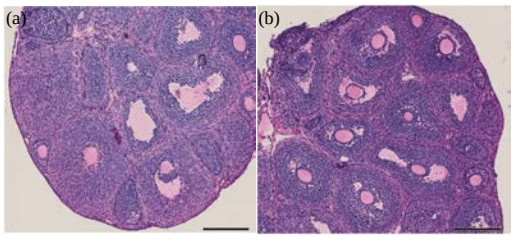

Fig. 4: The 46 h after PMSG treatment, many large follicles in knock out mouse transited into corpus lutea like tissue (star). Arrow show oocytes were trapped in corpus lutea-like tissue. Arrow head show oocytes seem normal in secondary follicles. a) knockout; b) control. Scale bar: 200 μm

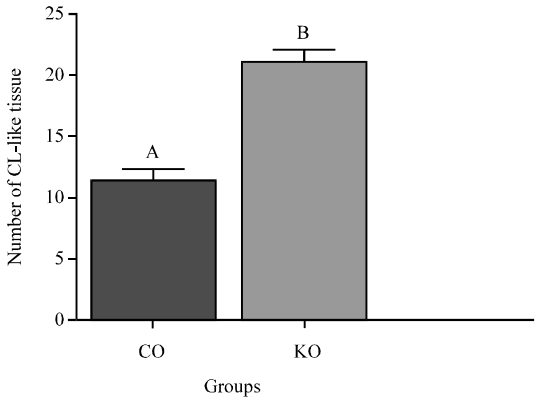

Fig. 5: The number of CL-like follicle. CO: Control; KO: Knockout

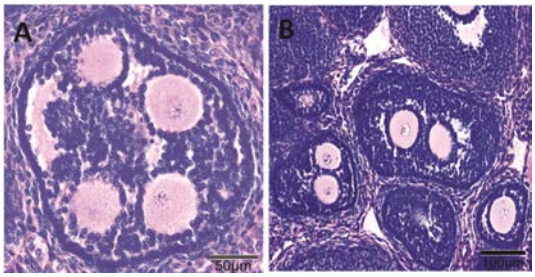

Fig. 6: Muti-oocyte follicles in ggpps knockout mouse ovary. Scale bar: A) 50 μm; B) 100 μm

Granulosa cell apoptosis in early secondary follicles of homozygous mice: By serial section and TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining, granulosa cells in secondary follicles of homozygous mice were TUNEL positive at the 4 weeks stage while granulosa cells in heterozygous mice had no change (Fig. 7) and oocytes demonstrated negative TUNEL staining. Granulosa cell apoptosis may be attributed to oocyte degeneration in ggpps$^{-/-}$ mice.

Interaction between oocyte and granulosa cells was changed in ggpps$^{-/-}$ mouse: During primary follicle development, gap junction was formed between oocyte and graulosa cells. Gap junction allows free flow of small molecules between adjacent cells. By electron microscope, researchers saw interaction between oocyte and granulosa cells was changed in ggpps$^{-/-}$ mouse. And configuration of granulosa cells around oocyte was changed and projections from granulosa cell was lost in ggpps$^{-/-}$ mouse (Fig. 8).

In this study, researchers genetically engineered ggpps knockout mice. After 4 months of long-term mating experiments it was found that homozygous ggpps$^{-/-}$ mice have low fecundity and no significant difference between

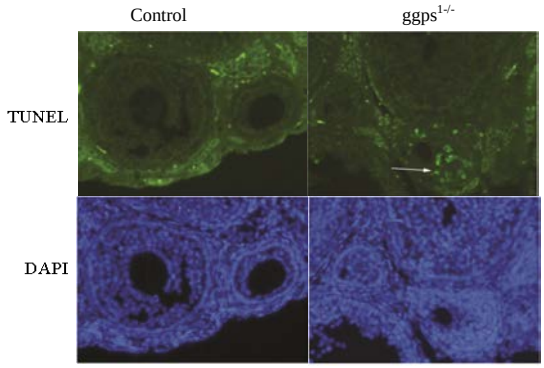

Fig. 7: Granulosa cells apoptosis by TUNEL in secondary follicle of ggpps$^{-/-}$ mouse. Arrow show apoptotic granulosa cells in in secondary follicle of ggpps$^{-/-}$ mouse

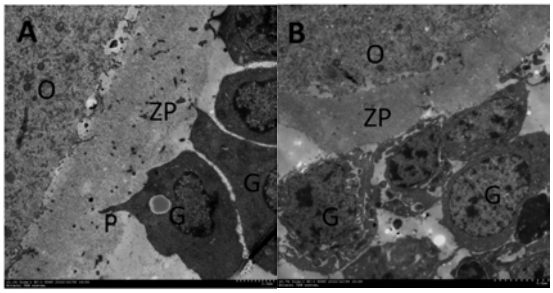

Fig. 8: Interaction between oocyte and granulosa cells was changed in ggpps$^{-/-}$ mouse. Notice granulosa cell configuration was changed and projections from granulosa cell was lost in ggpps$^{-/-}$ mouse. A) ggpps$^{+/-}$; B) ggpps$^{-/-}$. O: Oocyte; ZP: Zona Pellucida; G: Granulosa cell; Scale bar: A) 2 μm; B) 5 μm

heterozygote and wild-type exists. Homozygous mice have lower ovulation rate and lower oocyte developmental rate after IVF. Homozygous ggpps$^{-/-}$ mice formed follicular cavities early than the control group, accompanied by the advanced development of ovarian vessels, asymmetric follicular development and MOFs phenomenon. A large number of oocytes in secondary follicles of homozygous mice exhibited shrinkage or degradation and theca cell hypertrophy and granulosa cell luteinization. Though secondary follicles of homozygous mice look normal after PMSG treatment, a large part of antral follicles and preovulatory follicles form luteal-like structures. Interaction through gap junction between oocyte and granulosa cells was changed in ggpps$^{-/-}$ mouse.

Mouse oocytes cannot synthesize cholesterol directly but can regulate cholesterol metabolism by

promoting the synthesis of enzymes involved in cholesterol metabolism in granulosa cells (Su *et al*., 2008). Therefore, as a result of ggpps deletion, FPP accumulation unlikely affected cholesterol metabolism in ggpps$^{-/-}$ mouse oocytes but may be used directly as a substrate for protein isoprenylation.

In mammals, Growth Differentiation Factor 9 (GDF-9) is the most important regulatory factor during follicular development after the initiation of follicular development. The phenotype of GDF-9 knockout homozygous mice demonstrated (Dong *et al*., 1996; Carabatsos *et al*., 1998; Elvin *et al*., 1999; Spicer *et al*., 2008) no Graafian follicles asymmetric growth of granulosa cells in the follicles abnormal meiosis high FSH and LH levels in blood but their receptor mRNA levels are normal follicles with no theca cells or theca cells with no function follicles of small luteal-like structures granulosa cells in 3b type follicles will not proliferate. As a result, GDF-9 knockout homozygous mice are sterile.

Researchers found that ggpps$^{-/-}$ mice showed asymmetric growth of the follicles and abnormal meiosis and follicles with luteal-like structures. Such a phenotype is similar to GDF-9 knockout homozygous mice. Based on this, researchers hypothesize that oocyte deletion of the *ggpps* gene may affect the function of the *GDF-9* gene in follicular development in mice.

BMP-15 is another key gene that controls follicle development. It has a synergistic effect with GDF9 though there are differences in different mammals. BMP-15 knockout homozygous male mice (Yan *et al*., 2001) are normal and female homozygous mice also show normal follicular development, although the ovulation rate and fertilization rate of oocytes was undermined. Female mice of BMP-15 and GDF-9 double mutations (gdf9$^{+/-}$bmp15$^{-/-}$) (Yan *et al*., 2001; Su *et al*., 2004; Mottershead *et al*., 2012) show subfertility, multi-oocyte follicles, premature luteinization and decreased ovulation rate and fertilization rate. The ggpps mouse knockout phenotype such as premature follicle luteinization, decreased ovulation rate and oocyte fertilization rate are similar with the BMP15 knockout mouse phenotype suggesting that the lack of *ggpps* gene in oocytes may affect the function of BMP15 in follicular development.

After binding to its receptors, TGFβ (Transforming Growth Factor Beta) superfamily members can regulate cell proliferation, cell differentiation and immunity. TGFβ binding to its receptor is mediated by the SMAD (Sma and Mad collectively referred SMAD) family (Heldin *et al*., 1997). SMAD is divided into receptor activated SMAD (receptor regulated SMAD, R-SMAD) and the common SMAD (coSMAD), both capable of forming heterodimers which play biological functions. SMAD4 is the only coSMAD discovered at this time. The granulosa cell conditional knockout SMAD4 (AMH^{cre}SMAD4$^{-/-}$) mice (Pangas *et al*., 2006) demonstrate subfertility, low ovulation rate and oocytes trapped in the follicles of premature luteinization. These features are similar with reproduction traits of ggpps$^{-/-}$ mice. It is worth mentioning that hormone (FSH, LH and estradiol) levels were unchanged after SMAD4 deletion, consistent with the experimental results.

From the phenotypic analysis of ggpps$^{-/-}$ mice, researchers conclude that the ggpps$^{-/-}$ mouse phenotype resembles gdf9$^{-/-}$ mice, smad4$^{-/-}$ mice, bmp15$^{-/-}$ mice or bmp 15$^{-/-}$ gdf9$^{-/-}$ double-knockout mice (Elvin *et al*., 1999; Su *et al*., 2008). This suggests that deletion of ggpps may affect GDF9 and/or BMP15 even the entire TGFβ family members' functions or disturb its signalling.

Protein isoprenylation makes a hydrophilic protein becomes a hydrophobic protein and is conducive to anchoring the protein in the cell membrane. The most studied isoprenylated protein is the RAS family of small G protein superfamily (Fan *et al*., 2011; Kim *et al*., 2011). One explanation is that TGFβ family members (GDF9 and BMP15) may be disturbed by the activated RAS. The indirect evidence came from cancer research where RAS and TGF-beta exert antagonistic effects on extracellular matrix gene expression (Wisdom *et al*., 2005) or RAS has an inhibition effect on TGFβ (Liu *et al*., 2000; Mohamed *et al*., 2013). Another explanation is that isoprenylation may occur on GDF9 and/or BMP15. But there are no reports on protein isoprenylation of TGFβ family members. Protein isoprenylation need a carboxy-terminal structure CaaX (Moores *et al*., 1991; Zhang and Casey, 1996) wherein C is cysteine, a is lipid amino acid, X can be any amino acid. By blast, researchers observed that TGFβ family members have two conservative cysteine with RAS family members and meet the characteristics of CaaX (Fig. 9). Inhibitors of the mevalonate pathway can suppress protein isoprenylation modification while adding FPP can rescue suggesting that protein isoprenylation has an important role in the Xenopus oocytes (Kim *et al*., 1990). Inhibitors of the mevalonate pathway can increase BMP2 expression of TGFβ family in the studies of osteoblasts (Sugiyama *et al*., 2000; Ohnaka *et al*., 2001). Based on the experimental results, researchers speculate that the TGFβ family members may be modified by protein isoprenylation.

In human clinical studies, MOFs is one of the reasons for female infertility. And MOFs is often accompanied with high estrogen and estrogen receptor levels. Phytoestrogen genistein injection treatment of mice after birth can lead to multi-oocyte follicles (Jefferson *et al*., 2006, 2007; Tingen *et al*., 2009) and elevated estrogen receptor levels.

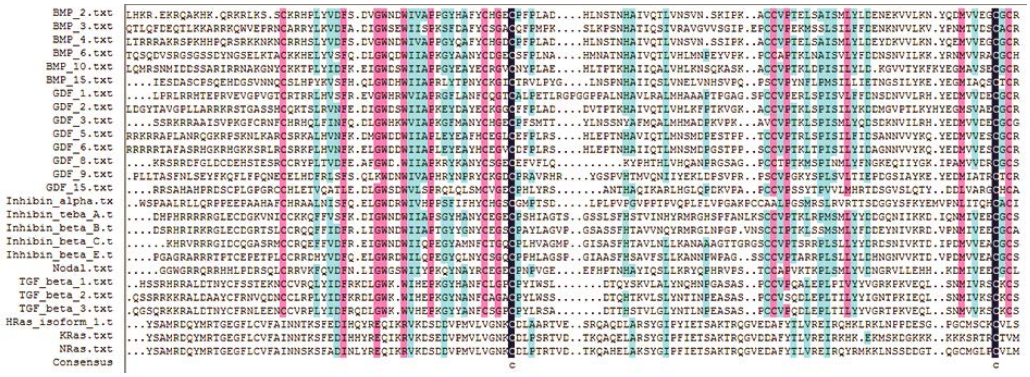

Fig. 9: Blast result of RAS and TGFβ superfamily. Notice the conserved cysteine residue in carboxy-terminal between RAS family and TGFβ superfamily

CONCLUSION

In the experiment, researchers tested unchanged FSH, LH and estrogen levels, so estrogen levels caused MOFs theory may not be suitable for ggpps$^{-/-}$ mice. The absence of T-synthase (Williams and Stanley, 2008) which is involved in protein glycosylation can also cause MOFs formation at late stages of folliculogenesis. Indeed, the researchers observed that multi-oocyte follicles are formed by increased breakdown of basal lamina and migration of theca cells and granule cells. The same phenomenon was observed in the experiment but the relationship between ggpps and T-synthase in MOFs yet need further study.

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