Modulation of Cathepsin S (CTSS) Regulates the Secretion of Progesterone and Estradiol, Proliferation, and Apoptosis of Ovarian Granulosa Cells in Rabbits

Guohua Song 1,4, Yixuan Jiang 1,4, Yaling Wang 1, Mingkun Song 1, Xuanmin Niu 2, Huifen Xu 1,* and Ming Li 1,*

1 College of Animal Science and Technology, Henan Agricultural University, Zhengzhou 450046, China; m13782525956@163.com (G.S.); J19203090@163.com (Y.J.); 15637410238@163.com (Y.W.); m1862951565@163.com (M.S.)
2 Yangguang Rabbit Technology Co., Ltd., Jiyuan 454650, China; damoguyue@163.com
* Correspondence: huifen221@126.com (H.X.); 13803849306@163.com (M.L.); Tel.: +86-0371-5699-0218 (M.L.)
† Guohua Song and Yixuan Jiang contributed equally to this paper.

Simple Summary: In goat and sheep, CTSS is reported to be important for the development and maturation of oocytes by regulating cell proliferation and apoptosis. The purpose of this study was to investigate the role of CTSS in regulating cell apoptosis and hormone secretion in rabbit granulosa cells. Our results suggested that the CTSS gene can promote the proliferation of granulosa cells and reduce its apoptosis in vitro, while overexpression of CTSS promoted the secretion of progesterone and estrogen in rabbit granulosa cells. Therefore, manipulation of CTSS may improve development of oocytes, and thus provide an approach for better manipulation of rabbit reproductive performance.

Abstract: Cathepsin S (CTSS) is a member of cysteine protease family. Although many studies have demonstrated the vital role of CTSS in many physiological and pathological processes including tumor growth, angiogenesis and metastasis, the function of CTSS in the development of rabbit granulosa cells (GCS) remains unknown. To address this question, we isolated rabbit GCS and explored the regulatory function of the CTSS gene in cell proliferation and apoptosis. CTSS overexpression significantly promoted the secretion of progesterone (P4) and estrogen (E2) by increasing the expression of STAR and CYP19A1 (p < 0.05). We also found that overexpression of CTSS increased GCS proliferation by up-regulating the expression of proliferation related gene (PCNA) and anti-apoptotic gene (BCL2). Cell apoptosis was markedly decreased by CTSS activation (p < 0.05). In contrast, CTSS knockdown significantly decreased the secretion of P4 and E2 and the proliferation of rabbit GCS, while increasing the apoptosis of rabbit GCS. Taken together, our results highlight the important role of CTSS in regulating hormone secretion, cell proliferation, and apoptosis in rabbit GCS. These results might provide a basis for better understanding the molecular mechanism of rabbit reproduction.

Keywords: CTSS; granulosa cells; proliferation; apoptosis; hormone secretion

1. Introduction

Rabbits are multi-purpose domestic animals, which can be used as pet animals or biomedical model animals for scientific research, but in people's lives, rabbits are mainly used for meat and fur production [1–3]. Rabbit meat has high nutritional value and is widely accepted by many people for its low content in fat, cholesterol, and high content in protein [4,5]. However, as the demand for rabbit meat increases rapidly, global rabbit meat production is still relatively low [6]. Accelerating the reproductive performance is an important strategy to increase rabbit meat production, thus it is vital to investigate the regulatory mechanism of factors affecting rabbit reproduction.

Ovarian granulosa cells (GCS) are an important component of follicles and play a vital role in follicle growth and development. Each follicle contains an oocyte, numerous
GCS and theca cells [7]. GCS deliver regulatory signals to oocytes through zone projections (TZPs), and also provide nutrition and metabolite for oocyte [8]. Many studies reported that apoptosis of GCS affect cellular connection between GCS and oocytes [9]. It causes ovarian follicular atresia [10], which occurs in more than 99% of developmental follicles in mammals [11,12]. Another important role of GCS is the secretion of progesterone and estradiol, both of which play an important role in animal breeding and reproduction. It was reported that estradiol can promote the expression of FSH receptors in GCS [13], and promote the formation of rat antral follicles [14]. In bovine, low concentration of progesterone enhanced the follicular development, and the diameter of follicular increased [15,16]. Moreover, after follicle ovulation, GCS differentiated into luteal granulosa cells to form the corpus luteum. The main function of corpus luteum is to secrete progesterone to maintain pregnancy. Therefore, both GCS progesterone secretion disorder and GCS apoptosis will result in a decrease in rabbit reproduction performance.

Cathepsin S (CTSS), which is located on chromosome 13 in rabbits, is one member of 11 cysteine proteases, and plays an important role in extracellular matrix degradation and remodeling, antigen presentation, inflammation, and angiogenesis [17]. In malignant tumors, CTSS induces tumor proliferation, invasion and metastasis through various mechanisms [18–20]. It was reported that CTSS might be a potential predictor of chemotherapy response because up-regulation of CTSS is associated with tumor progression and poor prognosis [21]. Studies in vitro also showed that CTSS promoted adipocyte differentiation by degrading fibrinolytic proteins [22]. Studies have also found that the polymorphisms of the CTSS gene are associated with obesity-related traits [23], and CTSS circulating levels are associated with triglycerides synthesis and accumulation [24,25]. CTSS can also regulate blood sugar by reducing glucose output [26].

The regulatory function of CTSS in cell proliferation has been widely investigated, while its function in the development and maturation of rabbits’ oocytes has not been reported. Our hypothesis was that CTSS plays an important role in follicle development and ovulation through regulating cell proliferation and hormone secretion. To verify this hypothesis, the present study aimed to investigate the functional role of the CTSS gene in cell proliferation and apoptosis, as well as progesterone and estradiol secretion in rabbit GCS. These results provide evidence with which to further uncover the regulatory mechanism which mediated the ovulation process and reproductive performance of rabbits.

2. Materials and Methods

2.1. Ethic Statement

The present study was designed and performed according to the guidelines of Institutional Animals Care and Use Committee College (IACUC) of College of Animal Science and Technology of Henan Agricultural University, China (Permit Number: 11-0085; Data: 06-2011).

2.2. Tissue Sample Collection

In this experiment, three female New Zealand white rabbits (180 days old) were selected from the animal house of Henan Agricultural University. The rabbits were anesthetized and slaughtered for ovary collection. The collected ovaries were placed in PBS (containing 1% penicillin/streptomycin, 37 °C) for rabbit granulosa cells isolation.

2.3. Isolation and Culture of Rabbits GCS

The rabbits’ GCS were isolated and cultured according to the previously published papers with few modifications. Briefly, the collected ovaries were washed three times with PBS (phosphate buffer saline) supplemented with 1% penicillin/streptomycin at 37 °C. Follicles were needled with a 1 mL syringe in a basal medium supplemented with DMEM/F12 medium (Gibco, MD, USA), 15% FBS (Gibco, CA, USA), and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA) [27]. The GCS were centrifuged (5 min, 1000 rpm/min) and incubated with basal medium at 37 °C in 5% CO2; cell medium was changed every 24 h [28]. The GCS isolated from the 3 rabbits were isolated individually and pooled together...
for experimental treatments. Before experiments, GCS were seeded in 6-well plate at a density of $3 \times 10^5$ cells per cm$^2$. After 24 h incubation (approximately 90% confluence), GCS were treated with siRNA and recombinant adenovirus for 24 h. All treatments were performed with 3 independent biological replicates.

2.4. Immunostaining

FSHR (follicle stimulating hormone receptor) protein is usually used as a marker protein to identify GCS. The cells were seeded on culture plates plated with cell-climbing slices. After reaching 70% confluence, cells were fixed with 4% paraformaldehyde and then permeated with 0.1% Triton X-100. For immunohistochemical staining, the cells were incubated with anti-FSHR (MAB65591, 1: 200, R&D Systems, Minneapolis, MN, USA) for 1 h at 37 °C, and then incubated with goat anti-rabbit IgG (bs-0296G-FITC, 1:400, Bioss, Beijing, China) at 37 °C for 45 min. Finally, the cells were taken out and mounted with DAPI (4',6-Diamidino-2-phenylindole, Dihydrochloride) medium (Solarbio, Beijing, China). The cells were then observed and imaged under a fluorescence microscope (Nikon Eclipse C1, Tokyo, Japan).

2.5. HE (Hematoxylin-Eosin) Staining

The morphology examination of GCS was measured by HE staining. GCS were seeded in 6-well culture plates plated with cell-climbing slices. Then, the cells were fixed with 95% ethanol for 20 min and stained with hematoxylin dye solution for 3 min. After dehydration with gradient alcohol, the cells were dyed with eosin dye solution for 5 min. Finally, the cells were mounted with neutral gum and observed by microscope (Nikon Eclipse E100, Tokyo, Japan).

2.6. SiRNA (Small Interfering RNA) Interference

GCS were seeded into 6-well plates and transfected with siRNA-CTSS and siRNA-NC by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The specific siRNA sequence targeting CTSS was designed and synthesized by GenePharma (Shanghai, China). siRNA-CTSS, sense: 5′-GGAAGAAAGCCUACG GCAATT-3′; antisense:5′-UUGCCGUAGGCUUUCUUCCTT-3′. siRNA-NC, sense: 5′-UUUCUCCGAACGUGUCACGUTT-3′, antisense: 5′-ACGUGACACGUUCCGGAGAATT-3′.

2.7. Recombinant Adenoviruses Generation

The production of recombinant adenovirus has been previously described [29]. In short, CTSS was amplified and subcloned into the shuttle vector pAdTrack-CMV. Then the linearized product of pAdTrack-CMV-CTSS was transformed into Escherichia coli BJ5183 competent cells containing the backbone vector pAdEasy-1 to obtain the positive recombinants of pAd-CTSS. Then, linearized pAd-CTSS fragment was transfected into 293 cells for adenovirus packaging and amplification. GCS were infected with Ad-CTSS at MOI (multiplicity of infection) value of 40.

2.8. RNA Extraction and RT-qPCR

Total RNA of rabbit GCS were extracted by using Trizol reagent (Invitrogen, CA, USA) according to the instructions of the manufacturer. Total RNA was quantified with a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized by using Prime Script RT reagent Kit (Takara, Tokyo, Japan) following the instructions. The SYBR Premix Ex Taq II kit (Takara, Tokyo, Japan) was used for RT-qPCR. Primers used for RT-qPCR were listed in Table 1. $\beta$-actin was selected as reference gene.
Table 1. Name, accession number, sequences, amplicon length of primer pairs of RT-qPCR.

| Gene/Accession No. | Primers | Sequence (5′ to 3′) | bp |
|--------------------|---------|---------------------|----|
| PCNA               | F:432   | TGCACGTATATGCCGAGACC | 240 |
|                    | R:671   | GTAGGAGAAGCGGAGTGGC |    |
| Notch 2            | F:3798  | CAACCGCCAGTGTGTTCA  |    |
|                    | R:4029  | TTCCCGCTTCTGTCACCTG |    |
| Cyclin D           | F:44    | GTAGGAGAAAGCGGAGTGG |    |
|                    | R:697   | CTTGACGCCTGACCTGAC  | 244 |
| STAR1              | F:477   | CATTGGGAAGGACACGCTA |    |
|                    | R:655   | ACACTCGTGACGCCCTTT  |    |
| NM_001170921.2     | F:463   | CCTAGGGGTCCTGAGATG  |    |
|                    | R:633   | ACTTTCGTCCATGGGGATGC | 171 |
| CYP19A1            | F:1259  | GCCGGAACACACTCTTCTT |    |
|                    | R:1413  | GATCTGTTCATCCACCTCG | 155 |
| BCL2               | F:492   | GACTGATACCTGAAACCGC |    |
| CTSS               | F:11    | CCCTCGAGAGACCTCCAGA |    |
|                    | R:65    | CACCTGTTTGAGCTGCTG  | 166 |
| Caspase-3          | F:1     | ATGGACAGAAGCGGGACGCC | 177 |
| NM_001802117.1     | R:191   | CCGGAGAAGACACTGGTG  | 191 |
| β-actin            | F:1140  | ATGACGAGAGAGATCCCGC |    |
|                    | R:191   | ACTCCCTCGGTCGCCACCC | 148 |
| CTSS               | F:128   | CTACAGCAAGACATACA   |    |
|                    | R:296   | TCTCAAGAACTCATAAA   | 187 |

Annealing temperature for all primers listed in this table is 60°C. F, forward primer; R, reverse primer. PCNA, proliferating cell nuclear antigen; STAR1, steroidogenic acute regulatory protein 1; CYP19A1, cytochrome P450 family 19 subfamily A member 1; CYP11A1, cytochrome P450 family 11 subfamily A member 1; BCL2, B-cell lymphoma-2.

2.9. Measurement of Progesterone and Estradiol Secretion

After 48 h of treatment with Ad-CTSS and siRNA-CTSS, cell culture medium was collected for determination of progesterone and estradiol concentration. Secretion levels of progesterone and estradiol were evaluated with enzyme linked immunosorbent assay (ELISA) according to the instructions of the manufacturer. The kits were all obtained from Nanjing Ruixin Biology (Quanzhou, China). The absorbance was measured using a microplate reader (Bio-Rad) at 450 nm.

2.10. Cell Viability Assay

GCS were seeded onto 96-well plates at 1 × 10^4/well and treated with recombinant adenovirus and siRNA for 0 h, 12 h, 24 h, 36 h and 48 h, separately. Cell proliferation was detected with Cell Counting Kit-8 (CCK8) (US Everbright® Inc., Nanjing, China). According to the instructions of the manufacturer, the cells were added to 10 µL of CCK8 solution and incubated at 37 °C for 2 h. Then, the optical density (OD) value at a wavelength of 450 nm was detected by a microplate reader (Bio-Rad, Hercules, CA, USA).

2.11. Cell Apoptosis Analysis

After treatment with recombinant adenovirus and siRNA for 48 h, GCS apoptosis was detected by Annexin V-Alexa Fluor 747/7-AAD Apoptosis Kit (4Abiotech., Beijing, China). According to the instructions of the manufacturer, the cells were digested with trypsin (without EDTA) and resuspended with cold PBS, and then mixed with 5 µL Annexin V/Alexa Fluor 647 at room temperature for 5 min. Finally, the cells were treated with 10 µL 7-AAD and 400 µL PBS. The apoptosis rate of the GCS was measured with a CytoFLEX flow cytometer (Beckman CytoFlex, CA, USA).

2.12. Statistical Analysis

All data analyses were conducted using SPSS 22.0 software (SPSS, Chicago, IL, USA). All the treatments were performed in three independent biological replicates and three
technical replicates. Statistical analyses were performed with one-way ANOVA to compare the effects of CTSS overexpression and knockdown relative to negative control. The fixed effect was treatment and the random effect was replicated in the statistic model. Relative mRNA expression of RT-qPCR was calculated by using $2^{-\Delta\Delta Ct}$ method, where Ct is the cycle threshold. $p$ value < 0.05 was indicative of a statistically significant difference, and data are presented as means ± SE.

3. Results

3.1. Isolation and Identification of Rabbit GCS

Rabbit GCS were identified by HE staining and immunofluorescence by using FSHR antibody, our HE staining results showed that the nucleus of GCS is blue and the cytoplasm is red, and the pseudopodia between the cells was closely connected (Figure 1A). As shown in Figure 1B, cell nuclei were stained with DAPI and presented to be blue; FSHR protein was green and located in the cytoplasm. The positive rate of FSHR was 99%, indicating that the isolated GCS were 99%.

![Figure 1. Isolation and identification of rabbit GCS: (A): GCS were stained with Hematoxylin and eosin (H&E) staining, (a-c): Original magnification, 40×, 100×, 200×. (B): The identification of rabbit GCS was measured by immunofluorescence staining with fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG. (d): Cell nucleus were stained by DAPI (blue), (e): FSHR were stained with fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG (green), (f): Merge of (d) and (e). (Scale bar = 100 µm).](image)

3.2. Efficiency of CTSS Overexpression and SiRNA Interference

RT-qPCR was performed to determine the efficiency of Ad-CTSS and siRNA-CTSS in rabbit GCS. Our results showed that CTSS expression was remarkably increased by Ad-CTSS compared with Ad-GFP (Figure 2A, $p < 0.05$), and its expression was significantly decreased by siRNA-CTSS relative to siRNA-NC (Figure 2B, $p < 0.05$).
Figure 2. The efficiency of Ad-CTSS and siRNA-CTSS in rabbit GCS: (A): mRNA expression changes of CTSS after infection with Ad-GFP. (B): mRNA expression changes of CTSS after transfection with siRNA-CTSS. Values are presented as means ± SEM for three biological replicates. ** p < 0.01.

3.3. CTSS Promotes the Secretion of Progesterone and Estradiol in Rabbit GCS

To explore the role of CTSS on rabbit reproduction, we measured the effects of CTSS overexpression and knockdown on hormone secretion in rabbit GCS. As shown in Figure 3A,D, genes responsible for steroid hormone synthesis (Steroidogenic acute regulatory protein, STAR) and estrogen synthesis (Cytochrome p450 family 19 subfamily A member 1, CYP19A1) were remarkably increased and decreased by CTSS overexpression and knockdown, respectively (p < 0.05). Consistently, levels of progesterone and estradiol in cell medium were significantly increased by CTSS overexpression (Figure 3B,C). While CTSS knockdown only decreased progesterone secretion (Figure 3E, p < 0.05), estradiol secretion was not changed by siRNA-CTSS (Figure 3F, p > 0.05).

Figure 3. CTSS overexpression and interference on progesterone and estradiol secretion in rabbit GCS: Effects of CTSS overexpression and knockdown on (A,D) mRNA expression changes of genes related to progesterone and estradiol secretion; (B,E) progesterone secretion and (C,F) estradiol secretion. Values are presented as means ± SEM for 3 biological replicates. * p < 0.05.

3.4. CTSS Promotes Cell Proliferation Activity in Rabbit GCS

After treatment with Ad-CTSS and siRNA-CTSS for 0 h, 12 h, 24 h, 36 h and 48 h, the proliferation activity of GCS was measured. As shown in Figure 4A, genes responsible for cell proliferation (PCNA, proliferating cell nuclear antigen) were significantly increased by CTSS overexpression (p < 0.05), and cell proliferation activity of the Ad-CTSS infected group was remarkably higher than the Ad-GFP infected group (Figure 4B, p < 0.05). CTSS
knockdown decreased Notch2 expression and cell proliferation activity in rabbit GCS (Figure 4C,D, p < 0.05).

![Figure 4](image)

**Figure 4.** CTSS overexpression and interference on cell proliferation in rabbit GCS: Effects of CTSS overexpression and knockdown on (A,C): mRNA expression changes of cell proliferation related genes and (B,D): cell proliferation activity. Values are presented as means ± SEM for 3 biological replicates. * p < 0.05; ** p < 0.01; *** p < 0.001.

### 3.5. Activation of CTSS Deceased Cell Apoptosis in Rabbit GCS

As shown in Figure 5A, compared with the Ad-GFP infected group, mRNA level of BCL2 (one of the main anti-apoptotic gene) was significantly increased by CTSS overexpression (p < 0.05). The other important pro-apoptotic gene, BAX, was decreased significantly by CTSS knockdown (Figure 5E, p < 0.05). Flow cytometry analysis revealed that CTSS overexpression inhibited the apoptosis of rabbit GCS significantly (Figure 5B–D, p < 0.01). As shown in Figure 5F–H, CTSS knockdown led to a remarkable increase in apoptotic cells in rabbit GCS (p < 0.05).

![Figure 5](image)

**Figure 5.** Effect of CTSS overexpression and interference on cell apoptosis in rabbit GCS: Relative mRNA expression changes of marker genes related to cell apoptosis after CTSS overexpression (A) and knockdown (E). Cell apoptosis rate was measured by using flow cytometry after CTSS overexpression (B–D) and knockdown (F–H). * p < 0.05; ** p < 0.01; *** p < 0.001.
4. Discussions

Reproductive performance is an important index for animal husbandry. GCS play an important role in oocyte development and affect female breeding performance. A number of studies have shown that small molecules including Vitamin D3 [30], FSH [31], Bisphenol A [32] and genes including CITED4 [33], CXCL12 [34], and STAT4 [35] regulate cell proliferation, apoptosis and hormone secretion in mammals, thus affecting estrus of female animals and oocyte maturation. However, as one species of the high fertility husbandry animals, the effect of CTSS gene on rabbit reproduction has been rarely studied.

Cathepsin S (CTSS), one kind of lysosomal protease, is a major participant in proteolysis [36], and participates in many physiological processes. Studies have shown that CTSS gene played an important role in lipid metabolism in tumor tissue. To further investigate the role of CTSS in rabbit reproduction, we isolated rabbit GCS. GCS exist in the follicle and play an important role in the development of the follicle, as it can provide nutrients to oocytes during follicular development [37]. Studies have reported many methods for in vitro isolation and culture of GCS in pigs [38], cattle [39], and mice [40]. Isolation of rabbit GCS in the present study was performed according to the published methods with little modifications [41], providing material for further study of the complex mechanism of rabbit reproduction.

Progestrone and estradiol are mainly secreted by GCS and play an important role in the breeding process of mammals. They can promote estrus of female rabbits and early fetal pregnancy. To investigate whether CTSS affects progesterone and estradiol secretion in GCS, we overexpressed and interfered the expression of CTSS gene in rabbit GCS. Our results demonstrated that CTSS gene can regulate the secretion of progesterone hormone, and that this effect might be mediated by STAR and CYP19A1. As summarized in Figure 6, activation of CTSS resulted in up-regulation of progesterone hormone-related genes (STAR and CYP19A1) and promoted the secretion of progesterone. In mammals, progesterone plays a key role in follicular development and early fetal pregnancy [40]. Progesterone promotes follicular development, and low levels of progesterone increase follicular diameter [16]. However, the insufficiency of progesterone secretion will cause the fetus to be unable to survive or, even worse, will result in the abortion phenomenon [42]. In this experiment, our results demonstrated that CTSS play an important role in regulating the secretion of progesterone and estradiol in GCS.

![Granulosa Cells](image)

**Figure 6.** Molecular mechanism of CTSS regulating cell apoptosis and progesterone secretion in rabbit GCS.

By providing nutrition and metabolite to oocytes, GCS play an important role in oocyte development. GCS also transmit signals and information for oocytes, and make oocytes mature. Apoptosis of GCS affects follicle development and leads to follicular atresia. In this study, we found that CTSS gene could affect the proliferation and apoptosis of GCS, and that the overexpression of CTSS gene in GCS increased the expression of proliferation gene (PCNA) and anti-apoptotic gene (BCL2), and promoted the proliferation of GCS while decreasing its apoptosis (Figure 6). Consistently, CTSS knockdown decreased the proliferation rate while increasing the apoptosis of rabbit GCS. These findings are in...
accordance with recent research reporting that CTSS interference inhibited the proliferation while inducing the apoptosis in mhCC97-H cells [43,44], and recent studies have also shown that CTSS plays a crucial role in the invasion and apoptosis of cancer cells [45,46]. Taken together, our results have demonstrated that CTSS can promote the proliferation while reducing the apoptosis of rabbit GCS.

5. Conclusions

In summary, our data suggest that CTSS regulates the proliferation and apoptosis of granulosa cells by regulating the expression of key genes involved in these processes. In addition, CTSS is also involved in the process of progesterone and estradiol secretion in rabbit GCS. Overall, the present study provided evidence that CTSS may play an important role in regulating follicular development by regulating cell apoptosis and hormone secretion in rabbits. However, whether manipulation of CTSS expression in vivo can improve the reproductive performance of rabbits needs further research.

Author Contributions: Conceptualization, H.X., M.L. and G.S.; funding acquisition, M.L.; supervision, X.N.; formal analysis, Y.W.; validation, Y.J. and M.S.; writing—original draft, G.S. and Y.J.; writing—review and editing, H.X. and M.L. All authors have read and agreed to the published version of the manuscript.

Funding: The research was jointly supported by the National Key Research and Development Program of China (2018YFD0502203) and the Special Fund for Henan Agriculture Research System (S2013-08-G01).

Institutional Review Board Statement: The study was conducted according to the guidelines of Declaration of Helsinki, and approved by the Ethics Committee of College of Animal Science and Technology of Henan Agricultural University, China (Permit Number: 11-0085; Data: 06-2011).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no competing financial interest.

References

1. Mage, R.G.; Esteves, P.J.; Rader, C. Rabbit models of human diseases for diagnostics and therapeutics development. Dev. Comp. Immunol. 2019, 92, 99–104. [CrossRef] [PubMed]
2. Esteves, P.J.; Abrantes, J.; Baldauf, H.-M.; Ben Mohamed, L.; Chen, Y.; Christensen, N.; González-Gallego, J.; Giacani, L.; Hu, J.; Kaplan, G.; et al. The wide utility of rabbits as models of human diseases. Exp. Mol. Med. 2018, 50, 1–10. [CrossRef] [PubMed]
3. Fontanesi, L. Rabbit Genetic Resources Can Provide Several Animal Models to Explain at the Genetic Level the Diversity of Morphological and Physiological Relevant Traits. Appl. Sci. 2021, 11, 373. [CrossRef]
4. Dalle Zotte, A. Rabbit farming for meat purposes. Anim. Front. 2014, 4, 62–67. [CrossRef]
5. Li, S.; Zeng, W.; Li, R.; Hoffman, L.C.; He, Z.; Sun, Q.; Li, H. Rabbit meat production and processing in China. Meat Sci. 2018, 145, 320–328. [CrossRef] [PubMed]
6. Trocino, A.; Cotozzolo, E.; Zomeño, C.; Petracci, M.; Xiccato, G.; Castellini, C. Rabbit production and science: The world and Italian scenarios from 1998 to 2018. Ital. J. Anim. Sci. 2019, 18, 1361–1371. [CrossRef]
7. Chang, H.-M.; Qiao, J.; Leung, P.C.K. Oocyte-somatic cell interactions in the human ovary–novel role of bone morphogenetic proteins and growth differentiation factors. Hum. Reprod. Update 2016, 23, 1–18. [CrossRef]
8. Li, R.; Albertini, D.F. The road to maturation: Somatic cell interaction and self-organization of the mammalian oocyte. Nat. Rev. Mol. Cell Biol. 2013, 14, 141–152. [CrossRef]
9. Emanuelli, I.P.; Costa, C.B.; Rafagnin Marinho, L.S.; Seneda, M.M.; Meirelles, F.V. Cumulus-oocyte interactions and programmed cell death in bovine embryos produced in vitro. Theriogenology 2019, 126, 81–87. [CrossRef]
10. Tilly, J.L.; Kowalski, K.I.; Johnson, A.L.; Hsueh, A.J. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinology 1991, 129, 2799–2801. [CrossRef]
11. Hughes, F.M.; Gorospe, W.C. Biochemical identification of apoptosis (programmed cell death) in granulosa cells: Evidence for a potential mechanism underlying follicular atresia. Endocrinology 1991, 129, 2415–2422. [CrossRef]
12. Manabe, N.; Goto, Y.; Matsuda-Minehata, F.; Inoue, N.; Maeda, A.; Sakamaki, K.; Miyano, T. Regulation mechanism of selective atresia in porcine follicles: Regulation of granulosa cell apoptosis during atresia. J. Reprod. Dev. 2004, 50, 493–514. [CrossRef]
13. Cai, L.; Sun, A.; Li, H.; Tsinkgou, A.; Yu, J.; Ying, S.; Chen, Z.; Shi, Z. Molecular mechanisms of enhancing porcine granulosa cell proliferation and function by treatment in vitro with anti-inhibin alpha subunit antibody. Reprod. Biol. Endocrin. 2015, 13, 26. [CrossRef] [PubMed]

14. Gore-Langton, R.E.; Daniel, S.A. Follicle-stimulating hormone and estradiol regulate antrum-like reorganization of granulosa cells in rat preantral follicle cultures. Biol. Reprod. 1990, 43, 65–72. [CrossRef] [PubMed]

15. Cerri, R.L.A.; Chebel, R.C.; Rivera, F.; Narciso, C.D.; Oliveira, R.A.; Thatcher, W.W.; Santos, J.E.P. Concentration of progesterone during the development of the ovulatory follicle: I. Ovarian and embryonic responses. J. Dairy Sci. 2011, 94, 3342–3351. [CrossRef] [PubMed]

16. Cerri RL, A.; Chebel, R.C.; Rivera, F.; Narciso, C.D.; Oliveira, R.A.; Thatcher, W.W.; Santos, J.E.P. Effect of progesterone concentrations, follicle diameter, timing of artificial insemination, and ovulatory stimulus on pregnancy rate to synchronized artificial insemination in postpubertal Nellore heifers. Theriogenology 2014, 81, 446–453. [CrossRef]

17. Zhao, P.; Lieu, T.M.; Barlow, N.; Metcalf, M.; Bunnnett, N.W. Cathepsin S causes inflammatory pain via biased agonism of PAR2 and TRPV4. J. Biol. Chem. 2014, 289, 35858. [CrossRef]

18. Karimkhankhoo, H.; Keenan, S.N.; Sun, E.W.; Wathchow, D.A.; Keating, D.J.; Montgomery, M.K.; Watt, M.J. Circulating cathepsin S improves glycemic control in mice. J. Endocrinol. 2011, 248, 167–179. [CrossRef]

19. Sevenich, L.; Bowman, R.L.; Mason, S.D.; Quail, D.F.; Rapaport, E.; Elie, B.T.; Brogi, E.; Brastianos, P.K.; Hahn, W.C.; Holsinger, L.J. Analysis of tumour- and stroma-supplied proteolytic networks reveals a brain-metastasis-promoting role for cathepsin S. Nat. Cell Biol. 2014, 16, 876–888. [CrossRef]

20. Joyce, J.A.; Baruch, A.; Chehade, K.; Meyer-Morse, N.; Giraudo, E.; Tsai, F.Y.; Greenbaum, D.C.; Hager, J.H.; Bogyo, M.; Hanahan, D. Cathepsin cysteine proteases are effectors of invasive growth and angiogenesis during multistage tumorigenesis. Cancer Cell. 2004, 5, 443–453. [CrossRef]

21. Harbeck, N.; Alt, U.; Berger, U.; Krüger, K.; Thomessen, C.F.J.; Höfler, H.; Kates, R.E.; Schmitt, M. Prognostic Impact of Proteolytic Factors (Urokinase-Type Plasminogen Activator, Plasminogen Activator Inhibitor 1, and Cathepsins B, D, and L) in Primary Breast Cancer Reflects Effects of Adjuvant Systemic Therapy. Clin. Cancer Res. 2001, 7, 2757. [CrossRef] [PubMed]

22. Taleb, S.; Cancello, R.; Clément, K.; Lacasa, D. Cathepsins promote human preadipocyte differentiation: Possible involvement of fibroblast degradation. Endocrinology 2006, 147, 4950–4959. [CrossRef] [PubMed]

23. Hooton, H.; Ångquist, L.; Holst, C.; Hager, J.; Rousseau, F.; Hansen, R.D.; Tjønneland, A.; Roswall, N.; Overvad, K.; Jakobsen, M.U.; et al. Dietary Factors Impact on the Association between CTSS Variants and Obesity Related Traits. PLoS ONE 2012, 7, e40394. [CrossRef] [PubMed]

24. Taleb, S.; Lacasa, D.; Bastard, J.P.; Poitou, C.; Cancello, R.; Pelloux, V.; Viguier, N.; Benis, A.; Zucker, J.D.; Bouillot, J.L. Cathepsin S, a novel biomarker of adiposity: Relevance to atherogenesis. Faseb. J. 2005, 19, 1540–1542. [CrossRef] [PubMed]

25. Nadia, N.; Christine, R.; Soraya, F.; Marie-Eve, L.; Christine, P.; Mayoura, K.; Delphine, E.; Steve, S.; Salwa, R.; Jean-Philippe, B. Cathepsins in Human Obesity: Changes in Energy Balance Predominantly Affect Cathepsin S in Adipose Tissue and in Circulation. J. Clin. Endocrinol. Metab. 2010, 95, 1861–1868. [CrossRef]

26. Xu, H.F.; Luo, J.; Zhao, W.S.; Yang, Y.C.; Tian, H.B.; Shi, H.B.; Bionaz, M. Overexpression of SREBP1 (sterol regulatory element binding protein 1) promotes de novo fatty acid synthesis and triacylglycerol accumulation in goat mammary epithelial cells. J. Dairy Sci. 2016, 99, 783–795. [CrossRef] [PubMed]

27. Lovasco, L.A.; Seymour, K.A.; Zafra, K.; O’Brien, C.W.; Schorl, C.; Freiman, R.N. Accelerated ovarian aging in the absence of the STS binding protein 1. M.U.; et al. Dietary Factors Impact on the Association between CTSS Variants and Obesity Related Traits. PLoS ONE 2012, 7, e40394. [CrossRef] [PubMed]

28. Shi, J.; Liu, C.; Chen, M.; Yan, J.; Wang, C.; Zuo, Z.; He, C. The interference effects of bisphenol A on the synthesis of steroid hormones in human ovarian granulosa cells. Environ. Toxicol. 2021, 36, 665–674. [CrossRef] [PubMed]

29. Yao, X.; Li-Samahy, M.A.; Xiao, S.; Wang, Z.; Meng, F.; Li, X.; Bao, Y.; Zhang, Y.; Wang, Z.; Fan, Y.; et al. CITED4 mediates proliferation, apoptosis and steroidogenesis of Hu sheep granulosa cells in vitro. Reproduction 2021, 161, 255–267. [CrossRef] [PubMed]

30. Jin, L.; Ren, L.; Lu, J.; Wen, X.; Zhuang, S.; Geng, T.; Zhang, Y. CXCL12 and its receptors regulate granulosa cell apoptosis in PCOS rats and human KGN tumor cells. Reproduction 2021, 161, 145–157. [CrossRef] [PubMed]

31. Jin, L.; Ren, L.; Lu, J.; Wen, X.; Zhuang, S.; Geng, T.; Zhang, Y. STAT4 targets KISS1 to promote the apoptosis of ovarian granulosa cells. J. Ovarian Res. 2020, 13, 135. [CrossRef]

32. Riese, R.J.; Mitchell, R.N.; Villadangos, J.A.; Shi, G.P.; Palmer, J.T.; Karp, E.R.; De Sanctis, G.T.; Ploegh, H.L.; Chapman, H.A. Cathepsin S activity regulates antigen presentation and immunity. J. Clin. Invest. 1998, 101, 2351–2363. [CrossRef]

33. Kim, S.; Jin, H.; Seo, H.-R.; Lee, H.J.; Lee, Y.S. Regulating BRCA1 protein stability by cathepsin S-mediated ubiquitin degradation. Cell Death Differ. 2019, 26, 812–825. [CrossRef]
37. Zhang, J.Q.; Gao, B.W.; Guo, H.X.; Ren, Q.L.; Wang, X.W.; Chen, J.F.; Wang, J.; Zhang, Z.J.; Ma, Q.; Xing, B.S. miR-181a promotes porcine granulosa cell apoptosis by targeting TGFBR1 via the activin signaling pathway. *Mol. Cell Endocrinol.* 2020, 499, 110603. [CrossRef]

38. Morrell, B.C.; Perego, M.C.; Maylem ER, S.; Zhang, L.N.; Schutz, L.F.; Spicer, L.J. Regulation of the transcription factor E2F1 mRNA in ovarian granulosa cells of cattle. *J. Anim. Sci.* 2020, 98, skz376. [CrossRef]

39. Yao, G.; Yin, M.; Lian, J.; Tian, H.; Liu, L.; Li, X.; Sun, F. MicroRNA-224 is involved in transforming growth factor-beta-mediated mouse granulosa cell proliferation and granulosa cell function by targeting Smad4. *Mol. Endocrinol.* 2010, 24, 540–551. [CrossRef]

40. Aisemberg, J.; Vercelli, C.A.; Bariani, M.V.; Billi, S.C.; Wolfson, M.L.; Franchi, A.M. Progesterone is essential for protecting against LPS-induced pregnancy loss. LIF as a potential mediator of the anti-inflammatory effect of progesterone. *PLoS ONE* 2013, 8, e56161. [CrossRef]

41. Sirotkin, A.V.; Petrak, J.; Alwasel, S.; Harrath, A.H. Apoptosis signal-regulating kinase (ASK1) and transcription factor tumor suppressor protein TP53 suppress rabbit ovarian granulosa cell functions. *Anim. Reprod. Sci.* 2019, 204, 140–151. [CrossRef]

42. Ke, R.W. Endocrine Basis for Recurrent Pregnancy Loss. *Obstet. Gynecol. Clin. N. Am.* 2014, 41, 103–112. [CrossRef]

43. Wang, X.; Xiong, L.; Yu, G.; Li, D.; Peng, T.; Luo, D.; Xu, J. Cathepsin S silencing induces apoptosis of human hepatocellular carcinoma cells. *Am. J. Transl. Res.* 2015, 7, 100–110. [CrossRef]

44. Fan, Q.; Wang, X.; Zhang, H.; Li, C.; Fan, J.; Xu, J. Silencing cathepsin S gene expression inhibits growth, invasion and angiogenesis of human hepatocellular carcinoma in vitro. *Biochem. Biophys. Res. Commun.* 2012, 425, 703–710. [CrossRef] [PubMed]

45. Ärnlöv, J. Cathepsin S as a biomarker: Where are we now and what are the future challenges? *Biomark. Med.* 2012, 6, 9–11. [CrossRef] [PubMed]

46. Small, D.M.; Burden, R.E.; Jaworski, J.; Hegarty, S.M.; Spence, S.; Burrows, J.F.; McFarlane, C.; Kissenpfennig, A.; McCarthy, H.O.; Johnston, J.A.; et al. Cathepsin S from both tumor and tumor-associated cells promote cancer growth and neovascularization. *Int. J. Cancer* 2013, 133, 2102–2112. [CrossRef] [PubMed]