FOXO1 mediates hypoxia-induced G0/G1 arrest in ovarian somatic granulosa cells by activating the TP53INP1-p53-CDKN1A pathway

Chengyu Li, Zhaojun Liu, Gang Wu, Ziyu Zang, Jia-Qing Zhang, Xiaoxuan Li, Jingli Tao, Ming Shen and Honglin Liu
DOI: 10.1242/dev.199453

Editor: Haruhiko Koseki

Review timeline

Original submission:	2 September 2020
Editorial Rejection:	7 September 2020
Resubmission:	21 January 2021
Editorial decision:	8 March 2021
First revision received:	27 May 2021
Accepted:	13 June 2021

Original submission

First decision letter

MS ID#: DEVELOP/2020/196410

MS TITLE: FOXO1 mediates hypoxia-induced G0/G1 arrest in ovarian somatic granulosa cells via activating the TP53INP1-p53- CDKN1A pathway

AUTHORS: Chengyu Li, Zhaojun Liu, Gang Wu, Jia-Qing Zhang, Jilong Zhou, Wang Yao, Jingli Tao, Ming Shen, and Honglin Liu

Many thanks for submitting your manuscript to Development. I have read it carefully and found the study potentially very interesting. Unfortunately, analyses were performed only by using primary granurosa cells and the model was not validated in vivo in this version. Moreover, the knockdown studies should be at least partly validated by using genetic mutants to avoid off- target effects. I am sorry to say that I am returning it without review. The guidelines to our reviewers would almost certainly lead to the rejection of the paper after the review process. I hope that this rapid decision will give you the opportunity to submit your work to a more suitable journal without further delay.

First revision

Please find attached a revised version of our manuscript entitled “FOXO1 mediates hypoxia-induced G0/G1 arrest in ovarian somatic granulosa cells via activating the TP53INP1-p53-CDKN1A pathway”, which original version was submitted as DEVELOP/2020/196410 in September 2, 2020. We would like to thank you for your thoughtful review of the manuscript. You raise important issues and your suggestions are very helpful for improving the quality of our manuscript. We have made our efforts to revise this manuscript by providing more necessary experimental data. In the following pages are our point-by-point responses to each of the comments. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in Development.

Editor comments to the author: I have read it carefully and found the study potentially very interesting.

Response: We are thankful to the editor for this encouraging and positive comment to our manuscript.

Editor comments to the author: Unfortunately, analyses were performed only by using primary granurosa cells and the model was not validated in vivo in this version.

Response: We thank the editor for his careful reading and thoughtful comments. In the revised version of manuscript, we have added new experiments to address the editor's concern by providing evidence to validate the mechanistic model for cell cycle regulation of GCs in vivo. We have now added a new figure (See Fig. 9) and refined the results part on this issue in our resubmitted manuscript as follows (See Line 293-316):
To further test whether the model of cell cycle regulation is applicable in vivo, GCs collected from developing follicles around 4 mm in diameter were classified into groups with high G0/G1 distribution (high groups) and low G0/G1 distribution (low groups). As expected, the elevated JNK activity associated with a more hypoxic status as reflected by the accumulation of HIF-1α in GCs with higher G0/G1 distribution (Fig. 9A). In addition, JNK activity was proportional to the intranuclear level of FOXO1, both of which were increased in high groups (Fig. 9B). Moreover, GCs with higher G0/G1 distribution showed upregulated level of phophorylated 14-3-3, along with an impaired binding affinity of 14-3-3 to FOXO1 (Fig. 9C). Of note, compared with low groups, the JNK activity, 14-3-3 phosphorylation, and the binding status between 14-3-3 and FOXO1 showed similar trend of change in high groups (Fig. 9D). These findings suggest the possible involvement of JNK-14-3-3-FOXO1 signaling in controlling GCs proliferation under hypoxia in vivo.
We next determined the downstream effectors of FOXO1. As shown in Fig. 9E, GCs with high G0/G1 proportion showed increased TP53INP1 expression, which is accompanied by the elevated level of intranulear FOXO1. Using ChIP-qPCR assay, we further observed increased binding of FOXO1 to the promoter of TP53INP1 in high groups (Fig. 9F).
Concomitantly, the expression of TP53INP1 was proportional to that of CDKN1A in above-mentioned GCs groups (Fig. 9G). Further investigation revealed that the upregulated expression of CDKN1A was accompanied by an enrichment of p53 at the promoter region of CDKN1A (Fig. 9H). Furthermore, TP53INP1 expression was proportional to the p53 binding level at CDKN1A promoter (Fig. 9I). Taken together, these results suggest that the FOXO1-TP53INP1-p53-CDKN1A axis might mediate hypoxia-induced G0/G1 arrest of ovarian GCs in vivo.

Editor comments to the author: The knockdown studies should be at least partly validated by using genetic mutants to avoid off-target effects.

Response: We agree with the editor's advice. More and more studies have confirmed that there is non-specificity in the action of siRNA, which can act on other genes other than target genes, resulting in the silencing of non-target genes, leading to the off-target effect of siRNA (Jackson et al, 2010). How to improve the reliability of the RNAi results? Studies have shown that targeting the same gene with multiple siRNAs is an effective method. Each siRNA might has a unique off-target type, but for the same target, the same phenotype could be observed. For multiple parallel siRNAs, if they affect the same phenotype, it implies that the effect is caused by interfering with the target gene rather than other genes. Therefore, targeting the same gene with multiple siRNAs could improve the credibility of the RNAi results (Cullen et al, 2006). Based on this method, we designed six siRNAs against the genes of interest. Two out of the candidate siRNAs in each group were identified as the most potent and specific siRNAs, and were used in parallel for the RNAi experiments. We have now refined the results part and corresponding figures on this issue in our resubmitted manuscript (See Fig. 3E and F, Fig. 6B, Fig. 7F and G, Fig. 8C, Fig. 8G and H, Fig. S2).

Reference

1. Jackson A L, Linsley P S. Recognizing and avoiding siRNA offtarget effects for target identification and therapeutic application. Nature Rev Drug Discov, 2010, 9(1) : 57-67
2. Cullen B R. Enhancing and confirming the specificity of RNAi experiments. Nature Methods,

2006, 3(9) : 677-681.

Honestly, we have tried our best efforts in modifying our resubmitted manuscript. Hopefully, the revised manuscript will meet your journal's standard. Thank you very much for considering our paper's publication.

Resubmission

Second decision letter

MS ID#: DEVELOP/2021/199453

MS TITLE: FOXO1 mediates hypoxia-induced G0/G1 arrest in ovarian somatic granulosa cells via activating the TP53INP1-p53-CDKN1A pathway

AUTHORS: Chengyu Li, Zhaojun Liu, Gang Wu, Ziyu Zang, Xiaoxuan Li, Jia-Qing Zhang, Jingli Tao, Ming Shen, and Honglin Liu

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

DEVELOP.2021.199453v1

FOXO1 mediates hypoxia-induced G0/G1 arrest in ovarian somatic granulosa cells via activating the TP53INP1-p53-CDKNiA pathway

Li et al

This manuscript provides detailed and carefully executed experiments to determine the pathways regulating the arrest of granulosa cells (GCs) in G0/G1 of the cell cycle and the onset if apoptosis in

response to hypoxia, a known stress factor. Experiments were mostly performed using porcine GCs in culture but were supported by isolating GCs from porcine follicles in vivo exhibiting low and high levels of G0/G1 based on flow cytometry. Using multiple biochemical and molecular analyses the investigators show (summarized in a nice diagram) that hypoxia activates the JNK pathway leading to the phosphorylation of 14-3-3. This prevents 14-3-3 from binding to FOXO1 allowing FOXO1 to remain in the nucleus where it activates the transcription of TP53INP1, leading to activation of p53 with then induces expression of the cell cycle suppressor CDKN1A (p21).

Some questions that need to be addressed:

What activates JNK? Perhaps MSK1?

Small antral follicles that are exposed in vivo to slighted higher levels of FSH/LH are recruited and rescued from apoptosis to become ovulatory follicles.
Would FSH overcome the effects of hypoxia?

Immunofluorescent analyses of TP53INP1 and CDKN1A to show their distribution in proliferating GCs with a follicle would be helpful because even within a given follicle, the dividing cells are not synchronized but rather scattered throughout the GC layer. This might provide a nice additional physiologically relevant contribution of interpreting in vivo processes.

FOXO1 is also a tumor suppressor; its disruption leads to GC tumor formation and elevated SMAD2/3 and FOXL2 levels in granulosa cells.
How does this fit into your model?

There are numerous grammatical errors that need to be corrected by careful proof-reading by an English speaking colleague.

Comments for the author

What activates JNK? Perhaps MSK1?

Small antral follicles that are exposed in vivo to slighted higher levels of FSH/LH are recruited and rescued from apoptosis to become ovulatory follicles.

Would FSH overcome the effects of hypoxia?

Immunofluorescent analyses of TP53INP1 and CDKN1A to show their distribution in proliferating GCs with a follicle would be helpful because even within a given follicle, the dividing cells are not synchronized but rather scattered throughout the GC layer. This might provide a nice additional physiologically relevant contribution of interpreting in vivo processes.

FOXO1 is also a tumor suppressor; its disruption leads to GC tumor formation and elevated SMAD2/3 and FOXL2 levels in granulosa cells.

How does this fit into your model?

There are numerous grammatical errors that need to be corrected by careful proof-reading by an English speaking colleague.

Reviewer 2

Advance summary and potential significance to field

Li et al., investigated the signaling pathway in granulosa cells under hypoxia condition during follicular development. They showed that the cell cycle arrest was induced in granulosa cells of growing follicles where cell cycle inhibitor and hypoxia-induced genes were highly expressed. From these results the authors made a hypothesis that follicular development would induce hypoxia condition, which activated FOXO1 to induce G0/G1 arrest. Using in vitro culture system, they did a lot of studies and then cleared their hypothesis.

Comments for the author

However, the reviewer did not understand the method how the authors judged whether each follicle around 4 mm diameter is healthy or atretic follicle. In the atretic follicles, the number of vessels is lower than those in healthy follicles. The condition of follicles is dependent on gonadotropins secreted from pituitary grand. The circular levels of gonadotropins are changed during estrus cycle.
However, the authors did not show any information about estrus stage even if the authors collected ovaries from mature sows. Moreover, the diameter of follicles is increased to more than 10 mm diameter in preovulatory follicles. The authors did not explain the mechanisms how to induce physiological follicular development. The authors should examine the cellular localization of FOXO1 in granulosa cells of each stage of developing follicles. These verification studies to show that this model in in vitro is adapted to in vivo physiological follicular development.
Thus, the reviewer doubt if the mechanisms are much to in vivo mechanisms.
In vitro study, the authors did numerous studies to clear their hypothesis hypoxia condition in granulosa cells during follicular development process.
However, the molecular mechanisms have been showed in other cell types. Thus, the reviewer thinks that this study has limited novel information about hypoxia in molecular levels.

Reviewer 3

Advance summary and potential significance to field

In this study, the authors have examined the mechanism governing G0/G1 arrest in ovarian granulosa cells in pigs. The proportion of granulosa cells in G0/G1 phase was significantly increased with hypoxic culture condition. This hypoxia-induced G0/G1 arrest seems to be mediated by JNK kinase-triggered nuclear translocation of FOXO1. In addition, the authors had identified a novel FOXO1 target, TP53INP1, which may act through p53-CDKN1A signaling to induce G0/G1 arrest in vitro. Moreover, the authors provided some evidence suggesting a similar mechanism exists in vivo. These results sound interesting; however, the overall significance of the present findings is not clear to this reviewer due to the reasons described below.

Comments for the author

First of all, the rationale for studying the mechanism of G0/G1 arrest in granulosa cells is not clear to this reviewer. Is there any evidence/literature supporting the idea that the G0/G1 arrest of granulosa cells is a critical event for, for example, normal folliculogenesis, granulosa cell development, or ovarian pathogenesis? Without having this information overall significance and broad interest of the present findings are questionable.
The interpretation of the results is too much speculative. Especially, the interpretation of western blot results is not convincing at all and, I feel, do not necessarily support the authors conclusions (especially, Figs 4A, 4H, 4I, 6B 8A, and 8C). The addition of quantitative protein measurements and statistics would strengthen the authors' argument.
Additional minor comments Some of the letters in the figures are so small that I could hardly read.

Second revision

Author response to reviewers' comments

We thank the reviewers for their valuable comments concerning our manuscript entitled **"FOXO1 mediates hypoxia-induced G0/G1 arrest in ovarian somatic granulosa cells via activating the TP53INP1-p53-CDKN1A pathway"** (ID: DEVELOP/2021/199453). The comments have greatly helped us in revising and improving our manuscript. We have studied reviewer's comments carefully and made revisions, which are highlighted in yellow font in the revised manuscript. In

the following pages are our point-by-point responses to each of the comments. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in Development. As the formatting and images included in the text will be lost when pasted into the box, we also uploaded a formatted file of the Response as Supplementary Information.

Reviewer 1:

Reviewer 1 Advance Summary and Potential Significance to Field:

This manuscript provides detailed and carefully executed experiments to determine the pathways regulating the arrest of granulosa cells (GCs) in G0/G1 of the cell cycle and the onset if apoptosis in response to hypoxia, a known stress factor. Experiments were mostly performed using porcine GCs in culture but were supported by isolating GCs from porcine follicles in vivo exhibiting low and high levels of G0/G1 based on flow cytometry. Using multiple biochemical and molecular analyses the investigators show (summarized in a nice diagram) that hypoxia activates the JNK pathway leading to the phosphorylation of 14-3-3. This prevents 14-3-3 from binding to FOXO1 allowing FOXO1 to remain in the nucleus where it activates the transcription of TP53INP1, leading to activation of p53 with then induces expression of the cell cycle suppressor CDKN1A (p21).

Response: We thank Reviewer 1 for his/her careful reading and valuable comments.

Reviewer 1 Comments for the Author:

Some questions that need to be addressed:

1. What activates JNK? Perhaps MSK1?

Response: This is a good question. According to the literature, MSK1 (Mitogen- and stress-activated kinase 1) is a kinase activated downstream of the p38 MAPK and ERK1/2, two other members of the MAPK family (Kathleen et al., 2016). But there are few reports on whether MSK1 is an upstream activator of JNK. Moreover, our data showed that p38 MAPK and ERK1/2 were inactive in hypoxic granulosa cells, suggesting that MSK1 might also be inactive. Therefore, JNK activation might be irrelevant to MSK1 in our experimental model. It is widely reported that the JNK signaling pathways are typically activated by environmental stresses such as cytokines, pH changes, UV irradiation, hypoxia, growth factor deprivation, and some chemotherapeutic agents. In each module, extracellular signals are relayed to the nucleus through protein kinase cascades consisting of a MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K), and MAP kinase (MAPK). Generally, phosphorylation and activation of JNK is carried out by two MAP2Ks, namely MKK4 and MKK7 (Davis et al., 2000). For the context of hypoxia, both MKK4 (Zhou et al., 2004) and MKK7 (Vasilevskaya et al., 2008) have been described for their contribution to JNK activation. Taken together, MKK4 and MKK7, rather than MSK1, is supposed to mediate hypoxia-induced JNK activation in granulosa cells. Since MKK4 and MKK7 are well-accepted activators of JNK, it might be redundant to repeat experiments for verifying the role of MKK4/MKK7 in activating JNK.

Reference:

1. Reyskens KM, Arthur JS. Emerging Roles of the Mitogen and Stress Activated Kinases MSK1 and MSK2. Frontiers in cell and developmental biology. (2016) 4:56.
2. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell. (2000) 103:239-252.
3. Zhou G, Golden T, Aragon IV, Honkanen RE. Ser/Thr protein phosphatase 5 inactivates hypoxia-induced activation of an apoptosis signal-regulating kinase 1/MKK-4/JNK signaling cascade. J Biol Chem. (2004) 279:46595-46605.
4. Vasilevskaya IA, Selvakumaran M, O'Dwyer PJ. Disruption of signaling through SEK1 and MKK7 yields differential responses in hypoxic colon cancer cells treated with oxaliplatin. Molecular pharmacology. (2008) 74:246-254.

2. Small antral follicles that are exposed in vivo to slighted higher levels of FSH/LH are recruited and rescued from apoptosis to become ovulatory follicles. Would FSH overcome the

effects of hypoxia?
Response: Thank you for your question. Our previous studies have confirmed that FSH promotes the proliferation of follicular granulosa cells and inhibits the apoptosis of granulosa cells by activating the hypoxic response pathway that centers on the hypoxic-inducible factor HIF-1α, thus maintaining the development and survival of ovarian follicles under hypoxic condition (Li et al., 2020a; Li et al., 2020b).

Reference:

1. Li C, Zhou J, Liu Z, Zhou J, Yao W, Tao J, Shen M, Liu H. FSH prevents porcine granulosa cells from hypoxia-induced apoptosis via activating mitophagy through the HIF-1α-PINK1-Parkin pathway. Faseb j. (2020) 34:3631-3645.
2. Li C, Liu Z, Li W, Zhang L, Zhou J, Sun M, Zhou J, Yao W, Zhang X, Wang H, et al. The FSH-HIF-1α-VEGF Pathway Is Critical for Ovulation and Oocyte Health but Not Necessary for Follicular Growth in Mice. Endocrinology. (2020) 161.

3. **Immunofluorescent analyses of TP53INP1 and CDKN1A to show their distribution in proliferating GCs with a follicle would be helpful because even within a given follicle, the dividing cells are not synchronized but rather scattered throughout the GC layer. This might provide a nice additional physiologically relevant contribution of interpreting in vivo processes.**

Response: We agree with the reviewer's suggestion. In the revised manuscript, using histological sections of porcine ovaries, we performed immunofluorescence staining to determine PCNA (reflecting cellular proliferation status) co-localization with TP53INP1 or CDKN1A in GCs within the same follicles. The results showed increased expression of both TP53INP1 and CDKN1A in inner layers of GCs, where the levels of PCNA tended to decrease in cells with higher abundance of TP53INP1 or CDKN1A. These data further confirmed the relevance of TP53INP1-CDKN1A axis to GCs proliferation retard at a single-cell level in vivo.

4. **FOXO1 is also a tumor suppressor; its disruption leads to GC tumor formation and elevated SMAD2/3 and FOXL2 levels in granulosa cells. How does this fit into your model?**
Response: Thank you for your question. As mentioned by the reviewer, FOXO1 functions as a suppressor of granulosa cell tumors (GCT). After searching the literature, we learnt that GCTs ubiquitously carry a somatic mutation in FOXL2 gene (C134W). FOXL2C134W acquires the ability to bind with SMAD2/3, forming a complex that leads to GC tumor formation (Weis-Banke et al., 2020). Overexpression of FOXO1 mitigates the carcinogenic effects of SMAD3/FOXL2C134W, while FOXO1 deficiency restores the activity of SMAD3/FOXL2C134W and promotes GCT development (Belli et al., 2019; Secchi et al., 2021). Therefore, FOXO1 is considered as a GCT tumor suppressor. However, granulosa cell tumor is a rare type of stromal cell malignant cancer of the ovary (account for only 2% of all ovarian cancers). Owing to the particularity of physiological structures, the granulosa cells are surrounded by an avascular environment and immersed in a hypoxia environment. During follicular development, the proliferation of GCs and thickening of the granulosa compartment further increases the distance from GCs to follicular vasculature, leading to progressively deficient supply of O2 in GCs. According to our model of cell cycle regulation in ovarian GCs, hypoxia represent a key factor that causes GCs proliferation arrest during follicular development. We demonstrated that hypoxia inhibits GCs proliferation by activating the FOXO1 signaling pathway. Since FOXO1 could block the carcinogenic effect of FOXL2 in GCs, this might explain why the incidence of GCT is low.

Reference:

1. Weis-Banke SE, Lerdrup M, Kleine-Kohlbrecher D, Mohammad F, Sidoli S, Jensen ON, Yanase T, Nakamura T, Iwase A, Stylianou A, et al. Mutant FOXL2(C134W) Hijacks SMAD4 and SMAD2/3 to Drive Adult Granulosa Cell Tumors. Cancer research. (2020) 80:3466-3479.
2. Belli M, Secchi C, Stupack D, Shimasaki S. FOXO1 Negates the Cooperative Action of FOXL2(C134W) and SMAD3 in CYP19 Expression in HGrC1 Cells by Sequestering SMAD3. Journal of the Endocrine Society. (2019) 3:2064-2081.
3. Secchi C, Benaglio P, Mulas F, Belli M, Stupack D, Shimasaki S. FOXO1 mitigates the

SMAD3/FOXL2(C134W) transcriptomic effect in a model of human adult granulosa cell tumor. Journal of translational medicine. (2021) 19:90.

5. There are numerous grammatical errors that need to be corrected by careful proof-reading by an English speaking colleague.
Response: We apologize for the grammatical errors in the manuscript. The paper has now been carefully revised by a professional language editing service to improve the grammar and readability.

Reviewer 2:

Reviewer 2 Advance Summary and Potential Significance to Field:

Li et al., investigated the signaling pathway in granulosa cells under hypoxia condition during follicular development. They showed that the cell cycle arrest was induced in granulosa cells of growing follicles where cell cycle inhibitor and hypoxia-induced genes were highly expressed. From these results the authors made a hypothesis that follicular development would induce hypoxia condition, which activated FOXO1 to induce G0/G1 arrest. Using in vitro culture system, they did a lot of studies and then cleared their hypothesis.
Response: We thank this reviewer for his/her thorough review of our manuscript.

Reviewer 2 Comments for the Author:

1. However, the reviewer did not understand the method how the authors judged whether each follicle around 4 mm diameter is healthy or atretic follicle. In the atretic follicles, the number of vessels is lower than those in healthy follicles. The condition of follicles is dependent on gonadotropins secreted from pituitary grand. The circular levels of gonadotropins are changed during estrus cycle. However, the authors did not show any information about estrus stage even if the authors collected ovaries from mature sows.
Response: We thank the reviewer for raising this important point. Indeed, we also recognized these issues when we collected porcine ovaries, but no detailed description was included in the original article. We have now added necessary information for sample collection in the section of materials and methods. Characteristic morphological difference between healthy follicles and atretic follicles includes follicular colour, abundance of blood vessels, clarity of follicular fluid, etc. Healthy follicles usually display pink or yellow in appearance, and the blood vessels are fine and evenly distributed. The follicular fluid is clear. The appearance of an atretic follicle is dark or black, with few or no capillary vessels, and there are floccules in the follicular cavity due to the dissociation of granulosa cells from the follicular wall. Therefore, based on follicular appearance (colour, vascular abundance, follicular fluid clarity), the healthy follicles were selected for our experiments. This procedure rules out the potential influence of follicular atresia on the proliferation of granulosa cells.

The estrous cycle of mature sows is divided into two phases: the follicular phase and the luteal phase. The follicular phase of the cycle could range from 5 to 7 days, while the luteal phase of the cycle lasts about 13 to 15 days. During follicular phase, follicles in the ovary develop from primary follicle to a fully mature preovulatory follicle in response to estrogen, FSH, and LH. In the luteal phase, the inhibition of gonadotropin secretion by progesterone hinders the further development of small antral follicles. Our research focused on the cell cycle regulation of granulsa cells during follicular development, so we collected ovaries at the stage of follicular phase. The visible difference between the follicular phase and the luteal phase is the presence/absence of the corpus luteum in the ovaries. In our study, ovaries without corpus luteum were selected for the experiments. Therefore, the estrus cycles are relatively synchronized at the follicular phase, rather than at the luteal phase.
We have now revised the part of sample collection in the materials and methods section. The corresponding text is presented as follows:
Ovaries from mature sows were collected at a local slaughterhouse and subsequently transferred to the laboratory within 2 h. Ovaries without corpus luteum were selected to isolate morphologically healthy follicles with small forceps. Generally, healthy follicles are rounded with evenly distributed blood vessels; they display pink or yellow in appearance and had a visible

cumulus–oocyte complex immersed in clear follicular fluid. After isolation, the follicles were subjected to size measurement, and then individually torn apart to obtain the mural GCs by scraping the follicular wall.

2. Moreover, the diameter of follicles is increased to more than 10 mm diameter in preovulatory follicles. The authors did not explain the mechanisms how to induce physiological follicular development. The authors should examine the cellular localization of FOXO1 in granulosa cells of each stage of developing follicles. These verification studies to show that this model in in vitro is adapted to in vivo physiological follicular development. Thus, the reviewer doubt if the mechanisms are much to in vivo mechanisms.

Response: We thank this reviewer for his/her thoughtful suggestion. To best fit the role of FOXO1 into the dynamics of follicle growth, in our revised manuscript, we performed additional experiments to investigate the relationship between GCs cell cycle and cellular localization of FOXO1 in follicles at various stages, including small antral follicles (1-3 mm), medium antral follicles (3-5 mm), large antral follicles (≥5 mm). The results is described as follows (see also in the corresponding text of the result section):

To validate the possible involvement of hypoxia and FOXO1 in modulating GC cell cycle, experiments were performed in porcine ovarian GCs harvested from growing follicles at various stages, including small antral follicles (1-3 mm), medium antral follicles (3-5 mm), large antral follicles (≥5 mm). As a transcription factor,

FOXO1 functions by entering the cell nucleus (Shen et al., 2012). For medium and large antral follicles, increased nuclear distribution of FOXO1 were observed in those GCs showing a higher prevalence in G0/G1 phase (Fig. S2F and K). Linear regression analysis showed that the fraction of the population arrested at G0/G1 was positively correlated with FOXO1 contents in the nuclei (Fig. S2G and L). To test whether hypoxia might be relevant to regulating GC cell cycle in vivo, the protein level of hypoxia inducible factor-1alpha (HIF-1α), a key endogenous marker of hypoxia, was determined in ovarian GCs. As shown in Fig. S2H-I and S2M-N, the elevated proportions of GCs at G0/G1 phase correlated strongly with a progressively more hypoxic status as reflected by the accumulation of cellular HIF-1α. Moreover, a significant positive correlation was determined between the nuclear distribution of FOXO1 and HIF-1α accumulation in ovarian GCs as mentioned above (Fig. S2J and O). However, no significant correlationship was detected among protein levels of intranuclear FOXO1, HIF-1α abundance, and cell cycle distribution in GCs collected from small antral follicles (Fig. S2A-E). Therefore, our in vivo findings indicated a potential interaction between hypoxia and FOXO1 signaling in controlling the cell cycle transition of GCs in medium and large antral follicles.

3. In vitro study, the authors did numerous studies to clear their hypothesis, hypoxia condition in granulosa cells during follicular development process. However, the molecular mechanisms have been showed in other cell types. Thus, the reviewer thinks that this study has limited novel information about hypoxia in molecular levels.

Response: Thank you for this valuable feedback. Admittedly, the mechanisms by which hypoxia regulates cell proliferation and cell cycle has been extensively investigated. Actually, our study elucidated the transcriptome profile of GCs proliferation retard during follicular development, and provided the first evidence suggesting hypoxia as a key stimulus of GCs proliferation retard during follicular development. To our knowledge, this discovery has never been reported by other reseachers, and might be a highlight of our manuscript. In addition, we revealed that the activation of FOXO1 by JNK-triggered 14-3-3 phosphorylation is responsible for hypoxia-induced G0/G1 arrest in ovarian GCs. Further investigations identified a novel transcriptional target of FOXO1, namely TP53INP1. We provided first evidence demonstrating that TP53INP1 functions as a major contributor of hypoxia-induced G0/G1 arrest in ovarian GCs. Moreover, our research is the first to show that TP53INP1 acts through the TP53INP1-p53 positive feedback and p53-CDKN1A signaling to mediate FOXO1-dependent G0/G1 arrest upon hypoxia exposure. We also discovered a novel regulation mode of FOXO1-mediated CDKN1A transcription through a TP53INP1-p53 dependent manner. We thus believed that our findings might provide novel insights into the functions and mechanisms of hypoxia in regulating GCs proliferation in developing follicles.

Reviewer 3:

Reviewer 3 Advance Summary and Potential Significance to Field:

In this study, the authors have examined the mechanism governing G0/G1 arrest in ovarian granulosa cells in pigs. The proportion of granulosa cells in G0/G1 phase was significantly increased with hypoxic culture condition. This hypoxia-induced G0/G1 arrest seems to be mediated by JNK kinase-triggered nuclear translocation of FOXO1. In addition, the authors had identified a novel FOXO1 target, TP53INP1, which may act through p53-CDKN1A signaling to induce G0/G1 arrest in vitro. Moreover, the authors provided some evidence suggesting a similar mechanism exists in vivo. These results sound interesting; however, the overall significance of the present findings is not clear to this reviewer due to the reasons described below.
Response: Thank you for your careful reading and helpful comments. We appreciate the positive remarks about the manuscript. We also consider it is very important and necessary to address the specific comments raised by the reviewer. As detailed in our response to the reviewer's specific comments below, we have revised our paper to address the reviewer's concern.

Reviewer 3 Comments for the Author:

1. First of all, the rationale for studying the mechanism of G0/G1 arrest in granulosa cells is not clear to this reviewer. Is there any evidence/literature supporting the idea that the G0/G1 arrest of granulosa cells is a critical event for, for example, normal folliculogenesis, granulosa cell development, or ovarian pathogenesis? Without having this information overall significance and broad interest of the present findings are questionable.
Response: We are grateful to reviewer 3 for raising this important point. To answer these questions, we first need to understand the relationship between cell proliferation and cell cycle arrest. Cell proliferation refers to the processes that result in an increase in the number of cells. Cell proliferation occurs through the four stages of cell cycle that includes G1 phase, S phase, G2 phase, and the M phase. Cell cycle arrest is a stopping point in the cell cycle, where it is no longer involved in the processes surrounding cell division, thereby preventing cell proliferation. In our present study, we discovered that hypoxia is responsible for the G0/G1 arrest in ovarian GCs. Since the progression from G1 to S phase is a rate-limiting step of cell proliferation (Bertoli et al., 2013), our data indicate that GC proliferation is inhibited by the hypoxia environment within ovarian follicles.
In the ovary, the growth rate of follicles is tightly related to the proliferation of granulosa cells. In primordial follicles, the oocyte is surrounded by a single layer of nondividing granulosa cells arrested in G0 phase. Primordial follicles leave this quiescent state and initiate a phase of slow growth in which the granulosa cells have entered the cell cycle but proliferation is exceedingly slow (Hirshfield, 1991). However, as these slowly dividing granulosa cells acquire enhanced responsiveness to FSH and LH and begin producing estradiol (Richards, 1980; Richards, 1975), exposure to these hormones triggers a rapid proliferation of granulosa cells that results in the formation of large preovulatory follicles (Rao et al., 1978). Granulosa cells of these preovulatory follicles not only are highly proliferative but are also differentiating and acquire LH receptors (Uilenbroek and Richards, 1979). The LH surge then triggers dramatic changes in both follicular structure and function. LH terminates follicular growth by causing granulosa cells of preovulatory follicles to exit the cell cycle (arrested in G0 phase) (Rao et al., 1978) and rapidly initiates a program of terminal differentiation (luteinization) in which the cells cease to divide (Richards, 1994; Richards et al., 1986). Therefore, granulosa cell proliferation is of ultimate importance not only for the growth of ovarian follicles but also for granulosa cell differentiation during folliculogenesis. Besides, granulosa cell proliferation is also closely related to ovarian disorders such as polycystic ovary syndrome (Das et al., 2008), premature ovarian insufficiency (Jiao et al., 2018), and granulosa cell tumours (Anttonen et al., 2014).
In summary, the G0/G1 arrest of granulosa cells, which inhibits granulosa cell proliferation, is believed to influence the pathophysiological processes including normal folliculogenesis, granulosa cell development, and ovarian pathogenesis.

Reference:

1. Bertoli, C., Skotheim, J. M. and de Bruin, R. A. (2013). Control of cell cycle transcription during G1 and S phases. Nat Rev Mol Cell Biol 14, 518-528.
2. Hirshfield, A. N. (1991). Development of follicles in the mammalian ovary. International review of cytology 124, 43-101.
3. Richards, J. S. (1980). Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol Rev 60, 51-89.
4. Richards, J. S. (1975). Estradiol receptor content in rat granulosa cells during follicular development: modification by estradiol and gonadotropins. Endocrinology 97, 1174-1184.
5. Rao, M. C., Midgley, A. R., Jr. and Richards, J. S. (1978). Hormonal regulation of ovarian cellular proliferation. Cell 14, 71-78.
6. Uilenbroek, J. T. and Richards, J. S. (1979). Ovarian follicular development during the rat estrous cycle: gonadotropin receptors and follicular responsiveness. Biol Reprod 20, 1159-1165.
7. Richards, J. S. (1994). Hormonal control of gene expression in the ovary. Endocr Rev 15, 725-751.
8. Richards, J. S., Hedin, L. and Caston, L. (1986). Differentiation of rat ovarian thecal cells: evidence for functional luteinization. Endocrinology 118, 1660-1668.
9. Das, M., Djahanbakhch, O., Hacihanefioglu, B., Saridogan, E., Ikram, M., Ghali, L., Raveendran, M. and Storey, A. (2008). Granulosa cell survival and proliferation are altered in polycystic ovary syndrome. J Clin Endocrinol Metab 93, 881-887.
10. Jiao, X., Ke, H., Qin, Y. and Chen, Z. J. (2018). Molecular Genetics of Premature Ovarian Insufficiency. Trends Endocrinol Metab 29, 795-807.
11. Anttonen, M., Pihlajoki, M., Andersson, N., Georges, A., L'Hôte, D., Vattulainen, S., Färkkilä, A., Unkila-Kallio, L., Veitia, R. A. and Heikinheimo, M. (2014). FOXL2, GATA4, and SMAD3 co-operatively modulate gene expression, cell viability and apoptosis in ovarian granulosa cell tumor cells. PLoS One 9, e85545.

2. The interpretation of the results is too much speculative. Especially, the interpretation of western blot results is not convincing at all and, I feel, do not necessarily support the authors conclusions (especially, Figs 4A, 4H, 4I, 6B, 8A, and 8C). The addition of quantitative protein measurements and statistics would strengthen the authors' argument.
Response: We agree with the reviewer's comment. We have now added the quantitative measurements and statistical analysis of the protein levels for the western blot data.

Additional minor comments

Some of the letters in the figures are so small that I could hardly read.

Response: Thank you for your reminding. We have now enlarged the letters in some of the figures.

Third decision letter

MS ID#: DEVELOP/2021/199453

MS TITLE: FOXO1 mediates hypoxia-induced G0/G1 arrest in ovarian somatic granulosa cells via activating the TP53INP1-p53-CDKN1A pathway

AUTHORS: Chengyu Li, Zhaojun Liu, Gang Wu, Ziyu Zang, Jia-Qing Zhang, Xiaoxuan Li, Jingli Tao, Ming Shen, and Honglin Liu
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The revised manuscript has been improved and is acceptable.

Comments for the author

this manuscript has been improved and is acceptable.

Reviewer 2

Advance summary and potential significance to field

This study showed the novel understanding the mechanisms how some follicles are developed and others are stayed at small antral follicle stage. The authors also found the novel functions of FOXO1 that bound TP53INP1. These information is well contributed for not only understanding the mechanisms of funicular development but also functions of FOXO1.

Comments for the author

According to reviewer's suggestions, the authors added some data and revised their manuscript. In the revised version, the authors' hypothesis has been substantiated. It also clearly mentions where the new discoveries are.

Reviewer 3

Advance summary and potential significance to field

In this study, the authors have examined the mechanism governing G0/G1 arrest in ovarian granulosa cells in pigs. The proportion of granulosa cells in G0/G1 phase was significantly increased with hypoxic culture condition. This hypoxia-induced G0/G1 arrest seems to be mediated by JNK kinase-triggered nuclear translocation of FOXO1. In addition, the authors had identified a novel FOXO1 target, TP53INP1 which may act through p53-CDKN1A signaling to induce G0/G1 arrest in vitro.

Moreover, the authors provided some evidence suggesting a similar mechanism exists in vivo.

Comments for the author

The authors have addressed all of my concerns. I do not have any further concerns.