Development and function of smooth muscle cells is modulated by *Hic1* in mouse testis

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DOI: 10.1242/dev.185884

Editor: Haruhiko Koseki

Review timeline

Original submission:	25 October 2019
Editorial decision:	28 November 2019
First revision received:	26 May 2020
Accepted:	9 June 2020

Original submission

First decision letter

MS ID#: DEVELOP/2019/185884

MS TITLE: Development and Function of Smooth Muscle Cells is Modulated by Hic1 in Mouse Testis

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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.

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

This manuscript examines the role of Hic1 in testicular peritubular cells, and finds that testicular structure and function are disrupted when Hic1 is deleted from smooth muscle cells. Little is known about how testicular peritubular cells, which are critical for spermatogonial stem cell niche

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function and immune regulation in the testis, are specifically regulated during development. The findings from this study could potentially contribute to our understanding of these important, but poorly-understood, testicular cells.

Comments for the author

In this manuscript, Uchida and colleagues examine the role of Hic1 in testicular smooth muscle cells. They observe that Hic1 expression is enriched in testicular peritubular cells and, using a Hic1-CreER lineage tracing/labeling method, they find that Hic1 expression is most prominent at 1 week of age; they also find that Hic1-expressing PMCs have high expression of the ECM component fibronectin. Using a tamoxifen-inducible aSma-CreER conditional KO (cKO) model to delete Hic1 from smooth muscle cells, they observe that cKO testes have dilated tubules that have an increased number of peritubular myoid cells (PMCs), reduced fibronectin expression, and reduced contractility; additionally, there appears to be a loss of differentiated germ cell types (although undifferentiated spermatogonia were increased in number). Based on these data, the authors conclude that Hic1 is a critical regulator of PMC function, which is subsequently necessary for testicular function.

Recent reports have demonstrated that peritubular cells, apart from well-appreciated roles in promoting tubule integrity and contractility, are also important players in the spermatogonial stem cell niche and in mediating immune and inflammatory responses in the testis. Therefore, studies that will increase our knowledge of PMCs and how they are regulated will be of interest to the field. In general, the assays performed in this study address the hypothesis posed and the data is presented clearly. However, there are some concerns regarding the experimental design of some of the assays, which impacts the interpretation of the data; additionally, some of the central claims made in the manuscript are not strongly supported by experimental evidence. There are several points that the authors could address to improve the manuscript:

1. One of the central claims made by the authors in the manuscript, which is that “deletion of Hic1 promoted proliferation of PMCs…which subsequently altered the size of seminiferous tubules and testis” (lines 282-283) is not strongly supported by the data presented. In particular, there is no data showing that Hic1 specifically affects proliferation. The Tomato lineage tracing data in Fig. 2 suggests that Hic1-expressing PMCs proliferate more than Hic1-negative cells; therefore, one might expect that deleting Hic1 from PMCs would decrease proliferation, not increase it. Also, the CCND1 data also does not reveal any differences in proliferation in mutant tubules, although this assay was done at 8 weeks old, which is likely not the time point when PMCs are proliferating, and was not done with cell-type-specific markers. The authors should perform Ki67 and BrdU assays at the appropriate time points (likely during postnatal stages) with cell-type-specific markers and in the Tomato lineage-tracing models. Such assays would definitely address the claims made the authors regarding proliferation of Hic1-expressing PMCs in both control and cKO conditions, and are critical to support their claims.

2. Related to the previous comment, the authors do not consider the possibility that Hic1 deletion only directly results in an increase in luminal fluid content, which causes a general expansion of tubule diameter; the increase in PMC number in the cKO testis may merely be a secondary effect of the increased tubule diameter and not a cell-autonomous effect of Hic1 deletion. Therefore, the increased PMC number may be an effect/symptom of the increased tubule diameter, not a cause of it. The authors should perform more specific and definitive assays to address these scenarios.

3. Even though vascular smooth muscle cells (VSMCs) are also a major target of the aSma-CreER driver used in this study, the authors did not mention these cells at all in this manuscript. Is Hic1 expressed in VSMCs? Did the Hic1-CreER lineage tracing label any VSMCs? Are blood vessels dilated in the cKO mice? Is there an increased number of VSMCs? Is their proliferation affected in cKO mice? Looking at VSMCs may shed additional light on the role of Hic1 in the testis, and whether it is specific to PMCs or not.

4. On line 100, the authors claim that there was “heterogeneous expression of Hic1 in the testicular smooth muscle cells…” Was this heterogeneity due to actual differences in Hic1 expression in individual cells, or was it due to inefficiency of the CreER line, or perhaps due to relatively low doses of tamoxifen (50 ug/g bw)? This could be addressed via in situ hybridization for Hic1, which

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was performed in Fig. 1D but the image was not entirely convincing to make any claims about specificity or homogeneity/heterogeneity of expression.

5. There are some technical concerns about using anti-SMA antibody to FACS-purify live cells. SMA should be an intracellular protein for the most part, so the antibody should not be able to penetrate live cells and provide specific fluorescence. That's why usually only cell-surface receptors or endogenous fluorescence are used for FACS purification of live cells. Otherwise, fixation and permeabilization with detergents is needed. Therefore, some may doubt if SMA antibody can be used for live cell purification and subsequent qPCR. At the very least, some flow cytometry plots with appropriate controls should be provided to satisfy readers that this is a technically sound, specific, and feasible assay.

6. Related to the previous comment, there are additional technical concerns using tdTomato-/SMA+ cells as the "negative population" of PMCs. If CreER activity is limited to the PMCs, then the tdTomato-/SMA+ cells will include PMCs and VSMCs, while the tdTomato+/SMA+ cells will only include PMCs. Therefore, these 2 populations will not be the same, which will confound the analyses.

7. The authors claim that there is differential cell proliferation between Hic1+ and Hic1- PMCs, but they do not address the role of cell death in potentially mediating the differential presence of certain PMC populations during development.

8. While examining the estrogen receptors is a good idea, the analyses shown are based on whole testis. If ERs are affected only in the PMCs in Hic1 cKO mice, then any changes would likely be diluted out by all the other cells in the testis that express ER (likely Leydig cells, and potentially others). Such analyses should be done in purified PMCs to obtain a more definitive and robust answer.

9. The authors mentioned that there is sperm in the epididymis, but do not perform any other functional assays to test testicular function, which are critical assays. Are these mice sterile, sub-fertile, or fertile? Do they have reduced sperm count? Reduced sperm motility? Do they have a disrupted hormonal (HPG) axis? Some basic information regarding testis function is needed to know whether Hic1 peritubular function is required for male fertility.

10. Can the authors provide some data (or least speculate) as to why there are more undifferentiated spermatogonia in the cKO testis even though there are fewer Sertoli cells per tubule? This seems to be unexpected, as Sertoli cells are often thought to be a critical limiting factor in determining the number of SSCs and undifferentiated spermatogonia in the testis.

11. The authors should provide some quantification for the images in Fig. 4D.

12. In Figure 5, the authors should provide information as to whether the increased number of PMCs in the cKO tubules are due to an increase in tdTomato+ cells only, or a general increase in both tdTomato+ and tdTomato-cells. This information would shed light on whether this phenomenon is cell-autonomous to Hic1-mutant cells or not.

13. Some of the SMA staining on whole-mount tubules is difficult to see and not convincing, mostly due to the fact that the cells are so thin and flat. An additional cell-surface marker to definitively outline the cells would be helpful for the reader in some cases to discern individual cells in whole-mount preparations.

14. In Fig. 6C, the authors look at SMA and Cnn1, but what about fibronectin, collagen IV, and Lama1?
These analyses would allow the authors to see if the fibronectin loss is cell-autonomous, rather than a general secondary disruption caused by tubule size disruption.

15. Can the authors provide more details about if there is any specific spermatogenic arrest? There are appear to be testicular and epididymal sperm, but there are also seems to be a reduced number of spermatocytes and spermatids. Is this a specific arrest or stage at which cells undergo cell death?

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Minor points:

1. I am not sure what is required by the journal, but the authors should likely adhere to standard nomenclature for gene names in the field as dictated by the mouse genomics database (MGI at JAX), such as Acta2 for Sma, Fn1 for fibronectin, etc..

2. Some more details are needed for the seminiferous epithelial stage analysis. In particular, it was stated how many tubules were counted, but not how many cells were analyzed.

3. The authors should provide some more explanation, either in the Methods section or Figure Legends regarding what the box plot and error bars signify in the qPCR graphs (for example, in Fig. 1G, 1H, and 6C).

4. It was unclear why the cell sorting analyses, e.g., in Fig. 1H, were done in adult testes (presumably 8 week old). There are so few Tomato+ cells in that stage, so it may be difficult to get any robust gene expression differences. Perhaps an earlier stage would be more informative.

5. On lines 205-206, the authors claim that SCP3 is a marker for "…more differentiated germ cells such as round spermatids…" However, SCP3 is more likely a marker for spermatocytes rather than spermatids.

6. A control image is needed for Fig. S1C.

7. A low-magnification image of Fig. 1C would be informative to show the specificity of Hic1-CreER activity and to show that is only in PMCs. A low-mag image would also be nice for Fig. 4A.

8. Can the authors comment on the efficiency of aSma-CreER targeting? What percent of PMCs are targeted, both in the control and Hic1-flox/flox background?

9. A germ cell co-stain in Fig. 6E would be informative.

10. Which cell types are CCND1+ in Fig. 4G? An image would be nice.

11. The CNN1 images in Fig. S2B are not very convincing to show the differences in expression between control and cKO. In general, Western blots would be a more robust way to show quantitative differences as compared to immunofluorescence.

Reviewer 2

Advance summary and potential significance to field

Compared to other cell types in the testis, relatively little is known about peritubular myoid cells, except that their contractibility helps moving the sperm produced towards the rete testis and that these cells produce factors that influence the behaviour of spermatogonial stem cells. The present manuscript now provides a wealth of new data on peritubular myoid cells. It was found that Hypermethylated in cancer 1 (Hic1) plays an important role in these cells, in such a way that it regulates architexture and contractility of the seminiferous tubules. In Hic1 deficient mice more PMCs are formed that miss fibronectin and have lower levels of several smooth muscle contractile proteins. The authors have studied various aspects of this investigations in a rather methodical way the experimental design is excellent and the results are very well described.
The results provide many interesting new details about how seminiferous tubule architexture and tubule function are regulated.

Comments for the author

There is one important aspect that has largely been overlooked. The authors show that in Hic1 deficient mice the numbers of Sertoli cells per tubule cross-section are decreased and in addition tubule circumference has increased. These data reinforce each other and as a result the density of the Sertoli cells on the basal lamina in Hic1 deficient testes likely is substantially lower than in wild

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type mice. As spermatogenesis critically depends on the support of Sertoli cells and as germ cells even may need contact with Sertoli cells, would a shortage of Sertoli cells not explain the poor spermatogenesis in the KO mice? Although the authors show that individual Sertoli cells behave normally, their low numbers may still cause too low levels of factors needed by germ cells.The authors should calculate how much lower the numbers of Sertoli cells are in KO mice and discuss the possibility that this is the cause both of the poor quality of spermatogenesis and the small height of the seminiferous epithelium in the absence of Hic1. Will the Sertoli cell barrier still be intact in KO mice?

Reviewer 3

Advance summary and potential significance to field

Hic1 encodes a member of the Kruppel/Zinc finger and BTB (POK/ZBTB) family of transcription factors, and is thought to act as a repressor and as a tumor suppressor.
The authors show that Hic1 is required in peritubular myoid cells (PMC) of the postnatal testis because when the gene is deleted specifically in the PMCs shortly after birth testis development and spermatogenesis are abnormal. In particular, when Hic1 is not present PMC number is increased (the normal function of Hic1 might be to prevent over-proliferation of PMCs). These PMC, though numerous, fail to produce fibronectin and expression of genes encoding contractile proteins alpha-SMA and Calponin1, are diminished. Presumably because of this defect, the testicular tubules are dilated and muscle contraction (required to facilitate movement of testicular sperm to the epididymus) is sluggish (as demonstrated in Supplementary videos). In terms of spermatogenesis, germ cells remain relatively undifferentiated although some progress through spermatogenesis and some spermatids are found in the epididymis.

Comments for the author

This is an interesting and well written and presented piece of work that I think will be of interest to those in the field of mammalian testis development, germ cell development and spermiogenesis. It is also likely to be of broad interest to the readership of Development.
There is quite a lot of attention to detail and most images are of high quality. I consider that all of the data data submitted in the Supplementary section are appropriate and essential for supporting the findings of the paper. The work is novel – relatively indeed little is known about the peritubular myoid cells and their contributions to testis structure and function.

1. The abstract mentions the spermatogonial stem cell niche in the first sentence, but then does not mention any results regarding spermatogenesis.

2. You do not refer to Figure 1B.

3. Figure 1D. How can we be sure that Hic1 is expressed in PTM cells here? They could be Sertoli cell nuclei? I am not sure you can say ‘validated the Hic1 expression in the testicular smooth muscle cells’.

4. Figure 3B – injected with 4-OH tamoxifem, not just tamoxifen. Throughout, tamoxifen is mostly used, other than in the figures. This is important for the reader I think (that you actually used the 4-OH form).

5. I don’t really follow the reasoning about the Hic1+ cells in different spermatogonial stages

6. “Hic1-tdTomato+ PMCs were observed in the seminiferous tubules at early (I-VI), mid (VII-VIII) and late (IX-XII) stages. The frequencies of Hic1+ PMCs to appear in early, mid and late stages were 51.14 (±2.76) %, 25.14 (±2.60) % and 23.72 (±2.86) % respectively, which were not significantly different from the frequency of the seminiferous epithelial stages reported in (Oakberg, 1956): 46.86%, 19.96% and 33.16%.” Aren’t we trying to find if Hic1 expression is particularly associated with a particular stage?

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7. Figure 4D – for SCP3 in the cKO, it looks like there are many normal looking Scp3+ cells (but you are not showing those in the high mag). Or is the apparent Scp3+ staining not real?

8. The areas of PMCs in Figure 5B are very tight, and no difference is found for wildtype compared with cKO – but in the images in Figure 5A they actually look quite different in size?

9. Does the blood-testis barrier form normally?

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

This manuscript examines the role of Hic1 in testicular peritubular cells, and finds that testicular structure and function are disrupted when Hic1 is deleted from smooth muscle cells. Little is known about how testicular peritubular cells, which are critical for spermatogonial stem cell niche function and immune regulation in the testis, are specifically regulated during development. The findings from this study could potentially contribute to our understanding of these important, but poorly-understood, testicular cells.

Reviewer 1 Comments for the Author:

In this manuscript, Uchida and colleagues examine the role of Hic1 in testicular smooth muscle cells. They observe that Hic1 expression is enriched in testicular peritubular cells and, using a Hic1-CreER lineage tracing/labeling method, they find that Hic1 expression is most prominent at 1 week of age; they also find that Hic1-expressing PMCs have high expression of the ECM component fibronectin. Using a tamoxifen-inducible aSma-CreER conditional KO (cKO) model to delete Hic1 from smooth muscle cells, they observe that cKO testes have dilated tubules that have an increased number of peritubular myoid cells (PMCs), reduced fibronectin expression, and reduced contractility; additionally, there appears to be a loss of differentiated germ cell types (although undifferentiated spermatogonia were increased in number). Based on these data, the authors conclude that Hic1 is a critical regulator of PMC function, which is subsequently necessary for testicular function.

Recent reports have demonstrated that peritubular cells, apart from well-appreciated roles in promoting tubule integrity and contractility, are also important players in the spermatogonial stem cell niche and in mediating immune and inflammatory responses in the testis.
Therefore, studies that will increase our knowledge of PMCs and how they are regulated will be of interest to the field. In general, the assays performed in this study address the hypothesis posed and the data is presented clearly. However, there are some concerns regarding the experimental design of some of the assays, which impacts the interpretation of the data; additionally, some of the central claims made in the manuscript are not strongly supported by experimental evidence. There are several points that the authors could address to improve the manuscript:

1. One of the central claims made by the authors in the manuscript, which is that "deletion of Hic1 promoted proliferation of PMCs...which subsequently altered the size of seminiferous tubules and testis" (lines 282-283) is not strongly supported by the data presented. In particular, there is no data showing that Hic1 specifically affects proliferation. The Tomato lineage tracing data in Fig. 2 suggests that Hic1- expressing PMCs proliferate more than Hic1-negative cells; therefore, one might expect that deleting Hic1 from PMCs would decrease proliferation, not increase it. Also, the CCND1 data also does not reveal any differences in proliferation in mutant tubules, although this assay was done at 8 weeks old, which is likely not the time point when PMCs are proliferating, and was not done with cell- type-specific markers. The authors should perform Ki67 and BrdU assays at the appropriate time points (likely during postnatal stages) with cell-type-specific markers and in the Tomato lineage-tracing models. Such assays would definitely address the

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claims made the authors regarding proliferation of Hic1-expressing PMCs in both control and cKO conditions, and are critical to support their claims.

Response: To answer this question, we performed Ki67 immunohistochemistry of WT and cKO mouse testes at 1wk-old, which is known as a time point for PMC proliferation (Nurmio et al., 2012). The proportion of testicular smooth muscle cells positive for Ki67 was higher in cKO mice compared to WT mice (see revised Fig.5E). We modified the manuscript throughout by reflecting this result.

2. Related to the previous comment, the authors do not consider the possibility that Hic1 deletion only directly results in an increase in luminal fluid content, which causes a general expansion of tubule diameter; the increase in PMC number in the cKO testis may merely be a secondary effect of the increased tubule diameter and not a cell-autonomous effect of Hic1 deletion. Therefore, the increased PMC number may be an effect/symptom of the increased tubule diameter, not a cause of it. The authors should perform more specific and definitive assays to address these scenarios.

Response: As we stated in the revised discussion section (lines 322-324, p.15), the secretion of luminal fluid starts around 2wk-old. Hence it is not likely that the proliferation of testicular smooth muscle cells we observed in 1wk-old time point is a secondary effect of the increased luminal fluid which expand the tubule diameter. The increased diameter of seminiferous tubule was only observed in adult mice, not in 4wk-old time point (Fig. 3D). To further explore whether the increase in seminiferous tubule diameter can affect the PMC number surrounding the seminiferous tubule, we ligated an efferent duct of wild-type mice at 7wk of age and examined its effect on PMCs at 8wk-old time point (see revised supplementary materials and methods). As a result, the number of PMCs around the seminiferous tubule did not significantly change (lines 255-259, p.12). We also ligated the efferent duct of mice at 1 and 4wk-old time points, yet it did not result in the fluid accumulation at 7wk-old time point (data not shown). Taken together, the increased PMC number around the seminiferous tubules observed in this study is unlikely to be induced by the increased seminiferous tubule diameter.

3. Even though vascular smooth muscle cells (VSMCs) are also a major target of the aSma- CreER driver used in this study, the authors did not mention these cells at all in this manuscript. Is Hic1 expressed in VSMCs? Did the Hic1-CreER lineage tracing label any VSMCs? Are blood vessels dilated in the cKO mice? Is there an increased number of VSMCs? Is their proliferation affected in cKO mice? Looking at VSMCs may shed additional light on the role of Hic1 in the testis, and whether it is specific to PMCs or not.

Response: Hic1-tdTomato signals were observed in the VSMCs, marked by adjacent PECAM-1-expressing endothelial cells at 1wk-old (see revised Fig.1F), suggesting that Hic1 is expressed both in PMCs and VSMCs. Lineage tracing experiments revealed the expression of tdTomato in 4wk-old vascular smooth muscle cells adjacent to PECAM-1+ endothelial cells (see revised Fig.2D). This result indicates that vascular smooth muscle cells also arise from Hic1 expressing cells present at 1wk of age. The proportion of testicular smooth muscle cells positive for Ki67, including PMCs and VSMCs, was higher in cKO mice compared to WT mice at 1wk of age (see revised Fig.5E, F). We could not evaluate potential vasodilation in the cKO mice, since blood vessels had a wide variety in diameter (lines 159– 161, p.8).

4. On line 100, the authors claim that there was “heterogeneous expression of Hic1 in the testicular smooth muscle cells...” Was this heterogeneity due to actual differences in Hic1 expression in individual cells, or was it due to inefficiency of the CreER line, or perhaps due to relatively low doses of tamoxifen (50 ug/g bw)? This could be addressed via in situ hybridization for Hic1, which was performed in Fig. 1D, but the image was not entirely convincing to make any claims about specificity or homogeneity/heterogeneity of expression.

Response: We optimized the protocol of in situ hybridization as shown in lines 488-489, page 22, and replaced the image of Hic1 in situ hybridization in Fig.1D. We further performed co-staining of Hic1 in situ hybridization and aSMA immunohistochemistry to confirm Hic1 expression in smooth muscle cells (see revised Fig.1E). The majority of testicular smooth muscle cells expressed Hic1 mRNA at 1wk of age, whereas approximately 55% of the PMCs were positive for tdTomato at 1wk

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of age (Fig.1C, H and Fig.2C). This can be due to the low efficiency of Cre or low doses of tamoxifen, or/and due to the heterogeneity of Hic1 expression in testicular smooth muscle cells at 1wk-old time point.

5. There are some technical concerns about using anti-SMA antibody to FACS-purify live cells. SMA should be an intracellular protein for the most part, so the antibody should not be able to penetrate live cells and provide specific fluorescence. That's why usually only cell- surface receptors or endogenous fluorescence are used for FACS purification of live cells. Otherwise, fixation and permeabilization with detergents is needed. Therefore, some may doubt if SMA antibody can be used for live cell purification and subsequent qPCR. At the very least, some flow cytometry plots with appropriate controls should be provided to satisfy readers that this is a technically sound, specific, and feasible assay.

6. Related to the previous comment, there are additional technical concerns using tdTomato-/SMA+ cells as the "negative population" of PMCs. If CreER activity is limited to the PMCs, then the tdTomato-/SMA+ cells will include PMCs and VSMCs, while the tdTomato+/SMA+ cells will only include PMCs. Therefore, these 2 populations will not be the same, which will confound the analyses.

Response to 5 and 6: We agree with these limitations, thus removed our data obtained by FACS sorting conducted with anti-aSMA antibody. We could not repeat this analysis with a reliable cell surface marker since there is no cell surface marker reported to distinguish smooth muscle cell populations to our knowledge. Also, tdTomato+ cells from aSMACreERT2:ROSAtdTomato include both PMCs and VSMCs, and it is a technical limitation to distinguish these two smooth muscle cell populations. As we described above, we now mention VSMCs in the whole manuscript.

7. The authors claim that there is differential cell proliferation between Hic1+ and Hic1- PMCs, but they do not address the role of cell death in potentially mediating the differential presence of certain PMC populations during development.

Response: We did TUNEL analysis at 1wk-old WT and cKO mice to examine the role of cell death in PMC proliferation, but we could not detect any PMCs positive for TUNEL (lines 251–252, p.12). In contrast, we observed some TUNEL+ germ cells within seminiferous epithelia of the testis section from 1wk-old WT and cKO mice, as reported previously (Wang et al., 1998).

8. While examining the estrogen receptors is a good idea, the analyses shown are based on whole testis. If ERs are affected only in the PMCs in Hic1 cKO mice, then any changes would likely be diluted out by all the other cells in the testis that express ER (likely Leydig cells, and potentially others). Such analyses should be done in purified PMCs to obtain a more definitive and robust answer.

Response: We performed qPCR analysis on the FACS sorted tdTomato-positive smooth muscle cells from WT and Hic1 cKO mice, which showed no significant difference in the expression of both Esr1 and Esr2. We modified the manuscript accordingly (lines 177-180, p.9).

9. The authors mentioned that there is sperm in the epididymis, but do not perform any other functional assays to test testicular function, which are critical assays. Are these mice sterile, sub-fertile, or fertile? Do they have reduced sperm count? Reduced sperm motility? Do they have a disrupted hormonal (HPG) axis? Some basic information regarding testis function is needed to know whether Hic1 peritubular function is required for male fertility.

Response: 4-OHT is an active metabolite of tamoxifen and a selective modulator of estrogen receptor, and its administration to juvenile males is reported to cause long-term effect (Patel et al., 2017), hence we cannot evaluate the effect of merely Hic1 deletion on their fertility. Nevertheless, we measured the weight of seminal vesicle as an indicator of testosterone (lines 168–169, p.8) and confirmed by CASA that cKO mice have motile sperm comparable to what has previously been reported for 4-OHT treated mice (lines 170–172, p.8).

10. Can the authors provide some data (or least speculate) as to why there are more undifferentiated spermatogonia in the cKO testis even though there are fewer Sertoli cells per

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tubule? This seems to be unexpected, as Sertoli cells are often thought to be a critical limiting factor in determining the number of SSCs and undifferentiated spermatogonia in the testis.

Response: In response to this comment, we added a new paragraph in discussion section in lines 338–360, p.16–17. The increased number of spermatogonia may reflect partial disruption or delay of meiotic differentiation of spermatogonia into spermatocytes. PMCs might also support spermatogonia considering their roles in supporting SSC niche. The functions of Sertoli cells and their tight junction were not severely affected (see revised Fig.S2), which may make them capable of supporting the spermatogonia.

11. The authors should provide some quantification for the images in Fig. 4D.

Response: We provide the quantitative data on both SCP3 and HSP70 counts in the revised manuscript (see revised Fig.4E, F, lines 217–219, p.10).

12. In Figure 5, the authors should provide information as to whether the increased number of PMCs in the cKO tubules are due to an increase in tdTomato+ cells only, or a general increase in both tdTomato+ and tdTomato-cells. This information would shed light on whether this phenomenon is cell-autonomous to Hic1- mutant cells or not.

Response: The majority of smooth muscle cells in adult cKO mice were tdTomato+ (Fig.S3A), yet some smooth muscle cells remained negative for tdTomato (Fig.5A). We modified the discussion section, referring to the possibility suggested by the reviewer (lines 332–336, p.15).

13. Some of the SMA staining on whole-mount tubules is difficult to see and not convincing, mostly due to the fact that the cells are so thin and flat. An additional cell-surface marker to definitively outline the cells would be helpful for the reader in some cases to discern individual cells in whole-mount preparations.

Response: aSMA antibody we used in this study was the best marker to visualize the testicular smooth muscle cells among the ones we tried. As we mentioned in lines 100–101 in page 5, aSMA is a widely-recognized and commonly used marker. To better visualize the smooth muscle cells in whole-mount preparation, we added the images taken by confocal microscopy to the figures (see right panels in the revised figures 1H, 2B and 5A).

14. In Fig. 6C, the authors look at SMA and Cnn1, but what about fibronectin, collagen IV, and Lama1? These analyses would allow the authors to see if the fibronectin loss is cell- autonomous, rather than a general secondary disruption caused by tubule size disruption.

Response: We performed qPCR analysis on fn1, col6a3 and lama1 as suggested, but none of these genes showed significant difference between WT and cKO mice (see revised Fig.S3C). As we show in revised Fig.S3A and B, fibronectin was present in vasculature, while not detected in basement membrane. The sorted tdTomato+ cells from WT and cKO mice include both PMC and VSMC populations, which might result in their similar gene expression levels of fn1. From this result, we cannot exclude the possibility that the loss of fibronectin in cKO PMCs could be secondary to the enlargement of cKO seminiferous tubules. We modified the manuscript accordingly (lines 386-497, p.18).

15. Can the authors provide more details about if there is any specific spermatogenic arrest? There are appear to be testicular and epididymal sperm, but there are also seems to be a reduced number of spermatocytes and spermatids. Is this a specific arrest or stage at which cells undergo cell death?

Response: To clarify which stage of germ cells are affected in cKO mice, we further quantified the numbers of c-KIT+ differentiating spermatogonia, SCP3+ spermatocyte and HSP70+ late spermatids (see revised Fig.4C-F). As a result, we found that germ cell population later than spermatocyte was decreased in cKO mice compared to WT mice (see revised Fig.4F). We also performed co-staining of TUNEL and immunohistochemistry as we show in revised Fig.4G, yet we could not clarify which cell population specifically is undergoing cell death due to the lack of marker expressed by TUNEL+ apoptotic cells (revised Fig.4G, lines 223–225, p.11). We added a discussion

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section regarding the increased spermatogonia / decreased spermatocyte and spermatids in lines 347–360, page 16-17 in the revised manuscript.

Minor points:

1. I am not sure what is required by the journal, but the authors should likely adhere to standard nomenclature for gene names in the field as dictated by the mouse genomics database (MGI at JAX), such as Acta2 for Sma, Fn1 for fibronectin, etc..

Response: We modified the throughout manuscript accordingly.

2. Some more details are needed for the seminiferous epithelial stage analysis. In particular, it was stated how many tubules were counted, but not how many cells were analyzed.

Response: The association of the cells within the seminiferous epithelia is the key to distinguish the stage (Meistrich and Hess, 2013). We added detailed explanation on how we distinguished seminiferous epithelial stages in Materials and methods section (see lines 471- 477, p.21–22).

3. The authors should provide some more explanation, either in the Methods section or Figure Legends, regarding what the box plot and error bars signify in the qPCR graphs (for example, in Fig. 1G, 1H, and 6C).

Response: The box plots in this study were all generated using R software, applying the R package of ggpubr. The median is shown as a line in the center of the box, and the whiskers show the range of sample distribution, excluding outliers. This information is now included (lines 514–516, p.23).

4. It was unclear why the cell sorting analyses, e.g., in Fig. 1H, were done in adult testes (presumably 8 week old). There are so few Tomato+ cells in that stage, so it may be difficult to get any robust gene expression differences. Perhaps an earlier stage would be more informative.

Response: In this study, we focused on the effect of Hic1 deletion on subsequent spermatogenesis after testicular development, hence conduced cell sorting on the mice at 8 weeks of age. However, as we pointed out above, we removed the results shown in original Fig.1 due to the lack of appropriate cell surface marker to sort testicular smooth muscle cells. We also tried to sort tdTomato+ cells from 1wk-old mice but we could not collect enough numbers of cells for conducting any qPCR analysis due to the small size of testis and smooth muscle cells.

5. On lines 205-206, the authors claim that SCP3 is a marker for “...more differentiated germ cells such as round spermatids...” However, SCP3 is more likely a marker for spermatocytes rather than spermatids.

Response: We modified the manuscript accordingly (see line 217-218, p.10).

6. A control image is needed for Fig. S1C.

Response: We provided a control image in revised Fig.S1C.

7. A low-magnification image of Fig. 1C would be informative to show the specificity of Hic1-CreER activity and to show that is only in PMCs. A low-mag image would also be nice for Fig. 4A.

Response: As requested, we added low-mag images into the revised figures 1C and 4A.

8. Can the authors comment on the efficiency of aSma-CreER targeting? What percent of PMCs are targeted, both in the control and Hic1-flox/flox background?

Response: The proportion of testicular smooth muscle cells labelled with aSMACreERT2:ROSA26tdTomato was 76.43% in this study (lines 141–142, p.7). Knock out efficiency of Hic1 by using the Hic1flox mouse strain was reported to be 92.4% in Scott et al., 2019. We describe aSMACreERT2:Hic1flox/flox:ROSA26tdTomato mouse strain in another paper focused on

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skin mesenchymal progenitor cells (Abbasi, Sinha, Labit et al., Cell Stem Cell, in revision). In this paper, we analyzed the Hic1 KO cells both in vitro and in vivo, and observed robust increase in their cell proliferation as we observed in this study.
Taken together, these studies suggest that recombination and gene deletion using this mouse strain is sufficiently high enough to effectively evaluate the impact of targeted deletion of Hic1.

9. A germ cell co-stain in Fig. 6E would be informative.

Response: We assume this was original Fig.4E. We added a new image showing co- staining of TUNEL and VASA as a general germ cell marker (see revised Fig.4G).

10. Which cell types are CCND1+ in Fig. 4G? An image would be nice.

Response: CCND1+ cells were negative for GATA4, which is a marker for Sertoli cells (see revised Fig.4I). Since seminiferous epithelia contain only germ cells and Sertoli cells, CCND1+ cells are germ cells, specifically spermatogonia due to their location within the basal compartment of seminiferous epithelia. We modified the manuscript accordingly (lines 227–230, p.11).

11. The CNN1 images in Fig. S2B are not very convincing to show the differences in expression between control and cKO. In general, Western blots would be a more robust way to show quantitative differences as compared to immunofluorescence.

Response: We performed Western blot analysis for CNN1 by using whole testis, but the protein expression levels of CNN1 showed no significant difference between WT and cKO mice (lines 296-300, p.14). It might be related to protein longevity, or, Western blot might not be sensitive enough to detect the difference.

Reviewer 2 Advance Summary and Potential Significance to Field:

Compared to other cell types in the testis, relatively little is known about peritubular myoid cells, except that their contractibility helps moving the sperm produced towards the rete testis and that these cells produce factors that influence the behaviour of spermatogonial stem cells. The present manuscript now provides a wealth of new data on peritubular myoid cells. It was found that Hypermethylated in cancer 1 (Hic1) plays an important role in these cells, in such a way that it regulates architexture and contractility of the seminiferous tubules. In Hic1 deficient mice more PMCs are formed that miss fibronectin and have lower levels of several smooth muscle contractile proteins. The authors have studied various aspects of this investigations in a rather methodical way, the experimental design is excellent and the results are very well described. The results provide many interesting new details about how seminiferous tubule architexture and tubule function are regulated.

Reviewer 2 Comments for the Author:

There is one important aspect that has largely been overlooked. The authors show that in Hic1 deficient mice the numbers of Sertoli cells per tubule cross-section are decreased and in addition tubule circumference has increased. These data reinforce each other and as a result the density of the Sertoli cells on the basal lamina in Hic1 deficient testes likely is substantially lower than in wild type mice. As spermatogenesis critically depends on the support of Sertoli cells and as germ cells even may need contact with Sertoli cells, would a shortage of Sertoli cells not explain the poor spermatogenesis in the KO mice? Although the authors show that individual Sertoli cells behave normally, their low numbers may still cause too low levels of factors needed by germ cells. The authors should calculate how much lower the numbers of Sertoli cells are in KO mice and discuss the possibility that this is the cause both of the poor quality of spermatogenesis and the small height of the seminiferous epithelium in the absence of Hic1. Will the Sertoli cell barrier still be intact in KO mice?

Response: As we show in Fig.4A, the number of Sertoli cells per cross-sectioned seminiferous tubule was 12.48 ± 0.37 in cKO mice, which is significantly smaller than both Het: 15.23 ± 0.28 and WT: 16.50 ± 0.20. This means that cKO mice have 24.26% reduction in the number of Sertoli cells compared to WT mice. From previous work, it is known that meiosis is supported by Sertoli

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cells (Chen and Liu, 2015), and the number of spermatids correlates with Sertoli cell number (Orth et al., 1988). Therefore, it is possible that the lower number of Sertoli cells in cKO mice is associated with the disrupted spermatogenesis. We discussed this possibility in the revised manuscript, in lines 356-360, p.16–17. Two major blood-testis barrier (BTB) components, Occludin and Claudin 11 (McCabe et al., 2016), were both present in cKO mice (see revised Fig.S2B–C), and the junction structure between Sertoli cells appeared to be normal in transmission electron micrographs (see revised Fig.S2D). From these results, we speculate that the BTB remains intact in cKO mice. We modified the result section accordingly (lines 192–202, p.9–10).

Reviewer 3 Advance Summary and Potential Significance to Field:

Hic1 encodes a member of the Kruppel/Zinc finger and BTB (POK/ZBTB) family of transcription factors, and is thought to act as a repressor and as a tumor suppressor. The authors show that Hic1 is required in peritubular myoid cells (PMC) of the postnatal testis because when the gene is deleted specifically in the PMCs shortly after birth testis development and spermatogenesis are abnormal. In particular, when Hic1 is not present, PMC number is increased (the normal function of Hic1 might be to prevent over- proliferation of PMCs). These PMC, though numerous, fail to produce fibronectin and expression of genes encoding contractile proteins alpha-SMA and Calponin1, are diminished. Presumably because of this defect, the testicular tubules are dilated and muscle contraction (required to facilitate movement of testicular sperm to the epididymus) is sluggish (as demonstrated in Supplementary videos). In terms of spermatogenesis, germ cells remain relatively undifferentiated although some progress through spermatogenesis and some spermatids are found in the epididymis.

Reviewer 3 Comments for the Author:

This is an interesting and well written and presented piece of work that I think will be of interest to those in the field of mammalian testis development, germ cell development and spermiogenesis. It is also likely to be of broad interest to the readership of Development. There is quite a lot of attention to detail and most images are of high quality. I consider that all of the data data submitted in the Supplementary section are appropriate and essential for supporting the findings of the paper. The work is novel – relatively indeed little is known about the peritubular myoid cells and their contributions to testis structure and function.

1. The abstract mentions the spermatogonial stem cell niche in the first sentence, but then does not mention any results regarding spermatogenesis.

Response: We modified the abstract, focusing more on the testicular smooth muscle cells instead of spermatogonial stem cell niche.

2. You do not refer to Figure 1B.

Response: We now refer to Fig.1B in lines 95-97, page 5 in the revised manuscript.

3. Figure 1D. How can we be sure that Hic1 is expressed in PTM cells here? They could be Sertoli cell nuclei? I am not sure you can say ‘validated the Hic1 expression in the testicular smooth muscle cells’.

Response: We performed co-staining of *Hic1* in situ hybridization and aSMA immunohistochemistry which show the co-localization of Hic1 and aSMA signals (see revised Fig.1D,E). This result provides evidence of *Hic1* mRNA expression in PMCs, not in Sertoli cells.

4. Figure 3B – injected with 4-OH tamoxifem, not just tamoxifen. Throughout, tamoxifen is mostly used, other than in the figures. This is important for the reader I think (that you actually used the 4-OH form).

Response: We modified the term “tamoxifen” into “4-OHT” throughout the revised manuscript.

5. I don’t really follow the reasoning about the Hic1+ cells in different spermatogonial stages

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Response: The purpose of this analysis is to see whether the Hic1 expression in PMCs is associated with specific seminiferous stages. We modified the sentence “To test a potential relationship between the seminiferous epithelial cycle and the distribution pattern of Hic1+ PMCs” (lines 122-123, p.5 in original manuscript) into “To see whether Hic1 expression in adult mouse testis is associated with specific seminiferous epithelial stage” (see revised manuscript, lines116-117, p.6).

6. “Hic1-tdTomato+ PMCs were observed in the seminiferous tubules at early (I-VI), mid (VII-VIII) and late (IX-XII) stages. The frequencies of Hic1+ PMCs to appear in early, mid and late stages were 51.14 (±2.76) %, 25.14 (±2.60) % and 23.72 (±2.86) % respectively, which were not significantly different from the frequency of the seminiferous epithelial stages reported in (Oakberg, 1956): 46.86%, 19.96% and 33.16%.” Aren’t we trying to find if Hic1 expression is particularly associated with a particular stage?

Response: As suggested, the purpose of this analysis is to see whether the Hic1 expression in PMCs is associated with specific seminiferous stages. We elaborated the explanation on the rationale to clarify this (see lines 116-117, p.6 in the revised manuscript).

7. Figure 4D – for SCP3 in the cKO, it looks like there are many normal looking Scp3+ cells (but you are not showing those in the high mag). Or is the apparent Scp3+ staining not real?

Response: As stated in lines180-182, p.8 in the original manuscript, cKO mice have seminiferous tubules with very severe to relatively mild phenotypes of aberrant spermatogenesis. Therefore, there are indeed normal looking SCP3+ cells in cKO mice. We have now included clearer staining images of SCP3, showing both a severely affected tubule (right) and a tubule with normal looking SCP3+ cells (left) in the panel for cKO (see revised Fig.4E). We further provide quantitative data on SCP3+ cells in WT and cKO mice (see revised Fig.4F).

8. The areas of PMCs in Figure 5B are very tight, and no difference is found for wildtype compared with cKO – but in the images in Figure 5A they actually look quite different in size?

Response: We observed various sizes of PMCs both in WT and cKO mice, which can be an interesting aspect of PMC heterogeneity. However, when we quantify the PMC area, the size of PMCs in WT and cKO are not significantly different as we showed in Fig.5B. We added new confocal images of PMCs in Fig.5 as it facilitates the visualization of PMC outlines.

9. Does the blood-testis barrier form normally?

Response: blood-testis barrier (BTB) appeared to form normally in cKO mice (see revised Fig.S2B-D). We mention BTB in the result section of revised manuscript (lines 192–202, p.9–10).

References:

[1] Patel, S.H., O'Hara, L., Atanassova, N., Smith, S.E., Curley, M.K., Rebourcet, D., Darbey, A.L., Gannon, A.L., Sharpe, R.M. & Smith, L.B. (2017). Low-dose tamoxifen treatment in juvenile males has long-term adverse effects on the reproductive system: implications for inducible transgenics. *Sci. Rep.* 7, 8991-017-09016-4.

[2] Wang, R.A., Nakane, P.K. & Koji, T. (1998). Autonomous cell death of mouse male germ cells during fetal and postnatal period. *Biol. Reprod.* 58, 1250-1256.

Second decision letter

MS ID#: DEVELOP/2019/185884

MS TITLE: Development and Function of Smooth Muscle Cells is Modulated by Hic1 in Mouse Testis

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AUTHORS: Aya Uchida, Sadman Sakib, Elodie Labit, Speideh Abbasi, Wilder Scott, Michael Underhill, Jeff Biernaskie, and Ina Dobrinski
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

In this study, the authors have described a gene, Hic1, that plays a key role in the development and function of peritubular cells in the testis. Very little is known about the functional role of these cells, so this research is a significant contribution to our knowledge of these cells and how they contribute to testicular development and fertility.

Comments for the author

The authors have very thoroughly addressed the reviewer's comments. There are no major outstanding scientific or technical concerns. However, there are 2 minor points that the authors should re-visit: 1) In the text, there appears to be an error on line 150, where the authors mistakenly stated that the 4-week WT testis/body weight ratio was 2.68, when it is clearly around 1.5 according to Figure 3G. 2) On line 168, the authors report the weight of seminal vesicles in cKO males, but it is unclear if this is significantly different from controls.
Citing a previous reference is fine but, given potential differences in organ weight due to genetic background, environment, etc., if the authors have this data, they should include it in the manuscript. Otherwise, the authors have done a comprehensive job in this revision and have significantly improved the manuscript.

Reviewer 2

Advance summary and potential significance to field

See my initial review

Comments for the author

The authors have responded well to my comments. I have no further comments.

Reviewer 3

Advance summary and potential significance to field

Hic1 encodes a member of the Kruppel/Zinc finger and BTB (POK/ZBTB) family of transcription factors, and is thought to act as a repressor and as a tumor suppressor.
The authors show that Hic1 is required in peritubular myoid cells (PMC) of the postnatal testis because when the gene is deleted specifically in the PMCs shortly after birth testis development and spermatogenesis are abnormal. In particular, when Hic1 is not present, PMC number is increased (the normal function of Hic1 might be to prevent over- proliferation of PMCs). These PMC though numerous, fail to produce fibronectin and expression of genes encoding contractile proteins alpha-SMA and Calponin1, are diminished. Presumably because of this defect, the testicular tubules are dilated and muscle contraction (required to facilitate movement of testicular sperm to the epididymus) is sluggish (as demonstrated in Supplementary videos). In terms of spermatogenesis germ cells remain relatively undifferentiated although some progress through spermatogenesis and some spermatids are found in the epididymis.

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Comments for the author

In my opinion the authors have satisfactorily addressed all comments from all reviewers.

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