nature portfolio

Peer Review File

Fallopian tube rheology regulates epithelial cell differentiation and function to enhance cilia formation and coordination

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The range of viscosity cited from the fallopian tube in the introduction is 1-100 mPa which is a quite wide range. It must be noted that the fallopian tube contains specialized regions with complex primary to tertiary folding of the epithelium an d with varying amount of ciliated and secretory cells. The activity of both cell types either in cilia movement or mucus secretion and resorption varies over the ovarian cycle and is under endocrine and paracrine control. This makes that the viscosity in the lumen of the oviduct is both varying at different time periods within the ovarian cycle but also in the longitudinal axis (fimbriae, ampulla, isthmus, uterine tubular junction) but also radially (for instance in the tertiary crypts versus at the center of the duct).

The current study provides detailed features of cilia formation and coordination in isolated epithelial cells of the ewe oviduct cultured on collagen coated multi wells plates in 2D-cell culture system.

Points of attention:

1. It is for me questionable whether the differentiated morphology of the cultured epithelium can be maintained: In our hands (oviduct cultures form other mammalian species) a rapid dedifferentiation, deciliation and flattening of oviduct epithelial cells occurs in 2D-cell culture systems (within a day of culture). The authors should demonstrate that the % of ciliated cells and the length of the cilia is not affected by the cell culture.

2. It is better to perform oviduct cell cultures in an trans-well system where a confluent columnar oviduct epithelium can be formed and cultured with a specific basolateral side with nutrient feeding medium and a apical side which can be manipulated with varying viscosity media. This resembles more the physiological topology of increased viscosity caused naturally by secretion of mucous. It also allows to measure whether the confluency can be maintained under the higher viscous conditions at the apical compartment. It would either be good to compare the results of this study with trans-well cell cultures or to discuss this way of cell culture properly.

3.I miss more precise information on in situ local viscosity throughout the fallopian tube as well as changes therein through the estrous cycle. In the discussion more attention can be paid to this as it highlights the potential importance of this in vitro study. For instance it may cause another coordinated movement of cilia allow different interactions of sperm that initially bind to the cilia to be released after a certain interaction period where these released sperm are supposed to have attained fertilization capability. Or likewise a change in coordinated beating may guide the oocyte/embryo to be transported towards the uterus. Of course aberrations in secretions may alter

viscosity parameters and thus also result in a mismatch in cilia formation/coordination affecting sperm binding and release properties and or correct oocyte/embryo migration toward the uterus. In the discussion also attention should be paid to fertility problems related to too high or too low fallopian viscosity. As it reads now the manuscript gives nice data on cilia properties of oviduct cultures under different viscosity media but lacks to relate this to the physiological relevance.

4. The major findings in viscosity dependency of formation cilia and coordinated activity are new to the field. The authors carefully and with rigor have performed analysis on the properties of cilia under various viscosity environments. Despite points 1-3 I think these study is important both for the field of reproductive biology but also more in general for studies on tissues that have a ciliated epithelium.

Reviewer #2 (Remarks to the Author):

The authors study the role of external fluid viscosity on ciliary beat differentiation and phase coordination. The authors conclude that high viscosity promotes ciliation and facilitates the transport of egg and embryo for successful fertilization outcomes.

I have a comment on how the authors regulate viscosity by increasing the concentration of methylcellulose in the external fluid. It should be noted that it's not just viscosity that is affected, but also the osmotic pressure of the fluid. Two parameters change, therefore, which cannot be separated. The effects observed therefore stem from the increase in methylcellulose concentration, but it is not clear whether they result exclusively from the increase in viscosity or also, for example, from the increase in osmotic pressure induced by the increase in methylcellulose concentration. In particular, it has been shown that osmotic pressure plays on cilia, which can collapse in the airways when osmotic pressure increases (Button et al Science 2012). The authors should comment.

The methodology is sound. Details provided in the methods are fine.

It is clearly shown that increasing methylcellulose concentration plays a role on the density and proportion of ciliated cells during development. However, there was no analysis of the final mature tissue. Ultimately, when the tissue is mature, are there more ciliated cells on the epithelium? What is the cell density on the mature epithelium obtained? These questions are important for egg transport.

The authors also show that linear rise in TRPV4 intensity is observed under viscous loading. It results in increasing intracellular Ca2+, subsequently decreasing the MMP level and increasing ATP production. The question then arises as to whether ciliary beating is limited by ATP or whether ATP is present in sufficient quantity to always allow cilia to beat efficiently. The authors should discuss.

I don't understand why the TRPV4 study is shown after the discussion paragraph. It doesn't seem logical.

As regards ciliary activity, it is clearly shown that the frequency of cilia beating decreases under viscous loading. However, the reason is not discussed. Is it a physical reason linked to the increase in hydrodynamic resistance as viscosity increases? The velocity of a cilium in a viscous medium is proportional to the force exerted divided by the viscosity (Stokes' law). If the force exerted by a cilium is constant (fixed by the molecular motors in the presence of ATP), then the velocity of the cilium and therefore its frequency decreases as the medium becomes more viscous. Or is it a biological effect? One consequence of increased viscosity could also be to reduce the amplitude of the beats (which seems to be observed on the movie). The authors should analyze the evolution of the beating pattern as a function of viscosity. This is an important parameter for transport. In both cases, the function would be rather degraded as viscosity increases.

Regarding metachronal waves, the results show that the phase coordination seems to be better upon increase in viscosity but this coordination is on a very small spatial scale (a few microns) and we do not fully understand how this could be very important. to improve transport over a centimeter distance. There are no waves that propagate long distances and the wave front is very narrow. Furthermore, the authors do not discuss the nature of the wave (antiplectic, symplectic, etc.). The nature of the wave plays a big role in the efficiency of transport. We therefore do not know whether the waves are effective in improving transport.

More troubling is that the authors do not look at the most basic parameter of transport, which is the direction of beating of the cilia. if the cilia do not beat in the same direction, it is very difficult to transport efficiently and beating direction coordination is more important for transport than phase coordination. If the directions are the same, transport will take place even in the absence of metachronal waves (like on airways where such waves are not really observed ref: Feriani, L. et al. Assessing the Collective Dynamics of Motile Cilia in Cultures of Human Airway Cells by Multiscale DDM. Biophys. J. 113, 109–119 (2017); and : Sanderson, M. J. & Sleigh, M. A. Ciliary activity of cultured rabbit tracheal epithelium: beat pattern and metachrony. J. Cell Sci. 47, 331–347 (1981)). On the other hand, if the directions are not coordinated, there will simply be no transport. However, on the films, we clearly see that the cilia do not beat in a single direction, whether the medium is viscous or not. It is first necessary to analyze the coordination of the beating directions as a function of viscous loading.

So, conrcerning the transport function, I'm not at all convinced of the role of viscous loading, since the analysis is based solely on metachronal waves.

Transport depends on

- the density of ciliated cells on the mature ciliated epihtelium

- directional coordination of ciliary beats

- beat pattern

- beat frequency

- and finally, to the second order, the presence and nature of metachronal waves.

The role of viscous loading on the first 3 points is not discussed by the authors, while the last two points do not seem to go in the same direction.

Metachronal waves can improve transport, but the first points are major. In particular, metachronal waves do not exist on airways on long scales, and when they are found, they are not of the same nature as those that optimize transport (Mesdjian et al. Phys Rev Lett, 2022). I find it very limiting to estimate the function only from metachronicity, especially as the space scale of phase coherence seems to be on one or two ciliated cells only and on a developing epithelium. This is not a dense, uniform carpet of cilia. It's also worth noting that the egg is over 100 microns in size, much larger than the metachronal wave and with a speed much slower than that of a cilium. The same cilia are going to beat any times under the egg, while it progresses. The image of an egg being carried from cilium to cilum by a coordinated movement is therefore erroneous. In my opinion, it is essential that the authors carry out a thorough analysis of the efficiency of transport, and not just on the basis of waves.

Reviewer #3 (Remarks to the Author):

This is an interesting study that explores the hypothesis that the ciliation and cilia beating pattern of fallopian tubes is responsive and could be controlled by the rheological properties of its environment, specifically its viscosity. The authors propose the important role of the TRPV4 channel

in this regulation. The manuscript is well-written and most of the data support the conclusions. This study is also physiologically important and provides important new insight into the regulation of this important process. However, the absence of essential controls, such as TRPV4 knockout mouse (which is available) or KO cell line, or at least an explanation of why these controls were not utilized dampens the enthusiasm for this work.

Editorial suggestions:

• The authors describe TRPV4's role in elevating intracellular Ca2+ and state that it results in mitochondrial depolarization events under different viscosity. However, how the Ca2+ level/mitochondrial depolarization is related to any of the FTEC characteristics (ciliation/beating behavior) is not stated well. A smooth connecting sentence is missed there.

• Moving Figure 5 to the result section could be beneficial, as it suggests the potential mechanism for TRPV4 involving in regulation of the cilia beating. The discussion section can spread out more on YAP and Notch signaling, which could be the potential regulatory pathway for ciliation. This part is good to stay in the discussion session as no following experiment data in the paper is provided to support this deduction. How YAP might relate to TRPV4 is not discussed in the paper either.

Other major issues:

• Figure 1/Suppl. Fig.1: the media was replaced every 24 hours during 72 hours of this proliferation assay and cells exposed to a higher viscosity proliferated much less. What if the latter suffer from poorer attachment to the surface and as a result were aspirated at a higher number? Was it controlled and if so, how? Similarly, in supplementary figure 1, the author provides under different culture conditions, the total number of dead cells is similar. They didn't show whether the aspiration of media can take any live cell or not. There is a possibility that ciliated cells attach to the dish better/stronger than secretory FTEC, so a high viscosity media changing process can take secretory FTEC cells off the plate, which makes false positive seeing under 200 mPa·s more cells on the plate are ciliated cell rather than secretory cell.

• Figure 2, panel A: As viscosity increases, the cell size under 50 mPa·s, 100 mPa·s, and 200 mPa·s looks larger (or flatter) than at 1 mPa·s, which is not very consistent with the bright field images in Figure 1 panel A and a statement that an area/cell does not change (Fig 1d). Why?

• Figure 2, panel B, C; Figure 3 (and the entire bar graphs): The bar graphs should include individual data points, and not only means +/- sd/sem. Including proliferation assay images alongside Ki-67 immunofluorescent staining could be beneficial.

• The proof of TRPV4 functional expression in FTEC needs to be shown. Suppl Fig.9 shows largely cytoplasmic immunostaining. TRPV4 is an ion channel exclusively plasma membrane protein. Either antibodies are nonspecific, or this is an autofluorescence. Clear membrane staining should indicate TRPV4 presence, which is not the case here. Western blot data would also be beneficial.

• Overall, not utilizing tissue from TRPV4 KO mice or TRPV4 KO cell lines is not justified. Data exploring the effect of TRPV4 knockdown on cilia beating coherence is missing in Figure 4.

• Vehicle control is missing from Figure 4e. Was the inhibitor dissolved in the DMSO or ethanol? Corresponding vehicle control should be used as a control condition.

• Figures 4 and 5: These experiments focus on proving TRPV4's role in maintaining the viability of the FTEC under various viscous conditions. It lacks the connection of TRPV4 to the cilia beating function. If the author just wants to state all the physiological relevant changes they found under various viscosity conditions, but not mention TRPV4 is important on cilia beating function, the abstract section needs to be reorganized. Otherwise, some further experiments need to be done:

a. Show under different viscosity conditions, TRPV4 expression levels on ciliated FTEC cells might be different (QPCR, western blot, and/or IHC).

b. Show adding an inhibitor of TRPV4 may lead to less vigorous movement of cilia, but by adding a TRPV4 activator the activity can be restored.

c. Show in vivo data, like collecting fallopian tube epithelial cells freshly dissected from mice at different estrus cycle stages and compare the expression level of TRPV4 (this data can be analyzed from the published literature).

Some minor issues:

• The dot position in "mPa·s" is not consistent in the paper. Some are "mPa.s"

• The first time mention YAP in the introduction should use the full name instead of an abbreviation. This is true for other abbreviations as well.

• Figure 1d: what exactly was measured here as "y-axis": 'area per cell' or actually "cell size"? I

• Figure 5 should be explained in the results, instead of the discussion

Reviewer 1

We appreciate the Reviewer's input, the result of which is an improved manuscript. Sections of the original manuscript that were revised (added, edited or reordered) are indicated both below and in the revised manuscript.

Comment 1 – 1

The range of viscosity cited from the fallopian tube in the introduction is 1-100 mPa which is a quite wide range. It must be noted that the fallopian tube contains specialized regions with complex primary to tertiary folding of the epithelium and with varying amount of ciliated and secretory cells. The activity of both cell types either in cilia movement or mucus secretion and resorption varies over the ovarian cycle and is under endocrine and paracrine control. This makes that the viscosity in the lumen of the oviduct is both varying at different time periods within the ovarian cycle but also in the longitudinal axis (fimbriae, ampulla, isthmus, uterine tubular junction) but also radially (for instance in the tertiary crypts versus at the center of the duct).
The current study provides detailed features of cilia formation and coordination in isolated epithelial cells of the ewe oviduct cultured on collagen coated multi wells plates in 2D-cell culture system.

Response 1 – 1

Thank you for your positive feedback and careful consideration of our work. We appreciate the key points mentioned by the reviewer and have revised the manuscript to more clearly highlight the highly dynamic nature of the tubal fluid viscosity, which varies both along the length of the tube and throughout the menstrual cycle.

Pg. 2

The female reproductive tract is a complex microenvironment lined with folded epithelium that provides a range of rheological and biochemical cues to facilitate fertilisation[1–3]. The geometrical complexity of the tract, including epithelial folds and crypts, considerably increases surface area to enhance nutrient transport, optimizing the environment for egg support and fertilization[4,5]. The tubal fluid in the fallopian tube originates from blood plasma transduction[6] and secretion of metabolic components and proteins via the lining epithelium[7,8]. The secretory products such as glucose, amino acids and oviduct-specific glycoprotein play important roles in reproductive events[9] by providing nutrients to promote gamete function and embryo development[10]. Steroid hormones, such as progesterone and estrogen, regulate the secretory activity of epithelial cells to modulate the composition and concentration of their secretions, thus altering rheology of the tubal fluid[11–13]. The epithelium lining the tract becomes less extensively folded and decreases in height from the ampulla to the isthmus[14], coupled with an increasing depth of crypts[15]. During the menstrual cycle, crypt epithelium consists mainly of secretory cells[15]. Hence, the viscosity of the mammalian tubal fluid varies considerably along the fallopian tube[16,17], radially (near the folds, crypts and lumen) and throughout the menstrual cycle[18,19], ranging from 1 mPa·s (similar to that of water) to over 10^3 mPa·s (similar to that of glycerine)[19–22]. The tubal …

Pg. 2,3

… The tubal fluid is more viscous at the utero-tubal junction - the port of entry to the fallopian tube[6] and decreases towards the ampulla and infundibulum at the distal end[7,9] – a mechanism to prevent sperm with poor motility from entering the fallopian tube[23,24]. The composition of oviductal secretion also varied over the menstrual cycle, induced by endocrine stimuli and influenced by both paracrine and autocrine effects[25]. The tubal fluid is most viscous …

Pg. 3

… embryo along the tube[34,39,40]. The ratio of ciliated to secretory cells changes during the menstrual cycle[39,41] and also increases along the length of the fallopian tube, from ~30% in the isthmus to ~80% closer to the site of fertilisation in the ampulla[34,42]. …

References

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Comment 1 – 2

Points of attention:
1. It is for me questionable whether the differentiated morphology of the cultured epithelium can be maintained: In our hands (oviduct cultures form other mammalian species) a rapid dedifferentiation, deciliation and flattening of oviduct epithelial cells occurs in 2D-cell culture systems (within a day of culture). The authors should demonstrate that the % of ciliated cells and the length of the cilia is not affected by the cell culture.

Response 1 – 2

We thank the reviewer for their valuable feedback. In response, we conducted new experiments to indicate how ciliation and cell morphology are maintained in high-viscosity culture systems, compared with cells cultured using Transwell inserts. The results indicate similar trends and cilia lengths when comparing the two culture systems. It is also noteworthy that while previous works have discussed the importance of 2D vs 2.5D vs 3D culture systems for long-term culture, our work investigates the effects of culture media viscosity for the first time.

Pg. 6,7

We also compared our well plate culture system with cells cultured in transwell systems under different viscosities (Supplementary Figure 2). For cell culture using transwell systems, the same overall trend as in traditional well plates was observed, where the proportion of ciliated cells increased significantly with increasing media viscosity (from 6.5% at 1 mPa·s to 18.5% at 200 mPa·s). These results indicate that high-viscosity culture also facilitates ciliation in epithelial cells. Moreover, the overall morphology of the cells was comparable between well plate and transwell systems (Supplementary Figure 2c), with no significant changes in cilia length ($p \geq 0.05$) between the two culture systems (well plate vs. Transwell). Both measurements were also directly comparable with the cilia length of cells from the epithelial tissue (4.9±0.3 µm). Interestingly, the percentage of ciliated cells under Transwell culture was higher compared to cells in a well plate. This lower percentage of ciliated cells in well plates is potentially attributed to the inherent nature of our 2D culture system, which promotes flattening and deciliation of epithelial cells[45,46]. In contrast, the viscoelastic and inherent 3D structure of MC polymeric chains in high-viscosity buffers may have the opposite effect, facilitating ciliation. This latter result is relevant to previous work where viscoelasticity and extracellular fluid density have been shown to influence the development and spreading behaviour of other cell types[51–53]. It is …

Pg. 8

… cultured at 200 mPa·s. While the cells demonstrated active proliferation by mitosis over multiple passages, the results suggest the potential role of high viscous loading on maintaining growth and differentiation competency of the FTEC over time.

Pg. 15

The significant increase in ciliation at higher viscosities (Fig. 1e), with comparable trends between well plate and transwell systems (Supplementary Figure 2), and cilia lengths from these systems similar to native tissue, indicate the crucial role of a high-viscosity environment in facilitating ciliation and maintaining the developmental behavior of epithelial cells. This increasing ciliation …

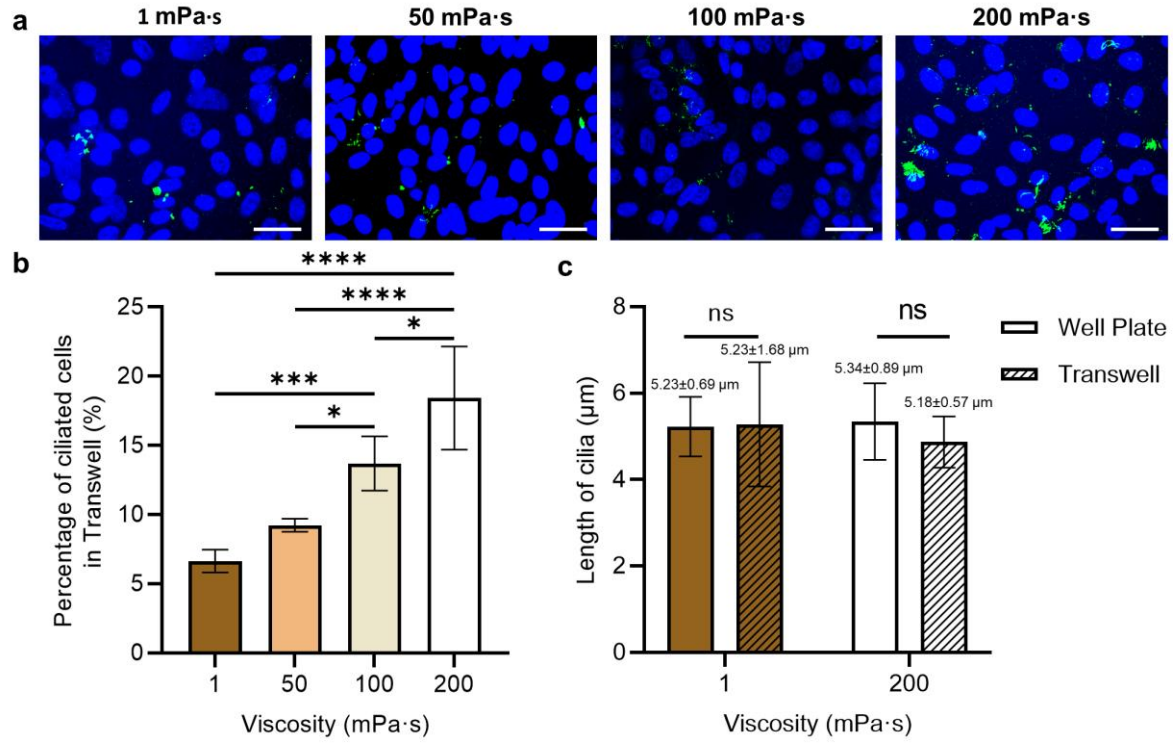
Pg. 20 - Methods

… analysis was carried out at 72-hour timepoint. The same culture protocol was used for culturing cells in transwells, with cells seeded in the insert and the culture media in the bottom well replaced over time.

Pg. 21 - Methods

… for TRPV4 assays, before being processed in ImageJ. Reconstructed confocal microscopy z-stacks were used to view the side profiles of ciliated cells and measure cilia length. The expression of TRPV4 channels …

Supplementary Figure 2a-c – *New Figure, New experiments*



Supplementary Figure 2 | Comparison of the effects of culture media viscosity between well plate and transwell cultures. (**a**) Representative immunofluorescent images of FTEC 72 hours post-seeding in transwell, where the green stain indicates the cilia and the blue stain indicates nuclei. Scale bars, 30 µm. (**b**) The proportion of ciliated cells as a function of viscosity, and (**c**) cilia length at 1 mPa·s and 200 mPa·s for cells cultured in well plates compared to transwell systems, after 72 hours of culture. (**d**) Cell density (number of cells per cm^2) for cells culture in transwell systems, and (**e**) nucleus size at 1 mPa·s and 200 mPa·s for cells cultured in well plates compared to transwell systems, after 72 hours of culture. Data are represented as mean ± s.d. and analyzed using one-way ANOVA with Tukey post hoc testing in b and d, and using student t-test in c and e, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$. Experiments were carried out on 3 different animals, with over 200 cells analyzed for each datapoint in b and d, and over 20 cells analyzed in c and e.

Comment 1 – 3

2. It is better to perform oviduct cell cultures in a transwell system where a confluent columnar oviduct epithelium can be formed and cultured with a specific basolateral side with nutrient feeding medium and an apical side which can be manipulated with varying viscosity media. This resembles more the physiological topology of increased viscosity caused naturally by secretion of mucous. It also allows to measure whether the confluency can be maintained under the higher viscous conditions at the apical compartment. It would either be good to compare the results of this study with transwell cell cultures or to discuss this way of cell culture properly.

Response 1 – 3

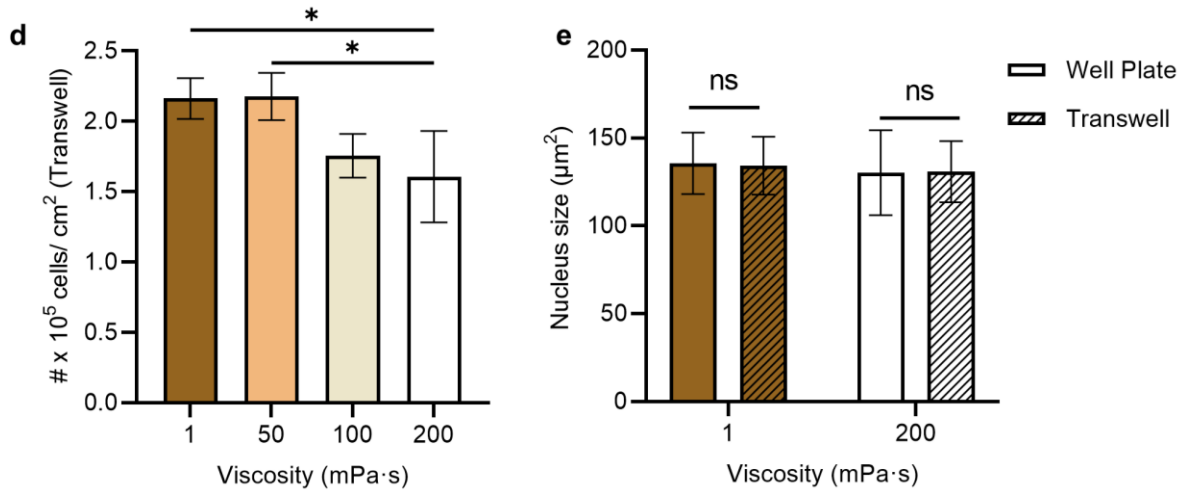
In agreement with the reviewer's suggestion, and as detailed in ***Response 1-2***, we conducted new experiments at different viscosities in transwell systems and compared our results, indicating similar ciliation trends, developmental behaviour, confluency trends, and nucleus size between transwell and well plate cultures.

Changes <u>additional</u> to those covered under ***Response 1-2*** are shown below.

Pg. 7

… spreading behaviour of other cell types[51–53]. It is noteworthy that the cell density (number of cells per unit area of the well) of cells cultured in transwell maintained the same decreasing trend as compared with well plate culture, decreasing from $2.2\pm0.1\times10^5$ cells/cm^2 at 1 mPa·s to $1.6\pm0.1\times10^5$ cells/cm^2 at 200 mPa·s (Supplementary Figure 2d), with no significant changes in nucleus size between well plate and transwell cultures (Supplementary Figure 2e). This comparable trend and similar behaviour of cells in the well plate and transwell systems suggest the role of high-viscosity fluidic environments in maintaining the developmental behaviour of ciliated epithelial cells, hindering their rapid dedifferentiation into secretory cells as traditional well plate culture systems at low viscosities do.

Supplementary Figure 2d,e – *New Figure from new experiments*



Supplementary Figure 2 | Comparison of the effects of culture media viscosity between well plate and transwell cultures. (**a**) Representative immunofluorescent images of FTEC 72 hours post-seeding in transwell, where the green stain indicates the cilia and the blue stain indicates nuclei. Scale bars, 30 µm. (**b**) The proportion of ciliated cells as a function of viscosity, and (**c**) cilia length at 1 mPa·s and 200 mPa·s for cells cultured in well plates compared to transwell systems, after 72 hours of culture. (**d**) Cell density (number of cells per cm^2) for cells culture in transwell systems, and (**e**) nucleus size at 1 mPa·s and 200 mPa·s for cells cultured in well plates compared to transwell systems, after 72 hours of culture. Data are represented as mean ± s.d. and analyzed using one-way ANOVA with Tukey post hoc testing in b and d, and using student t-test in c and e, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$. Experiments were carried out on 3 different animals, with over 200 cells analyzed for each datapoint in b and d, and over 20 cells analyzed in c and e.

Comment 1 – 4

3. I miss more precise information on in situ local viscosity throughout the fallopian tube as well as changes therein through the estrous cycle. In the discussion more attention can be paid to this as it highlights the potential importance of this in vitro study. For instance it may cause another coordinated movement of cilia allow different interactions of sperm that initially bind to the cilia to be released after a certain interaction period where these released sperm are supposed to have attained fertilization capability. Or likewise a change in coordinated beating may guide the oocyte/embryo to be transported towards the uterus. Of course aberrations in secretions may alter viscosity parameters and thus also result in a mismatch in cilia formation/coordination affecting sperm binding and release properties and or correct oocyte/embryo migration toward the uterus. In the discussion also attention should be paid to fertility problems related to too high or too low fallopian viscosity. As it reads now the manuscript gives nice data on cilia properties of oviduct cultures under different viscosity media but lacks to relate this to the physiological relevance.

Response 1 – 4

Thank you. We have revised the discussion section to more clearly highlight the importance of our findings with respect to their relevance to viscosity changes *in vivo* and how they might contribute to influencing sperm migration and egg/embryo transport.

Pg. 18

In the fallopian Tube, viscosity varies from 1 mPa·s to over 200 mPa·s[19–22] along the tract, radially across the cross-section of the tract, and more importantly, throughout the menstrual cycle. These changes in the tubal fluid viscosity, combined with our findings, highlight the potential role of these rheological changes in altering sperm migration behaviour *in vivo*, coordinating the timing of fertilisation, and facilitating egg and embryo transport in the fallopian tube. The interactions between sperm and ciliated surfaces are suggested to provide storage sites for sperm[123,124], stimulate capacitation, and influence the timing of fertilisation[1,34]. Our findings suggest that increased viscosity of tubal fluid, peaking at the time of ovulation[19,112], can enhance cilia beating and coordination to encourage metachronal wave formation. This enhanced cilia activity may act as a mechanism to release sperm from storage sites when they have gained fertilisation competence through direct physical contact with epithelial tissue[125], and also to coordinate this timing with egg transport in the tract for optimal meeting at the fertilization site. Moreover, our findings are relevant to infertility cases where tubal flushing with oil-based media has been shown to significantly improve fertilisation outcomes, although the underlying mechanism of this fertility-enhancing effect has been unknown[126]. Our results suggest a potential mechanism for facilitating cilia beating and gametes transport within the reproductive tract due to an increase in tubal fluid viscosity from the introduction of oil-based media[126].

References

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126. Roest, I. *et al.* What is the fertility-enhancing effect of tubal flushing? A hypothesis article. *J. Obstet. Gynaecol. (Lahore).* **42**, 1619–1625 (2022).

Comment 1 – 5

4. The major findings in viscosity dependency of formation cilia and coordinated activity are new to the field. The authors carefully and with rigor have performed analysis on the properties of cilia under various viscosity environments. Despite points 1-3 I think these studies is important both for the field of reproductive biology but also more in general for studies on tissues that have a ciliated epithelium.

Response 1 – 5

Thank you. We have modified the manuscript to also emphasize the significance of our findings in these areas.

Pg. 19

…. This highlights the importance of matching the viscosity of tubal fluid in *in vitro* culture models to accurately replicate the natural developmental and functional properties of the epithelial cells, offering additional insights into studies of other tissues with ciliated epithelium. Our findings also offer additional insights into the underlying causes of infertility where abnormal changes in the tubal fluid viscosity can inhibit ciliation. …

We thank the reviewer again for the positive assessment with very helpful input.

Reviewer 2

We appreciate the Reviewer's input, the result of which is an improved manuscript. Sections of the original manuscript that were revised (added, edited or reordered) are indicated both below and in the revised manuscript.

Comment 2 – 1

The authors study the role of external fluid viscosity on ciliary beat differentiation and phase coordination. The authors conclude that high viscosity promotes ciliation and facilitates the transport of egg and embryo for successful fertilization outcomes.

I have a comment on how the authors regulate viscosity by increasing the concentration of methylcellulose in the external fluid. It should be noted that it's not just viscosity that is affected, but also the osmotic pressure of the fluid. Two parameters change, therefore, which cannot be separated. The effects observed therefore stem from the increase in methylcellulose concentration, but it is not clear whether they result exclusively from the increase in viscosity or also, for example, from the increase in osmotic pressure induced by the increase in methylcellulose concentration. In particular, it has been shown that osmotic pressure plays on cilia, which can collapse in the airways when osmotic pressure increases (Button et al Science 2012). The authors should comment.

The methodology is sound. Details provided in the methods are fine.

Response 2 – 1

Thank you for your feedback and your careful consideration of our work.

In light of the reviewer's comment, we have measured the osmolarity of the culture media across the range of viscosities, indicating changes in osmolarity within the physiologically relevant range for tubal fluid and embryo culture media. We have also modified the manuscript to highlight the potential contribution of these even minor changes on epithelial cell behavior.

Pg. 5

…. We also measured the osmolarity of the culture media (Supplementary Table 1, see Methods), which deacreased from 312 mOsm/kg at 1 mPa·s (0% MC) to 257 mOsm/kg at 200 mPa·s (1% MC), within the physiologically relevant range of osmolarity for oviductal fluid in mammals and embryo cculture media (rainging from 250 to over 355 mOsm/kg)[68,69]. This change in osmolarity might also contribute to the effects observed here. Our results …

Pg. 15

…. independent of the proliferation rate. It is noteworthy that slight change in the osmolarity of culture media, within the physiologically relevant range (from 312 mOsm/kg to 257 mOsm/kg, Supplementary Table 1), due to the increasing MC concentration, might also contribute to the regulation of epithelial cell behaviour. While considerably more significant changes in osmolarity has been reported to regulate airway epithelial cells behaviour and cilia length[94], this effect is potentially negligible here as we do not see changes in cilia length (Supplementary Figure 2). Previous studies using up to 2% MC or 1% Dextran (with similar osmolarity to 1% MC) have also shown insignificant changes in cell behaviour due to this range of changes in osmolarity[53].

Supplementary Table 1 – *New*

Supplementary Table 1 | Osmolarity of culture media across viscosity range. Values are reported as mean ± s.d. from 3 measurements.

Viscosity (mPa·s)	Methyl cellulose (%)	Osmolarity (mOsm/kg)
1	0	312 ± 1
50	0.7	271± 2
100	0.9	265 ± 2
200	1	257± 4

Pg. 19 – Method

Viscous media preparation. … was adjusted to 7.4. The osmolarity of the culture media was measured using an osmometer (John Morris and Advanced instruments, Model 2020) based on the manufacturer guidelines.

References

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Comment 2 – 2

It is clearly shown that increasing methylcellulose concentration plays a role on the density and proportion of ciliated cells during development. However, there was no analysis of the final mature tissue. Ultimately, when the tissue is mature, are there more ciliated cells on the epithelium? What is the cell density on the mature epithelium obtained? These questions are important for egg transport.

Response 2 – 2

It is noteworthy that this study is an important step towards replicating the 3D complex architecture of the female reproductive tract. However, due to the nature of obtaining sheep tissues from a local abattoir, accurately determining the developmental stage of the tissues at the time of collection was not possible.

In response to the reviewer's comment, we have compared the trend observed here with reported changes in the ratio of ciliated to secretory cells and viscosity changes during the menstrual cycle, indicating consistent behavior that facilitates ciliation as the tissue matures and the viscosity of the tubal fluid increases, both peaking at the time of ovulation.

Pg. 15 – Underlined sentences have been added to address this comment

… maintaining the developmental behavior of epithelial cells. This increasing ciliation trend is consistent with how the proportion of ciliated cells and viscosity change during the menstrual cycle as the epithelial tissue matures. Similar to our results, the ratio of ciliated to secretory cells increases during the menstral cycle along with the tubal fluid viscosity, both peaking at the time of ovulation[26,95].

References

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Comment 2 – 3

The authors also show that linear rise in TRPV4 intensity is observed under viscous loading. It results in increasing intracellular Ca2+, subsequently decreasing the MMP level and increasing ATP production. The question then arises as to whether ciliary beating is limited by ATP or whether ATP is present in sufficient quantity to always allow cilia to beat efficiently. The authors should discuss.

Response 2 – 3

We have updated the manuscript to more carefully discuss the interplay between TRPV4 expression level, Ca^{2+} influx and ATP influence cilia beating.

Pg. 18

Our findings suggest that the interplay between TRPV4 expression levels and subsequent Ca^{2+} influx to increase ATP production is crucial for regulating cilia beating under viscous loading. When comparing cells with blocked TRPV4 channels to untreated cells (Fig. 4e,f), we observed a drop in cilia beating frequency and coherence, indicating that the reduction in ATP production due to lower Ca^{2+} influx[119,120] influences cilia beating. While ciliated cells can self-generate ATP through either oxidative phosphorylation in mitochondria or glycolysis within the cilium itself[121], the activation of relevant signalling pathways is required to achieve this. Hence, enhanced TRPV4 activity and mitochondrial function trigger more ATP production at higher viscosities to ensure persistent cilia activity. These findings are consistent with previous studies where ciliated cells incubated with TRPV4 ion channel activators (instead of inhibitors, as in our case) or ATP showed an increased cilia beating frequency[77], also confirming that additional ATP is required under increased viscous loading[122] to maintain cilia beating.

References

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120. Nakahari, T. Regulation of ciliary beat frequency in airways: Shear stress, ATP action, and its modulation. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **292**, 612–613 (2007).
121. Acevedo, C., Blanchard, K., Bacigalupo, J. & Vergara, C. Possible ATP trafficking by ATP-shuttles in the olfactory cilia and glucose transfer across the olfactory mucosa. *FEBS Lett.* **593**, 601–610 (2019).
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Comment 2 – 4

I don't understand why the TRPV4 study is shown after the discussion paragraph. It doesn't seem logical.

Response 2 – 4

In agreement with the reiviewer's comment, we have moved the TRPV4 study to the Results section and now only discuss our key findings in the Discussion section.

Pg. 12 – *The entire paragraph was moved to the end of the Results section with a new heading*

TRPV4 expression is regulated in response to viscous loading. TRPV4 channel is a calcium-permeable cation channel that is sensitive to osmotic and mechanical stimulation[78,79]. To assess the role of the TRPV4 channel in regulating intracellular calcium levels, we monitored calcium uptake over a period of 120 seconds for cells treated with RN-1734 (to block the TRPV4 channel) as compared with a control group of vehicle-treated cells (Fig. 5a, see Methods). Calcium uptake was reduced by up to 40% in cells with blocked TRPV4 channels, indicating the crucial role of this channel in modulating calcium influx into the cytosol in response to viscous loading. In non-blocked cells, increase in intracellular Ca^{2+} level elevates the influx of cytosolic Ca^{2+} into the mitochondria[80,81], reducing the mitochondrial membrane potential (MMP)[82,83] and increasing ATP production[84–86]. To test the cellular response under viscous loading, cultured FTEC were stained with JC-1 dye to evaluate the MMP level (Fig. 5b-d). JC-1 dye exhibits a potential-dependent accumulation in mitochondria such that low MMP cells with JC-1 monomer emit green fluorescence and high MMP cells with JC-1 aggregate emit red fluorescence (Fig. 5b,c). Consequently, mitochondrial depolarization can be indicated by an increase in the ratio of cells with low MMP to high MMP[87] (see Methods). As shown in Fig. 5d, our results demonstrate a 38% increase in the low MMP/high MMP ratio (from 0.13±0.02 to 0.18±0.02) by increasing the culture viscosity from 50 to 100 mPa·s (*$P \leq 0.05$; ANOVA followed by a post hoc Tukey test). To evaluate ATP production, cells cultured in different viscosities were also stained using MitoTracker deep red (Supplementary Figure 10, see Methods) that specifically labels respiring mitochondria[88]. Epithelial cells cultured at higher viscosities showed a significantly higher level of mitochondrial respiration (63% increase when comparing cultured cells at 1 mPa·s with 200 mPa·s), indicating that more mitochondria are respiring under viscous loading to convert the intracellular energy to ATP. The results also agree with previous findings where a decrease in MMP increases mitochondria ATP production[84–86]. To further elucidate the effects of viscosity on TRPV4 activity, we evaluated the expression of TRPV4 using a TRPV4 antibody (see Methods)[89]. A linear rise in TRPV4 intensity (R^2=0.99) was observed, with a 5-fold increase in intensity (i.e. active TRPV4 channels) when the culture viscosity was elevated from 1 to 200 mPa·s (Fig. 5e, Supplementary Figure 11). It is noteworthy that, while TRPV4 is an ion channel, it can be immunolocalised at the cytoplasm due to the potential trafficking pathways of the ion channel to and from the plasma membrane, as confirmed by previous studies using the same immunofluorescent staining antibodies[89–91]. Since TRPV4 channels can also be activated via agonists such as phorbol ester (4α-PDD) and GSK1016790A, Baratchi *et al.*[90] reported that GSK1016790A induces the translocation of TRPV4 from the plasma membrane to the cytoplasm, where the channels formed vesicular structures with concentration-dependent cytoplasmic aggregation. Differentiated mouse mammary epithelial cells (HC11) cultured in growth medium also demonstrated TRPV4 protein pooled in intracellular cytoplasmic compartments for both single and clustered cells[92].

Pg. 13 – *The entire paragraph was moved to the end of the Results section with a new heading*

We also compared TRPV4 expression for cells at different viscosities using Western blot (see Methods). TRPV4 protein expression increased under high-viscosity conditions (Supplementary Figure 12), specifically by about 1.5-fold when media viscosity increased from 1 mPa·s to 200 mPa·s. This is supported by our calcium uptake results (Fig. 5a), showing that inhibition of TRPV4 with RN-1734, a well-established TRPV4 channel inhibitor[93], leads to a significant reduction in calcium influx into the cell. Overall, our results indicate that viscous loading leads to increased TRPV4 expression and encourages ciliation. Conversely, blocking TRPV4 channel restricts cilia beating frequency and coherence (Fig. 4e,f), consistent with similar previous work including those that used knockout mouse models[52,77]. Therefore, viscous loading activates TRPV4 channel to increase intracellular Ca^{2+}, subsequently decreasing the MMP level and increasing ATP production to maintain cell viability and functionality at higher viscosities.

Pg. 17&18 – *Discussion section*

We also indicated that TRPV4 expression increases by 5-fold when the culture viscosity is elevated from 1 to 200 mPa·s (Fig. 5e). Since TRPV4 channels modulate calcium influx into the cytosol (Fig. 5a), this increase results in additional Ca^{2+} influx into the mitochondria[80,81], reducing the MMP ratio by 38% in response to viscous loading (Fig. 5c,d). This reduction in MMP leads to a 63% increase in mitochondrial respiration (Supplementary Figure 10) and increases ATP production[84–86]. The increase in Ca^{2+} influx through the TRPV4 channel subsequently mediate cilia beating frequency (Fig. 4e), with cells treated with RN-1734 showing reduced cilia beating. Moreover, the higher influx of Ca^{2+} has been shown to sustain ciliary beating behaviour at high viscosities without collapse[55]. TPRV4 activity has also been shown to regulate mechanotransduction via the YAP/TAZ nuclear-shutting mechanism in various cell types, such as cellular epithelial-mesenchymal transition (EMT) in keratinocytes, mesenchymal stem cells and HUVEC endothelial cells[113–115]. Hence, viscous loading activates the TRPV4 channel in FTEC to increase intracellular Ca^{2+}, possibly via the regulation of YAP, subsequently decreasing the MMP level and increasing ATP production to maintain cell viability and functionality at higher viscosities. Previous work has also demonstrated a correlation between TRPV4 expression and the menstrual cycle[116,117]. It has been shown that TRPV4 expression peaks at the time of ovulation[116], as does the tubal fluid viscosity[19,112], which is then downregulated post-ovulation as the progesterone level increases. The increase in progesterone level during the menstrual cycle has been suggested to supress oviductal ciliation[118] and inhibit TRPV4 protein expression using mammary gland epithelial cells[117]. Therefore, our findings suggest how the interplay of changes in tubal fluid viscosity, progesterone level, and TRPV4 expression during the menstrual cycle might influence ciliation and cilia activity.

Pg. 18

Our findings suggest that the interplay between TRPV4 expression levels and subsequent Ca^{2+} influx to increase ATP production is crucial for regulating cilia beating under viscous loading. When comparing cells with blocked TRPV4 channels to untreated cells (Fig. 4e,f), we observed a drop in cilia beating frequency and coherence, indicating that the reduction in ATP production due to lower Ca^{2+} influx[119,120] influences cilia beating. While ciliated cells can self-generate ATP through either oxidative phosphorylation in mitochondria or glycolysis within the cilium itself[121], the activation of relevant signalling pathways is required to achieve this. Hence, enhanced TRPV4 activity and mitochondrial function trigger more ATP production at higher viscosities to ensure persistent cilia activity. These findings are consistent with previous studies where ciliated cells incubated with TRPV4 ion channel activators (instead of inhibitors, as in our case) or ATP showed an increased cilia beating frequency[77], also confirming that additional ATP is required under increased viscous loading[122] to maintain cilia beating.

References

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122. Noguchi, M., Sawada, T. & Akazawa, T. ATP-regenerating system in the cilia of Paramecium caudatum. *J. Exp. Biol.* **204**, 1063–1071 (2001).

Comment 2 – 5

As regards ciliary activity, it is clearly shown that the frequency of cilia beating decreases under viscous loading. However, the reason is not discussed. Is it a physical reason linked to the increase in hydrodynamic resistance as viscosity increases? The velocity of a cilium in a viscous medium is proportional to the force exerted divided by the viscosity (Stokes' law). If the force exerted by a cilium is constant (fixed by the molecular motors in the presence of ATP), then the velocity of the cilium and therefore its frequency decreases as the medium becomes more viscous. Or is it a biological effect? One consequence of increased viscosity could also be to reduce the amplitude of the beats (which seems to be observed on the movie). The authors should analyze the evolution of the beating pattern as a function of viscosity. This is an important parameter for transport. In both cases, the function would be rather degraded as viscosity increases.

Response 2 – 5

We have now updated the discussion section to more carefully explain how a decrease in cilia beating frequency at high viscosities is attributed to both hydrodynamic resistance and the mechanoresponse of the cilia (also see ***Response 2 – 3***). Furthermore, to better support our discussion and as suggested, we have quantified changes in cilia beating amplitude (distance) as a function of media viscosity.

Pg. 16

… for viscosities higher than 100 mPa·s. The combination of hydrodaynamic effects, changes in ATP production rate, and potential regulation of the molecular motor activity that governs the movement of cilia may contribute to influence cilia beating behaviour at different viscosities[28,102,103]. The increased drag at higher viscosities can reduce the beating velocity of the cilia[104], and consequently their beating frequency. This increase in hydrodynamic resistance also leads to a decrease in cilia beating amplitude (Supplementary Figure 13) and a higher energy consumption rate per beat cycle[28,103]. Moreover, similar to other flagellated eukaryotic cells, under viscous loading, the higher force production rate of dynein arms that govern the beating waveform may act as a molecular level mechanism to regulate motor activity[105]. The higher Ca^{2+} influx and TRPV4 channel activity at higher viscvosities, and the subsequent increase in ATP production, support this later mechanism.

Pg. 23 – Methods

… patches. To quantify cilia beating amplitude, the movement of the tip of the cilia in the top-view widefield microscopy videos was manually tracked, and the average maximum traveled distance over multiple cycles was reported. …

References

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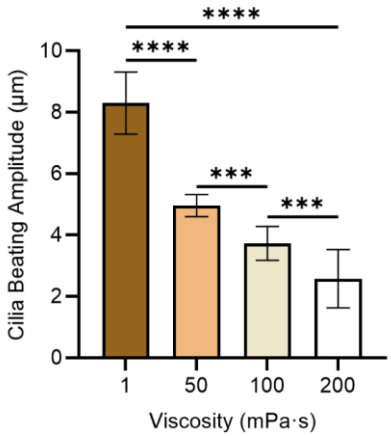
New Supplementary Figure - *Cilia Beating Amplitude*



Supplementary Figure 13 | Cilia beating amplitude as a function of culture media viscosity. Experiments were carried out on samples from 3 different animals, and at least 15 individual cilia were traced manually for each datapoint. Data are represented as mean ± s.d. and analyzed using one-way ANOVA with Tukey post hoc testing, ***$P \leq 0.001$, ****$P \leq 0.0001$.

Comment 2 – 6

Regarding metachronal waves, the results show that the phase coordination seems to be better upon an increase in viscosity but this coordination is on a very small spatial scale (a few microns) and we do not fully understand how this could be very important to improve transport over a centimetre distance. There are no waves that propagate long distances and the wavefront is very narrow. Furthermore, the authors do not discuss the nature of the wave (antiplectic, symplectic, etc.). The nature of the wave plays a big role in the efficiency of transport. We therefore do not know whether the waves are effective in improving transport. More troubling is that the authors do not look at the most basic parameter of transport, which is the direction of beating of the cilia. if the cilia do not beat in the same direction, it is very difficult to transport efficiently and beating direction coordination is more important for transport than phase coordination. If the directions are the same, transport will take place even in the absence of metachronal waves (like on airways where such waves are not really observed ref: Feriani, L. et al. Assessing the Collective Dynamics of Motile Cilia in Cultures of Human Airway Cells by Multiscale DDM. Biophys. J. 113, 109–119 (2017); and: Sanderson, M. J. & Sleigh, M. A. Ciliary activity of cultured rabbit tracheal epithelium: beat pattern and metachrony. J. Cell Sci. 47, 331–347 (1981)). On the other hand, if the directions are not coordinated, there will simply be no transport. However, on the films, we clearly see that the cilia do not beat in a single direction, whether the medium is viscous or not. It is first necessary to analyze the coordination of the beating directions as a function of viscous loading.

Response 2 – 6

In agreement with the reviewer's comment, (*i*) we now evaluate the cilia beating direction, demonstrating stable unidirectional movement under viscous loading that facilitates effective transport; (*ii*) we highlight the importance of this unidirectional beating rather than just phase coordination in providing effective transport, as suggested by previous studies using airway epithelial cells; (*iii*) we delay further discussion of the implications of our findings for the formation of metachronal waves and egg transport until the end of the paper in the discussion section, where we provide a more nuanced discussion; (*iv*) we acknowledge the existing spatial limitations of our analysis (which is the subject of a current study in our group); (v) we updated

our heading to put less emphasis on the formation of metachronal waves and more on the observed coordinated cilia beating behavior; and (*vi*) we have softened our language, particularly in the abstract and throughout the paper.

Abstract

> … Further, cilia manifest a coordinated beating pattern that can facilitate the formation of metachronal waves. At the cellular level, viscous loading activates the TRPV4 channel in the epithelial cells to increase intracellular Ca^{2+}, subsequently decreasing the mitochondrial membrane potential level for ATP production to maintain cell viability and function. Our findings provide new insights into the role of elevated tubal fluid viscosity in promoting ciliation and coordinating their beating, as a potential mechanism to facilitate the transport of egg and embryo for successful fertilisation, suggesting possible therapeutic opportunities for infertility treatment.

Pg. 9 – *Updated heading*

> **Viscous loading facilitates self-organisation and coordination of ciliated cells.** The dominant factors of ciliary transport are the beating frequency and direction which can be regulated by chemical and mechanical cues[72] to form metachronal waves for transporting egg and embryo along the fallopian tube[34,39,40]. To quantitatively…

Pg. 10

> … with a similar wavelength of 2.13±0.74 μm was observed. To better evaluate how this coordinated cilia movement might facilitate the formation of metachronal waves or enable effective transport, we manually traced cilia beating direction and used kymographs as the common approach[73,76] to evaluate whether metachronal wave-like activity was present (Supplementary Figure 9, Supplementary Movie 2). While cilia beat randomly relative to each other at 1 mPa·s, our results indicate that cilia not only coordinate in phase but also in direction, beating in a coordinated manner towards one direction at 200 mPa·s. Our kymograph results (Supplementary Figure 9b) also show how cilia movement results in a smooth wave with a stable direction over time at high viscosity, suggesting the formation of metachronal waves.

Pg. 16

> Moreover, our results indicate that viscous loading regulates the biophysics of cilia beating and coordination to potentially facilitate the formation of metachronal waves at higher viscosities (>100 mPa·s). Specifically, …

Pg. 16, 17

At such high viscosities, ciliated cells also self-organised their beating behaviour to induce a phase shift between neighbouring cilia (Fig. 4b) while beating with a similar wave direction (Fig. 4c), cilia beating direction (Supplementary Figure 9), wavelength (Fig. 4d), and frequency (Fig. 4e). This spatial heterogeneity in cilia beating (*i.e.* separate patches with a gradual phase shift), but with a similar beating frequency, wavelength and direction, is crucial to encourage the formation of metachronal waves[73,74], and has been shown to produce a unidirectional flow towards the uterus[106,107]. The coordinated movement of cilia to enable effective transport is further demonstrated by evaluating cilia beating direction and kymographs (Supplementary Figure 9, Supplementary Movie 2). The results indicate that cilia self-organize to beat in a unidirectional manner with a stable wave direction over time at 200 mPa·s, while they beat randomly at 1 mPa·s. While our study is limited by the number of ciliated cells observed and our limited spatial resolution, this metachronal wave-like activity with unidirectional cilia movement under viscous loading can result in effective transport. The observed increase in the tendency of cilia to beat in the same direction has been shown to be an effective transport mechanism for airway epithelial cells[108,109], even in the absence of metachronal waves or beating coordination. This mechanism, together with fluid flow and muscle contractions[1,110], provides an effective means for the transport of the egg and embryo, which are up to 10 times larger than the cilia[111], at the time of ovulation when the viscosity peaks *in vivo*[19,112].

Pg. 23 – Methods

… segmented patches. To quantify cilia beating amplitude, the movement of the tip of the cilia in the top-view widefield microscopy videos was manually tracked in ImageJ, and the average maximum traveled distance over multiple cycles was reported. Cilia beating direction was eavulated for 750 ms and the wave propagation was analyzed using the kymograph plugin in ImageJ. For TRPV4 inhibition …

References

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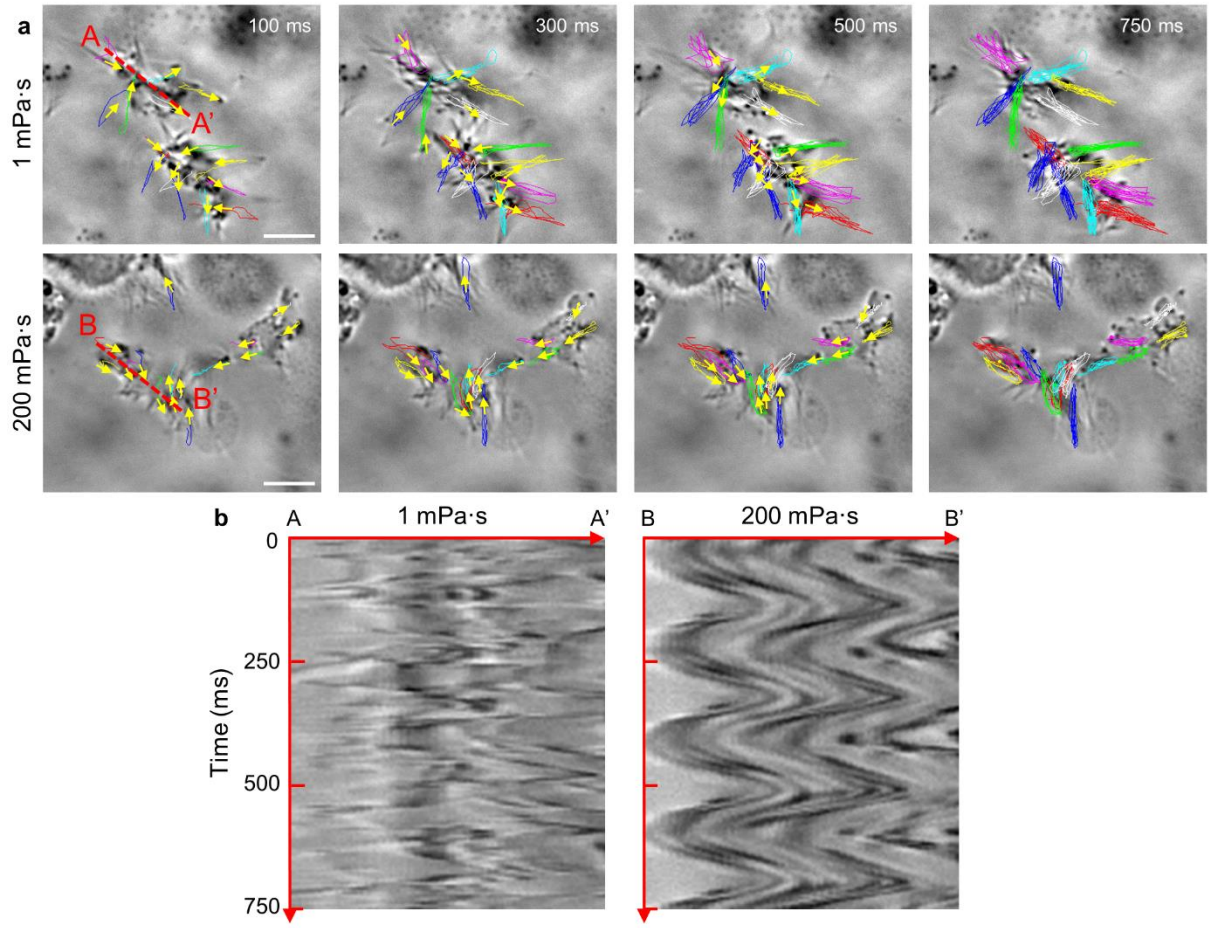
New Supplementary Figure 9 - Cilia Beating Direction



Supplementary Figure 9 | Cilia beating direction. (**a**) Cilia beat in a random manner in all directions at 1 mPa·s, while cilia beat in a coordinated and unidirectional manner at 200 mPa·s. Scale bars, 5 µm. (**b**) Kymographs of cilia beating along A-A' at 1 mPa·s and along B-B' at 200 mPa·s.

Comment 2 – 7

So, concerning the transport function, I'm not at all convinced of the role of viscous loading, since the analysis is based solely on metachronal waves.
Transport depends on
- the density of ciliated cells on the mature ciliated epithelium
- directional coordination of ciliary beats
- beat pattern
- beat frequency
- and finally, to the second order, the presence and nature of metachronal waves.

The role of viscous loading on the first 3 points is not discussed by the authors, while the last two points do not seem to go in the same direction.

Response 2 – 7

As detailed under ***Response 2 – 6*** (and also ***Responses 2 – 3 & 2 – 5***), we have revised the manuscript based on the reviewer's valuable feedback to better indicate the roles of viscous loading in facilitating a coordinated, unidirectional movement as a potential mechanism to enable effective transport, while also acknowledging the limitations of our model with respect to the number of cilia analyzed and spatial resolution, which is the subject of a current study in our group.

Comment 2 – 8

> Metachronal waves can improve transport, but the first points are major. In particular, metachronal waves do not exist on airways on long scales, and when they are found, they are not of the same nature as those that optimize transport (Mesdjian et al. Phys Rev Lett, 2022). I find it very limiting to estimate the function only from metachronicity, especially as the space scale of phase coherence seems to be on one or two ciliated cells only and on a developing epithelium. This is not a dense, uniform carpet of cilia. It's also worth noting that the egg is over 100 microns in size, much larger than the metachronal wave and with a speed much slower than that of a cilium. The same cilia are going to beat any times under the egg, while it progresses. The image of an egg being carried from cilium to cilium by a coordinated movement is therefore erroneous. In my opinion, it is essential that the authors carry out a thorough analysis of the efficiency of transport, and not just on the basis of waves.

Response 2 – 8

As detailed under ***Response 2 – 6***, we have revised the manuscript to include new data on cilia beating direction, acknowledge the importance of this stable unidirectional movement in facilitating effective transport, and acknowledge the limitations of our model with respect to the number of cilia analyzed and spatial resolution (which is the subject of a current study in our group). We have also revised the discussion to indicate how this wave works in coordination with flow direction and muscle contractions for transporting the relatively large egg or embryo.

We thank the reviewer for the detailed assessment with very helpful input.

Reviewer 3

We appreciate the Reviewer's input, the result of which is an improved manuscript. Sections of the original manuscript that were revised (added, edited or reordered) are indicated both below and in the revised manuscript.

Comment 3 – 1

> This is an interesting study that explores the hypothesis that the ciliation and cilia beating pattern of fallopian tubes is responsive and could be controlled by the rheological properties of its environment, specifically its viscosity. The authors propose the important role of the TRPV4 channel in this regulation. The manuscript is well-written and most of the data support the conclusions. This study is also physiologically important and provides important new insight into the regulation of this important process. However, the absence of essential controls, such as TRPV4 knockout mouse (which is available) or KO cell line, or at least an explanation of why these controls were not utilized dampens the enthusiasm for this work.

Response 3 – 1

Thank you for your positive feedback and careful consideration of our work.

Indeed, employing a TRPV4 knockout mouse and KO cell line would be helpful. However, given the focus of our work to study primary cells and due to the lack of access to required resources in Australia, these experiments are outside the scope of this current study and will be pursued in future work. Specifically, the importation of mice and establishment of a colony would take >1 year. Equally, the CRISPR edition of primary ovine lines is not established, let alone protocols for editing and then transformation into ciliated cells. Despite these limitations, to better support our results, we provided experimental results for cells with blocked TRPV4 channel (cells treated with RN-1734) and also conducted western blot experiments to compare TRPV4 expression in cells at different viscosities, as detailed under ***Responses 3-7, 3-8 and 3-10***. These provide the necessary controls and data to support our findings, adding novelty and sustaining enthusiasm for this work.

Comment 3 – 2

> The authors describe TRPV4's role in elevating intracellular Ca2+ and state that it results in mitochondrial depolarization events under different viscosity. However, how the Ca2+ level/mitochondrial depolarization is related to any of the FTEC characteristics (ciliation/beating behavior) is not stated well. A smooth connecting sentence is missed there.

Response 3 – 2

Thank you. We have modified the manuscript to emphasize the significance of Ca^{2+} and mitochondrial depolarisation.

Pg. 17 – *Underlined sentences have been added to address this comment*

… modulate calcium influx into the cytosol (Fig. 5a), this increase results in additional Ca^{2+} influx into the mitochondria[80,81], reducing the MMP ratio by 38% in response to viscous loading (Fig. 5c,d). This reduction in MMP leads to a 63% increase in mitochondrial respiration (Supplementary Figure 10) and increases ATP production[84–86]. The increase in Ca^{2+} influx through the TRPV4 channel subsequently mediate cilia beating frequency (Fig. 4e), with cells treated with RN-1734 showing reduced cilia beating. Moreover, the higher influx of Ca^{2+} has been shown to sustain ciliary beating behaviour at high viscosities without collapse[55]. TPRV4 activity …

Comment 3 – 3

Moving Figure 5 to the result section could be beneficial, as it suggests the potential mechanism for TRPV4 involving in regulation of the cilia beating. The discussion section can spread out more on YAP and Notch signaling, which could be the potential regulatory pathway for ciliation. This part is good to stay in the discussion session as no following experiment data in the paper is provided to support this deduction. How YAP might relate to TRPV4 is not discussed in the paper either.

Response 3 – 3

In agreement with the reiviewer's comment, we have moved the TRPV4 study (Figure 5) to the Results section and now only discuss our key findings in the Discussion section. We also modified the discussion section to better explain how YAP and TRPV4 might be related.

Pg. 12 – *The entire paragraph was moved to the end of the Results section with a new heading*

TRPV4 expression is regulated in response to viscous loading. TRPV4 channel is a calcium-permeable cation channel that is sensitive to osmotic and mechanical stimulation[78,79]. To assess the role of the TRPV4 channel in regulating intracellular calcium levels, we monitored calcium uptake over a period of 120 seconds for cells treated with RN-1734 (to block the TRPV4 channel) as compared with a control group of vehicle-treated cells (Fig. 5a, see Methods). Calcium uptake was reduced by up to 40% in cells with blocked TRPV4 channels, indicating the crucial role of this channel in modulating calcium influx into the cytosol in response to viscous loading. In non-blocked cells, increase in intracellular Ca^{2+} level elevates the influx of cytosolic Ca^{2+} into the mitochondria[80,81], reducing the mitochondrial membrane potential (MMP)[82,83] and increasing ATP production[84–86]. To test the cellular response under viscous loading, cultured FTEC were stained with JC-1 dye to evaluate the MMP level (Fig. 5b-d). JC-1 dye exhibits a potential-dependent accumulation in mitochondria such that low MMP cells with JC-1 monomer emit green fluorescence and high MMP cells with JC-1 aggregate emit red fluorescence (Fig. 5b,c). Consequently, mitochondrial depolarization can be indicated by an increase in the ratio of cells with low MMP to high MMP[87] (see Methods). As shown in Fig. 5d, our results demonstrate a 38% increase in the low MMP/high MMP ratio (from 0.13±0.02 to 0.18±0.02) by increasing the culture viscosity from 50 to 100 mPa·s (*$P \leq 0.05$; ANOVA followed by a post hoc Tukey test). To evaluate ATP production, cells cultured in different viscosities were also stained using MitoTracker deep red (Supplementary Figure 10, see Methods) that specifically labels respiring mitochondria[88]. Epithelial cells cultured at higher viscosities showed a significantly higher level of mitochondrial respiration (63% increase when comparing cultured cells at 1 mPa·s with 200 mPa·s), indicating that more mitochondria are respiring under viscous loading to convert the intracellular energy to ATP. The results also agree with previous findings where a decrease in MMP increases mitochondria ATP production[84–86]. To further elucidate the effects of viscosity on TRPV4 activity, we evaluated the expression of TRPV4 using a TRPV4 antibody (see Methods)[89]. A linear rise in TRPV4 intensity (R^2=0.99) was observed, with a 5-fold increase in intensity (i.e. active TRPV4 channels) when the culture viscosity was elevated from 1 to 200 mPa·s (Fig. 5e, Supplementary Figure 11). It is noteworthy that, while TRPV4 is an ion channel, it can be immunolocalised at the cytoplasm due to the potential trafficking pathways of the ion channel to and from the plasma membrane, as confirmed by previous studies using the same immunofluorescent staining antibodies[89–91]. Since TRPV4 channels can also be activated via agonists such as phorbol ester (4α-PDD) and GSK1016790A, Baratchi *et al.*[90] reported that GSK1016790A induces the translocation of TRPV4 from the plasma membrane to the cytoplasm, where the channels formed vesicular structures with concentration-dependent cytoplasmic aggregation. Differentiated mouse mammary epithelial cells (HC11) cultured in growth medium also demonstrated TRPV4 protein pooled in intracellular cytoplasmic compartments for both single and clustered cells[92].

Pg. 13 – *The entire paragraph was moved to the end of the Results section with a new heading*

We also compared TRPV4 expression for cells at different viscosities using Western blot (see Methods). TRPV4 protein expression increased under high-viscosity conditions (Supplementary Figure 12), specifically by about 1.5-fold when media viscosity increased from 1 mPa·s to 200 mPa·s. This is supported by our calcium uptake results (Fig. 5a), showing that inhibition of TRPV4 with RN-1734, a well-established TRPV4 channel inhibitor[93], leads to a significant reduction in calcium influx into the cell. Overall, our results indicate that viscous loading leads to increased TRPV4 expression and encourages ciliation. Conversely, blocking TRPV4 channel restricts cilia beating frequency and coherence (Fig. 4e,f), consistent with similar previous work including those that used knockout mouse models[52,77]. Therefore, viscous loading activates TRPV4 channel to increase intracellular Ca^{2+}, subsequently decreasing the MMP level and increasing ATP production to maintain cell viability and functionality at higher viscosities.

Pg. 17 – *the underlined setences explain how YAP and TRPV4 might be related*

.. influx of Ca^{2+} has been shown to sustain ciliary beating behaviour at high viscosities without collapse[55]. TPRV4 activity has also been shown to regulate mechanotransduction via the YAP/TAZ nuclear-shutting mechanism in various cell types, such as cellular epithelial-mesenchymal transition (EMT) in keratinocytes, mesenchymal stem cells and HUVEC endothelial cells[113–115]. Hence, viscous loading activates the TRPV4 channel in FTEC to increase intracellular Ca^{2+}, possibly via the regulation of YAP, subsequently decreasing the MMP level and increasing ATP production to maintain cell viability and functionality at higher viscosities. Previous work has also demonstrated a correlation between TRPV4 expression …

Pg. 17&18 – *Discussion section*

We also indicated that TRPV4 expression increases by 5-fold when the culture viscosity is elevated from 1 to 200 mPa·s (Fig. 5e). Since TRPV4 channels modulate calcium influx into the cytosol (Fig. 5a), this increase results in additional Ca^{2+} influx into the mitochondria[80,81], reducing the MMP ratio by 38% in response to viscous loading (Fig. 5c,d). This reduction in MMP leads to a 63% increase in mitochondrial respiration (Supplementary Figure 10) and increases ATP production[84–86]. The increase in Ca^{2+} influx through the TRPV4 channel subsequently mediate cilia beating frequency (Fig. 4e), with cells treated with RN-1734 showing reduced cilia beating. Moreover, the higher influx of Ca^{2+} has been shown to sustain ciliary beating behaviour at high viscosities without collapse[55]. TPRV4 activity has also been shown to regulate mechanotransduction via the YAP/TAZ nuclear-shutting mechanism in various cell types, such as cellular epithelial-mesenchymal transition (EMT) in keratinocytes, mesenchymal stem cells and HUVEC endothelial cells[113–115]. Hence, viscous loading activates the TRPV4 channel in FTEC to increase intracellular Ca^{2+}, possibly via the regulation of YAP, subsequently decreasing the MMP level and increasing ATP production to maintain cell viability and functionality at higher viscosities. Previous work has also demonstrated a correlation between TRPV4 expression and the menstrual cycle[116,117]. It has been shown that TRPV4 expression peaks at the time of ovulation[116], as does the tubal fluid viscosity[19,112], which is then downregulated post-ovulation as the progesterone level increases. The increase in progesterone level during the menstrual cycle has been suggested to supress oviductal ciliation[118] and inhibit TRPV4 protein expression using mammary gland epithelial cells[117]. Therefore, our findings suggest how the interplay of changes in tubal fluid viscosity, progesterone level, and TRPV4 expression during the menstrual cycle might influence ciliation and cilia activity.

Pg. 18

Our findings suggest that the interplay between TRPV4 expression levels and subsequent Ca^{2+} influx to increase ATP production is crucial for regulating cilia beating under viscous loading. When comparing cells with blocked TRPV4 channels to untreated cells (Fig. 4e,f), we observed a drop in cilia beating frequency and coherence, indicating that the reduction in ATP production due to lower Ca^{2+} influx[119,120] influences cilia beating. While ciliated cells can self-generate ATP through either oxidative phosphorylation in mitochondria or glycolysis within the cilium itself[121], the activation of relevant signalling pathways is required to achieve this. Hence, enhanced TRPV4 activity and mitochondrial function trigger more ATP production at higher viscosities to ensure persistent cilia activity. These findings are consistent with previous studies where ciliated cells incubated with TRPV4 ion channel activators (instead of inhibitors, as in our case) or ATP showed an increased cilia beating frequency[77], also confirming that additional ATP is required under increased viscous loading[122] to maintain cilia beating.

References

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Comment 3 – 4

Figure 1/Suppl. Fig.1: the media was replaced every 24 hours during 72 hours of this proliferation assay and cells exposed to a higher viscosity proliferated much less. What if the latter suffer from poorer attachment to the surface and as a result were aspirated at a higher number? Was it controlled and if so, how? Similarly, in supplementary figure 1, the author provides under different culture conditions, the total number of dead cells is similar. They didn't show whether the aspiration of media can take any live cell or not. There is a possibility that ciliated cells attach to the dish better/stronger than secretory FTEC, so a high viscosity media changing process can take secretory FTEC cells off the plate, which makes false positive seeing under 200 mPa·s more cells on the plate are ciliated cell rather than secretory cell.

Response 3 – 4

Thank you for this careful feedback. We have modified the manuscript to more clearly explain that the cells were first seeded using 1 mPa·s culture media for 24 hours, resulting in a similar number of cells attached per well and confluency levels to ensure a valid comparison when switching to higher viscosity fluids. We also conducted a new viability test on the aspirated cells across all viscosities, and there were no significant changes in the number of aspirated cells or their viability. We have also updated the caption for Supplementary Figure 1 to reflect the total number of aspirated cells, as suggested by the reviewer. It is also noteworthy that previous literature has demonstrated a considerably higher preference for attachment to plates by secretory cells compared to ciliated cells.

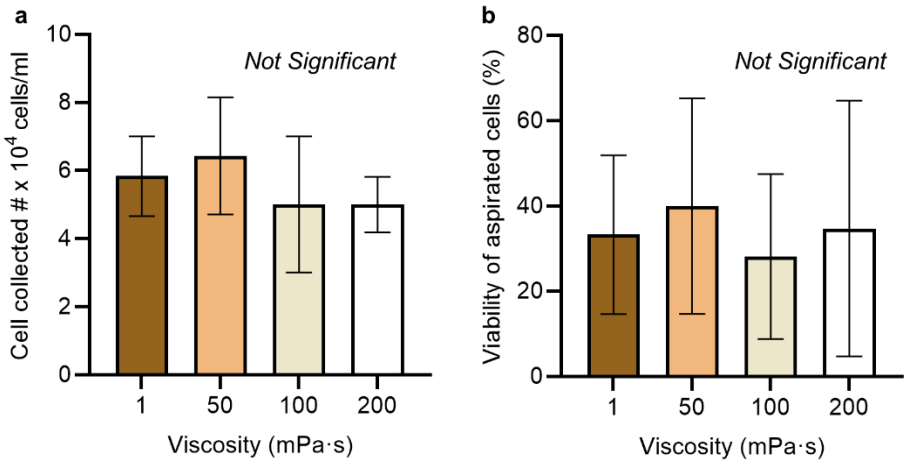
Pg. 5

To facilitate cell attachment and ensure a valid comparison, we first seeded the cells at 8×10^5 cells per well on collagen coated plates using 1 mPa·s culture media (0% MC) for 24 hours (Fig. 1a). This resulted in a similar number of cells attached per well and similar confluency levels before switching to culture media of varying viscosities. Upon cell attachment, we then cultured the cells in media of different viscosities for another 48 hours, replacing the media every 24 hours. While the number and viability of aspirated cells remained similar (Supplementary Figure 1), the confluency of cultured cells decreased considerably by increasing the media viscosity at the 72-hour timepoint (Fig. 1a). Specifically, the 1 mPa·s culture demonstrated a high level of confluency, but the confluency level decreased by approximately 54% at 200 mPa·s (Fig. 1a).

Pg. 5 - *The underlined sentence* was added to address this comment

... by increasing media viscosity from 1 to over 100 mPa·s. This reveals the influence of media viscosity on epithelial cells ciliation, despite a lower preference for attachment to plates by ciliated cells compared to secretory cells[45,67]. We also …

Supplementary Figure 1 – Updated caption and new results in part b



Supplementary Figure 1 | (a) Total number of cells aspirated when replacing the culture media, and (b) percentage of viability of the aspirated cells at the 72-hour timepoint. Data are represented as mean ± s.d. and analyzed using one-way ANOVA with Tukey post hoc testing., Experiments were carried out from 3 different animals.

Comment 3 – 5

Figure 2, panel A: As viscosity increases, the cell size under 50 mPa·s, 100 mPa·s, and 200 mPa·s looks larger (or flatter) than at 1 mPa·s, which is not very consistent with the bright field images in Figure 1 panel A and a statement that an area/cell does not change (Fig 1d). Why?

Response 3 – 5

Thank you for pointing this out. Previously, we reported the average area per cell by tracing the total area covered by cells and then dividing it by the number of cells within that area. Upon your comment, we more carefully evaluated the cell area by manually tracing at least 200 individual cells for each condition. As noted by the reviewer, the new *area per cell* results indicate an increasing trend, and the manuscript has been revised accordingly.

Pg. 5

… (Fig. 1b, c; Methods). However, the area covered by each cell increased from 431±139 μm^2 to 1052±356 μm^2 by increasing the culture media viscosity from 1 mPa·s to 200 mPa·s (Fig. 1d). This indicates that increased fluid viscosity encourages cell spreading, as also reported for human liver cancer cells under viscous loading[51]. Interestingly …

Pg. 6 – Figure 1 – Updated part d

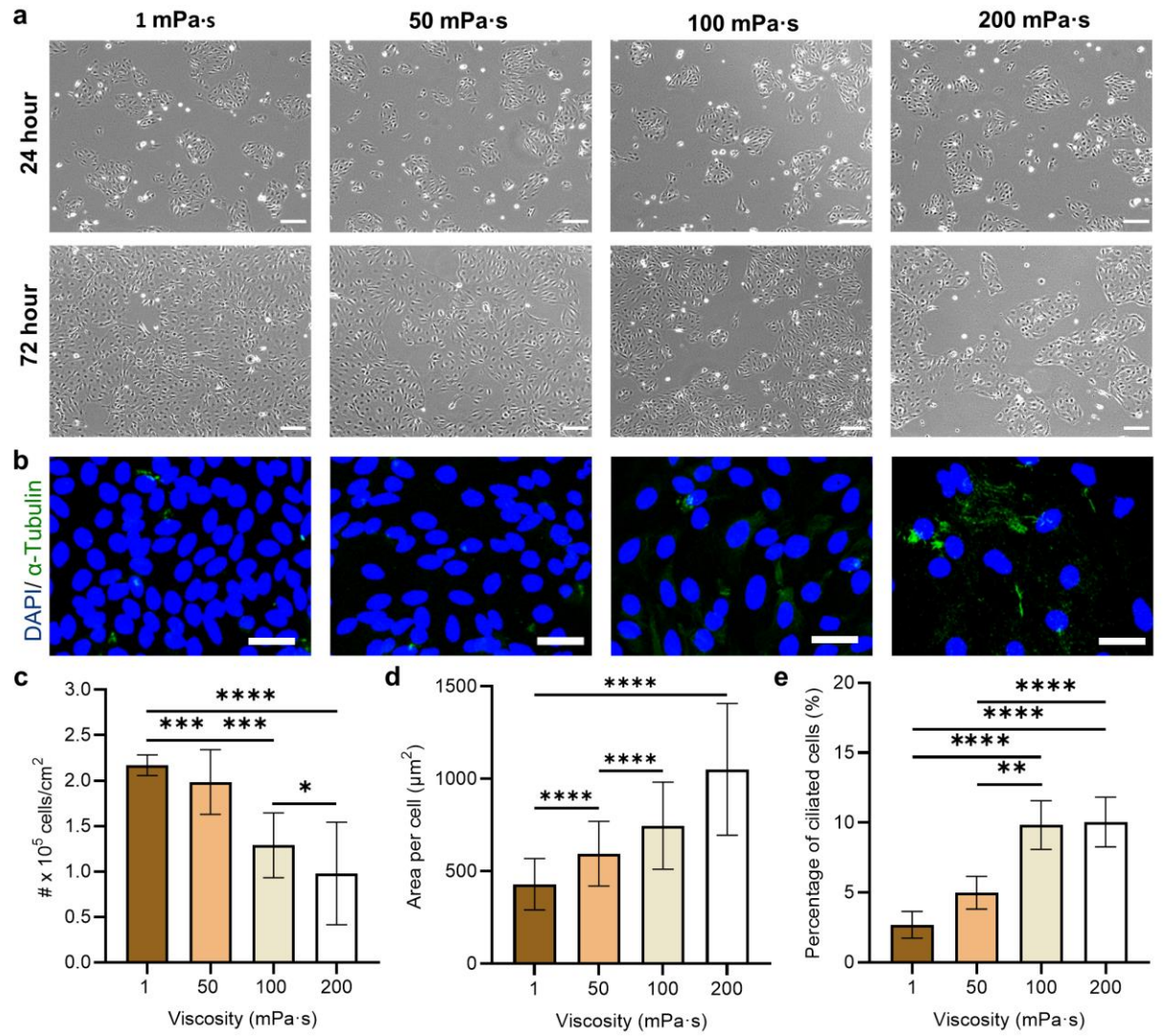


Fig. 1 | Effect of culture media viscosity on the development of the Fallopian tube epithelial cells (FTEC). a Representative phase contrast images of FTEC 24 hours (top) and 72 hours (bottom) post-seeding as a function of culture media viscosity ranging from 1 mPa·s to 200 mPa·s. **b** Representative immunofluorescent images of FTEC 72 hours post-seeding, where the green stain indicates the cilia and the blue stain indicates nuclei. Scale bars, 30 μm.**c** Epithelial cell density (number of cells per cm^2), **d** area per cell, and **e** the proportion of ciliated cells as a function of viscosity, after 72 hours of culture. Data are represented as mean ± s.d. and analyzed using one-way ANOVA with Tukey post hoc testing, *P ≤ 0.05, **P≤ 0.01, ***P≤ 0.001 and ****P≤ 0.0001. Experiments were carried out from 3 different animals and >200 cells were analyzed for each datapoint.

Pg. 20

… analysed per measurement. The area covered per cell was quantified by manually tracing at least 200 individual cells in ImageJ for each condition for cells stained with Calcein AM.

Comment 3 – 6

Figure 2, panel B, C; Figure 3 (and the entire bar graphs): The bar graphs should include individual data points, and not only means +/- sd/sem. Including proliferation assay images alongside Ki-67 immunofluorescent staining could be beneficial.

Response 3 – 6

In response to the reviewer's comments, we have updated these figure to also indicate individual data points. Figure 3 was also supplemented with Ki-67 immunofluorescent staining images.

Figure 2 with individual data points included.

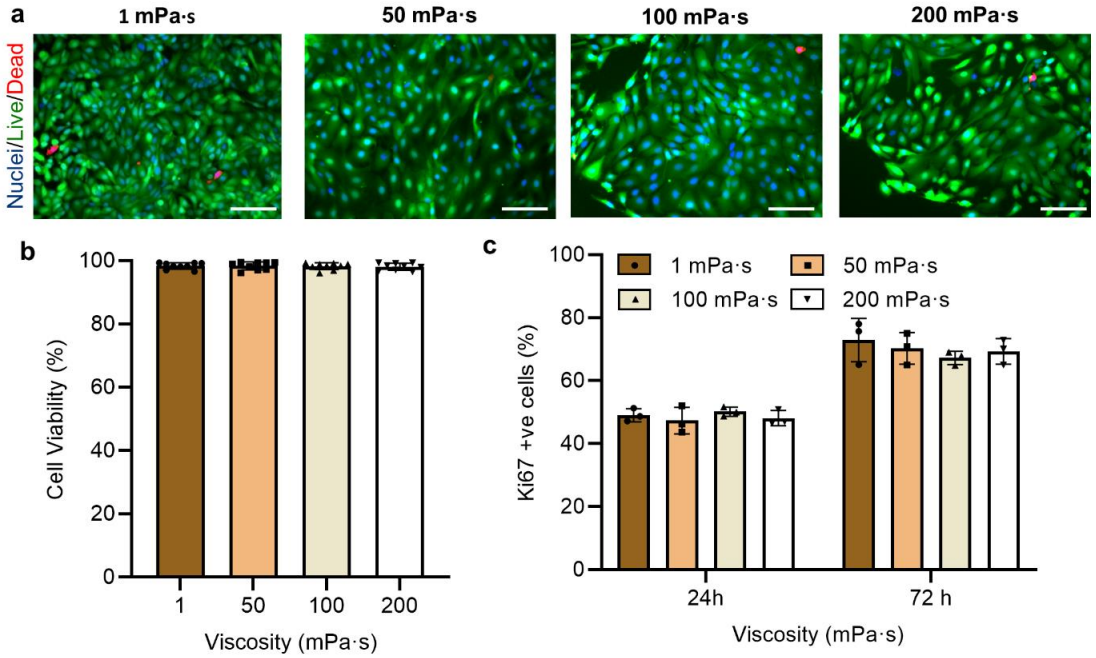


Fig. 2 | Effect of culture media viscosity on FTEC viability and proliferation. a Representative live/dead (green/red) fluorescence staining images of cells at the 72-hour timepoint and the **b** percentage of live cells (cell viability). Cell nuclei were stained using blue-fluorescent Hoechst 33342. Scale bar, 50 µm. **c** The percentage of Ki-67 positive cells at the 24-hour and 72-hour time points. Data are represented as mean ± s.d., experiments were done from 3 different animals, triplicate samples, >200 cells per timepoint for each condition tested. Scale bars, 100 µm.

Figure 3 – New immunostaining images, with individual data points included.

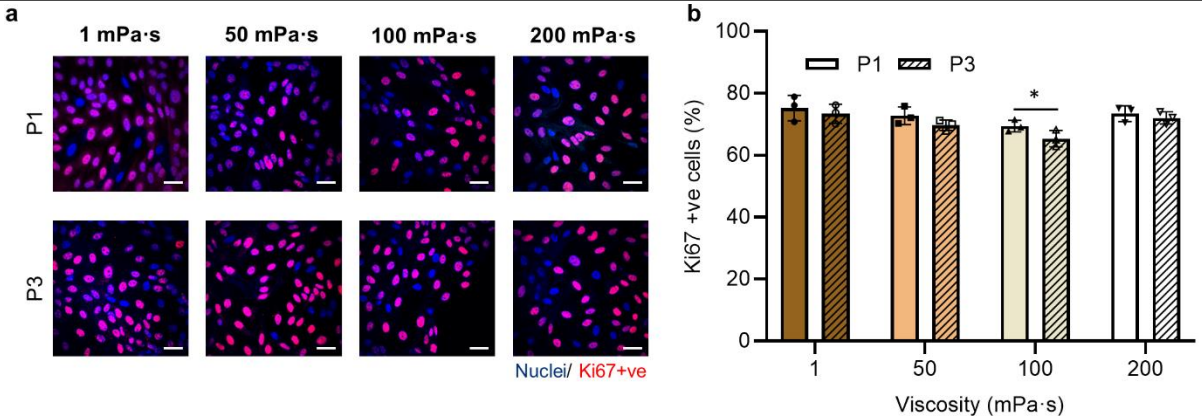


Fig. 3 | Comparison of FTEC proliferation between the initial culture and after 3 passages as a function of viscosity at 72-hour time point for each passage. a Representative Ki67 positive staining images of cells at the 72-hour timepoint for passage 1 and 3, and the **b** percentage Ki67 positive cells. Scale bar, 30 µm. Experiments were carried out on 3 different animals and ≥200 cells for each condition were analyzed. Data are represented as mean ± s.d. and analyzed using one-way ANOVA with Tukey post hoc testing, $*P \leq 0.05$.

Comment 3 – 7

The proof of TRPV4 functional expression in FTEC needs to be shown. Suppl Fig. 9 shows largely cytoplasmic immunostaining. TRPV4 is an ion channel exclusively plasma membrane protein. Either antibodies are nonspecific, or this is an autofluorescence. Clear membrane staining should indicate TRPV4 presence, which is not the case here. Western blot data would also be beneficial.

Response 3 – 7

In response to the reviewer's comment, we have conducted new immunofluorescent staining experiments with negative control for results presented in Supplementary Figure 9 and supplemented our TRPV4 findings with western blot analysis. The new results confirm TRPV4 expression at higher viscosities to regulate cellular behaviour and functions.

Pg. 12&13

… To further elucidate the effects of viscosity on TRPV4 activity, we evaluated the expression of TRPV4 using a TRPV4 antibody (see Methods)[89]. A linear rise in TRPV4 intensity (R^2=0.99) was observed, with a 5-fold increase in intensity (i.e. active TRPV4 channels) when the culture viscosity was elevated from 1 to 200 mPa·s (Fig. 5e, Supplementary Figure 11). It is noteworthy that, while TRPV4 is an ion channel, it can be immunolocalised at the cytoplasm due to the potential trafficking pathways of the ion channel to and from the plasma membrane, as confirmed by previous studies using the same immunofluorescent staining antibodies[89–91]. Since TRPV4 channels can also be activated via agonists such as phorbol ester (4α-PDD) and GSK1016790A, Baratchi *et al.*[90] reported that GSK1016790A induces the translocation of TRPV4 from the plasma membrane to the cytoplasm, where the channels formed vesicular structures with concentration-dependent cytoplasmic aggregation. Differentiated mouse mammary epithelial cells (HC11) cultured in growth medium also demonstrated TRPV4 protein pooled in intracellular cytoplasmic compartments for both single and clustered cells[92].

Pg. 13

We also compared TRPV4 expression for cells at different viscosities using Western blot (see Methods). TRPV4 protein expression increased under high-viscosity conditions (Supplementary Figure 12), specifically by about 1.5-fold when media viscosity increased from 1 mPa·s to 200 mPa·s. This is supported by our calcium uptake results (Fig. 5a), showing that inhibition of TRPV4 with RN-1734, a well-established TRPV4 channel inhibitor[93], leads to a significant reduction in calcium influx into the cell. Overall, our results indicate that viscous loading leads to increased TRPV4 expression and encourages ciliation. Conversely, blocking TRPV4 channel restricts cilia beating frequency and coherence (Fig. 4e,f), consistent with similar previous work including those that used knockout mouse models[52,77]. Therefore, viscous loading activates …

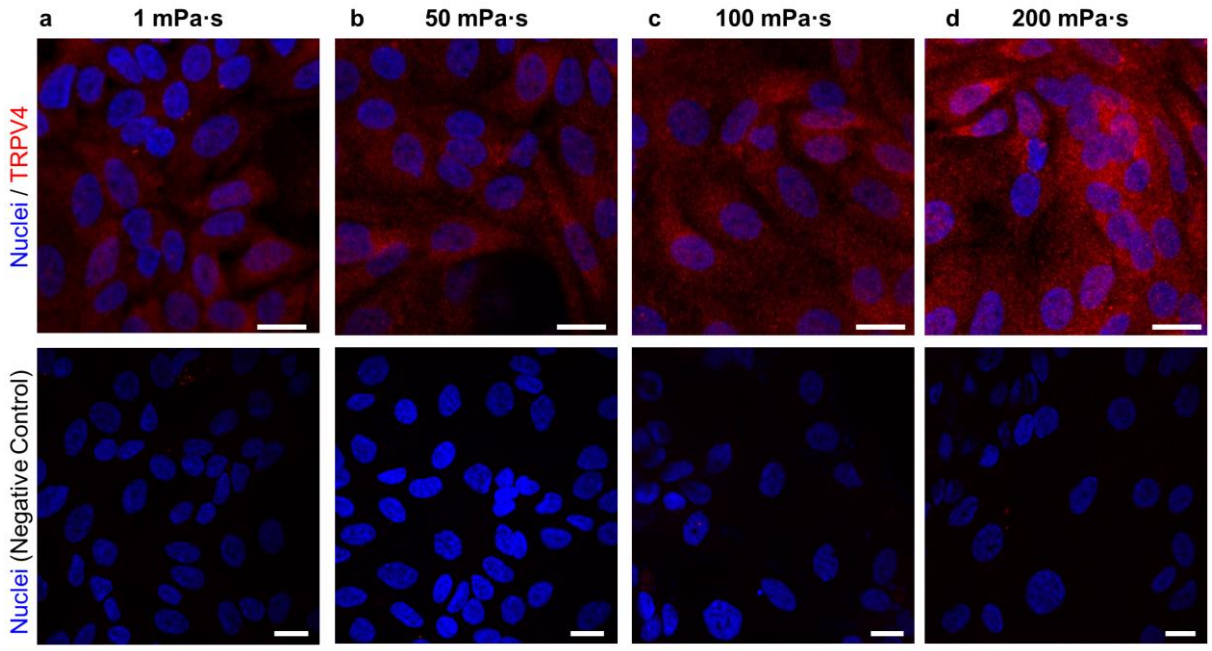
Pg. 21

… quantified using rabbit polyclonal TRPV4 antibody (Abcam, ab39260, 1:180 dilution) as the primary antibody and Rhodamine–conjugated goat anti-rabbit IgG (Proteintech, SA00007-2, 1:200 dilution) as the secondary antibody, as described previously[89]. This primary antibody has been shown to specifically target TRPV4[89,90]. To ensure the secondary antibody does not result in nonspecific binding or autofluorescence, we also conducted a negative control experiment where the cells were only stained with the secondary antibody, resulting in no fluorescent signal (Supplementary Figure 11). The average fluorescence was …

Pg. 23 – Methods – Western Blot analysis

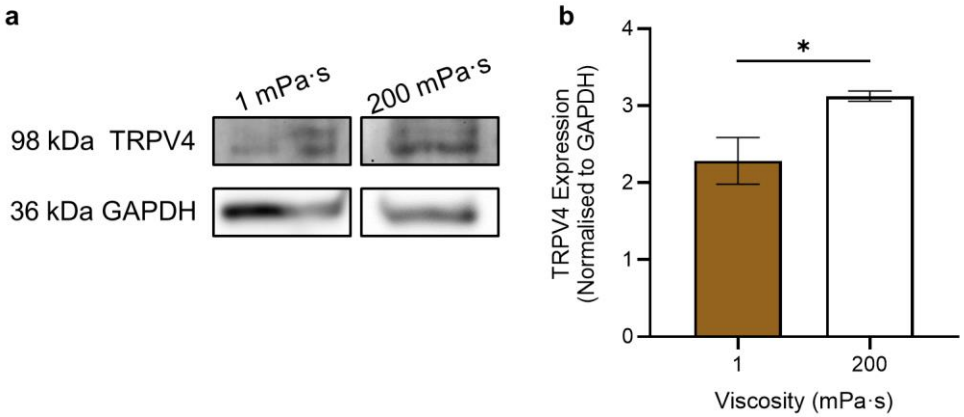
Western Blot Analysis. Cell pellets were collected at the 72-hour timepoint and lysed in Laemmli Sample Buffer (Bio-Rad). Samples were boiled at 95 °C for 5 minutes, then diluted and loaded into 10-well gels (Bio-Rad). The gels were run for 1 hour, after which the protein was then transferred to a PVDF membrane (Immobilon®). The membrane was blocked in 5% non-fat milk for 1 hour, then incubated with primary antibodies overnight (TRPV4, Abcam ab39260, GAPDH, Invitrogen, MA5-15738). Secondary antibodies against the primary (Goat Anti-Rabbit HRP conjugate, Invitrogen, 31460, and Goat Anti-Mouse HRP conjugate, Invitrogen, A16066) were incubated with the blot for 1 hour. Blots were imaged using a Uvitec Chemiluminescence Imaging System and analysed with ImageJ. Two bands with molecular weights of ~98 kDa were detected, with the higher molecular weight band representing the glycosylated form[127,128], consistent with previous studies[129,130], confirming the expression of TRPV4. Protein expression for TRPV4 was normalized to GAPDH, as previously described[131].

Updated Supplementary Figure11 – TRPV4 immunostaining with negative control



Supplementary Figure 11 | Comparing TRPV4 expression as a function of viscosity using immunofluorescent sating. Representative images of FTEC stained with a TRPV4 antibody (indicating expression of TRPV4 activity in red fluorescence) and Hoechst 33342 (indicating nuclei with blue fluorescence) for cells cultured in (**a**) 1 mPa·s, (**b**) 50 mPa·s, (**c**) 100 mPa·s, and (**d**) 200 mPa·s. Staining images with both primary and secondary antibodies are shown at the top, and cells only stained with the secondary antibody (in the absence of the primary antibody) are shown as the negative control at the bottom. Scale bars, 15 μm.

New Supplementary Figure 12 – Western Blot analysis



Supplementary Figure 12 | Comparison of TRPV4 protein expression for FTEC as a function of viscosity. (**a**) Representative images of the Western blot analysis and (**b**) quantification of TRPV4 protein expression in FTEC at 1 mPa·s and 200 mPa·s (normalised to GAPDH). n =3, * P <0.05 by Student's t-test.

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Comment 3 – 8

Overall, not utilizing tissue from TRPV4 KO mice or TRPV4 KO cell lines is not justified. Data exploring the effect of TRPV4 knockdown on cilia beating coherence is missing in Figure 4.

Response 3 – 8

We thank the reviewer for highlighting the potential use of TRPV4 knockouts as controls in our study. As detailed in ***Response 3 – 1***, due to lack of access to required resources in Australia, these experiments are outside the scope of this current study and will be pursued in future work.

We have also updated Figure 4 to also include cilia beating coherence results for cells with blocked TRPV4 channels, as suggested by the reviewer.

Pg. 10

… To test the hypothesis that TRPV4 channel contributes to maintaining cilia beating frequency at higher viscosities, we repeated our experiments in the presence of the TRPV4 antagonist RN-1734 (see Methods). At 200 mPa·s, the cilia beating frequency further decreased to 5.3±0.5 Hz for cells treated with RN-1734 (Fig. 4e). The results indicate that ciliary beating is sustained at high viscosity through the activation of TRPV4 channels, which subsequently increase the intracellular calcium level. …

Pg. 10

… coordination and the overall beating consistency. In contrast, for cells with blocked TRPV4 channel (treated with RN-1734), the coherence level was considerably lower at 200 mPa·s (Fig. 4f), highlighting the role of this channel in facilitating cilia beating and coordination. The results also confirm previous work that, using a a TRPV4 activator, indicate TRPV4 regulates ciliary beating in both oviduct ciliated cells and tracheal cilia at high viscosities[55,77], further supporting that TRPV4 regulates ciliary activity. These results indicate ...

Pg. 11- *Coherence results for cells with blocked TRPV4 channels in Figure 4f*

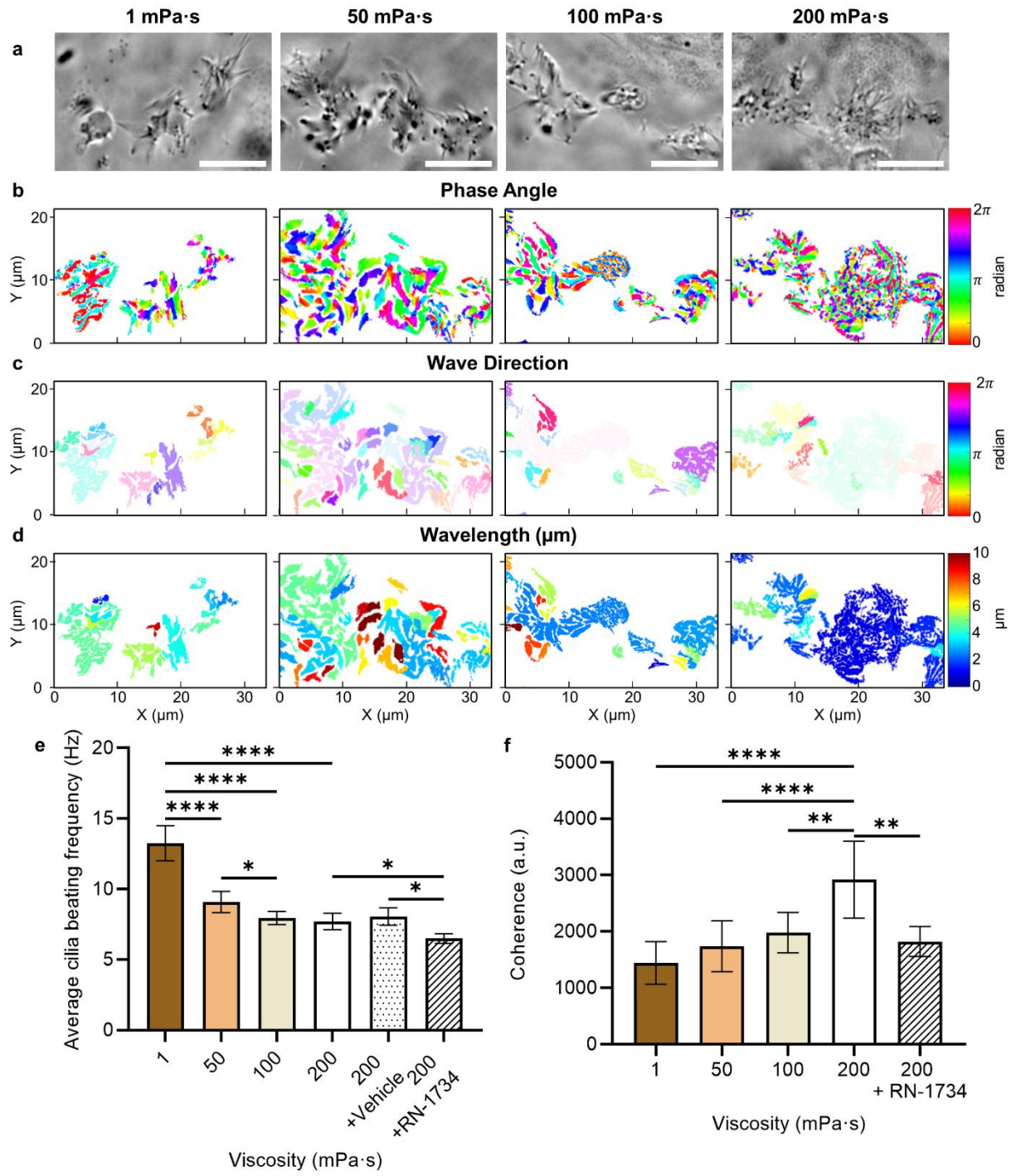


Fig. 4 | Characterization of cilia beating and coordination under viscous loading. a Representative widefield images of live ciliated cells, and the corresponding (**b**) phase angle**,** (**c**) wave direction and (**d**) wavelength of beating cilia as a function of media viscosity. Scale bars, 10 µm. **e** Average cilia beating frequency, and **f** coherence (a measure of the cilia coordination) as a function of culture media viscosity and for cells treated with RN-1734 to block the TRPV4 channel, and the vehicle control. Experiments were carried out using 3 different animals and 3 replicates per animal. Data are represented as mean ± s.d. and analyzed using one-way ANOVA with Tukey post hoc testing, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$.

References

77. Lorenzo, I. M., Liedtke, W., Sanderson, M. J. & Valverde, M. A. TRPV4 channel participates in receptor-operated calcium entry and ciliary beat frequency regulation in mouse airway epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 12611–12616 (2008).

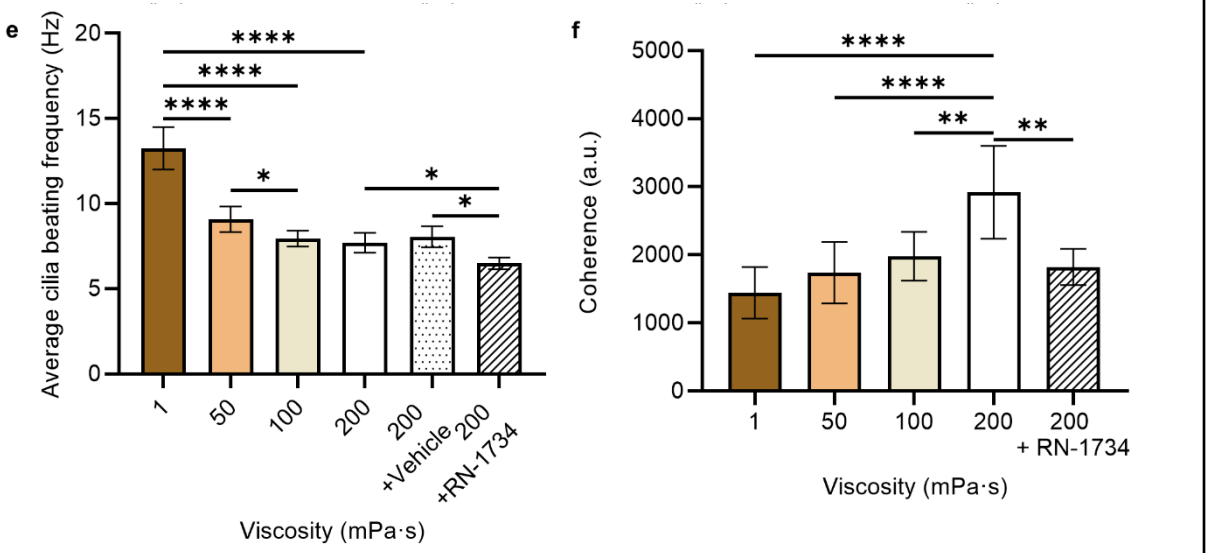
Comment 3 – 9

Vehicle control is missing from Figure 4e. Was the inhibitor dissolved in the DMSO or ethanol? Corresponding vehicle control should be used as a control condition.

Response 3 – 9

The inhibitor was dissolved in DMSO. We have included the details in the Methods section and updated Figure 4e to also include the control condition.

Pg. 11- New results – *Vehicle control in Figure 4e*



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Pg. 10

.. decreased to 5.3±0.5 Hz for cells treated with RN-1734 (Fig. 4e), while the results for the vehicle control were comparable to untreated cells. The results indicate …

Pg. 23 – *Methods*

… For TRPV4 inhibition experiment, cells were incubated with 10 µM of TRPV4 antagonist RN-1734 (Sigma-Aldrich, R0658) dissolved in DMSO prior to experiments. The inhibitor dissolved in DMSO for the 200 mPa·s media was used as the vehicle control.

Comment 3 – 10

Figures 4 and 5: These experiments focus on proving TRPV4's role in maintaining the viability of the FTEC under various viscous conditions. It lacks the connection of TRPV4 to the cilia beating function. If the author just wants to state all the physiological relevant changes they found under various viscosity conditions, but not mention TRPV4 is important on cilia beating function, the abstract section needs to be reorganized. Otherwise, some further experiments need to be done:

a) Show under different viscosity conditions, TRPV4 expression levels on ciliated FTEC cells might be different (QPCR, western blot, and/or IHC).
b) Show adding an inhibitor of TRPV4 may lead to less vigorous movement of cilia, but by adding a TRPV4 activator the activity can be restored.
c) Show *in vivo* data, like collecting fallopian tube epithelial cells freshly dissected from mice at different estrus cycle stages and compare the expression level of TRPV4 (this data can be analyzed from the published literature).

Response 3 – 10

We thank the reviewer for the detailed feedback. As suggested, we have conducted new experiments to compare TRPV4 protein expression using western blot analysis (as detailed under ***Response 3 – 7***), confirming higher expression at higher viscosities. We also provided new results and more carefully analysed our data on cilia beating and coordination for cells with blocked TRPV4 channels (treated with with RN-1734), confirming a decrease in beating ferequency and coherence in cells with blocked channel compared with untreated cells. This substantiates the role of this channel in coordinating cilia beating at higher viscosities. Additionally, we conducted a thorough literature review to more clearly explain how TRPV4 expression can dynamically change throughout the menstrual cycle.

Pg. 10

… To test the hypothesis that TRPV4 channel contributes to maintaining cilia beating frequency at higher viscosities, we repeated our experiments in the presence of the TRPV4 antagonist RN-1734 (see Methods). At 200 mPa·s, the cilia beating frequency further decreased to 5.3±0.5 Hz for cells treated with RN-1734 (Fig. 4e). The results indicate that ciliary beating is sustained at high viscosity through the activation of TRPV4 channels, which subsequently increase the intracellular calcium level. …

Pg. 10

… the overall beating consistency. In contrast, for cells with blocked TRPV4 channel (treated with RN-1734), the coherence level was considerably lower at 200 mPa·s (Fig. 4f), highlighting the role of this channel in facilitating cilia beating and coordination. The results also confirm previous work that, using a a TRPV4 activator, indicate TRPV4 regulates ciliary beating in both oviduct ciliated cells and tracheal cilia at high viscosities[55,77], further supporting that TRPV4 regulates ciliary activity. These results indicate …

Pg. 11- *Updated results for Figure 4e and 4f*

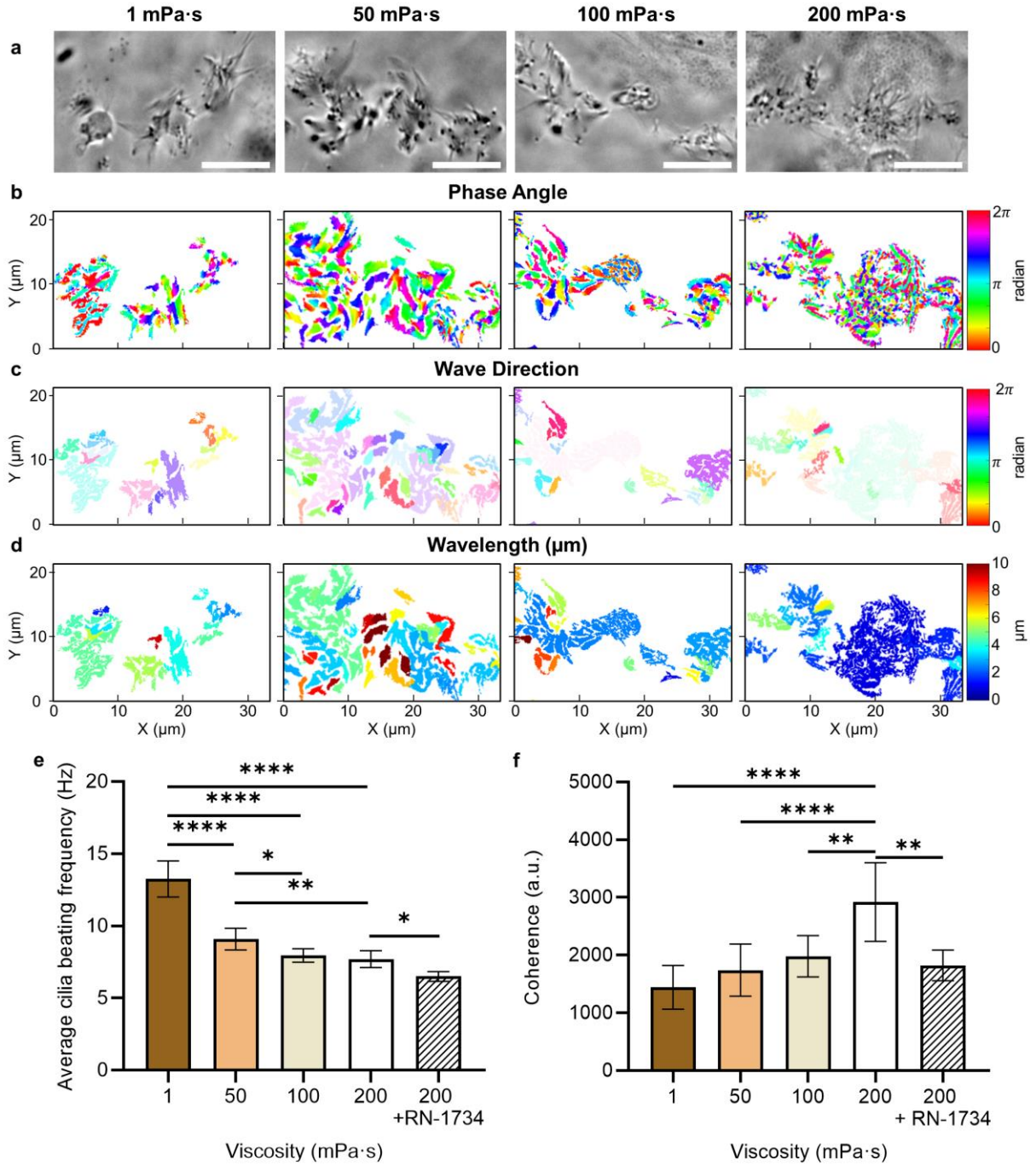

Fig. 4 | Characterization of cilia beating and coordination under viscous loading. a Representative widefield images of live ciliated cells, and the corresponding (**b**) phase angle**,** (**c**) wave direction and (**d**) wavelength of beating cilia as a function of media viscosity. Scale bars, 10 μm. **e** Average cilia beating frequency, and **f** coherence (a measure of the cilia coordination) as a function of culture media viscosity and for cells treated with RN-1734 to block the TRPV4 channel. Experiments were carried out using 3 different animals and 3 replicates per animal. Data are represented as mean ± s.d. and analyzed using one-way ANOVA with Tukey post hoc testing, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$.

Pg.17 – *Underlined sentences have been added to address this comment*

… to maintain cell viability and functionality at higher viscosities. Previous work has also demonstrated a correlation between TRPV4 expression and the menstrual cycle[116,117]. It has been shown that TRPV4 expression peaks at the time of ovulation[116], as does the tubal fluid viscosity[19,112], which is then downregulated post-ovulation as the progesterone level increases. The increase in progesterone level during the menstrual cycle has been suggested to supress oviductal ciliation[118] and inhibit TRPV4 protein expression using mammary gland epithelial cells[117]. Therefore, our findings suggest how the interplay of changes in tubal fluid viscosity, progesterone level, and TRPV4 expression during the menstrual cycle might influence ciliation and cilia activity.

References

77. Lorenzo, I. M., Liedtke, W., Sanderson, M. J. & Valverde, M. A. TRPV4 channel participates in receptor-operated calcium entry and ciliary beat frequency regulation in mouse airway epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 12611–12616 (2008).
116. De Clercq, K. *et al.* Functional expression of transient receptor potential channels in human endometrial stromal cells during the luteal phase of the menstrual cycle. *Hum. Reprod.* **30**, 1421–1436 (2015).
117. Jung, C. *et al.* The progesterone receptor regulates the expression of TRPV4 channel. *Pflugers Arch. Eur. J. Physiol.* **459**, 105–113 (2009).
118. Slayden, O. D., Luo, F. & Bishop, C. V. Physiological Action of Progesterone in the Nonhuman Primate Oviduct. *Cells* **11**, 1534 (2022).

Comment 3 – 11

Some minor issues:
The dot position in "mPa·s" is not consistent in the paper. Some are "mPa.s"

Response 3 – 11

Thank you. This has been double-checked and fixed throughout the paper.

Comment 3 – 12

The first time mention YAP in the introduction should use the full name instead of an abbreviation. This is true for other abbreviations as well.

Response 3 – 12

Thank you for your comment. We have revised the manuscript to ensure that the full form of all abbreviations, including YAP, is used the first time they appear in the text.

Pg. 4

… This is potentially attributed to a mechanistic response of FTEC, possibly via activation of the yes-associated protein (YAP) transcription factor to inhibit Notch signalling pathway. …

… increase their mitochondria activity for adenosine triphosphate (ATP) production to maintain their viability and functionality. ….

Comment 3 – 13

Figure 1d: what exactly was measured here as “y-axis”: ‘area per cell’ or actually “cell size”?

Response 3 – 13

We updated Figure 1d and the relevant discussion, as detailed under ***Response 3-5***, to report the average area per cell.

Comment 3 – 14

Figure 5 should be explained in the results, instead of the discussion

Response 3 – 14

As detailed in ***Response 3 – 3***, Fig. 5 is now explained at the end of the Results section.

We thank the reviewer for the detailed assessment with very helpful input.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed points made by me and by the other two reviewers in a very detailed and satisfactory way.

Reviewer #2 (Remarks to the Author):

The authors responded very carefully to my many comments and amended the manuscript to take account of my criticisms. They have done a substantial job.

They answered most of my questions.

I'm not sure that the osmometer that uses the freezing point depression method is the most suitable, but the authors have verified that cilium length is not affected.

I think the manuscript can now be accepted.

Reviewer #3 (Remarks to the Author):

The authors addressed all questions and modified the manuscript accordingly. This manuscript has been significantly improved.

Reviewer 1

Comment

The authors have addressed points made by me and by the other two reviewers in a very detailed and satisfactory way.

We appreciate the reviewer's positive feedback and constructive input, the result of which is a substantially improved manuscript.

Reviewer 2

Comment

The authors responded very carefully to my many comments and amended the manuscript to take account of my criticisms. They have done a substantial job. They answered most of my questions.
I'm not sure that the osmometer that uses the freezing point depression method is the most suitable, but the authors have verified that cilium length is not affected. I think the manuscript can now be accepted.

We thank the reviewer for the careful consideration of our work.

In addition to vapor pressure osmometers, the freezing point depression osmometer has also been commonly and routinely used to measure the osmolarity of culture media [1, 2]. Given that the cilium length and the trend of ciliation in both 2D culture and Transwell under elevated viscosity remain unchanged, as confirmed by the reviewer, the impact of osmolarity change is minimal.

1. Perez-Camps, M. & Garcia-Ximenez, F. Osmolarity and composition of cell culture media affect further development and survival in zebrafish embryos. *Animal* **2**, 595–599 (2008).

2. Tarhan, D., Özsobaci, N. P., Ergün, D. D. & Ercan, A. M. Investigation of extracellular medium osmolality depending on zinc application and incubation time on A549 cancer cells. *J. Biol. Phys.* **48**, 215–226 (2022).

Reviewer 3

Comment

The authors addressed all questions and modified the manuscript accordingly. This manuscript has been significantly improved.

We thank the reviewer for the positive feedback.