nature portfolio

Peer Review File

Single-cell transcriptomics reveals hair growth retardation mediated by aberrant CTS contraction in male androgenetic alopecia

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is a very intriguing study that shifts attention in human AGA research from the role of androgens to the CTS by proposing that continuous, hyperactive CTS contraction eventually causes the PIEZO1-dependent loss of epithelial HF progenitor cells, mainly through excessive & ectopic apoptosis. This seems to be somewhat in line with previous work from the lab of M Rendl on the importance of CTS contraction in murine hair follicle (HF) biology, whose clinical relevance has remained obscure, and as such enriches the human hair research literature substantially. However, in its current form, the study has many shortcomings.

1. Some statements in the Introduction & Discussion question how familiar the authors really are with human hair follicle biology and AGA, which (avoidably) mitigates enthusiasm for this study. For example: "In AGA, the growth phase decreases to only days or weeks, followed by premature entry into hair regression; this leads to an increase in the percentage of resting HFs, generating microscopic hairs in the balding scalp as the HF cycles(8, 9). In HF cycle, the growth of HFs relies on the proliferation and differentiation of HF stem/progenitor cells, whose activities are mainly regulated by the components of HF niche (including dermal papilla (DP), dermal sheath (DS), adipocytes and blood vessels etc.)". These statements require correction, and need to be better supported by optimally chosen citations.
a. Even in AGA, anagen typically lasts much longer than days or weeks.
b. Premature catagen entry does not cause HF miniaturization, but telogen effluvium. In fact, HF miniaturization can only occur during anagen, and can happen even within a single ongoing anagen phase (Whiting JAAD 2001). Instead, HF miniaturization is driven mainly by a declining size, cell number and inductive potential of the dermal papilla, while a reduction in the pool of epithelial progenitor cells seems to be only one of several additional relevant signals leading to HF miniaturization.
c. HF growth, or more precisely: the duration of anagen and the speed of hair shaft production, primarily relies on interactions between the rapidly proliferating cells of the of anagen hair matrix and inductive signals arising from/communication with the fibroblasts of the dermal papilla (epithelial stem cells and extrafollicular signals all prominently play into this, but are not the recognized driving forces).
d. In the Results, it is stated "Our data demonstrate that bulge cells first differentiate into ORS and IRS cells..." Yet, it is common knowledge that only hair matrix cells can terminally differentiate into IRS keratinocytes.
e. Keratin 6A is not a specific marker for the IRS, but is also expressed prominently throughout the ORS.
These selected examples, of which one could cite many more, suggest strongly that this study would profit from recruiting an expert hair biologist and AGA researcher as additional co-authors for professional text editing and literature synthesis and to assist with data interpretation.

2. The statement (Introduction) "To date, the pathogenetic study of AGA has been scant and limited to histomorphology, global gene analysis and immunohistochemistry due to the fact that most laboratory models are not fully representive of AGA(15, 16)" ignores that there is now an excellent humanized mouse model for preclinical AGA research, in which the authors' main working hypotheses could and should be experimentally probed: human AGA scalp xenotransplants on SCID mice (e.g., Zeltzer AA et al. Acta Derm Venereol. 2024, Gilhar et al. Acta Derm Venereol. 2024). This model permits exactly those functional long-term studies directly in human scalp skin that are required to convincingly support the authors' main claims.

3. The Introduction fails to clearly state the specific research questions addressed, and does not sufficiently explain the rationale for the chosen research design. For example, since scRNAseq analyses always struggle with gene expression profiling artifacts caused by enzymatic digestion and cell dispersion (which are not even discussed here) the use of spatial transcriptomics, combined with CellChat analysis, would overcome the limitations of scRNAseq. This would also have provided better insights into spatiotemporal interactions of unmanipulated HF cell populations within the native tissue habitat of AGA-affected versus AGA-non-affected scalp HFs (occipital versus frontotemporal).

4. That the gene expression profile of all investigated cell types "had differences in various degree (Fig. S2N, Supplementary Table 2)" is entirely expected, but does not characterize (and much less so explain) important transcriptional changes the precede the early stages of hair miniaturization in AGA - not the least since these early stages (=intermediate HFs, see Miranda et al. FASEB J 2018) do not seem to have been specifically studied. The latter, however, is mandatory if one wishes to make any plausible claims reg. the molecular controls that drive the early stages of HF miniaturization in AGA.

5. May be I just missed the points the authors tried to make here, but I fail to recognize in which way exactly this study advances the field fundamentally over what has already been published on HF stem cells and their immediate progeny, their heterogeneity, and corresponding AGA-associated abnormalities in the Garza/Cotsarelis study and later work (e.g., Purba et al. Sci Rep 2017). It is essential to clarify this.

6. The subclusters of DP cells and related changes in AGA reported here are interesting and potentially important - if the transcriptomic data are backed up on the decisive protein expression level by.

7. It is a general shortcoming of this study that too few transcriptional changes reported here that are critical for supporting the authors' main working hypothesis have been confirmed by IF/IHC and quantitatively analyzed at the protein level. It is strongly recommended that the authors expand these protein data sets, including but not restricted to verifying the reported changes in immune cell populations by quantitative immunohistomorphometry, following published standard protocols.

8. Measurements of changes in HF cycling seem to largely rely on macroscopic assessment. This is so unreliable as to be unacceptable. For hair cycle staging of organ-cultured human HFs, standard hair cycle histomorphometry needs to be employed (Kloepper et al. Exp Dermatol 2010), analyzing a sufficient number of HFs from at least 3 different donors.

9. It remains too unclear on which data exactly the differences between murine pelage and human scalp HF CTS that are depicted in the cartoon (Fig. 4A) are based. What exactly is the take home message from this cartoon, and how does this relate to the authors' main hypothesis? Please explain this more lucidly and corroborate the main claims contained in this cartoon by convincing evidence.

10. While the postulate that "hyperactive CTS contraction drives premature hair regression via inducing apoptosis of progenitor cells in AGA" is very intriguing, I am unconvinced by the currenly presented evidence. In human terminal anagen scalp HFs, the early stages of catagen development occur in the anagen hair matrix, i.e. very far distant from the bulge stem cells and their immediate progeny. While - in line with the Garza/Cotsarelis AGA study - it is theoretically conceivable that excessive progenitor apoptosis might result from hyperactive CTS contraction and may somehow contribute to HF miniaturization, it is therefore highly implausible that this should also induce premature catagen induction. To support this claim, much more direct evidence would have to be provided, such as the demonstration that key anagen-maintaining growth factors (IGF-1, KGF, HGF, Wnt activity) are reduced, while key drivers of catagen (TGFb1/2, SFRP1 etc.) are increased.

11. I am also intrigued by the reported hair growth promotion by ML-7, which would represent a welcome novel AGA treatment principle. However, convincing evidence that ML-7 is indeed more effective than minoxidil is best generfated in the humanized AGA model after long-term appliucation in vivo - especially since human HF organ culture is a notoriously unreliable assay for evaluating minoxidil activities (Magerl et al. Exp Dermatol 2004).

Minor issues:
-- The main concepts emanating from this study should be synthesized in an instructive cartoon (presented as part of the last core figure or as a supplementary fig.).
-- Complex statements like "To investigate the trajectories among all 20 cell types, monocle3(36) was employed to perform the pseudotime analysis with the root node setting at start point of bulge on the SOX9+ HF branch and start point of IFE basal on the SOX9- IFE branch separately...." (Results) will not be easily digestible by many non-expert readers and should be rephrased to be more instructive & self-explanatory.
-- "For the SOX9- IFE branch, IFE basal cells can directly differentiate into SOX9- PR and IFE spinous cells which finally develop into IFE granular cells (Fig. 1E). These patterns are consistent with the knowledge from previous studies(26, 35)." Please explain what exactly these results add to the published literature, how they advance the field, and why they are even relevant in the context of AGA and CTS contraction.
-- What exactly do the authors feel to be the role of DHT and AR in promoting the presumed hyperactive CTS contraction? Can they generate direct evidence to support or exclude such a role, e.g. in HF organ culture?

Reviewer #2

(Remarks to the Author)
The authors Li et al provide a single cell analysis of androgenic alopecia human skin. Androgenic alopecia is still largely

unexplored and, although being a “cosmetic” disease, harbors a high burden on many affected patients concerning their quality of life. Thus, high throughput investigations of this conditions are urgently mandated. Comparing occipital with frontal skin (affected and unaffected) is a reasonable approach.

Major

In the beginning and in the abstract, the authors need to more clearly state their sample size, which seems quite low. Also, it needs to be clearer whether investigations have been performed in same patients or independent cohorts.

The authors need to make clear in abstract, title and throughout the manuscript that they are investigating male patients, as balding patterns strongly differ between males and females.

The authors might want to add a table with QC parameters such as genes per cell, cells per sample obtained, as well as a table showing how many cells were filtered out during QC, and how many cells are present for each cluster and each sample. Also, a baseline table with patients age etc needs to be provided to assess whether the biopsies are appropriately matched.

A cutoff for DEGs of avg_log2FC>0.25 is very low, I would recommend to increase to 0.5 or 0.75 if feasible.

The authors need to better label their figures, the figures are very difficult to follow. Particularly also in supplements, the authors need to write what cells have been subclustered etc, right now plots are too sparsely labelled and partly incomprehensible.

When sublcustering, authors need to explain (or label in figure) what they obtained. For example for endothelial cells, they perform subclustering and obtain 4 clusters, but do not explain what they find. Arteriolar or venular or capillary? If they perform subclustering, they should elaborate on them. This needs to be done for all figures.

In lymphocyte subclustering, the “unknown” clusters seem keratinocyte contaminations.

Reviewer #3

(Remarks to the Author)
The manuscript entitled Single-cell transcriptomics reveals cellular hierarchies and aberrant CTS contraction-mediated premature hair regression in androgenetic alopecia revealed aberrant CTS contraction in AGA patients, which is the crucial factor that causes premature hair regression. Focusing on intracellular contraction force in HF is quite interesting. However, this work missed critical evidence to confirm the enhanced mechanical stress, and lacked further exploration in the detailed molecular mechanism of how contraction force led to hair regression.

Major concerns
1. The authors suggested enhanced connective tissue sheath as the significant driver of premature hair regression. However, bioinformatic analysis and immunostaining of p-MLC2 couldn’t fully support this hypothesis. Atomic force microscope is appreciated to examine and confirm the hyperactive contraction force in CTS.
2. Piezo1 is one of the most investigated mechanosensitive ion channel proteins in recent years. How did the authors come to consider exploring Piezo1, but not other proteins? Is the expression pattern of Piezo1 upregulated, or other mechanosensitive pathways functionally enriched, in the single cell sequence metadata of frontal balding, especially the CD200 positive subset?
3. The results indicated that Piezo1 inhibitor GsMTX4 could ameliorate hair regeneration. While in the next section, the authors also showed the positive effect of myosin activation inhibitor ML-7. From the authors’ perspective, inhibiting mechanosensitive ion channels via GsMTX4, or hindering cytoskeleton contraction via ML-7, which had a more significant regulatory effect on CTS contraction, and possibly be a therapeutic target. Please compare, verify, and illustrate.
4. The authors showed that ML-7 had a better effect on treating androgenetic alopecia. I would also prefer to know the therapeutic effect of ML-7 comparing with finasteride. And could the combination of ML-7 with minoxidil/finasteride acquire a better clinical outcome?
5. The authors didn’t explain the molecular mechanism of how Piezo1/myosin activation-induced CTS contraction induces apoptosis and thus causes premature hair regression, which is critical for an in-depth understanding of AGA progression.

Minor concerns
1. Before conducting scRNA-seq, the HFs were confirmed in anagen phase (line 117). Please describe the biomarker and briefly illustrate the sorting protocol for preparing single-cell suspension in the main body.
2. The results showed that all cell types had differences in various degree (Fig. S2N, Supplementary Table 2), suggesting that anagen HFs undergo significant changes at the early stage of hair miniaturization in AGA (line 163-165). Please explain the change in gene expression pattern and the corresponding biological meanings.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I wholeheartedly commend the authors for their extensive and very satisfactory efforts to greatly improve their study on multiple levels. In its current form, this study has matured into a major contribution that significantly advances the field: congratulations!

I have only minor suggestions for some additional improvements the authors may wish to consider.

Response 1a
The authors are still not quoting the currently most relevant literature and should read and cite at least Altendorf et al. Physiol Rev 2025.

Response 1b
The additional data generated are most welcome. Yet, as the authors appear to recognize, they do not provide sufficient evidence for explaining HF miniaturization, which is primarily driven by a reduction in the volume, cell number and inductive properties of the dermal papilla and secondarily by a reduction in the capacity of bulge epithelial stem cells to generate progeny (as explained in Altendorf et al. 2025). Therefore, to convincingly explain how CTS-related events may lead to HF miniaturization, it would have to be documented that these CTS events reduce the volume, cell number and inductive properties of the dermal papilla and/or the capacity of K15+ epithelial stem cells to generate CD34+ progeny. This should be stated more clearly in the Discussion.

Though I find the authors' working hypothesis how aberrant CTS contraction may exert differential effects on spatially distinct ORS and matrix cells plausible, in the Discussion, the authors should 1) openly acknowledge that the study provides no mechanistic evidence in support of this hypothesis and 2) very briefly suggest some key experiments to probe it.

The revised Discussion would profit from dissecting even more clearly in which respects exactly the current study a) is in line with, b) conflicts with, and c) advances the field compared to the previous CTS work published by the Rendl Lab in mice.

Reviewer #2

(Remarks to the Author)
The authors have answered all the reviewers questions. However, sample size and DEG cutoffs are very small, raising the question of reproducibility. Also, their reasoning that most of their DEGs disappeared with higher threshold indicates the weakness of their data. They need to reproduce their findings in independent sample sets.

Particularly, their reasoning to choose a FCH cutoff of 0.25 as it is the "default" in Seurat is a weak argument. They need to choose a threshold that is biologically meaningful. With such a small sample size and such a very low threshold, they need to more convincingly show that their detected genes are found in a reproducible fashion, which is impossible at n=4 and at their threshold.

Reviewer #3

(Remarks to the Author)
I appreciate the extensive amount of work the authors have undertaken in this revision, including new in vitro and ex vivo experiments, re-analysis of scRNA-seq data, AFM-based mechanical measurements, calcium imaging, and the humanized AGA mouse model. The revised manuscript shows substantial improvements in mechanistic depth, experimental rigor, and conceptual clarity. Most of the concerns I raised in the first round have been addressed thoroughly. However, several important issues still require clarification or further refinement to ensure that the manuscript reaches the level of completeness and scientific soundness expected for publication.
Below are my detailed second-round comments.
Major Comments
1. Further clarification needed regarding evidence for hyperactive CTS contraction
The authors have added AFM measurements of dermal sheath (DS) cell contractility and provided real-time imaging data indicating stronger contraction of DS cells from balding scalp under stimulation. These additions largely address my earlier concerns.
Remaining issues requiring clarification:
- Were DS cells from balding and non-balding follicles obtained from the same individual donors in the AFM measurements?
- Are the baseline (unstimulated) contraction forces also altered in DS cells from balding scalp?

2. Justification for prioritizing PIEZO1 over other mechanotransducers
The authors have added scRNA-seq evidence, TSA-amplified immunofluorescence data, and YODA1 functional assays.
Remaining suggestion:
- Briefly explain why other mechanotransducers were not further explored in the Discussion.

3. Comparison between ML-7 and GsMTX4 as therapeutic strategies
The new data show both agents rescue MCH-induced inhibition, with ML-7 showing stronger evidence in vivo.

4. Clarification regarding comparison with finasteride
Please add a clear explanation in the Discussion section regarding why finasteride cannot be meaningfully compared within the current model system.

5. Recommendation to include a schematic summarizing the proposed mechanistic pathway
A visual summary would enhance conceptual clarity.

Overall Assessment
The manuscript has improved significantly. I recommend minor revision before acceptance.

Dear reviewers:

We would like to express our deepest appreciation for your time and effort in reviewing our manuscript. Please find our point to point responses to the comments as detailed below. We are hopeful that all of the additional data, analyses and information we have provided satisfy your concerns.

Reviewer #1

Overall comments:

This is a very intriguing study that shifts attention in human AGA research from the role of androgens to the CTS by proposing that continuous, hyperactive CTS contraction eventually causes the PIEZO1-dependent loss of epithelial HF progenitor cells, mainly through excessive & ectopic apoptosis. This seems to be somewhat in line with previous work from the lab of M Rendl on the importance of CTS contraction in murine hair follicle (HF) biology, whose clinical relevance has remained obscure, and as such enriches the human hair research literature substantially. However, in its current form, the study has many shortcomings.

Overall response:

We thank the reviewer for the recognition of our work, and the helpful and constructive comments, all of which have been addressed, as detailed below.

Comment 1:

1. Some statements in the Introduction & Discussion question how familiar the authors really are with human hair follicle biology and AGA, which (avoidably) mitigates enthusiasm for this study. For example: "In AGA, the growth phase decreases to only days or weeks, followed by premature entry into hair regression; this leads to an increase in the percentage of resting HFs, generating microscopic hairs in the balding scalp as the HF cycles (8, 9). In HF cycle, the growth of HFs relies on the proliferation and differentiation of HF

stem/progenitor cells, whose activities are mainly regulated by the components of HF niche (including dermal papilla (DP), dermal sheath (DS), adipocytes and blood vessels etc.)". These statements require correction, and need to be better supported by optimally chosen citations.

Response 1:

We thank the reviewer for the kind suggestions, and apologize for the unprecise statements. As suggested, in the revised manuscript we have made the corresponding corrections according to optimally established literatures (J Am Acad Dermatol. 2001 Sep;45(3 Suppl):S81-6; PMID: 25905192; Exp Dermatol. 2022 Jun;31(6):980-982.) published by David A. Whiting and Ralf Paus, both world-renowned experts in AGA research, as detailed below.

a. Even in AGA, anagen typically lasts much longer than days or weeks.

Response 1a:

As suggested, we have corrected the unprecise statement.

b. Premature catagen entry does not cause HF miniaturization, but telogen effluvium. In fact, HF miniaturization can only occur during anagen, and can happen even within a single ongoing anagen phase (Whiting JAAD 2001). Instead, HF miniaturization is driven mainly by a declining size, cell number and inductive potential of the dermal papilla, while a reduction in the pool of epithelial progenitor cells seems to be only one of several additional relevant signals leading to HF miniaturization.

Response 1b:

We sincerely thank the reviewer for the constructive and helpful comments. Based on these comments and the established literatures, we have carefully re-analyzed and reconsidered our results. We agree that our initial interpretation may have introduced confusion or inaccuracies, particularly regarding the direct causality between premature catagen entry and hair follicle (HF) miniaturization, and we acknowledge that premature catagen entry does

not directly cause HF miniaturization.

Following the reviewer's suggestions, we performed a series of additional experiments to examine the proliferation of HF cells enveloped by CTS, including the outer root sheath (ORS) cells and matrix cells surrounding the dermal papilla. Our results demonstrated that excessive CTS contraction not only induces apoptosis in progenitor cells, but also significantly suppresses the proliferation of matrix cells and ORS cells (including progenitor cells) (**Shown in new Figure 4n, o; new Figure 4w-z; new Figure 5l, m; new Figure 5u, v**). Moreover, we have provided additional evidence showing that activating PIEZO1 in human hair follicle ORS cells directly with YODA1 in vitro not only significantly suppressed cell proliferation, but also induced apoptosis in a subset of cells (**Shown in new Supplementary Figure 14i-m**)—consistent with our observations from ex vivo organ-cultured human hair follicles. These alterations, especially the significant suppression of matrix cell proliferation, lead to hair growth retardation. We propose that this retardation may progressively contribute to HF miniaturization over time. Furthermore, in a subset of follicles, the combined effects of increased progenitor cell apoptosis and significantly reduced proliferation of both the matrix and progenitor cells appear to culminate in premature entry into catagen. With additional experiments and analyses, we observed that this premature catagen entry is reflected by the onset of apoptosis in matrix cells. Critically, this matrix cell apoptosis occurs after the observed increase in progenitor cell apoptosis and decrease in matrix/ORS cell proliferation (**Shown in new Supplementary Figure 9g-i**). This temporal sequence suggests that premature catagen entry is likely a consequence of these preceding events (apoptosis and proliferation defects), rather than a direct cause of HF miniaturization.

Therefore, consistent with these insights and new data, we have revised the description of the impact of excessive CTS contraction throughout the manuscript. References to "premature catagen entry" have been replaced with "hair follicle growth retardation".

Notably, our study provides no mechanistic evidence elucidating why aberrant CTS contraction exerts differential effects on spatially distinct ORS and matrix cells within human hair follicles. Specifically, aberrant CTS contraction simultaneously induces apoptosis and inhibits proliferation in ORS cells, whereas matrix cells exhibit only proliferation suppression at early stage. We postulate that this heterogeneity may correlate with spatially varying magnitudes of mechanical forces generated by CTS contraction along the hair follicle. However, validation of this hypothesis will require more sophisticated biomechanical experimentation in future studies. This limitation has been explicitly addressed in the revised discussion section of our manuscript.

c. HF growth, or more precisely: the duration of anagen and the speed of hair shaft production, primarily relies on interactions between the rapidly proliferating cells of the of anagen hair matrix and inductive signals arising from/communication with the fibroblasts of the dermal papilla (epithelial stem cells and extrafollicular signals all prominently play into this, but are not the recognized driving forces).

Response 1c:

As suggested, we have corrected the unprecise statement.

d. In the Results, it is stated "Our data demonstrate that bulge cells first differentiate into ORS and IRS cells..." Yet, it is common knowledge that only hair matrix cells can terminally differentiate into IRS keratinocytes.

Response 1d:

We apologize for the inaccurate result interpretation. To more accurately define hair follicle cell populations in our single-cell dataset, we performed spatial transcriptomics analysis using non-balding occipital scalp tissue from AGA patients. Based on these spatial transcriptomics results, we re-analyzed the cell clustering within our single-cell data. While the re-analysis largely confirmed our original findings, it revealed that cells previously identified as IRS cells

actually corresponded to ORS suprabasal cells (**Shown in new Figure 1b-e; new Supplementary Figure 3**). However, IRS cells were not detected in our single-cell dataset. We attribute this absence of IRS cells to limitations during the single-cell suspension preparation phase of our workflow, where these particular cells may not have been captured or preserved. Since this specific finding (**shown in former Figure 1E**) was not novel but consistent with existing literature (PMID: 35606346), a point also noted by another reviewer, we have removed this specific result from the revised manuscript.

Consequently, we have corrected the corresponding interpretations throughout the revised manuscript and ensured alignment with these updated findings.

e. Keratin 6A is not a specific marker for the IRS, but is also expressed prominently throughout the ORS.

Response 1e:

We thank the reviewer for the helpful comments, and apologize for the inaccurate result interpretation. Indeed, keratin 6A is not a specific marker for the IRS, but is expressed prominently throughout the ORS, which is comfirmed by our spatial transcriptomics analysis results (**Shown in new Figure 1b-e; new Supplementary Figure 3**). Therefore, we have corrected the corresponding interpretations throughout the revised manuscript.

These selected examples, of which one could cite many more, suggest strongly that this study would profit from recruiting an expert hair biologist and AGA researcher as additional co-authors for professional text editing and literature synthesis and to assist with data interpretation.

Response:

We thank the reviewer for the kind and helpful suggestion. As suggested, we have recruited professor Mingxing Lei (Chongqing University), an experted hair biologist and AGA researcher as an additional co-author for professional text

editing and literature synthesis and to assist with data interpretation. With the help from professor Mingxing Lei, we have tried our best to improve the manuscript.

Comment 2:

2. The statement (Introduction) "To date, the pathogenetic study of AGA has been scant and limited to histomorphology, global gene analysis and immunohistochemistry due to the fact that most laboratory models are not fully representive of AGA(15, 16)" ignores that there is now an excellent humanized mouse model for preclinical AGA research, in which the authors' main working hypotheses could and should be experimentally probed: human AGA scalp xenotransplants on SCID mice (e.g., Zeltzer AA et al. Acta Derm Venereol. 2024, Gilhar et al. Acta Derm Venereol. 2024). This model permits exactly those functional long-term studies directly in human scalp skin that are required to convincingly support the authors' main claims.

Response 2:

We appreciate the reviewer highlighting the utility of the humanized mouse model for preclinical AGA research. As suggested, we first established the humanized AGA mouse model by transplanting human scalps from non-balding and balding regions of AGA patients on SCID mice according to previous studies (e.g., Zeltzer AA et al. Acta Derm Venereol. 2024; Gilhar et al. Acta Derm Venereol. 2023; Gilhar et al. Exp Dermatol. 2022). Using this humanized AGA mouse model, we demonstrated that administration of MCH or DHT: (1) enhances CTS contraction, (2) induces apoptosis in the ORS cells (including progenitor cells), (3) significantly suppresses the proliferation of matrix cells and ORS cells, and (4) over extended observation periods, inhibits hair growth. Conversely, application of ML-7 effectively countered these effects, markedly improving the phenotypes induced by both MCH and DHT within xenografted hair follicles derived from the non-balding scalps of AGA patients (**Shown in new Figure 6a-r; new Supplementary Figure 16**). Most importantly, our

longitudinal observations revealed that ML-7, administered for over one month, prominently improved hair growth even within xenografted hair follicles derived from the balding scalps of AGA patients (**Shown in new Figure 6s-x**). These compelling in vivo findings are in agreement with the results we previously obtained in ex vivo HF organ culture assays.

We wish to clarify that the use of DHT and its relationship with CTS contraction have been specifically addressed in both our response to Minor issue 4 below and the corresponding Results section of the revised manuscript (**Shown in new Supplementary Figure 10 and 11**; **new Figure 6g-r**).

Comment 3:

3. The Introduction fails to clearly state the specific research questions addressed, and does not sufficiently explain the rationale for the chosen research design. For example, since scRNAseq analyses always struggle with gene expression profiling artifacts caused by enzymatic digestion and cell dispersion (which are not even discussed here) the use of spatial transcriptomics, combined with CellChat analysis, would overcome the limitations of scRNAseq. This would also have provided better insights into spatiotemporal interactions of unmanipulated HF cell populations within the native tissue habitat of AGA-affected versus AGA-non-affected scalp HFs (occipital versus frontotemporal).

Response 3:

We appreciate the reviewer's insightful comments regarding the need for clearer research questions and a stronger rationale for the research design in the Introduction which have been improved in the revised manuscript, as well as the inherent limitations of scRNA-seq analysis, including potential artifacts introduced by enzymatic digestion and cell dispersion, which we regrettably did not explicitly discuss previously. Due to significant financial constraints, conducting large-scale spatial transcriptomics on multiple AGA-affected samples was unfortunately not feasible for this study. However, we directly

followed the reviewer's advice and performed spatial transcriptomics analysis on scalp tissue from the non-balding occipital region of a single AGA patient. Based on this spatial transcriptomics data, published cell markers, and known hair follicle spatial organization, we meticulously re-analyzed our scRNA-seq cell clusters. This re-analysis largely corroborated our initial clustering results. Nonetheless, it also revealed a specific error: we had previously misclassified ORS suprabasal cells as IRS cells (**Shown in new Figure 1b-e; new Supplementary Figure 3**). We have corrected this misclassification throughout the revised manuscript based on the spatial transcriptomics evidence. Importantly, this revision does not alter the core conclusions of our study. Furthermore, we recognize the limitations of the scRNA-seq approach more broadly. We have therefore added a detailed discussion of these limitations (including sample preparation artifacts and spatial context loss) within the revised manuscript. To definitively address the spatiotemporal interactions crucial for AGA pathogenesis, as strongly suggested by the reviewer, we plan future studies utilizing spatial transcriptomics on intact scalp tissue specimens. This will specifically compare balding (frontotemporal) versus non-balding (occipital) regions from AGA patients, enabling a direct investigation within the native tissue habitat.

Comment 4:

4. That the gene expression profile of all investigated cell types "had differences in various degree (Fig. S2N, Supplementary Table 2)" is entirely expected, but does not characterize (and much less so explain) important transcriptional changes the precede the early stages of hair miniaturization in AGA - not the least since these early stages (=intermediate HFs, see Miranda et al. FASEB J 2018) do not seem to have been specifically studied. The latter, however, is mandatory if one wishes to make any plausible claims reg. the molecular controls that drive the early stages of HF miniaturization in AGA.

Response 4:

We appreciate the reviewer for the constructive comments. We also sincerely apologize for our previous inappropriate wording and fully acknowledge that our current results indeed cannot make any plausible claims regarding the molecular controls that drive the early stages of hair follicle miniaturization in AGA. Therefore, we have revised the relevant descriptions in the revised manuscript. Additionally, we have performed an in-depth analysis of transcriptional changes within our single-cell RNA sequencing dataset. This comprehensive re-analysis specifically included conducting differentially expressed gene (DEG) analyses and pathway enrichment analyses for the two critical comparisons: B (Balding) vs. NB (Non-balding), and B (Balding) vs. HS (Healthy Scalp), aiming to better characterize the specific transcriptional alterations in balding anagen HFs of AGA patients. To reflect these new findings, we have incorporated the full results of this in-depth transcriptional analysis into the revised manuscript. A detailed description of these results, including the newly identified DEGs and enriched pathways relevant to AGA development, now appears in the Results section (**Shown in new Supplementary Figure 4b, c; new Supplementary Table 5 and 6**).

Comment 5:

5. May be I just missed the points the authors tried to make here, but I fail to recognize in which way exactly this study advances the field fundamentally over what has already been published on HF stem cells and their immediate progeny, their heterogeneity, and corresponding AGA-associated abnormalities in the Garza/Cotsarelis study and later work (e.g., Purba et al. Sci Rep 2017). It is essential to clarify this.

Response 5:

We acknowledge that our finding of reduced HF stem cells (HFSCs) and their immediate progeny (progenitor cells) in the balding scalp of AGA patients indeed aligns with and is not unprecedented in the field, as consistently reported in previous seminal studies (J Clin Invest. 2011 Feb;121(2):613-22;

Sci Rep. 2017 Nov 9;7(1):15197). However, our study provides mechanistic insights and novel observations. Firstly, we demonstrate that CTS contraction -mediated cellular apoptosis might be a contributing mechanism to this progenitor cell reduction, a point not previously confirmed. Secondly, we provide evidence that aberrant CTS contraction suppresses the proliferation of matrix cells and ORS cells (**Shown in new Figure 4n, o; new Figure 4w-z; new Figure 5l, m; new Figure 5u, v**), potentially representing a key driver underlying HF miniaturization in AGA. Critically, as suggested by the reviewer, we rigorously tested our main findings using the humanized AGA mouse model (human AGA scalp xenografts on SCID mice). This in vivo validation offers robust, clinically oriented preclinical evidence supporting the targeting of CTS contraction as a potential therapeutic strategy for AGA (**Shown in new Figure 6; new Supplementary Figure 16**). Furthermore, our preliminary analysis via single-cell trajectory modeling suggests that HFSCs in AGA exhibit a differentiation bias towards epidermal and sebaceous gland lineages (**Shown in new Supplementary Figure 6a-f**). While this potential shift in fate commitment requires further functional and mechanistic investigation in future studies, it adds another layer to our understanding of HFSC dysregulation in AGA pathogenesis.

We have now included a direct comparison and discussion of our findings relative to the aforementioned foundational work within the revised Discussion section to clarify the specific advancements made by this study.

Comment 6:

6. The subclusters of DP cells and related changes in AGA reported here are interesting and potentially important - if the transcriptomic data are backed up on the decisive protein expression level by.

Response 6:

We sincerely appreciate the reviewer's constructive and insightful comments regarding the potential significance of DP cell subclusters and their changes in

AGA. Following your suggestion, we carefully reanalyzed the DP data. While our analysis identified five distinct DP subclusters, no statistically significant differences in their proportion were observed between B vs. HS or B vs. NB comparisons. Moreover, due to the very limited cell counts within each individual DP subcluster, conducting reliable subcluster-specific differential gene expression (DEG) analysis risked substantial technical bias. Consequently, we performed DEG analysis for total DP cells in B vs. HS and B vs. NB. This yielded 19 DEGs (Padj < 0.05) for B vs. HS and 101 DEGs (Padj < 0.05) for B vs. NB, with no overlap between the two lists (**Shown in new Supplementary Table 5**). Collectively, our results (subcluster proportions and aggregate DEGs) reveal no robust transcriptional signature differentiating AGA balding DP cells from either HS or NB controls within this dataset, suggesting no strong candidate pathway for immediate protein-level validation. We speculate this may result from the inherently low abundance of captured DP cells, potentially exacerbated by transcriptomic perturbations during single-cell suspension preparation. To address this limitation, we plan future studies utilizing multiple samples (B, NB, HS) through spatial transcriptomics or optimized suspension protocols to rigorously explore DP heterogeneity in AGA. We have explicitly acknowledged these limitations in the revised Discussion, advising readers to interpret our DP-related findings with caution.

Comment 7:

7. It is a general shortcoming of this study that too few transcriptional changes reported here that are critical for supporting the authors' main working hypothesis have been confirmed by IF/IHC and quantitatively analyzed at the protein level. It is strongly recommended that the authors expand these protein data sets, including but not restricted to verifying the reported changes in immune cell populations by quantitative immunohistomorphometry, following published standard protocols.

Response 7:

We appreciate the reviewer's kind suggestion regarding the need for greater protein-level validation. As suggested, we conducted additional co-immunostaining for CD4 and IL-17A, and performed quantitative analysis of both the number of CD4-positive cells and the proportion of IL-17A-positive cells within the total CD4-positive population across the study groups (**Shown in new Supplementary Figure 5n, o**), which is consistent with our transcriptomic findings. Furthermore, we have provided immunostaining of Cleaved-Caspase 3 with quantitative analysis of apoptotic cell number in progenitor/matrix cells, and p-MLC2 and α-SMA co-immunostaining with quantitative assessment of p-MLC2 intensity validating CTS hypercontraction—all critical evidence strengthening our mechanistic conclusions (**Shown in revised Figure 4c, d**).

Comment 8:

8. Measurements of changes in HF cycling seem to largely rely on macroscopic assessment. This is so unreliable as to be unacceptable. For hair cycle staging of organ-cultured human HFs, standard hair cycle histomorphometry needs to be employed (Kloepper et al. Exp Dermatol 2010), analyzing a sufficient number of HFs from at least 3 different donors.

Response 8:

We thank the reviewer for this constructive critique and clarify that our hair follicle cycling assessments were not solely based on macroscopic evaluation, but rather adopted histomorphometric methods detailed in the previous publication (Exp Dermatol. 2015 Dec;24(12):903-11)—a methodology concurrent with the approach mentioned by the reviewer (Kloepper et al. Exp Dermatol 2010), both originating from the Ralf Paus (a world-renowned expert in AGA research) research group. Our original **Supplementary Figure 1** already demonstrated cycle staging for HS, NB, and B groups using this standardized framework, showing that compared to catagen HFs, anagen HFs exhibited hair matrix (HM) with larger volume, more onion-shaped DP and

maximal melanin content; besides, the HM cells displayed obvious proliferative activity and no apoptotic signals. As suggested, to further validate our findings, we performed additional analyses on organ-cultured HFs treated with MCH ± ML-7, including: Cleaved-Caspase 3 immunostaining in matrix cells, Hematoxylin staining for melanin content, and RT-qPCR quantification of anagen-maintaining factors (IGF-1, KGF) and catagen drivers (TGFβ1/2, SFRP1)—characterized across HFs from ≥3 donors per condition. These comprehensive datasets reinforce our conclusions (**Shown in revised Supplementary Figure 8b, c; new Supplementary Figure 9g-i; new Supplementary Figure 10r; new Supplementary Figure 11h, i; new Supplementary Figure 16d**), with full methodological details now explicitly citing the literature (Kloepper et al. Exp Dermatol 2010) in our revised Methods section and experimental specifics integrated throughout Results, Figures, and Figure legends.

Comment 9:

9. It remains too unclear on which data exactly the differences between murine pelage and human scalp HF CTS that are depicted in the cartoon (Fig. 4A) are based. What exactly is the take home message from this cartoon, and how does this relate to the authors' main hypothesis? Please explain this more lucidly and corroborate the main claims contained in this cartoon by convincing evidence.

Response 9:

We regret any confusion caused by Fig. 4A, and clarify that this cartoon—modified from prior literature (Exp Dermatol. 2021 Apr;30(4):512-521.)—visually summarizes the key structural differences of CTS between murine pelage and human scalp HF, specifically illustrating that ORS (including progenitor cells) in mice is enveloped by DS structure (mainly composed of a layer of elastic DS cells), whereas the human scalp CTS (the homolog of murine DS) forms a multilayer structure comprising DS cells, collagens, and vascular elements (including vSMCs and endothelial cells), as detailed in our Results

section. To avoid any confusion, we have described it more clearly in the result section, and explicitly noted the cartoon's origin in the revised figure legend. Crucially, this cartoon contextualizes our functional findings that both DS cells and vSMCs exhibit augmented contractility in balding HFs (**Shown in revised Figure 2m, p**)—directly relevant as these cells constitute the primary contractile units of the human CTS, thereby underscoring how CTS hypercontraction mechanistically contributes to AGA pathogenesis.

Comment 10:

10. While the postulate that "hyperactive CTS contraction drives premature hair regression via inducing apoptosis of progenitor cells in AGA" is very intriguing, I am unconvinced by the currenly presented evidence. In human terminal anagen scalp HFs, the early stages of catagen development occur in the anagen hair matrix, i.e. very far distant from the bulge stem cells and their immediate progeny. While - in line with the Garza/Cotsarelis AGA study - it is theoretically conceivable that excessive progenitor apoptosis might result from hyperactive CTS contraction and may somehow contribute to HF miniaturization, it is therefore highly implausible that this should also induce premature catagen induction. To support this claim, much more direct evidence would have to be provided, such as the demonstration that key anagen-maintaining growth factors (IGF-1, KGF, HGF, Wnt activity) are reduced, while key drivers of catagen (TGFb1/2, SFRP1 etc.) are increased.

Response 10:

We sincerely thank the reviewer for the insightful comments. Based on these comments, comment 1 also provided by the reviewer and the established literatures, we have carefully re-analyzed and reconsidered our results. We acknowledge that our initial interpretation may have introduced confusion or inaccuracies, particularly regarding the direct causality between excessive progenitor apoptosis resulting from hyperactive CTS contraction and hair follicle (HF) miniaturization. In order to provide a clearer explanation, we performed a

series of additional experiments to examine the proliferation of HF cells enveloped by CTS, including the outer root sheath cells and matrix cells surrounding the dermal papilla. Our results demonstrated that excessive CTS contraction not only induces apoptosis in progenitor cells, but also significantly suppresses the proliferation of matrix cells and ORS cells (including progenitor cells) (**Shown in new Figure 4n, o; new Figure 4w-z; new Figure 5l, m; new Figure 5u, v**). Moreover, we have provided additional evidence showing that activating PIEZO1 in human hair follicle ORS cells directly with YODA1 in vitro not only significantly suppressed cell proliferation, but also induced apoptosis in a subset of cells (**Shown in new Supplementary Figure 14i-m**)—consistent with our observations from ex vivo organ-cultured human hair follicles. These alterations, especially the significant suppression of matrix cell proliferation, lead to hair growth retardation. We propose that this retardation may progressively contribute to HF miniaturization over time. Furthermore, in a subset of follicles, the combined effects of increased progenitor cell apoptosis and significantly reduced proliferation of both the matrix cells and ORS cells (including progenitor cells) appear to culminate in premature entry into catagen. With additional experiments and analyses suggested by the reviewer, including Cleaved-Caspase 3 immunostaining in matrix cells, Hematoxylin staining for melanin content, and RT-qPCR quantification of anagen-maintaining factors (IGF-1, KGF) and catagen drivers (TGFβ1/2, SFRP1), we observed that a portion of HFs treated with MCH or DHT indeed prematurely entered the catagen phase (**Shown in new Supplementary Figure 9g-i; new Supplementary Figure 10r; new Supplementary Figure 11h, i; new Supplementary Figure 16d**). Critically, this matrix cell apoptosis occurs after the observed increase in progenitor cell apoptosis and decrease in matrix/ORS cell proliferation (**Shown in new Supplementary Figure 9g-i**). This temporal sequence suggests that premature catagen entry is likely a consequence of these preceding events (apoptosis and proliferation defects), rather than a direct cause of HF miniaturization.

Therefore, consistent with these insights and new data, we have revised the description of the impact of excessive CTS contraction throughout the manuscript. References to "premature catagen entry" have been replaced with "hair follicle growth retardation".

Notably, our study provides no mechanistic evidence elucidating why aberrant CTS contraction exerts differential effects on spatially distinct ORS and matrix cells within human hair follicles. Specifically, aberrant CTS contraction simultaneously induces apoptosis and inhibits proliferation in ORS cells, whereas matrix cells exhibit only proliferation suppression at early stage. We postulate that this heterogeneity may correlate with spatially varying magnitudes of mechanical forces generated by CTS contraction along the hair follicle. However, validation of this hypothesis will require more sophisticated biomechanical experimentation in future studies. This limitation has been explicitly addressed in the revised discussion section of our manuscript.

Comment 11:

11. I am also intrigued by the reported hair growth promotion by ML-7, which would represent a welcome novel AGA treatment principle. However, convincing evidence that ML-7 is indeed more effective than minoxidil is best generfated in the humanized AGA model after long-term appliucation in vivo - especially since human HF organ culture is a notoriously unreliable assay for evaluating minoxidil activities (Magerl et al. Exp Dermatol 2004).

Response 11:

We thank the reviewer for the positive feedback and insightful suggestions regarding ML-7's therapeutic potential for AGA. As suggested, we extended our experiments in the humanized AGA mouse model, demonstrating that both ML-7 and minoxidil (MNX) significantly counteracted DHT-induced growth suppression in transplanted non-balding AGA hair follicles, with no statistically significant efficacy difference observed between them. Notably, combination treatment yielded superior improvement (**Shown in new Figure 6m-r**). More

strikingly, ML-7 administration prominently stimulated hair growth in xenografts derived from balding AGA scalp regions (**Shown in new Figure 6s-x**), further underscoring its translational relevance.

Minor issue 1:

The main concepts emanating from this study should be synthesized in an instructive cartoon (presented as part of the last core figure or as a supplementary fig.).

Response 1:

We thank the reviewer for this excellent suggestion. As recommended, we have now synthesized our main findings into an instructive cartoon (**Presented as new Supplementary Figure 17**).

Minor issue 2:

Complex statements like "To investigate the trajectories among all 20 cell types, monocle3 (36) was employed to perform the pseudotime analysis with the root node setting at start point of bulge on the SOX9+ HF branch and start point of IFE basal on the SOX9- IFE branch separately...." (Results) will not be easily digestible by many non-expert readers and should be rephrased to be more instructive & self-explanatory.

Response 2:

We sincerely appreciate the reviewer's constructive suggestion regarding the accessibility of our pseudotime analysis description. Considering that this particular finding (former Figure 1E) lacked novelty and similar with previously reported studies—a point also noted by another reviewer—we have removed this result from the revised manuscript. Concurrently, we have diligently refined similar technical descriptions throughout the manuscript to enhance clarity and ensure self-contained explanatory value for non-specialist readers.

Minor issue 3:

"For the SOX9- IFE branch, IFE basal cells can directly differentiate into SOX9-PR and IFE spinous cells which finally develop into IFE granular cells (Fig. 1E). These patterns are consistent with the knowledge from previous studies(26, 35)." Please explain what exactly these results add to the published literature, how they advance the field, and why they are even relevant in the context of AGA and CTS contraction.

Response 3:

We appreciate the reviewer's constructive critique regarding the significance of our SOX9– IFE trajectory findings and their relevance to AGA pathogenesis. In reconsidering this result, we recognized it confirmed known epidermal differentiation paradigms without introducing conceptual novelty, was peripherally related to our core focus on CTS contraction mechanisms in AGA, and was rendered obsolete by subsequent analysis—after integrating spatial transcriptomics data, all cell clustering was redefined without SOX9-based guidance. Thus, aligning with another reviewer's assessment, we have removed this analysis from the revised manuscript.

Minor issue 4:

What exactly do the authors feel to be the role of DHT and AR in promoting the presumed hyperactive CTS contraction? Can they generate direct evidence to support or exclude such a role, e.g. in HF organ culture?

Response 4:

We thank the reviewer for the interest in the potential role of DHT and AR in hyperactive CTS contraction, which indeed raises an intriguing question. Prompted by a recent Cell study demonstrating DHT promotes muscle cell contraction via the membrane receptor GPR133 (Cell. 2025 Mar 20;188(6):1589-1604.e24), we investigated whether DHT might similarly stimulate CTS contraction through GPR133 in AGA pathogenesis. Our new data revealed that GPR133 is indeed expressed in human HF CTS cells (**Shown in new Supplementary Figure 10a**). In vitro assays using isolated

dermal sheath (DS) cells—a key CTS component—demonstrated that DHT potently enhances DS cell contraction; this effect was markedly attenuated by GPR133 knockdown with siRNA (**Shown in new Supplementary Figure 10b-f**). Further HF organ culture experiments showed DHT not only induces CTS hypercontraction but also triggers progenitor cell apoptosis, suppresses matrix and ORS cell proliferation, and ultimately inhibits hair growth—all of which were significantly reversed by either GPR133 knockdown or ML-7-mediated contractility inhibition (**Shown in new Supplementary Figure 10g-r; new Supplementary Figure 11a-i**). Importantly, while AR knockdown alleviated DHT's inhibitory effects on hair follicle growth, it did not alter CTS contraction (**Shown in new Supplementary Figure 11k-m**). These findings suggest that DHT contributes to AGA pathogenesis through two distinct mechanisms: (1) canonical AR signaling in follicular cells (e.g., DP and ORS cells), and (2) GPR133-mediated hypercontraction in CTS components (e.g., DS cells). This DHT-GPR133-dependent contractile mechanism might represent a key consideration for AGA therapeutic development, particularly regarding AR-targeting therapies since AR blockade alone may not address this pathway. We have incorporated these mechanistic insights in the revised manuscript's Discussion section.

Reviewer #2

Overall comments:

The authors Li et al provide a single cell analysis of androgenic alopecia human skin. Androgenic alopecia is still largely unexplored and, although being a "cosmetic" disease, harbors a high burden on many affected patients concerning their quality of life. Thus, high throughput investigations of this conditions are urgently mandated. Comparing occipital with frontal skin (affected and unaffected) is a reasonable approach.

Overall response:

We thank the reviewer for the recognition of our work, and the insightful and helpful comments, all of which have been addressed, as detailed below.

Comment 1:

In the beginning and in the abstract, the authors need to more clearly state their sample size, which seems quite low. Also, it needs to be clearer whether investigations have been performed in same patients or independent cohorts.

Response 1:

We sincerely appreciate the reviewer's kind suggestion. Accordingly, we have clarified the sample size in the revised manuscript. Due to word constraints in the abstract, we now explicitly detail sample information in the beginning of Results section, Figure legends and "Human scalp and HF samples" subsection of Methods. Specifically, we have stated that scRNA-seq analysis utilized matched frontal balding/occipital non-balding HF units from 4 AGA patients and frontal HF units from 4 healthy donors, while HF organ culture and humanized mouse model experiments employed specimens from distinct cohorts. To enhance transparency, we provide a supplementary table cataloging all specimens used across experiments, including patient/donor sources, biopsy sites, and respective methodologies (**Shown in new Supplementary Table 18**).

Comment 2:

The authors need to make clear in abstract, title and throughout the manuscript that they are investigating male patients, as balding patterns strongly differ between males and females.

Response 2:

We sincerely appreciate the reviewer's constructive suggestion. As suggested, we have clarified throughout the revised manuscript—including the abstract, title, and main text—that this study exclusively investigates male patients, thereby addressing potential ambiguities regarding sex-specific balding

patterns. Also, we have discussed this limitation in the Discussion section of the revised manuscript.

Comment 3:

The authors might want to add a table with QC parameters such as genes per cell, cells per sample obtained, as well as a table showing how many cells were filtered out during QC, and how many cells are present for each cluster and each sample. Also, a baseline table with patients age etc needs to be provided to assess whether the biopsies are appropriately matched.

Response 3:

We thank the reviewer for this valuable suggestion. As suggested, we have added two comprehensive tables in the revised manuscript: (1) a QC metrics table detailing genes per cell, cells per sample, and the number of cells filtered during quality control (**Shown in new Supplementary Table 1**); and (2) a cluster distribution table specifying cell counts per cluster and sample (**Shown in new Supplementary Table 4**). Additionally, a baseline characteristics table including patient age, biopsy site, and clinical parameters has been provided to confirm appropriate cohort matching across all specimens (**Shown in new Supplementary Table 18**).

Comment 4:

A cutoff for DEGs of avg_log2FC>0.25 is very low, I would recommend to increase to 0.5 or 0.75 if feasible.

Response 4:

We appreciate the reviewer's suggestion regarding the DEG cutoff threshold. For clarity, the selection criterion (avg_log2FC > 0.25) aligns with Seurat's default parameters, reflecting that scRNA-seq datasets typically yield fewer DEGs than bulk RNA-seq (where avg_log2FC > 1 is common). When testing stricter thresholds (avg_log2FC > 0.5/0.75), we observed a dramatic reduction in detected DEGs, with many cell clusters exhibiting minimal or no significant

DEGs. This may reflect our use of early-stage AGA follicular specimens—collected prior to marked miniaturization—where transcriptomic alterations are inherently subtle. Notwithstanding this lower threshold, we exclusively prioritized the top 20 DEGs ranked by avg_log2FC when identifying cell/subpopulation markers to ensure biological relevance.

Comment 5:

The authors need to better label their figures, the figures are very difficult to follow. Particularly also in supplements, the authors need to write what cells have been subclustered etc, right now plots are too sparsely labelled and partly incomprehensible.

Response 5:

We apologize for the insufficient labeling in our original figures and sincerely appreciate the reviewer's constructive suggestion. Accordingly, we have comprehensively relabeled all figures throughout the manuscript with detailed annotations, particularly highlighting cell-type specifications and subclustering information to ensure clarity and interpretability.

Comment 6:

When sublcustering, authors need to explain (or label in figure) what they obtained. For example for endothelial cells, they perform subclustering and obtain 4 clusters, but do not explain what they find. Arteriolar or venular or capillary? If they perform subclustering, they should elaborate on them. This needs to be done for all figures.

Response 6:

We thank the reviewer for this insightful suggestion. Accordingly, in the revised manuscript, we have provided definitive labeling and textual interpretation of all cellular subclustering results across figures, specifically delineating subtypes of each cell type in relevant annotations, legends, and results sections to ensure comprehensive biological clarity.

Comment 7:

In lymphocyte subclustering, the "unknown" clusters seem keratinocyte contaminations.

Response 7:

We fully agree with the reviewer's insightful observation. The "unknown" cluster expresses high levels of keratinocyte marker genes (e.g., *KRT14*, *KRT15*, *KRT1, KRT10*), suggesting these likely represent dying keratinocyte-derived debris (or other dying cell-derived debris containing a large amount of mRNA from dead keratinocytes) inadvertently encapsulated into droplets during single-cell processing. Although we cannot definitively verify this contamination source, these data points are retained in UMAP projections to ensure data transparency.

Reviewer #3

Overall comments:

The manuscript entitled Single-cell transcriptomics reveals cellular hierarchies and aberrant CTS contraction-mediated premature hair regression in androgenetic alopecia revealed aberrant CTS contraction in AGA patients, which is the crucial factor that causes premature hair regression. Focusing on intracellular contraction force in HF is quite interesting. However, this work missed critical evidence to confirm the enhanced mechanical stress, and lacked further exploration in the detailed molecular mechanism of how contraction force led to hair regression.

Overall response:

We thank the reviewer for the recognition of our work, and the insightful and helpful comments, all of which have been addressed, as detailed below.

Major concern 1:

1. The authors suggested enhanced connective tissue sheath as the significant driver of premature hair regression. However, bioinformatic analysis and immunostaining of p-MLC2 couldn't fully support this hypothesis. Atomic force microscope is appreciated to examine and confirm the hyperactive contraction force in CTS.

Response 1:

We greatly appreciate the reviewer's constructive suggestion. As suggested, we performed atomic force microscopy (AFM) measurements directly comparing the contraction force of primary dermal sheath (DS) cells—key cellular components of the CTS—isolated from balding versus non-balding hair follicles (HFs) of AGA patients. These experiments demonstrated that stimulators (e.g., MCH or DHT) significantly enhanced DS cell contractility, with DS cells from balding HFs exhibiting stronger stimulator-induced contraction forces versus non-balding counterparts (**Shown in new Supplementary Figure 8h, i; new Supplementary Figure 10d; new Supplementary Figure 8d-g; new Supplementary Figure 10a-c**). Complementarily, real-time confocal imaging of primary DS cells and human hair follicle organ cultures consistently revealed that stimulators robustly promote contraction in both systems; notably, DS cells and hair follicles derived from balding scalp of AGA patients showed markedly amplified contractile responses upon stimulation (**Shown in new Figure 4e, f; new Supplementary Figure 8j, k; new Supplementary Figure 9b, c; new Supplementary Figure 10e-h**). These data directly validate hyperactive CTS contraction in AGA pathophysiology, strengthening our initial mechanistic hypothesis.

Major concern 2:

2. Piezo1 is one of the most investigated mechanosensitive ion channel proteins in recent years. How did the authors come to consider exploring Piezo1, but not other proteins? Is the expression pattern of Piezo1 upregulated, or other mechanosensitive pathways functionally enriched, in the single cell sequence

metadata of frontal balding, especially the CD200 positive subset?

Response 2:

We thank the reviewer for the insightful comments and constructive suggestions. In fact, our prior analysis of scRNA sequencing data had already assessed the expression profiles of multiple mechanosensitive ion channels (including PIEZO1, PIEZO2, TRPV4, KCNK2, KCNK4, KCNK10, STOML3 and TRPA1), revealing PIEZO1 and TRPV4 as the predominant channels in hair follicle stem cells (HFSCs). However, since published evidence (PMID: 34624205) established PIEZO1—but not TRPV4—as essential for murine HFSC function, we prioritized PIEZO1 for mechanistic validation in human AGA. This rationale led us to initially verify PIEZO1 upregulation via immunofluorescence in balding HFSCs (including progenitor cells) of AGA patients, rather than presenting our scRNA-seq data. In direct response to this comment, we have now: (1) added these scRNA-seq analyses to the revised manuscript, confirming PIEZO1's enrichment in the HFSC subset and other mechanosensitive cell populations (e.g., ORS, matrix) in human hair follicles; and (2) performed enhanced immunofluorescence using tyramide signal amplification (TSA) on balding versus non-balding AGA hair follicles, and our quantitative results directly validate PIEZO1 upregulation within ORS (including progenitor cells), and matrix of balding HFs (**Shown in new Figure 5a-d; new Supplementary Figure 14a, b**). Simultaneously, we have provided additional evidence showing that inhibiting TRPV4 by its specific inhibitor RN1734 fails to rescue the MCH-induced growth suppression of human hair follicles (**Shown in new Supplementary Figure 14n, o**). These results firmly establish the pathological relevance of PIEZO1-mediated mechanotransduction in AGA.

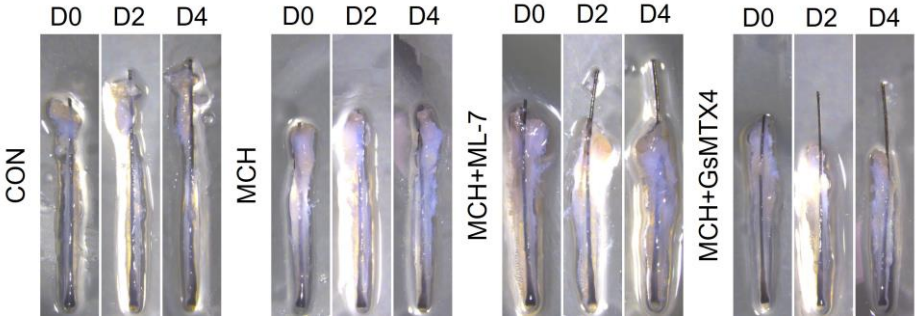
Major concern 3:

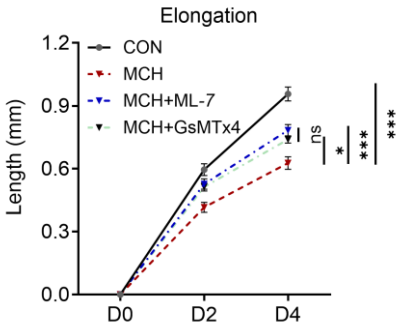
3. The results indicated that Piezo1 inhibitor GsMTX4 could ameliorate hair regeneration. While in the next section, the authors also showed the positive

effect of myosin activation inhibitor ML-7. From the authors' perspective, inhibiting mechanosensitive ion channels via GsMTX4, or hindering cytoskeleton contraction via ML-7, which had a more significant regulatory effect on CTS contraction, and possibly be a therapeutic target. Please compare, verify, and illustrate.

Response 3:

We are grateful for the reviewer's insightful comments on therapeutic target prioritization. To directly answer this question, we conducted additional comparative functional assays using MCH-treated human hair follicles, and demonstrated that both GsMTX4 and ML-7 effectively rescued growth inhibition, with ML-7 exhibiting a modest but non-significant advantage in efficacy (**Data presented below, for review only**). Crucially, our mechanistic model indicates that pathological CTS hypercontraction in AGA balding follicles (inhibited by ML-7) drives downstream PIEZO1 activation in HF cells (e.g., progenitor cells, matrix; inhibited by GsMTX4), thereby inducing apoptosis, suppressing proliferation, and ultimately impairing HF growth. Thus, ML-7's blockade of the proximal mechanical trigger provides broader therapeutic leverage by targeting the root cause, whereas GsMTX4 cannot rectify the primary CTS defect despite ameliorating its consequences. This premise is strongly supported by compelling preclinical validation: in humanized mouse models, ML-7 exhibited robust efficacy (**Shown in new Figure 6**), underscoring its enhanced translational promise. We maintain that topical/follicle-targeted delivery of either agent—rather than systemic administration—represents the optimal strategy to mitigate potential adverse effects in clinical settings.





Major concern 4:

4. The authors showed that ML-7 had a better effect on treating androgenetic alopecia. I would also prefer to know the therapeutic effect of ML-7 comparing with finasteride. And could the combination of ML-7 with minoxidil/finasteride acquire a better clinical outcome?

Response 4:

We appreciate the reviewer's constructive suggestions. To address this, we compared the therapeutic efficacy of ML-7 versus minoxidil (MNX) and their combination in humanized mouse models. Our new results showed that both ML-7 and MNX significantly rescued DHT-induced hair growth suppression, while their combination demonstrated superior therapeutic outcomes (**Shown in new Figure 6m-r**). Notably, no statistically significant difference was observed between ML-7 and MNX monotherapies in vivo, contrasting with our prior ex vivo human hair follicle organ culture data where ML-7 outperformed MNX (**Shown in revised Supplementary Figure 15e-j**). This discrepancy may stem from MNX's potential pro-angiogenic/vasodilatory effects within the cutaneous microenvironment—benefits not fully realized in isolated HF cultures. Moreover, given that finasteride primarily functions by inhibiting 5α-reductase to suppress DHT synthesis—and our current HF organ culture/humanized AGA mouse models (established by using exogenous DHT or MCH) do not target 5α-reductase activity or upstream regulatory pathways—we have not performed direct efficacy assessments of finasteride in these systems.

Major concern 5:

5. The authors didn’t explain the molecular mechanism of how Piezo1/myosin activation-induced CTS contraction induces apoptosis and thus causes premature hair regression, which is critical for an in-depth understanding of AGA progression.

Response 5:

We appreciate the reviewer's constructive comments. Previous studies have established that PIEZO1 activation in murine hair follicle stem cells elevates intracellular calcium, triggering mitochondrial membrane permeabilization to release pro-apoptotic factors and ultimately induce apoptosis (Cell Stem Cell. 2022;29:70-85); concurrently, PIEZO1-mediated calcium signaling suppresses murine HFSC activation and impedes hair regeneration (Sci Adv. 2025;11:eadt2771). To elucidate whether this mechanism underlies AGA, we performed calcium imaging using Cal520, a more specific calcium influx indicator than Fluo-4, in hair follicles from AGA patients (balding vs. non-balding regions) and MCH-treated human HF organs. This revealed significantly intensified calcium flux in the ORS (including progenitor cells) and matrix cells of balding HFs and MCH-treated HF organs (**Shown in new Figure 5c, d; and new Supplementary Figure 14c, d, f, g**). Crucially, experiments on isolated primary human ORS cells demonstrated that the PIEZO1 agonist YODA1 directly elevated intracellular calcium, inhibited proliferation, compromised mitochondrial function, and upregulated the apoptotic executioner molecule cleaved caspase-3, culminating in apoptosis (**Shown in new Supplementary Figure 14i-m**). Thus, pathological CTS contraction—or the consequential PIEZO1 activation in ORS/matrix cells—drives aberrant calcium signaling that simultaneously induces apoptosis (via mitochondrial-caspase axis) and suppresses proliferation, collectively propagating AGA pathogenesis. Although our preliminary evidence suggests that PIEZO1-mediated dysregulated calcium signaling may play a contributory role in this process, , the detailed molecular

mechanisms by which PIEZO1 hyperactivation induces progenitor cell apoptosis and suppresses proliferation of ORS/matrix cells in AGA need further study to clarify. These new experimental findings and supporting literature citations have been incorporated into the revised manuscript and discussed in depth in the Discussion section.

Minor concern 1:

1. Before conducting scRNA-seq, the HFs were confirmed in anagen phase (line 117). Please describe the biomarker and briefly illustrate the sorting protocol for preparing single-cell suspension in the main body.

Response 1:

We thank the reviewer for this kind suggestion. As suggested, we have described the relevant biomarker for confirming the anagen phase in the Result section, and briefly illustrated the sorting protocol for preparing single-cell suspensions in the “Preparation of single-cell suspensions of HF units” of Methods section of the revised manuscript.

Minor concern 2:

2. The results showed that all cell types had differences in various degree (Fig. S2N, Supplementary Table 2), suggesting that anagen HFs undergo significant changes at the early stage of hair miniaturization in AGA (line 163-165). Please explain the change in gene expression pattern and the corresponding biological meanings.

Response 2:

We sincerely appreciate the reviewer's valuable suggestion. As suggested, we have provided explanations for the change in gene expression pattern and the corresponding biological meanings in the Results section (Detailed data was also Shown in Supplementary Table 5 and 6).

Again, we appreciate the constructive and insightful comments, and helpful

suggestions from the reviewers. We hope that with the revisions the manuscript will be acceptable for publication.

Best wishes.

Sincerely

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Dear reviewers:

We would like to express our deepest appreciation for your time and effort in handling our manuscript. Please find our point-to-point responses to the reviewers' comments as detailed below. We are hopeful that all of the additional data, analyses and information we have provided satisfy the reviewers' concerns.

REVIEWERS' COMMENTS

Reviewer #1:

I wholeheartedly commend the authors for their extensive and very satisfactory efforts to greatly improve their study on multiple levels. In its current form, this study has matured into a major contribution that significantly advances the field: congratulations!

I have only minor suggestions for some additional improvements the authors may wish to consider.

Comment#1a:

The authors are still not quoting the currently most relevant literature and should read and cite at least Altendorf et al. Physiol Rev 2025.

Response:

We thank the reviewer for this valuable suggestion. We have now cited Altendorf et al., *Physiological Reviews*, 2025, together with several recent high-impact studies published in the past two years that are highly relevant to androgenetic alopecia pathogenesis, and have incorporated these references into the Introduction and Discussion.

Comment#1b:

The additional data generated are most welcome. Yet, as the authors appear to recognize, they do not provide sufficient evidence for explaining HF miniaturization, which is primarily driven by a reduction in the volume, cell number and inductive properties of the dermal papilla and secondarily by a

reduction in the capacity of bulge epithelial stem cells to generate progeny (as explained in Altendorf et al. 2025). Therefore, to convincingly explain how CTS-related events may lead to HF miniaturization, it would have to be documented that these CTS events reduce the volume, cell number and inductive properties of the dermal papilla and/or the capacity of K15+ epithelial stem cells to generate CD34+ progeny. This should be stated more clearly in the Discussion.

Though I find the authors' working hypothesis how aberrant CTS contraction may exert differential effects on spatially distinct ORS and matrix cells plausible, in the Discussion, the authors should 1) openly acknowledge that the study provides no mechanistic evidence in support of this hypothesis and 2) very briefly suggest some key experiments to probe it.

The revised Discussion would profit from dissecting even more clearly in which respects exactly the current study a) is in line with, b) conflicts with, and c) advances the field compared to the previous CTS work published by the Rendl Lab in mice.

Response:

We thank the reviewer for these insightful comments. We have revised the Discussion to more clearly situate our findings within the established dermal papilla–centric framework of hair follicle miniaturization, explicitly acknowledge the mechanistic limitations of the present study, briefly outline key future experimental directions, and clarify how our work relates to and extends previous CTS studies from the Rendl laboratory. We thank the reviewer again for these important and constructive comments.

Reviewer #2

Comment:

The authors have answered all the reviewers questions. However, sample size and DEG cutoffs are very small, raising the question of reproducibility. Also,

their reasoning that most of their DEGs disappeared with higher threshold indicates the weakness of their data. They need to reproduce their findings in independent sample sets.

Particularly, their reasoning to choose a FCH cutoff of 0.25 as it is the "default" in Seurat is a weak argument. They need to choose a threshold that is biologically meaningful. With such a small sample size and such a very low threshold, they need to more convincingly show that their detected genes are found in a reproducible fashion, which is impossible at n=4 and at their threshold.

Response:

We thank the reviewer for raising these important concerns regarding sample size, DEG thresholds, and reproducibility. We have clarified in the revised manuscript that the avg_log2FC cutoff of 0.25 was used as an inclusive screening threshold to generate ranked gene lists for downstream gene set enrichment analyses, which are designed to detect coordinated transcriptional shifts rather than strong individual gene effects. Accordingly, we have limited gene-level interpretations and emphasize that our biological conclusions are supported by pathway-level enrichment, cross-cell-type consistency, spatial transcriptomic validation, and multiple orthogonal functional assays, rather than reliance on individual DEGs alone.

Reviewer #3:

I appreciate the extensive amount of work the authors have undertaken in this revision, including new in vitro and ex vivo experiments, re-analysis of scRNA-seq data, AFM-based mechanical measurements, calcium imaging, and the humanized AGA mouse model. The revised manuscript shows substantial improvements in mechanistic depth, experimental rigor, and conceptual clarity. Most of the concerns I raised in the first round have been addressed thoroughly. However, several important issues still require clarification or further refinement

to ensure that the manuscript reaches the level of completeness and scientific soundness expected for publication.

Below are my detailed second-round comments.

Major Comments:

1. Further clarification needed regarding evidence for hyperactive CTS contraction

The authors have added AFM measurements of dermal sheath (DS) cell contractility and provided real-time imaging data indicating stronger contraction of DS cells from balding scalp under stimulation. These additions largely address my earlier concerns.

Remaining issues requiring clarification:

- Were DS cells from balding and non-balding follicles obtained from the same individual donors in the AFM measurements?
- Are the baseline (unstimulated) contraction forces also altered in DS cells from balding scalp?

Response 1:

We thank the reviewer for this important clarification request. We have now explicitly clarified in the Methods that DS cells used for AFM measurements were derived from matched balding and non-balding follicles from the same individual donors. In addition, we have clarified in the Results and Discussion that baseline (unstimulated) DS contractility did not show a consistent difference, whereas hypercontractility became evident under defined contractile stimulation, supporting a stimulus-dependent pathological sensitization rather than constitutive activation.

2. Justification for prioritizing PIEZO1 over other mechanotransducers

The authors have added scRNA-seq evidence, TSA-amplified immunofluorescence data, and YODA1 functional assays.

Remaining suggestion:

- Briefly explain why other mechanotransducers were not further explored in the

Discussion.

Response 2:

We thank the reviewer for this helpful suggestion. We have revised the Discussion to briefly clarify that PIEZO1 was prioritized based on its relatively higher expression, consistent activation signature, and functional responsiveness in our system, while other mechano-transducers were not excluded but were beyond the scope of the current study.

3. Comparison between ML-7 and GsMTX4 as therapeutic strategies

The new data show both agents rescue MCH-induced inhibition, with ML-7 showing stronger evidence in vivo.

Response 3:

We thank the reviewer for the positive assessment and for recognizing the strength of the comparative evidence presented.

4. Clarification regarding comparison with finasteride

Please add a clear explanation in the Discussion section regarding why finasteride cannot be meaningfully compared within the current model system.

Response 4:

We thank the reviewer for this important point. We have added a clarification in the Discussion explaining that finasteride acts upstream by inhibiting dihydrotestosterone production, whereas the hair follicle organ culture and humanized androgenetic alopecia models used in this study rely on exogenous dihydrotestosterone or MCH to induce AGA-like phenotypes. As a result, these models do not engage 5α-reductase activity, precluding a meaningful or mechanistically informative comparison of finasteride within the current experimental framework.

5. Recommendation to include a schematic summarizing the proposed mechanistic pathway

A visual summary would enhance conceptual clarity.

Response 5:

We thank the reviewer for this helpful suggestion. We have now added a schematic model summarizing the proposed CTS contraction–PIEZO1 mechano-transduction pathway and its impact on progenitor cell survival and hair follicle growth in Fig S17.

Overall Assessment:

The manuscript has improved significantly. I recommend minor revision before acceptance.

Response:

We thank the reviewer for this positive overall assessment and recommendation.

Thank you again for your time and insightful comments and suggestions.

Yours sincerely,

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