Dear Reviewers,

We sincerely thank you for your time and dedication in reviewing our manuscript. Your insightful comments and constructive suggestions have been instrumental in improving the quality and clarity of our work.

We have carefully addressed all the points raised and have made substantial revisions to the manuscript accordingly. In the following pages, we provide point-by-point responses to each comment. We believe these changes have significantly enhanced the scientific rigor, clarity, and overall presentation of our research.

We appreciate the opportunity to revise our manuscript and look forward to your further review.

Sincerely,

Zhijun Feng

Southern Medical University

Comments from Reviewer 3:

Comment 1: What techniques were used to investigate the heterogeneity of epithelial cells in ageing skin?

Reply to Comment 1: Thank you for your important question. In our study, to comprehensively investigate the heterogeneity of keratinocytes in aging skin, we employed multiple complementary technical approaches. First, we used single-cell RNA sequencing (scRNA-seq) as our primary technique, extracting 2323 keratinocytes from the GSE130973 dataset, and performed data normalization and batch effect correction using the Seurat package and Harmony integration. We applied the Louvain algorithm for cell clustering and identified subgroups such as basal cells and squamous cells based on the expression patterns of known marker genes including KRT5, KRT14, KRT1, and KRT10. Second, we constructed cell differentiation trajectories using Monocle2, revealing developmental trajectory changes of keratinocytes during the aging process. Additionally, we analyzed cell-cell communication networks using CellChat and validated our findings with the GSE67098 transcriptome dataset, which contains samples from young (<35 years) and elderly (>60 years) skin. Finally, we integrated GWAS data on facial skin aging to

explore the association between genetic variations and skin aging. In the limitations section of our revised manuscript (Lines 668-679), we specifically emphasized that a significant limitation of our current study is the lack of integration between genetic variants (GWAS) and epigenomic analyses. Although we identified aging-associated cell subpopulations and their transcriptional signatures, we could not determine how specific SNPs affect epigenetic modifications, transcription factor binding, or 3D chromatin architecture, which might drive this cellular heterogeneity. Furthermore, our study could not capture the differences in epigenetic landscapes among different keratinocyte subpopulations, which may cause functionally identical cells to adopt specialized states with age.

Thank you again.

Comment 2: Was it not clear from the text the relationship between the loss of NOTCH signaling and skin aging?

Reply to Comment 2: Thank you for your suggestion. Regarding the comment on the relationship between NOTCH signaling and skin aging, we understand your concern about the clarity of our explanation. In our study, we did observe significant changes in the NOTCH signaling pathway during the progression from BC-ES to BC-AS2 and IFI27+ subpopulations, including the loss of JAG1-NOTCH1/NOTCH2/NOTCH3 and DLL1-NOTCH related pathways. However, as you correctly pointed out, due to our lack of experimental validation, the exact mechanism remains unclear. This is precisely why we adopted a relatively conservative approach in our manuscript. We proposed the potential role of NOTCH signaling in maintaining epidermal homeostasis and the possible consequences of its loss, but avoided making definitive conclusions. In the revised version, we will more clearly indicate that these are observations based on computational analysis and emphasize the need for further functional experiments to validate the direct causal relationship between NOTCH signaling loss and skin aging. We greatly appreciate your comment which helps us improve the scientific rigor of our paper.

Comment 3: How does the remodeling of the extracellular matrix influence skin aging? and how could external factors, such as U|V rays, influence this study?

Reply to Comment 3: Thank you for these important questions about extracellular matrix (ECM) remodeling and external factors in skin aging.

ECM remodeling significantly influences skin aging through multiple mechanisms revealed in our study. Our analysis of ECM receptor features (Figure 4) shows that both BC-AS2 and IFI27+ subpopulations exhibit significant alterations in collagen-integrin and collagen-CD44 signaling during aging. Specifically, the IFI27+ subpopulation demonstrates upregulation of multiple collagen types (COL1A1, COL1A2, COL4A1, COL4A2, COL6A1-COL6A3), while BC-AS2 primarily expresses COL1A1, COL1A2, COL6A1, and COL6A2. This differential collagen expression is coupled with enhanced COL-ITGA1/ITGA2/ITGB1 interactions and strengthened COL-CD44 direct-binding signals.

Concerning external factors like UV radiation, while our current study primarily focused on intrinsic aging mechanisms, UV exposure would significantly influence our findings in several ways. UV radiation accelerates ECM degradation through upregulation of matrix metalloproteinases (MMPs), which could enhance the ECM remodeling patterns we observed. UV exposure also induces oxidative stress and DNA damage, likely amplifying the inflammatory signatures we detected in the IFI27+ subpopulation. The GSE67098 validation dataset included comparisons between sun-exposed and sun-protected skin areas, allowing us to partially account for these effects, but a comprehensive analysis of UV-specific impacts would require additional experimental designs specifically controlling for UV exposure variables.

We are looking forward to your further feedback. Thank you again.

Comment 4: What basis for therapeutic products do the authors believe could be viable for slowing down skin ageing?

Reply to Comment 4: Thank you for your comments. Based on our integrated analysis of keratinocyte heterogeneity, developmental trajectories, and intercellular communication networks in aging skin, we believe several promising therapeutic approaches could be developed to slow skin aging.

First, our study identified aberrant NOTCH signaling in aging keratinocytes, particularly the loss of JAG1-NOTCH and DLL1-NOTCH pathways during the transition from healthy basal cells to pro-inflammatory phenotypes. Therapeutics that restore or maintain proper NOTCH signaling in keratinocytes could potentially preserve epidermal homeostasis and prevent inflammatory

reprogramming. This might include topical formulations containing NOTCH pathway agonists or compounds that stabilize NOTCH receptor expression.

Second, our analysis revealed specific ECM alterations in aging skin, with BC-AS2 and IFI27+ subpopulations producing abnormal collagen profiles and altered integrin-collagen interactions. Products targeting these aberrant ECM interactions, perhaps through selective inhibition of specific collagen types or modulation of integrin signaling, could help maintain proper basement membrane structure and keratinocyte-ECM relationships.

Third, the prominent inflammatory signature we identified in IFI27+ cells suggests anti-inflammatory compounds targeting specific cytokine pathways (IL1, IL6, TGFB) could be effective. Rather than broad anti-inflammatory agents, our work points to precise targeting of these pathways specifically in keratinocyte subpopulations that have undergone inflammatory reprogramming.

Finally, our developmental trajectory analysis indicates that preventing the transition of keratinocytes toward senescent phenotypes represents a viable approach. Compounds that maintain the proliferative capacity of basal cells or prevent their diversion toward inflammatory fates could be particularly effective. This might involve senolytic agents specifically designed for epidermal application or factors that promote healthy basal cell renewal.

These targeted approaches, informed by our single-cell resolution of keratinocyte aging mechanisms, offer more precise interventions than current general strategies focused on antioxidants or broad moisturization.

We also reported these related text in the revised MS (Lines 600-636), and we are looking forward to your further guidance. Thank you again.

Comment 5: Do the authors believe that future biomarkers and methodologies could be developed based on these results? And what are the limitations of this study? What else needs to be done? It would be good to create a topioc of future perspectives.

Reply to Comment 5: Thank you for your feedback. Yes, our results provide several promising avenues for developing biomarkers and methodologies for skin aging assessment and intervention. The keratinocyte subpopulations we identified, particularly the IFI27+ inflammatory phenotype

and the BC-AS2 transitional state, could serve as cellular biomarkers for aging progression. Specifically, the ratio of these specialized subpopulations to normal keratinocytes might indicate biological age more accurately than chronological age.

The distinct molecular signatures we uncovered—such as altered NOTCH pathway components, specific ECM proteins (COL1A1, COL6A1-3), and inflammatory mediators—could be developed into non-invasive diagnostic panels. Future methodologies might include skin tape sampling coupled with targeted transcriptomic analysis to quantify these markers without requiring biopsies.

As noted in manuscript (lines 638-679), we've addressed the limitations of the study, including technical constraints of single-cell analysis and the need for integration with epigenomic data. In future perspectives section (lines 680-706), we've outlined additional work needed, which likely includes validation in diverse skin types and ages, functional studies of the identified pathways, and development of intervention strategies.

We are looking forward to your further feedback. Thank you again.