

Peer Review File

Manuscript Title: Prenatal skin atlas reveals immune regulation of human skin morphogenesis

Redactions – unpublished data

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

In this manuscript, Gopee et al. present a tour-de force molecular profiling and analysis of human prenatal skin with a large time series of weeks PCW 7-16 that encompass the key steps of skin and hair follicle appendage development. The state-of-the-art single cell RNAseq, spatial transcriptomics and multiplex FISH datasets are integrated with datasets of hair-bearing ES- and iPS-derived skin organoids and of adult skin samples for comparison. This allowed the authors to quite effectively and comprehensively define the cellular composition and transcriptional landscape over time for 1) describing the molecular events during human embryonic hair formation (largely matching previous murine studies); 2) tracking the expression profiles of fibroblast subtypes thereby identifying potential factors causing scarring; and 3) studying the dynamics of genetic skin diseases.

As a main fourth point in the paper, they further identify potentially functional interactions of immune cells with many other skin cell types, focusing here to a large extent on macrophages. In doing so, they propose a role of macrophages in fibroblast homeostasis and wound healing, which was not functionally validated. Finally, with further co-localization and molecular interaction profiles they also discover a role of macrophages in angiogenesis during early skin development, which they functionally validate with a 2D angiogenesis assay and a blood vessel analysis in 3D skin organoids.

Major strengths of the work are the high quality and clear presentation of the data, the compelling easy-to-follow writing of the manuscript, and the future sharing of a comprehensive and easy to use human prenatal skin gene expression atlas. Additionally, the rigorous comparison of human embryonic skin with the human skin-bearing organoids dissects the cellular and transcriptional similarities and differences, thereby establishing the organoid model, which lacks immune cells, as a powerful functional assay to study the identified macrophage-endothelial cell molecular interactions in their role in sprouting angiogenesis.

Overall, this work presents a great resource for the scientific community to study mechanisms of skin and hair development, discovers roles of immune cells in skin development, and highlights the

potential of human PS-derived skin organoids in functional studies. There are a few major concerns and many minor suggestions that when addressed and implemented would help ascertain the findings and further improve the presentation of the data and the usefulness of the paper for the future readers.

Major issues and suggestions:

1) One weakness is that the conclusions drawn from gene expression dynamics are largely based on pre-existing knowledge about candidate genes, which is only mildly distracting from the rigor of the overall analysis. As they are presented and discussed in an appealing high-quality way, they are meaningful and compelling. Nevertheless, this is why it is especially important that the only two functional validating experiments, i.e. the 2D angiogenesis assay (Fig. 4h,i) and the 3D angiogenesis readout in the skin organoids (Fig. 4j) in vitro, are presented with high rigor and quality, which should be further improved to shore up the findings.

2D angiogenesis assay.

a. It is a bit surprising that branching was maximal already after 4 or 6 hours of endothelial settling and then declined without macrophages. The exact timing is unclear as the main text describes network formation at 4 hours, while the figure and methods section describes taking images “2 hours after initial culture”. Only assuming the “2 hours” refers to co-culture time, then the control endothelial cells were imaged at 6 hours total time. A narrower time course that rigorously demonstrates in control ECs the ramp up of branching and the decrease by 24 hours would be very helpful. Maybe test every 2 hours for the first 8 hours and then at 12 and 24 hours (nothing changes at 48 and 72 hours)? How do the authors explain the absence of a further decrease at 48 or 72 hours?

b. What time point is the “Branching point” image at and which condition is it? It would be better to show both conditions and at two key time points.

c. What do the n=3 data points stand for in Fig 4i? 3 wells of one experiment? Or 3 experiments with replicate wells and multiple data measurements? It is not described and addressing this concern would increase the confidence in the data. Scatter plots for replicates showing underlying measurements would help very much.

3D organoids.

a) A quantification of microvessel density (MVD, a classic angiogenesis readout) and then of replicate experiments will be essential. Ideally, there would be a higher MVD close to macrophages to make a more convincing case.

b) The CD206 staining for macrophages looks a bit unconvincing as in the green channel there appear to be long multi-cell spanning processes. Would a broad CD45 stain or additional macrophage-specific markers work better?

c) Also, as there appears to be a green overall hue (background?) in the image of the single organoid with macrophages, how is it that the image of the organoid without macrophages has no green hue at all? Was the green channel omitted by accident or the sample not stained for CD206?

2) It would be great if the authors could validate and further confirm the proposed role of macrophages in wound healing and scarless healing. Expression analysis identified an upregulation of immune suppressing genes in early prenatal fibroblast subsets compared to adult fibroblasts. In contrast, immune activation genes are upregulated in the adult. Together with co-localization and cell-cell interaction analysis of macrophages with fibroblasts, the authors make the strong suggestions of LYVE1 macrophages contributing to WNT2+ fibroblast maintenance as well as of TML macrophages playing an important role in supporting WNT2+ fibroblasts in scarless healing.

However, while this data in combination with the literature suggests a potential functional contribution of macrophages, the proof of principal is missing. In order to make those strong claims, additional experimental data are necessary, e.g. at least demonstrating validation of co-localization at high resolution with IF imaging/RNAScope, and ideally with functional assays for an identified signaling pathway(s) in wounding experiments.

3) The microenvironments in Fig 1d seem to be an important concept. However, it is quite confusing that the dermal fibroblast subtypes are distributed among different microenvironments, while most macrophages are located within the same environment. Do the microenvironment definitions depend on developmental timepoints rather than on cell type or location, or a combination thereof?

Importantly, why are WNT2+ fibroblasts alone in their own microenvironment and not with TMLM? Why is there no joint microenvironment of WNT2+ fibroblasts with LYVE1 macrophages?

Generally, it would be very helpful for Fig. 1d if the authors could provide a distinct name/label for the microenvironments instead of just numbers.

4) To gain insights into the epidermal trajectory the authors only used the organoid data set. It would be great if the authors could confirm the result within the prenatal data or the integrated data set (as in Extended Data Fig 2b). Or are some epidermal cell types underrepresented in the prenatal samples?

It would be great if the authors could provide much more informative feature plots for the key genes of the pseudotime analysis, for both the ORS/CL and the placode/IRS trajectory.

Why is the placode/matrix represented as one cluster? In the Extended Data Fig 3d, which shows the integrated but split prenatal and adult skin, it appears that the placode (cluster 6) has a subcluster (pink) that nicely overlays with the matrix cluster (cluster 9) from adult skin. Considering an ~1:1 distribution of prenatal placode and matrix cells, why is the correlation with adult matrix cells rather low (Extended Data Fig. 3c)?

5) To study the dermal trajectories the authors relied solely on prenatal data. For consistency the authors should here also use the organoid data and show the prenatal or integrated prenatal/organoid dataset in the extended data.

From comparing the PAGA plot (Fig 2f, top) with the CellRank UMAP (Fig 2f bottom), it appears that one would draw the trajectories differently as there are no direct arrows from HOXC5+ fibroblasts to Pre-DC. How the authors explain that the Dermal Rank plot would not show clear directions towards the Pre-DC leaving the HOXC5 state?

WIF1 is only expressed in early HOXC5 fibroblasts (Extended Data Fig 4c), but it is also a DP signature gene. Why is it not expressed in the DP?

6) Inspection of the prenatal skin UMAP on the web portal appears to show a huge difference between male and female. In particular, the entire FRZB+ fibroblast cluster is just from male samples. Can the authors please explain how and whether this could be an issue that needs to be addressed experimentally?

General improvements and minor issues:

1. Genes of interest. A general improvement would be if the authors could highlight in the Figures the genes of interest that are mentioned in the text, thereby guiding and helping the reader to find the important factors within the long lists of genes.

2. It would be great if the authors could also improve the web portal such that they offer only the latest annotations. Alternatively, they could provide description of the various refinements so it can be followed better. Please highlight the annotations used in manuscript.

3. It would improve the validity of some discussed key genes if the authors would present feature plots in addition to dot plots. Feature plots would provide a great visual way to demonstrate expression levels by specific cell types compared to others. In the manuscript, feature plots were only used once in Extended Data Fig 3g.

4. Along the same lines, when outlining hair follicle development in prenatal skin, it would be helpful to also show the feature plots of the key markers (i.e. SHH, SLC26A7, POSTN...) used for identifying the various cell types in the prenatal epidermis and dermis.

5. It would be very useful if the authors could show the cell composition of the skin organoid model for the corresponding timepoint equivalent to prenatal skin. Unless missed, it appears that only the cell composition chart derived from UMAPs of prenatal skin is presented in Extended Data Fig. 2b.

6. The Figure legend 2 has multiple (c) labeling.

7. As the authors identified Treg markers related to the placode and concluded a role of Tregs in placode differentiation it would be great if the authors could provide their expression heatmaps or violin plots (CXCL14 and FOXP3), especially as the detection of Tregs using RNAscope appears rather low (Fig. 2d). How do the authors explain the large differences in the timing of Treg detection (23 PCW vs 15 PCW)?

8. Fig. 2h and others. Highlighting in the CellPhoneDB heatmaps the ligand-receptor pairs that are discussed in the manuscript would be beneficial.

9. Why do POSTN expression levels show a strikingly different result in the Milo plot (Fig 3c, consistent up) compared to the pseudotime heatmap (Fig.3b, down up down)?

10. Why is the DP not represented in any the microenvironments in Fig 1d?

11. A few suggested corrections for nomenclature and used terms: Line 74. It should be “epithelial hair sheath”. The hair is not “epidermis”. Lines 137/138. It should be “suprabasal epidermal and hair follicle epithelial cells...” Line 146. Better call it “maturation” and not “evolution”?

Referee #2 (Remarks to the Author):

Gopee et al. present a study on prenatal human skin, focusing on macrophages and their potential role in angiogenesis. The team has created a comprehensive atlas of prenatal human skin using various data sources, including single-cell and spatial transcriptomics. Their findings suggest that interactions between immune and non-immune cells are crucial for proper skin development. To validate the findings, a skin organoid model was compared to prenatal and adult skin.

Additionally, the research employs the human prenatal skin cell atlas to gain insights into the pathogenesis of genetic hair and skin disorders. The data from the skin atlas indicated that macrophages and the growth factors they produce play a significant role in prenatal endothelial cell development. As proof of principle, a co-culture model and the organoid model were taken to show that macrophages indeed enhance angiogenesis. In summary, the study strengthens the long-standing fact (mainly observed in mice) that particularly macrophages have additional roles in tissue development beyond their traditional immune functions.

The study is thoroughly done and will be a fantastic resource for many fields (e.g. developmental biology, macrophage biology). The bioinformatics analysis is easy to follow, despite its complexity. A major strength is the observation and validation of macrophage function in human tissue and a human organoid model. However, validation of the major findings on protein level and in situ is required to strengthen the statements that completely rely on transcriptome data so far. Additionally, the organoid model dataset should be expanded to strengthen the manuscript, which will certainly revolutionize the view of many scientists working on skin development and related pathophysiologies.

1. The authors group Langerhans cells into DCs. However, it is well-accepted that these cells are macrophages. It may well be that the MHCII+ macrophage fraction belongs together with LCs, especially considering that the authors used 1 single gene (MMP19) to designate this cell population in their Excel sheet (why is the dot plot for macrophages included so late during the study? One wonders about the specific genes for all the populations already in Figure 1). Further, only 2 genes are shown in the dotplot in Extended Data Fig. 1c to indicate the expression of specific genes in all cell types. The study completely relies on a proper cell population designation. Thus, the genes used for the 'manual designation' of cell types should be shown for all subpopulations or a better overview as Excel table should be provided.

2. In Figure 1d/e the authors use spatial transcriptomics data published in a preprint to show neighbourhoods. The authors should show actual data to show the reader where (anatomically) in the tissue they see these neighbourhoods. This is especially true for macrophage neighbourhoods that become important later in the study.

3. ACKR3 is an atypical receptor that does not signal the same way as CXCR4, the receptor that has been implicated in skin development. If the authors would want to keep the claim that the interaction of CXCL12 with ACKR3 mediates migration of pre-Dc (lines 295-299), they would need to prove this experimentally. In general, pure gene expression without validation on protein level and/or experimental data does not provide the basis for any claims. The authors should be very cautious in phrasing any 'conclusions' when only transcriptome data is the basis for their results.

4. The RNAScope data is not of such a quality that would allow a co-localization study of single macrophage subpopulations with endothelial cells. The authors have identified 4 different macrophage populations and before claiming that all of them are in close proximity with endothelial cells, they could do (at least) a 4 colour IF staining where one can appreciate the cell borders and the actual interaction with endothelial cells. Multiplex proteomics (e.g. CODEX) are nowadays broadly available and should be used to validate the most important findings claimed in this study.

5. Lines 486-488: Just because the lack of macrophages in an organoid model does not result in proper angiogenesis, does not mean that macrophages are actually responsible for this biological process. Many organoid models where macrophages are being introduced still lack endothelial cells. Nevertheless, the hypothesis is obvious and the data at the end of the manuscript support this point. However, the authors should use cryosections in addition to the whole mounts shown to assess macrophage-endothelial cell interaction and maybe use also Iba1 as macrophage marker since the CD206 antibody seems unspecific or rather staining endothelial cells that are negative for CD31. Further, the addition of macrophages to organoids is very variable and n = 3 with only 1 iPSC line is not the standard procedure in the organoid field. For some experiments, the figure legends say even only n=1 or n=2, which is not acceptable for in vitro experiments. Please include the same set of experiments with at least one more iPSC line and add more n numbers showing in how many % of the cases you end up with more vasculature. Finally, perform flow cytometry on the organoids with and without macrophages using the same flow cytometry markers as for macrophages before adding them to the organoids to obtain the ratio of macrophages within each organoid. Only this will show the reproducibility and consistency of the results.

6. Adipocytes typically become too big for scRNAseq and are prone to cell death after enzymatic digestion and are simply not found in the cell pellet after centrifugation. The data should be checked for previously described pre-adipocyte populations, which may be the actual predominant cell type in the data set.

7. Why are B progenitors and HSCs in the skin? This is likely a contamination with fetal liver or bone marrow, which are the only organs that harbour these precursor and progenitor cells.

Referee #3 (Remarks to the Author):

A human prenatal skin cell atlas reveals immune cell regulation of skin morphogenesis

Main Findings

Gopree, Huan, Admane, et al. report a novel and interesting investigation into the single-cell and spatial transcriptome of fetal skin development. As a product of this work, the investigators present a rich resource consisting of fetal human skin tissue spanning multiple timepoints and compelling data from a skin organoid model. This work constitutes the earliest and most complete atlas of human fetal skin at single cell resolution. The authors demonstrate important advancements in understanding the differentiation of cell types that contribute to hair follicle formation and demonstrate an unconventional role for innate immune cells in promoting angiogenesis. This resource will undoubtedly impact the field by driving hypothesis development and motivating many follow-up analyses.

However, there are a few areas where the authors can meaningfully improve the manuscript and increase readability. Many data panels are presented in extended data figures; the authors should consider moving more panels to the main figures to decrease the amount of cross-referencing that is currently required of the reader. The authors should also carefully consider conjectures vs experimentally-derived conclusions based on their data particularly in regards to the analysis of fibroblasts and macrophages in promoting scarless healing. Additional specific suggestions are outlined below:

Major Comments

1. It is not always clear which datasets are new and which are from a previous study. To infer the novelty of this dataset, it will be useful to have a table indicating the source of each dataset.
2. Could the authors clarify concerns of maternal cell contamination? Only 49 cells seems surprising – Perhaps this could be rectified with more details on sample preparation techniques that were applied to decrease contamination and specific details on implementation of souporecell.

3. Annotations could use more explanation, especially when marker genes that have not previously been referred to in the literature were chosen (particularly: fibroblast subpopulations (WNT2, PEAR1) and macrophages)

4. While Fig. 2d does show red FOXP3+ dots, no quantification is performed and there is no comparison to a control region further away from the hair follicles. Please perform a quantitative analysis showing preferred localization of FOXP3+ cells near the hair follicles or edit your statement “We identified FOXP3+ Tregs primarily within and around hair follicles” to be more inclusive of other regions of the skin.

5. It would help to increase readability if the authors could clarify details about how they determined pre-dc and dc using information from prior work in murine skin. Did the authors explicitly compare pre-Dc and/or Dc between fetal skin and published mouse data? Because the authors claim this is the first time that Dc has been identified in human skin, an explicit analysis (Harmony integration, UMAP co-embedding) addressing discernable differences (if any) between human Dc and previously described murine Dc.

6. Receptor-ligand interaction analyses could benefit from increased detail in methods.

a. The authors note that they down-sampled to 200 cells – why this threshold and what about cell types with fewer than 200 cells?

b. Were the results validated in any way? The down-sampling approach seems really amenable to bootstrapping.

7. Genes2Genes Analysis/Figure 2J – Please expand on the methodology used.

a. I am confused on how the authors can have an “Average 100% alignment” at the cell level (especially with 95% at the TF level).

b. For pseudotime trajectory, how were pseudotime bins picked? Do each of the bins have (roughly) equivalent numbers of cells?

c. A y-axis should be added to the barplots in the axes in 2.

8. The Figure 3A main caption claims are not supported by experimental evidence.

9. The analysis in Figure 3A analysis feels like a bit of a stretch and is at risk of identifying technical rather than biological differences due to batch/study biases. What steps did the authors take to

mitigate this concern? The differences presented between fetal and adult fibroblast populations are interesting, but this analysis oversimplifies established adult fibroblastic heterogeneity.

9. Several recent studies (Sole-Boldo et al, Reynolds et al, Ascension et al) of single cell dermal fibroblasts have identified multiple fibroblast subpopulations, including the published data used by the authors in this analysis.

a. It would be worthwhile for the authors to identify the fibroblast populations that are most similar between the fetal and adult human dermal populations.

b. Consider adding in an analysis like Ext. Data Figs 3C or 7B and a coembedded UMAP.

10. In Figure 3, are the predicted cell interactions between fibroblast populations and macrophages conserved between fetal and adult samples? The authors should speculate on whether a loss of a specific fibroblast population (WNT2+) or the changes in cell signaling/interactions play a role in loss of scarless healing.

11. The concluding paragraph from lines 444-448 is not supported by the evidence. The manuscript shows good evidence that the prenatal skin fibroblasts are not yet expressing genes required for ECM formation and collagen deposition, but doesn't show their involvement in tissue remodeling specifically. The fibroblasts themselves may just be developing without actively performing remodeling activities in the tissue. Additionally, while colocalization and likely signaling between TMLM cells and WNT2+ fibroblasts is established by the data, a clear role of this interaction in scarless healing remains conjecture based on data from other tissues. This should be made more clear. If Schwann cells are also co-localizing with TMLMs (and WNT2+ fibroblasts, by extension), were the cell-cell interactions investigated to see if there could be a role for Schwann cells in promoting scarless healing? This was not addressed.

12. The gene modules discussed in relation to Extended Data Fig. 7c don't line up with what is displayed by the figure. Gene module scoring – this approach has a lot of benefits for normalizing scores; but I am curious about controlling for sample variation. Was the same reference gene list used for all comparisons? Were any genes included in both the reference gene list and the gene list of interest?

13. The images in Fig 4j are quite striking. Please add information about replicates and a quantification of increased endothelium.

Minor Comments

1. For figures 1d-e, when discussing the microenvironments, please discuss whether these microenvironments have been previously observed through other methodologies such as histology or if they are novel.
2. Extended Data Fig. 2a is hard to interpret, please include facets for each sample in which only the cells from each individual sample are highlighted/colored.
3. For Extended Data Fig. 3e, does GO term enrichment back up the claims about differences in function between the adult and prenatal/organoid matrix cells? Specifically the authors should carefully consider and better explain their reasoning behind their heavy reliance on the use of the following genes:
 - a. CXCL14 – implicating Treg recruitment and immune protection (CXCL14 has been implicated in many biological activities)
 - b. LGALS1 indicating cell proliferation – consider evaluating MKI67, TOP2A, etc to back up this claim
4. Figure 2C – How many embryos were used in this analysis? Do the cell proportion percentages represent an average?
5. Figure 2E – What do the different lines indicate? (color, thickness)
6. Ext. Data Fig 3g,h – How were genes identified from the trajectory analysis? If same as Ext Data Fig 4c, the authors should consider adding this type of figure.
7. Were both prenatal skin and organoid cells used to infer fibroblast differentiation trajectory? Is the decreasing proportion of HOXC5+ fibroblasts a consistent finding between prenatal and organoid datasets?
8. When discussing the presence of genes involved in hair/skin disease, the statement “suggesting that these disorders result from dysfunctional hair follicle development” is not supported. The presence of a gene(s) by RNA-seq does not always mean that it is functional/required at that specific

space and time. You also don't discuss if these genes are found elsewhere in the dataset or are exclusive to these pseudotime trajectories.

9. When discussing the differences in immune gene expression between the prenatal and adult fibroblasts, only cherry picked genes are included. Please perform GO term enrichment or other similar analysis to show the unbiased detection of differentially expressed genes that fall into these categories.

10. The statement "WNT2+ fibroblasts downregulated IL6 expression compared to adult fibroblasts" should be edited to "WNT2+ fibroblasts had downregulated IL6 expression compared to adult fibroblasts" or similar. The current version implicates that the WNT2+ fibroblasts are a causative factor in downregulating IL6 expression broadly which you are not showing here.

11. Figure 3D,F would benefit from titles explaining what the correlation coefficient is measuring.

12. In Figure 4a, the dots are quite small/hard to see. Please make two different insets (one for each macrophage type) from smaller regions of the whole field.

13. For Extended Data Fig. h, please reference the UMAP panel which cell types are defined (Fig 4b) in the figure legend.

14. The legend for Extended Data Fig. 10g does not match the data. Please update this legend.

15. In Figure 2, what should be panels b-e are all labeled as c.

16. In Figure 3, there are two panels labeled as c.

17. Fig 4I - How were branches determined/quantitated?

18. Did the authors perform co-localization analysis for all pairs of cell types? The authors should consider showing the full analysis and summarize the major patterns.

19. Please include a brief discussion of other organoid systems in which co-culturing of immune cells has been successful. (E.g. Huang et al 2022 Arthritis Rheumatology (DOI: 10.1002/art.42097) in the skin and the use of microglia in brain organoids reviewed by Zhang et al. 2023 Mol Psychiatry (DOI: <https://doi.org/10.1038/s41380-022-01892-1>)

Referee #4 (Remarks to the Author):

The manuscript authored by Gopee et al. presents a study focusing on the role of immune cells in skin morphogenesis. To the best of my knowledge, this represents the first single-cell analysis of human prenatal skin, revealing intricate interplay among epithelial cells, dermal fibroblasts, and immune cells. The authors created a comprehensive atlas of prenatal human skin utilizing advanced methods and tried to demonstrate that communication between immune and non-immune cells is crucial for the formation of hair follicles, scarless wound healing, and skin angiogenesis. In addition, they introduced a skin organoid model that mimics hair follicle development and provides insights into skin angiogenesis in the absence of immune cells by comparing it with prenatal skin. However, it seems highly descriptive without conceptual advancement in skin morphogenesis.

Major points:

1. Most of the data heavily relies on transcriptomic analysis, which may not adequately elucidate the intricate mechanodynamic processes underlying skin development, particularly in scarring. Transcriptional regulation of scarring is complex and subject to influence from the surrounding microenvironment. For instance, Mascharak et al. (Science 2021;372(6540)) demonstrated the interplay between mechanical forces, fibroblast subsets, and transcriptional regulation in tissue regeneration and scarring. A simplistic comparison between prenatal and adult skin, along with two-dimensional transcriptomic analysis, may prove insufficient in unraveling the underlying mechanisms and yielding novel insights into the scarless healing observed in prenatal skin.

2. In terms of hair biology, while the manuscript offers abundant and valuable information on the early development of human skin, the scientific breakthrough is minimal. The authors discuss the formation of dermal papillae, hair placode induction, and their interplay during human skin development. However, these descriptions largely overlap with and are partly dependent on previous findings that have been extensively studied in a mouse model. The new atlas study merely confirms that similar processes take place in humans as well. It would be great if the study could identify human-specific features of embryonic skin development. As noted by the authors in the

introduction, human skin is distinct from mouse skin in several ways, with differences in the coexistence of hair follicles and sweat glands, as well as regional variations in hair follicles (i.e., scalp vs. body). However, the current human embryonic skin atlas study fails to answer key questions in human skin biology, such as how hair follicles and sweat glands develop in the same location, how the regionally heterogeneous pattern of hair follicles is established, and how human-specific features can be explained from an evolutionary perspective.

3. A significant finding of the study is that macrophages play a crucial role in skin angiogenesis. However, as the authors have pointed out, macrophages have been implicated in various other contexts, such as embryonic tissues and adult wound healing and cancer, in their pro-angiogenic roles. Therefore, the impact of this finding is not surprising. The authors used a human skin organoid model that contained developing hair follicles to validate the pro-angiogenic roles of macrophages. However, the results presented in the figure (Fig 4j) were not quantified, which makes the argument invalid. In addition, the authors used an antibody (against CD206) to detect macrophages in skin organoids. However, the staining pattern does not look like immune cells, but fibroblasts or neurons. The existence of macrophages should be checked with other antibodies such as CD45, CD11b, LYVE-1, etc. Furthermore, the data regarding the effect of co-culture with macrophages on the formation of hair follicles should be presented properly. Even though the authors mentioned in the text that co-culture with macrophages did not affect the formation of hair follicles, no hair follicles were presented in Fig 4j and Extended Fig 10g.

4. The authors propose that the expression of 'immune activation' signatures in adult fibroblasts contributes to scarring, while 'immune suppression' profiles are associated with scarless healing. They further highlight that TMLM (TREM2+ macrophages) in prenatal skin express genes related to immunomodulation, suggesting that the interaction between fibroblasts and these immunosuppressive TMLM cells may contribute to scarless healing. However, it's important to note that the impact of inflammation on scarring and tissue remodeling is dual-sided. While inflammation stimulates the initial stages of scar formation, anti-inflammatory cytokines and the anti-inflammatory properties of macrophages play a role in fibrotic scar development in the later stages of scarring. Although TMLM and other subsets of macrophages may differ from those in adult skin, the evidence presented in the manuscript is insufficient to conclusively establish the role of macrophages in scarless healing in prenatal skin.

5. While the authors aimed to illuminate novel roles of immune cells in skin morphogenesis and scarring, the available data may not be robust enough to substantiate these claims. Besides transcriptomic analysis, there is a paucity of evidence to corroborate their conclusion. The imaging data, including RNAScope, reveals a scarcity of immune cells, raising questions about the potential impact of such a small population on the regulation of skin morphogenesis. To strengthen their argument, it would be advisable for the authors to employ organoid co-culture systems, akin to the angiogenesis assay, in order to provide further support for the involvement of immune cells in skin development.

6. The study provides correlative and suggestive evidence. The authors rely solely on single-cell RNA-seq data for their explanations and descriptions, without proper validation. To confirm their findings, the expression of key factors that they newly discovered and described should be tested using in-situ hybridization and/or immunostaining. It would be helpful to perform protein expression analyses since RNA expression and protein expression do not always match. Furthermore, the authors inferred molecular interactions among cell populations using computational prediction tools, but it would be better if they functionally tested some of the interactions, particularly since a skin organoid system is available.

Minor comments

7. In line 74, "an epidermal hair sheath and a dermal root sheath" is corrected to "an outer root sheath and a dermal sheath".

8. In Fig 2d, Foxp3+ Treg cells are not visible (maybe just one dot?). To make the analysis convincing, it is highly recommended to use convincing immunofluorescence staining (IFS) to display intact tissue structure. Good IFS antibodies against FOXP3 (Tregs), SOX9 or Krt14 (outer root sheath), LEF1 or SOX2 (dermal papilla) are readily available.

9. To ensure easy comprehension by skin biologists and general readers, clear presentation of figures is crucial. For instance, the message conveyed by Fig 2j is difficult to comprehend.

10. There are multiple typos in figure numbering within the figure legend (e.g., Fig.2, where there are four (c)s in the legend, as indicated in Line 1564).

Author Rebuttals to Initial Comments:

We thank the reviewers for their positive and constructive feedback.

We provide our response which addresses every point raised by the reviewers (table below).

The skin organoid culture takes ~4/5 months from iPS cells to fully differentiated organoid and as such we needed the full six months to complete the work.

	Reviewer 1 comments - major	Response to reviewer
1.1	In this manuscript, Gopee et al. present a tour-de force molecular profiling and analysis of human prenatal skin with a large time series of weeks PCW 7-16 that encompass the key steps of skin and hair follicle appendage development. The state-of-the-art single cell RNAseq, spatial transcriptomics and multiplex FISH datasets are integrated with datasets of hair-bearing ES- and iPS-derived skin organoids and of adult skin samples for comparison. This allowed the authors to quite effectively and comprehensively define the	We thank the reviewer for their appreciation and positive comments on our manuscript, in particular the resource value of our high quality human prenatal skin datasets and the rigorous benchmarking with a hair-bearing skin organoid model. We have implemented the suggestions by the reviewer and address them point by point in detail below.

	cellular composition and transcriptional landscape over time for 1) describing the molecular events during human embryonic hair formation (largely matching previous murine studies); 2) tracking the expression profiles of fibroblast subtypes thereby identifying potential factors causing scarring; and 3) studying the dynamics of genetic skin diseases. As a main fourth point in the paper, they further identify potentially functional interactions of immune cells with many other skin cell types, focusing here to a large extent on macrophages. In doing so, they propose a role of macrophages in fibroblast homeostasis and wound healing, which was not functionally validated. Finally, with further co-localization and molecular interaction profiles they also discover a role of macrophages in angiogenesis during early skin development,	
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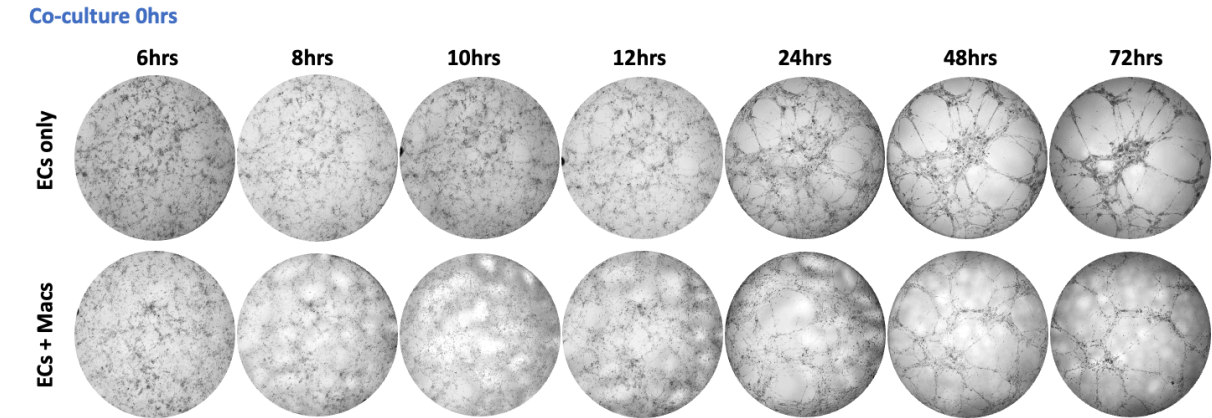
	which they functionally validate with a 2D angiogenesis assay and a blood vessel analysis in 3D skin organoids. Major strengths of the work are the high quality and clear presentation of the data, the compelling easy-to-follow writing of the manuscript, and the future sharing of a comprehensive and easy to use human prenatal skin gene expression atlas. Additionally, the rigorous comparison of human embryonic skin with the human skin-bearing organoids dissects the cellular and transcriptional similarities and differences, thereby establishing the organoid model, which lacks immune cells, as a powerful functional assay to study the identified macrophage-endothelial cell molecular interactions in their role in sprouting angiogenesis. Overall, this work presents a great resource for the scientific community to study	
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	mechanisms of skin and hair development, discovers roles of immune cells in skin development, and highlights the potential of human PS-derived skin organoids in functional studies. There are a few major concerns and many minor suggestions that when addressed and implemented would help ascertain the findings and further improve the presentation of the data and the usefulness of the paper for the future readers.	
1.2	One weakness is that the conclusions drawn from gene expression dynamics are largely based on pre-existing knowledge about candidate genes, which is only mildly distracting from the rigor of the overall analysis. As they are presented and discussed in an appealing high-quality way, they are meaningful and compelling. Nevertheless, this is why it is especially important that the only two functional validating experiments, i.e. the 2D angiogenesis assay (Fig.	We agree with the reviewer on the importance of unbiased analysis of gene expression dynamics. We highlight the following measures we have taken to ensure our analysis follows an unsupervised approach to minimise any bias in making conclusions from gene expression dynamics: 1. We sequenced all cells isolated from the human developing skin over time using scRNA-seq to comprehensively identify cellular states. In addition, in our revised manuscript, we have added 3 additional donors to increase the study's robustness. 2. Computational analysis of the scRNA seq data employed unbiased Leiden clustering to identify cell populations and states. Annotation of Leiden clusters was then performed based on general population identifying genes (into fibroblasts, keratinocytes and the broader lineages). 3. Our unbiased scRNA sequencing and data analysis approach identified gene signatures for individual cell populations in the developing human skin. We have used these gene

	4h,i) and the 3D angiogenesis readout in the skin organoids (Fig. 4j) in vitro, are presented with high rigor and quality, which should be further improved to shore up the findings.	signatures to annotate the more refined cell states, also guided by the existing literature on human skin. If no similar subpopulation had been previously described, we based our annotations on highly expressed and relevant genes for the cell state, such as the <i>HOXC5</i>⁺ early fibroblasts, or the <i>POSTN</i>⁺ basal cells. Additionally, our inferred pseudotime gene signature was computed in an unbiased way using CellRank. 4. In addition to emphasising genes that were previously published for a particular cell state, in our revised manuscript we have also made a concerted effort to further highlight novel genes and populations. These novel genes are crucial components of our findings and form a valuable resource to expand current understanding of human prenatal skin. Together, the previously published and novel marker genes act as supportive evidence, confirming the robustness of our results as well as our annotations. 5. The supplemental table (Supplementary Table 3) accompanying our manuscript provides a comprehensive list of DEGs of each cell population as well as genes relevant for differentiation trajectories, offering readers a detailed exploration of the populations. We have also performed many additional validation experiments, particularly expanding on the 3D organoid angiogenesis analysis, and included these new findings in our revised manuscript. Details on these 3D skin organoid validation experiments are provided in points 1.5 and 1.6.
1.3	2D angiogenesis assay. It is a bit surprising that branching was maximal already after 4 or 6 hours of endothelial settling and then declined without macrophages. The exact timing is unclear as the main text describes network formation at 4 hours, while the figure and methods section describes taking images “2	We thank the reviewer for the suggestions to improve the presentation quality and rigour of the 2D angiogenesis assay. We have repeated the 2D angiogenesis assay including more imaging timepoints at every 2 hours for the first 12 hours, then at 24, 48 and 72 hours (with timepoint 0 hours being the initial seeding of the endothelial cells and macrophages being added at timepoint 4 hours). According to our previous experiments, endothelial cells spontaneously started forming readily visible networks between 2 and 12 hours (which varied across different experiments). This is in accordance with the literature, in which network formation can vary from 2 to 24 hours depending on the cell line used (PMID: 27194743). 2D angiogenesis assays typically use HUVEC cell lines, whereas we are using iPSC-derived endothelial cells which do not perform as uniformly as HUVEC in culture

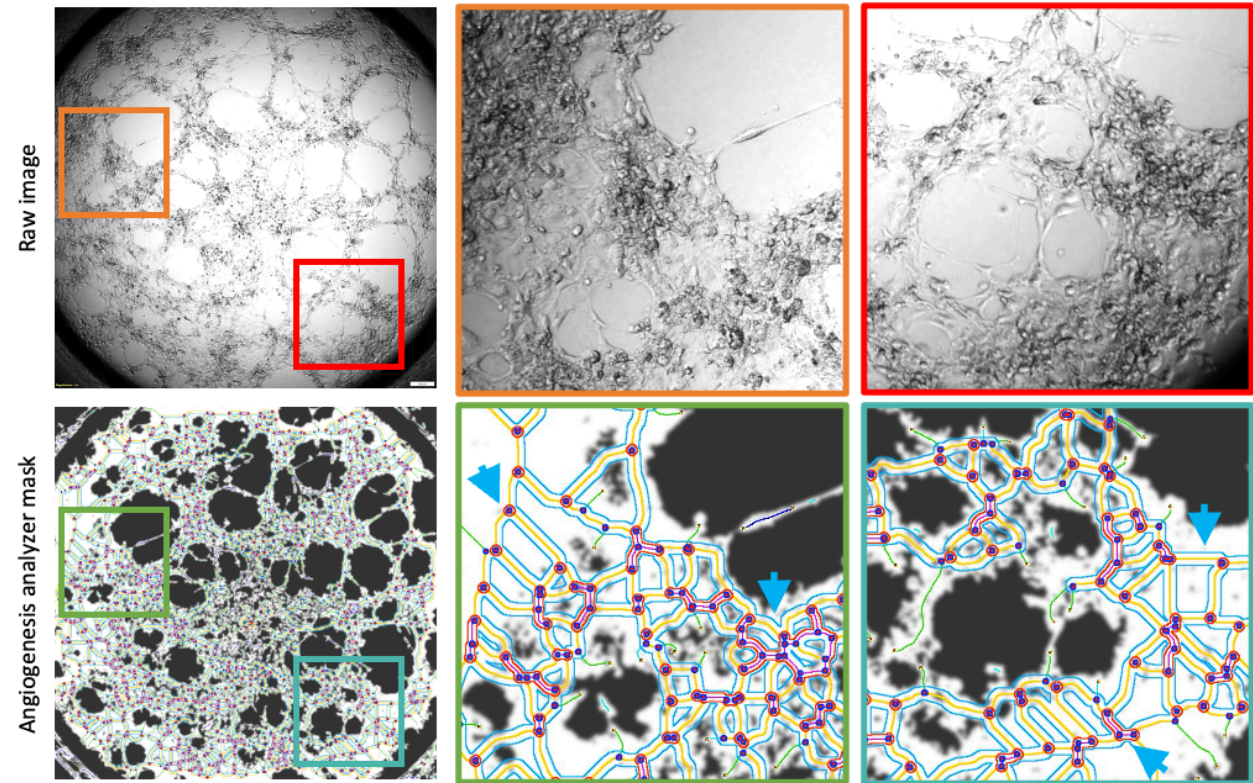
hours after initial culture”. Only assuming the “2 hours” refers to co-culture time, then the control endothelial cells were imaged at 6 hours total time. A narrower time course that rigorously demonstrates in control ECs the ramp up of branching and the decrease by 24 hours would be very helpful. Maybe test every 2 hours for the first 8 hours and then at 12 and 24 hours (nothing changes at 48 and 72 hours)? How do the authors explain the absence of a further decrease at 48 or 72 hours?

(PMID: 29681541). Over time (up to the 72-hour time-point), we observed that the network continued to remodel (**Reviewer Fig 1.3a**).



Reviewer Fig 1.3a. *Representative images of the full angiogenesis assay time-course over 72 hours for endothelial cells only (top) and endothelial cells with macrophages (bottom).*

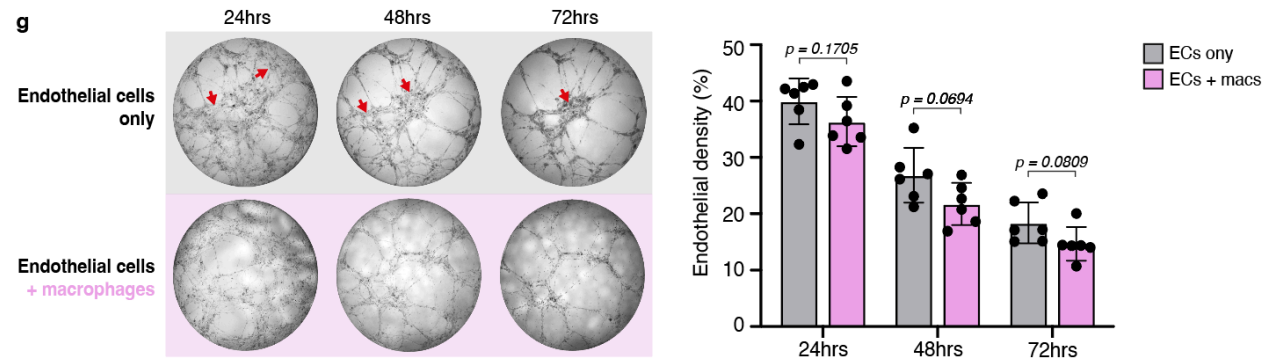
To quantify the angiogenesis assay in a more robust manner over the full time-course of the experiment, we used an automated image analysis pipeline. Briefly, we pre-processed the images to set the analysis area and segment the endothelial cell network, and then used the “Angiogenesis Analyser” plugin for ImageJ to carry out the network quantification. However, due to the fact that the software has been developed for HUVEC cells which form more uniform networks than iPSC-derived endothelial cells, we failed to reliably detect and obtain robust readouts (such as branch points shown as red dots below) for the endothelial cells network in our system (**Reviewer Fig 1.3b**).



Reviewer Fig 1.3b. Representative images of the “Angiogenesis Analyser” output with the raw image (top left) and analysis mask (bottom left). Example output of miscalculated “branching points” or “segments” is highlighted by blue arrows (bottom middle and right).

Despite the limits of the software to adequately quantify the endothelial cell network, we consistently observed that the addition of macrophages consistently changed the appearance of the endothelial network. In line with the 3D assay, the endothelial cell network on its own appeared more disorganised (i.e. as a mesh-like formation of endothelial cells), while the addition of macrophages resulted in a refined and elaborate network (**Extended Data Fig. 10g and shown below**).

To quantify this observation in an unbiased manner, consistent with the analysis of the endothelial cell network in the 3D organoid system (**discussed in reviewer point 1.6**), we quantified the area of the cell culture dish covered by endothelial cells after segmentation using intensity thresholding and normalisation at time-points where a network was readily detectable across different experimental repeats, i.e. after 12 hours, at 24, 48 and 72 hours. However, we note this approach will not readily distinguish between the area covered by endothelial cells from macrophages and therefore potentially overestimate the density where macrophages are co-cultured with endothelial cells. This analysis showed a consistent reduction in the area covered by endothelial cells, suggesting that macrophages act to improve vascular network formation (**Extended Data Fig. 10g** and shown below). This is in keeping with the refined vascular network formation seen in the 3D skin organoid co-culture with macrophages discussed in **reviewer point 1.6**.



Extended Data Fig. 10g. Representative images of angiogenesis assay of endothelial cells without (top) and with macrophages (bottom). Quantification of endothelial density in 2D cultures of iPSC-derived endothelial cells without ($n=6$, grey) and with macrophages ($n=6$; magenta) at 24, 48 and 72 hours of culture from 2 independent differentiation batches. Data are mean $\pm$ SD and statistics generated with an unpaired t -test. (red arrows indicate disorganised network).

1.4	What time point is the “Branching point” image at and which condition is it? It would be better to show both conditions and at two key time points.	As highlighted in reviewer point 1.3, we have now carried out an unbiased image analysis to robustly quantify the differences in endothelial network coverage with and without macrophages over time Extended fig 10g.
1.5	What do the n=3 data points stand for in Fig 4i? 3 wells of one experiment? Or 3 experiments with replicate wells and multiple data measurements? It is not described and addressing this concern would increase the confidence in the data. Scatter plots for replicates showing underlying measurements would help very much.	Please see point 1.3. We have carried out the 2D angiogenesis assay two more times and for each experiment we included three technical repeats. The data has been presented as a scatter plot in Extended Data Fig. 10g shown in point 1.3.

1.6	3D organoids. A quantification of microvessel density (MVD, a classic angiogenesis readout) and then of replicate experiments will be essential. Ideally, there would be a higher MVD close to macrophages to make a more convincing case.	We thank the reviewer for the suggestion to quantify the 3D skin organoid imaging. We repeated the 3D skin organoid cultures with and without the addition of macrophages and performed whole-mount labelling of endothelial cells (CD31) and macrophages (CD45). Previously, we imaged slice sections of the skin organoid which did not provide 3D volume analysis. To assess the vasculature in the entire skin organoid, we have now performed confocal spinning disc imaging of the vasculature in skin organoids cultured with (n=5) and without (n=5) macrophages. We observed clear differences in the 3D vascular network. Skin organoids without macrophages consistently displayed a disorganised mesh-like aggregation of endothelial cells reminiscent of a vascular plexus (5/5). The addition of macrophages resulted in a more refined and elaborate network formation and the striking absence of the disorganised mesh-like endothelial cell aggregation (0/5), representative images Fig. 4h and below. To quantify the endothelial network, we measured the area of the skin organoid covered by CD31⁺ endothelial cells displayed as endothelial density. In keeping with our visual observation, endothelial cell coverage was higher in skin organoids cultured without macrophages, due to the presence and abundance of the mesh-like disorganised endothelial plexus. The addition of macrophages to the skin organoid led to a refined network with pruned thin endothelial branches, resulting in an overall lower endothelial density per skin organoid area analysed (Fig. 4i). Overall, these data suggest a critical role for macrophages in enhancing vascular network remodelling during angiogenesis.
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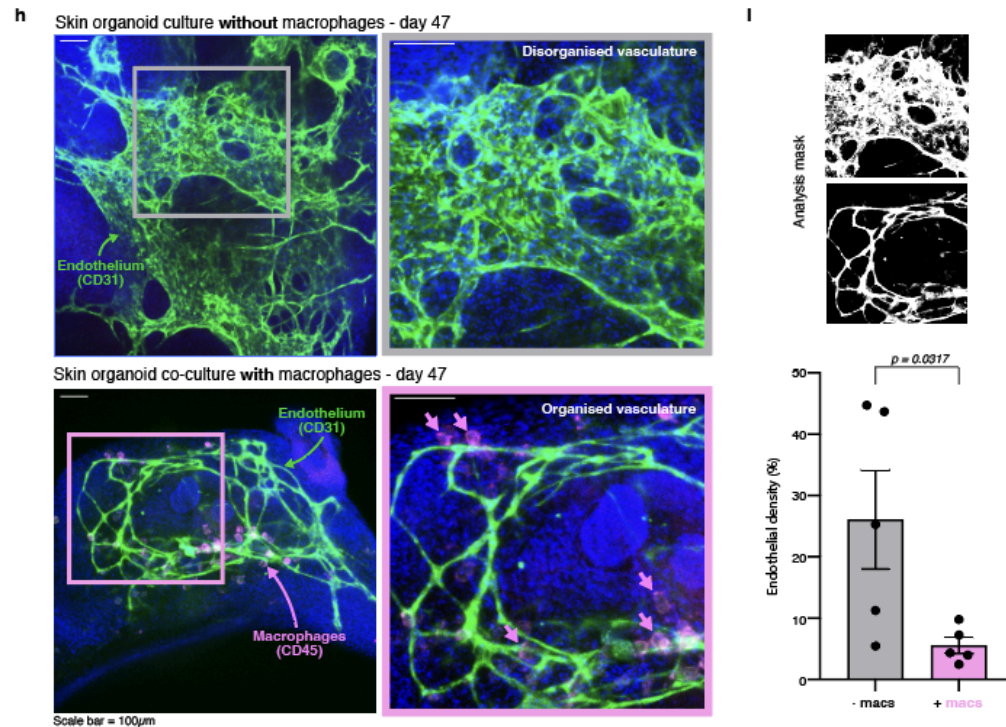
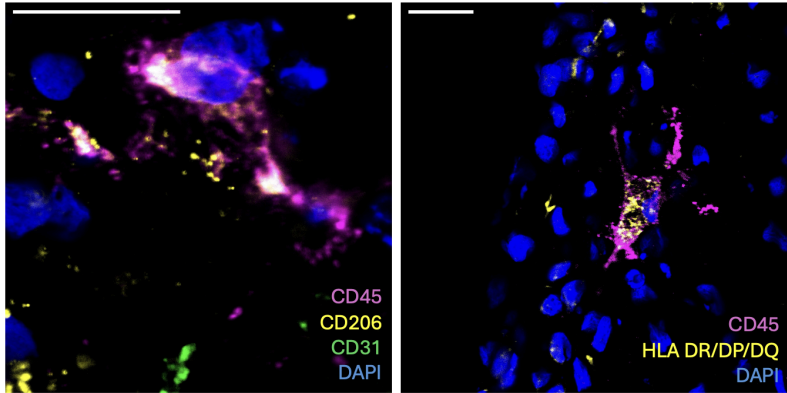


Fig. 4h: Representative whole-mount immunofluorescence images of the skin organoid at day 47 with and without macrophage co-culture (day 35 of co-culture), showing macrophages (CD45) in magenta, endothelium (CD31) in green and DAPI nuclei stain in blue. Scale bars = 100µm.

Fig. 4i: Quantification of endothelial cell coverage in skin organoids at day 47 cultured with (n=5, magenta) and without macrophages (n=5, grey) from 2 batches of differentiation of skin organoids and macrophages. Data are mean ± SD and statistics generated with an unpaired t-test.

1.7 The CD206 staining for macrophages looks a bit unconvincing as in the green channel there appear to be long multi-cell spanning processes. Would a broad CD45 stain or additional macrophage-specific markers work better?	As suggested by the reviewer, we have used CD45 antibody to identify macrophages in all whole-mount microscopy analysis of the skin organoid co-culture and show the imaging results in Fig. 4h (Please see reviewer point 1.6) To further validate this, we have also performed immunofluorescence on cryosections of skin organoids co-cultured with macrophages, revealing a CD45⁺CD206⁺ and CD45⁺HLA-DR/DP/DQ⁺ macrophage phenotype as shown in Reviewer Fig. 1.7 below.  Reviewer Fig. 1.7: <i>Immunofluorescence of cryosections of day 47 skin organoid co-cultured with macrophages (day 35 of co-culture) demonstrating CD45⁺CD206⁺ macrophages and CD45⁺HLA-DR/DP/DQ⁺ macrophages. Scale bars = 20μm.</i>
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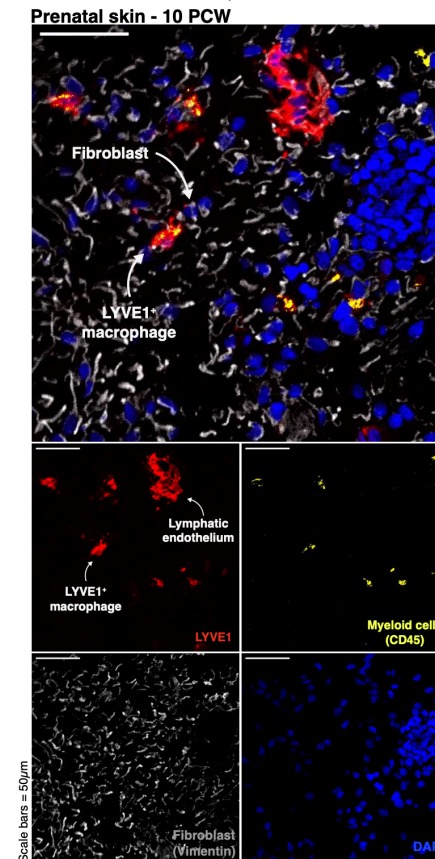
1.8	Also, as there appears to be a green overall hue (background?) in the image of the single organoid with macrophages, how is it that the image of the organoid without macrophages has no green hue at all? Was the green channel omitted by accident or the sample not stained for CD206?	We have now performed all 3D skin organoid imaging analysis using the following antibody panel: CD45 to label macrophages, CD31 to label endothelial cells and DAPI to label nuclei. We chose CD45 as suggested by the reviewer to be more specific than CD206, which we had previously used. During imaging of 3D skin organoid, we used secondary antibodies conjugated to AF647 for CD45 and secondary antibodies conjugated to AF488 for CD31. For every experiment fluorescence from all channels were simultaneously acquired for each laser excitation. We used the same immunolabeling protocol for skin organoids with and without macrophages. In these experiments, we no longer observed any background contamination.
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1.9

It would be great if the authors could validate and further confirm the proposed role of macrophages in wound healing and scarless healing. Expression analysis identified an upregulation of immune suppressing genes in early prenatal fibroblast subsets compared to adult fibroblasts. In contrast, immune activation genes are upregulated in the adult. Together with co-localization and cell-cell interaction analysis of macrophages with fibroblasts, the authors make the strong suggestions of LYVE1 macrophages contributing to WNT2+ fibroblast maintenance as well as of TML macrophages playing an important role in supporting WNT2+ fibroblasts in scarless healing.

We have now performed additional validation experiments and analysis to support the role of embryonic macrophages in prenatal scarless healing.

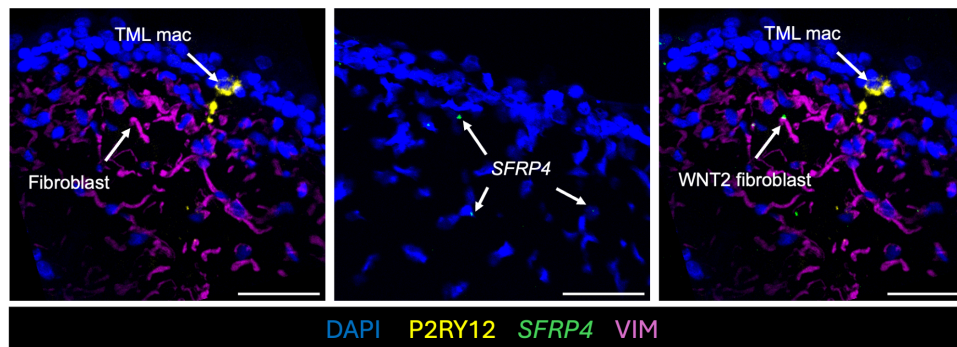
1. We demonstrated the **in situ co-location of macrophages with fibroblasts** in prenatal skin by immunofluorescence microscopy (**Fig. 3f, shown below**) and immunofluorescence/RNAscope (**Reviewer Fig. 1.9a**) analysis. Anti-LYVE1 and anti-CD45 immunostaining identifies *LYVE1*⁺ macrophages and anti-VIM is used as a pan-fibroblast marker (**Fig. 3f, shown below**).



However, while this data in combination with the literature suggests a potential functional contribution of macrophages, the proof of principal is missing. In order to make those strong claims, additional experimental data are necessary, e.g. at least demonstrating validation of co-localization at high resolution with IF imaging/RNAScope, and ideally with functional assays for an identified signaling pathway(s) in wounding experiments.

Fig. 3f: Immunofluorescence (representative images from 10 PCW prenatal skin cryosections) showing *LYVE1*⁺ macrophage (CD45⁺LYVE1⁺) co-locating with fibroblasts (VIM⁺). Scale bars = 50μm.

We then used anti-P2RY12 to label TML macrophages and anti-VIM to label fibroblasts, and on a consecutive section performed in situ hybridisation analysis (RNAscope) with a probe against *SFRP4* to label *WNT2*⁺ fibroblasts. Image overlay shows the proximity of TML macrophages with *WNT2*⁺ fibroblasts (**Reviewer Fig. 1.9a below**)

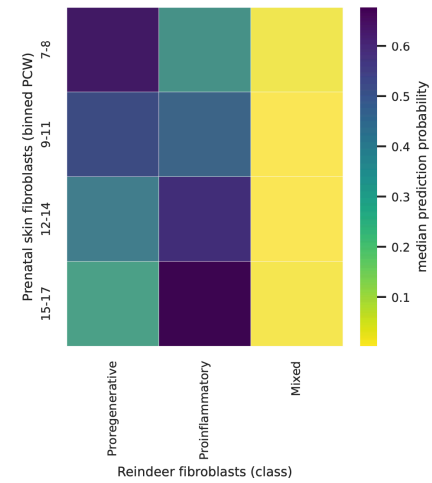


Reviewer Fig. 1.9a: Immunofluorescence (left) and RNAscope (middle) staining (representative images from consecutive 10 PCW skin sections) showing TML macrophages (P2RY12⁺) in close proximity to fibroblasts (VIM⁺, SFRP4⁺) on merged image (right). Scale bars = 50μm.

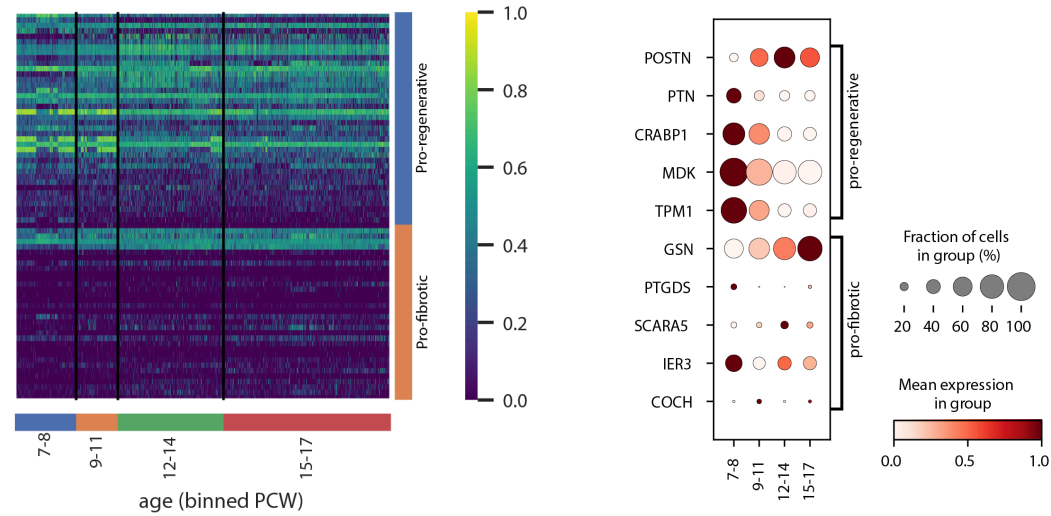
2. Comparison between prenatal human skin fibroblasts and reindeer skin fibroblasts.

In reindeers, antler velvet skin heals without scarring whilst back skin scars. This was reported to be due to differences in velvet and back skin fibroblast and macrophage populations during wound healing (PMID: 36493752). Velvet fibroblasts express pro-regenerative genes. These fibroblasts recruit and interact with ‘early macrophages’ and confer the non-scarring phenotype. In contrast, back skin fibroblasts express pro-inflammatory gene programs and secreted immunostimulatory factors to engage mature macrophages, predisposing to a pro-fibrotic wound environment.

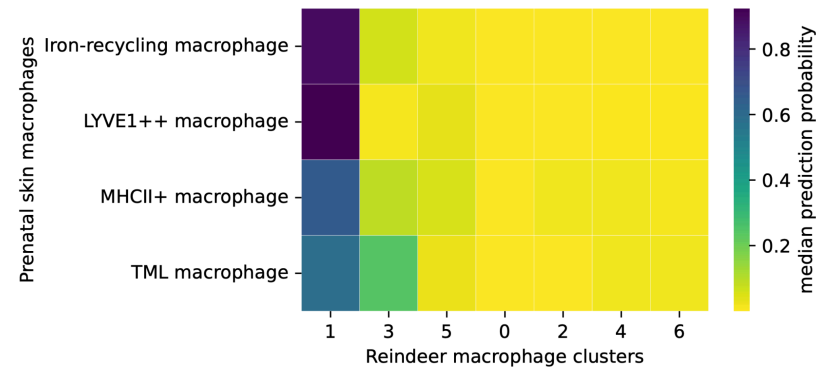
		By logistic regression (LR) analysis, we show that prenatal skin fibroblasts in early gestation have higher probability of correspondence to pro-regenerative reindeer fibroblasts. In contrast, human prenatal fibroblasts from later gestation have a higher probability of matching to pro-fibrotic fibroblasts (Extended Data Fig. 7d, shown below). Accordingly, prenatal skin fibroblasts had overall higher expression of pro-regenerative genes compared to pro-fibrotic genes (gene list used from PMID: 36493752 provided in supplementary table 20) with several key pro-regenerative genes (<i>MDK</i>, <i>CRABP1</i>, <i>PTN</i> and <i>TPMI</i>) downregulated in later gestation (Extended Data Fig. 7e, shown below), coinciding with loss of scarless healing potential. Importantly, comparison of the macrophage populations showed that human prenatal skin macrophages most closely resembled reindeer ‘early macrophages’ enriched in velvet skin (clusters 1, 3 and 5) which contribute to scarless healing, and do not resemble reindeer back skin macrophages (clusters 0, 2 and 4) (Extended Data Fig. 7f, shown below). This lends further support to our conclusions that the distinct gene expression profiles of prenatal skin fibroblasts, characterised by lack of pro-inflammatory genes, combined with interactions with macrophages, contribute to scarless healing potential in human prenatal skin.
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Extended Data Fig. 7d: Heatmap showing prediction probabilities from the LR model trained on classes of reindeer fibroblasts (pro-regenerative, pro-inflammatory and mixed populations) (x-axis), projected onto prenatal skin fibroblasts grouped by age (y-axis). Colour scale indicates median prediction probabilities.

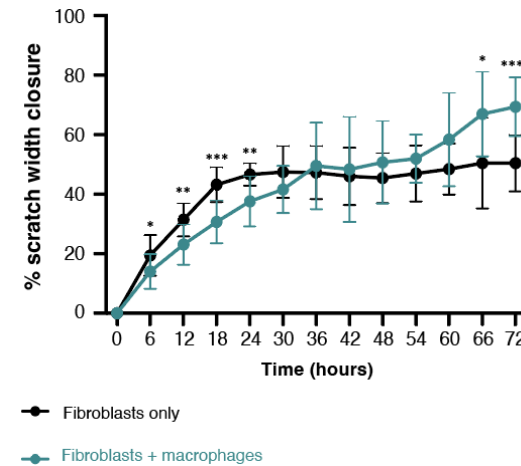


Reviewer Fig. 1.9b: (left) Heatmap showing variance-scaled expression (color scale) of pro-regenerative and pro-fibrotic genes in prenatal skin fibroblasts (grouped by PCW). **Extended Data Fig. 7e: (right)** Dot plot showing the variance-scaled, mean expression (dot colour) and percent of expressing cells (dot size) of selected pro-regenerative and pro-fibrotic genes in prenatal skin fibroblasts by gestational age (grouped PCW).



Extended Data Fig.7f: Heatmap showing prediction probabilities from the LR model trained on reindeer macrophage clusters (x-axis), projected onto prenatal skin macrophage subsets (y-axis). Colour scale indicates median prediction probabilities.

3. We performed an **in vitro scratch assay** with skin organoid-derived fibroblasts (n=6, isolated from day 79 of differentiation) cultured with or without autologous iPSC-derived macrophages. We observed improved scratch wound healing in fibroblasts in the presence of macrophages as demonstrated by a higher percentage of scratch width closure over the 72 hour assay (**Extended Data Fig. 7g, shown below**). By 66 and 72 hours, there was significantly higher scratch wound closure achieved in fibroblasts cultured with macrophages compared to fibroblasts alone.



Extended Data Fig. 7g: *Percentage scratch width closure (y-axis) quantified over time (x-axis) for fibroblasts cultured with macrophages (green) or in isolation (black).*

Collectively, these three orthogonal approaches provide further evidence for the role of macrophages in wound healing and their close association with prenatal skin fibroblasts to contribute to scarless skin healing.

1.10	The microenvironments in Fig 1d seem to be an important concept. However, it is quite confusing that the dermal fibroblast subtypes are distributed among different microenvironments, while most macrophages are located within the same environment. Do the microenvironment definitions depend on developmental timepoints rather than on cell type or location, or a combination thereof?	We thank the reviewer for raising the potentially confusing use of the term microenvironment in the manuscript. To improve clarity, we have defined microenvironment (ME) to refer to a specific grouping of different cell types, computationally predicted to have a high probability of occurring in the same spatial location based on their abundance profiles across tissue sections. ME is computed using non-negative matrix factorization (NMF) of celltype spatial abundance profiles (inferred by cell2location). The NMF is an unsupervised algorithm that only uses as input a matrix of spot by cell type from cell2location. The data from all tissue sections from different anatomical sites and stages (6-10 PCW) were processed together, thus both gestational stage and location contributed to the overall composition of the MEs. This analysis includes tissue spots regardless of their developmental time points, hence, there might be MEs that define time-dependent associations of cell types and spatial locations. We have not disentangled them at this point due to the complexity of considering both spatial and temporal axes together (e.g. lack of consistent developmental time point representation across different regions). We have modified the text to make sure that we define and use “microenvironment” only in this specific meaning (relevant text below): Lines 160-165: <i>We assessed if specific cell types were more likely to be co-located within microanatomical tissue niches using non-negative matrix factorisation (NMF) to computationally predict microenvironments in conjunction with correlation analyses (see methods). Co-location was indicated by a high proportion of two or more cell types sharing a microenvironment (Fig. 1d) and/or by positive correlation coefficient between cell pairs (Fig. 1e, Extended Data Fig. 1e).</i> Lines 169-175: <i>Our analysis revealed that predicted microenvironments in prenatal skin comprised epidermal, dermal, vascular, and neural cells (Fig. 1d, e, Extended Data Fig. 1e). Each of these microenvironments included specific types of immune cells (Fig. 1d). For example, macrophages co-located with endothelial and neuronal cells in ‘early and late neurovascular microenvironments’ (ME1 and ME5 respectively), while pre-dermal condensate (pre-Dc) co-located with dendritic cells and lymphoid cells based on correlation analyses (Fig. 1d, e, Extended Data Fig. 1e).</i>
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		Lines 436-438: <i>In prenatal skin, macrophage subsets (Extended Data Fig. 6d, e) were predicted to co-locate with fibroblasts, neural and vascular cells in distinct tissue microenvironments in early gestation (Fig. 1d, e).</i> Lines 492-493: <i>TML macrophages were also predicted to co-locate with Schwann cells in prenatal skin ('early neurovascular microenvironment', ME1)</i> Lines 512-514: <i>Visium deconvolution analysis predicted co-location of prenatal skin macrophages with endothelial cells ('early and late neurovascular microenvironments', ME1 and ME5) (Fig. 1d, 1e).</i> Based on how MEs are inferred, one cell type can contribute to different MEs and both macrophages and fibroblasts are part of different MEs. In Fig. 1d (shown below), the cell types are grouped (coloured bars) by the ME they contribute the highest to: Iron-recycling and <i>LYVE1</i>⁺ macrophages to ME2, TML macrophages to ME1, MHC-II⁺ macrophages to ME5, <i>WNT2</i>⁺ fibroblasts to ME6, <i>FRZB</i>⁺ early fibroblast to ME1 and <i>PEAR1</i>⁺ fibroblast to ME4 and <i>HOXC5</i>⁺ early fibroblasts to ME9.
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		 Fig. 1d: Dotplot showing spatial microenvironments. Cell type to microenvironment coefficients are normalised by cell type sums; cell type to microenvironment assignment is shown by colour. Microenvironment 5, which shows co-locating macrophages and endothelial cells, is highlighted (grey).
1.11	Importantly, why are WNT2+ fibroblasts alone in their own microenvironment and not with TMLM? Why is there no joint microenvironment of WNT2+ fibroblasts with LYVE1 macrophages?	As we explained, in Fig. 1d (shown in point 1.10) the cell types are grouped (coloured bars) by the microenvironment (ME) towards which they contribute the highest proportion. The NMF method also allows the assessment of whether each cell type contributes to different MEs. <i>WNT2</i>⁺ fibroblasts primarily contribute to MEs 4 and 6 whilst <i>LYVE1</i>⁺ macrophages primarily contribute to ME2 but also MEs 4 and 5. TML macrophages primarily contribute to ME1 with smaller contributions to MEs 2, 3, 4, 5 and 8. The co-location of <i>LYVE1</i>⁺ and TML macrophages with <i>WNT2</i>⁺ fibroblasts were based mainly on

their contributions to ME 4. This was further supported by correlation analysis respectively for *WNT2*⁺ fibroblast-*LYVE1*⁺ macrophages and *WNT2*⁺ fibroblast-TML macrophages (**Fig. 3d, shown below**). In the revised manuscript, we demonstrate their co-location in situ based on Visium data of skin sections in **Fig. 3e (shown below)**. This shows the parallel distributions of *WNT2*⁺ fibroblasts and *LYVE1*⁺ macrophages along the dermal-epidermal axis indicating enrichment of these cell types in the same areas in the skin, as well as for *WNT2*⁺ fibroblasts and TML macrophages.

In addition, we validated the co-location of macrophages (*LYVE1*⁺ and TML) with fibroblasts by immunofluorescence and RNAscope analysis as shown in response to point 1.9.

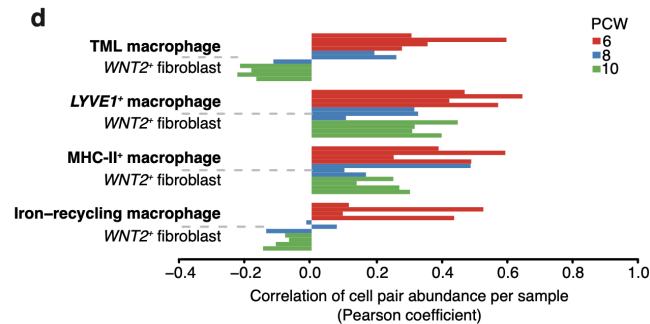
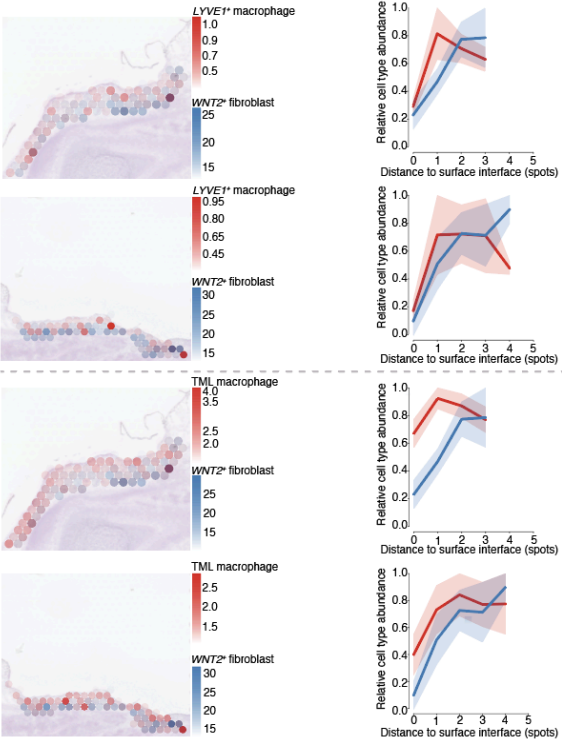
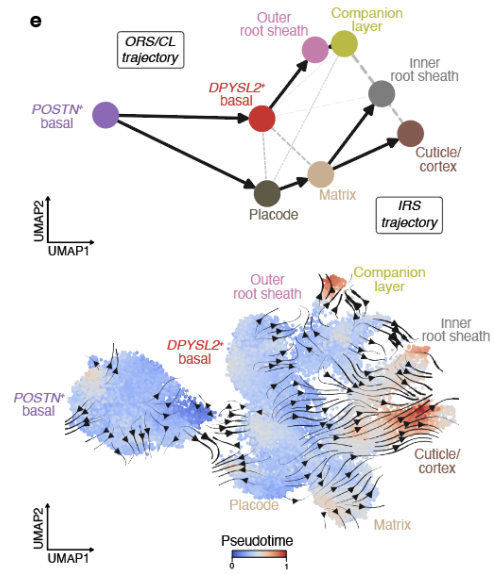


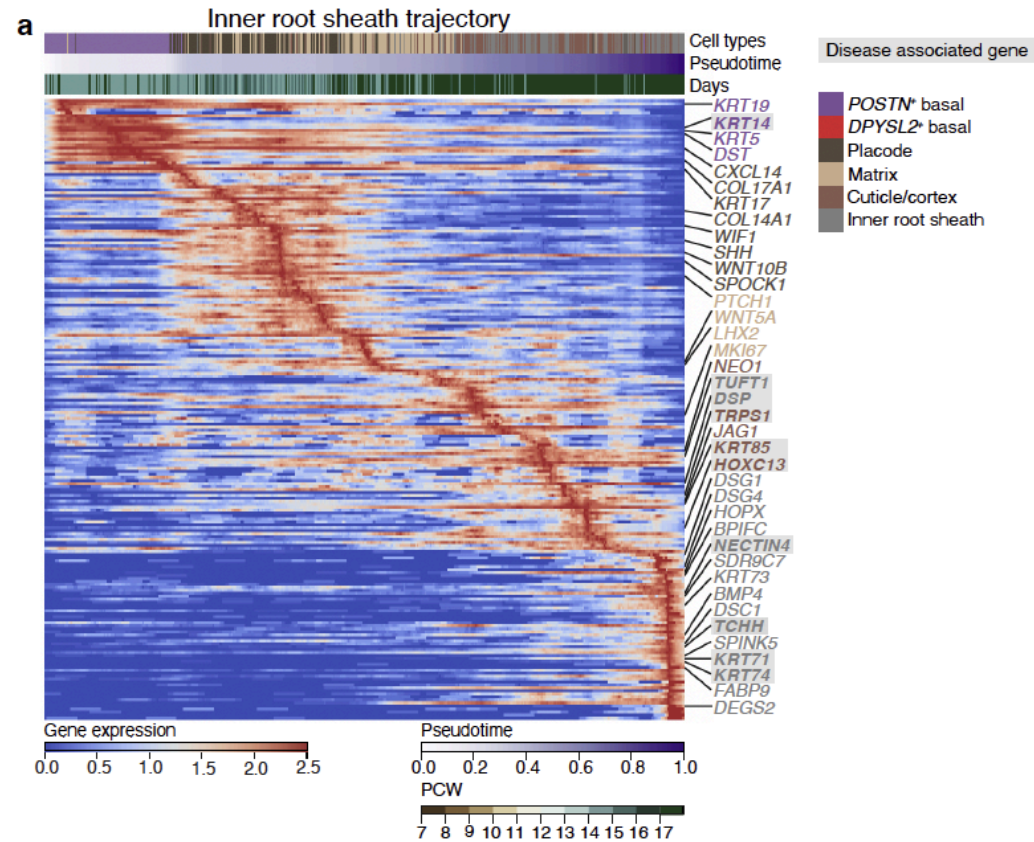
Fig. 3d: Bar plot showing cell type co-location, indicated by positive Pearson correlation coefficients calculated between per-spot normalised cell type abundances, for selected cell type pairs (macrophages and *WNT2*⁺ fibroblasts). Pearson correlation coefficients were calculated across all skin-covered spots of Visium samples; each sample is shown by an individual bar.

		 Fig. 3e: Curve plots showing the mean per-spot normalised abundance of LYVE1⁺ macrophages and WNT2⁺ fibroblasts (top) and of TML macrophages and WNT2⁺ fibroblasts (bottom) along the distance to skin surface (2 representative samples, 8 PCW). Shaded regions represent 2 SD confidence intervals. Accompanying H&E images show abundance of the same cell types overlaid on top of tissue sections.
1.12	Generally, it would be very helpful for Fig. 1d if the authors could provide a distinct name/label for the microenvironments instead of	As the microenvironments are predicted cell type groupings, we initially retained the way they are labelled (as numbered factors) during the analysis, in keeping with previous publications (PMID: 35027729; PMID: 35549310), except for specific microenvironments which are discussed in more detail in the manuscript.

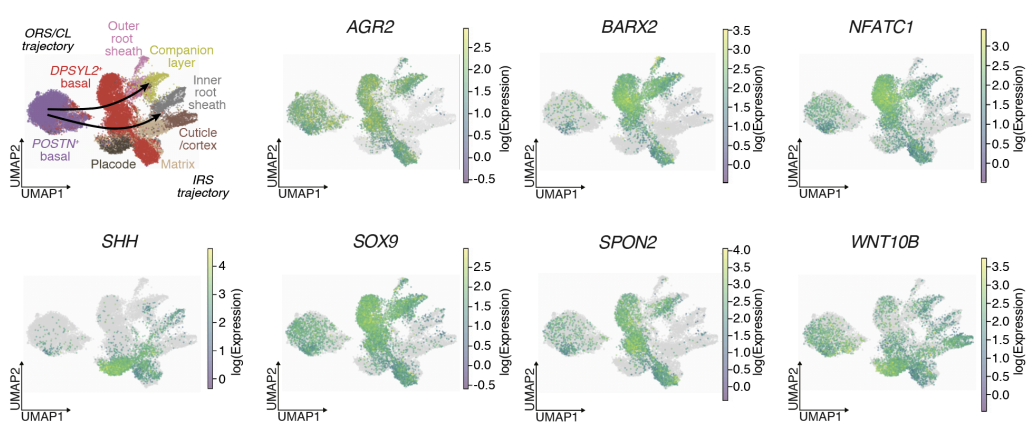
	just numbers.	We provide below suggested labels for each microenvironment as requested, based on the predominant celltype composition and/or spatial or age distribution. For ease of discussion in the manuscript, we have opted to keep the labels as numbers except for the ones we focus on which are ‘early neurovascular’ (ME1) and ‘late neurovascular’ microenvironments (ME5). 1: Early neurovascular 2: Myeloid/Megakaryocyte-erythroid enriched 3: Epidermal 4: Deep dermal 5: Late neurovascular 6: Mid-to-deep dermal 7: Immune-enriched 8: Lymphatic-enriched 9: Superficial dermal 10: Neural-enriched Please see below the amended text for new changes made to the revised manuscript on this point: Lines 169-175: <i>Our analysis revealed that predicted microenvironments in prenatal skin comprised epidermal, dermal, vascular, and neural cells (Fig. 1d, e, Extended Data Fig. 1e). Each of these microenvironments included specific types of immune cells (Fig. 1d). For example, macrophages co-located with endothelial and neural cells in ‘early and late neurovascular microenvironments’ (ME1 and ME5 respectively), while pre-dermal condensate (pre-Dc) co-located with dendritic cells and lymphoid cells based on correlation analyses (Fig. 1d, e, Extended Data Fig. 1e).</i> Lines 492-493: <i>TML macrophages were also predicted to co-locate with Schwann cells in prenatal skin (‘early neurovascular microenvironment’, ME1)</i>
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		Lines 512-514: <i>Visium deconvolution analysis predicted co-location of prenatal skin macrophages with endothelial cells ('early and late neurovascular microenvironments', ME1 and ME5) (Fig. 1d, 1e).</i>
1.13	To gain insights into the epidermal trajectory the authors only used the organoid data set. It would be great if the authors could confirm the result within the prenatal data or the integrated data set (as in Extended Data Fig 2b). Or are some epidermal cell types underrepresented in the prenatal samples?	We now confirm the inferred trajectory on the integrated prenatal and skin organoid scRNA-seq data as suggested by the reviewer. For the revision, we generated new scRNA-seq data from an additional three prenatal skin samples (from 14-17 PCW where hair follicles are actively forming). We profiled all cells and performed FACS-enrichment for keratinocytes by isolating the non-immune cell fraction (using CD45) and excluding fibroblasts and endothelial cells (using CD34). We created a combined latent embedding of prenatal skin and skin organoid (~23,000 keratinocytes of which ~9,000 cells are from human prenatal skin), and inferred and analysed the pseudotime developmental trajectory on this integrated embedding using palantir and RNA velocity under the CellRank framework. This analysis shows good capture of transitory cells, and now clearly shows the trajectory of <i>POSTN</i>⁺ basal keratinocytes along <i>DPYSL2</i>⁺ basal, into the outer root sheath and companion layer, and a second trajectory along Placode, into Matrix and then cuticle/cortex or inner root sheath as in Fig. 2e, shown below:

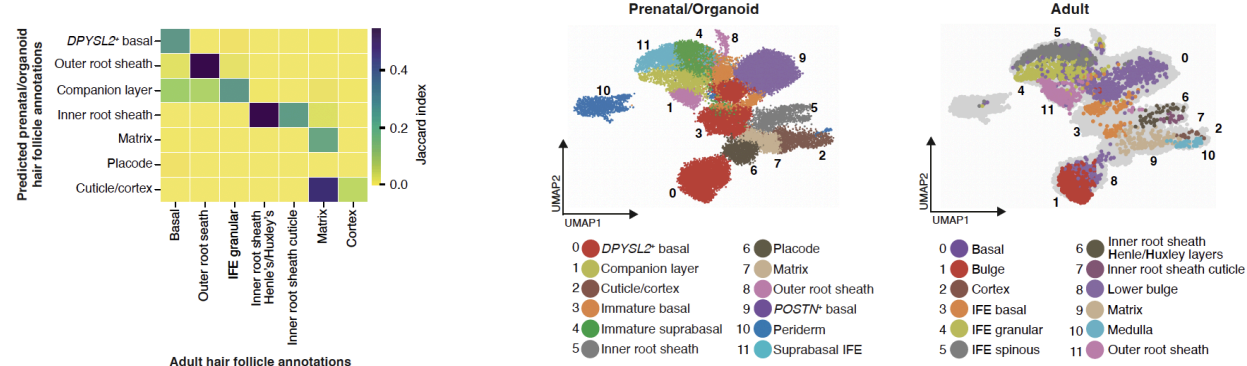
		 Fig. 2e: Inferred pseudotime trajectory of prenatal skin and skin organoid epidermal cell states differentiating along the ‘ORS/CL’ and ‘IRS’ trajectories; UMAP overlaid with PAGA and coloured by cell state (top) and overlaid with the cell directionality (shown in arrows) as inferred over the cell-to-cell transition probability matrix from running CellRank and coloured by pseudotime (bottom). Shown are connectivities (dashed) and transitions (solid/arrows). Threshold for Cellrank PAGA was kept at default.
1.14	It would be great if the authors could provide much more informative feature plots for the key genes of the pseudotime analysis, for both the ORS/CL and the placode/IRS trajectory.	The key genes of the pseudotime analysis are shown in Extended data Figure 5a (shown below), 5b, Extended data Figure 3i (shown below), j and all relevant genes are in Extended data table 7. We have decided against showing further feature plots due to the limited space in the extended figures.



Extended data Fig 5a: Heat map showing differentially expressed genes across pseudotime along the 'Inner root sheath trajectory'. Genes associated with genetic hair disorders are highlighted in grey.

		 Extended data Fig 3i: <i>Inferred pseudotime trajectory of skin organoid epidermal cell states differentiating along the ‘ORS/CL’ and ‘IRS’ trajectories, coloured by gene expression (log-transformed).</i>
1.15	Why is the placode/matrix represented as one cluster? In the Extended Data Fig 3d, which shows the integrated but split prenatal and adult skin, it appears that the placode (cluster 6) has a subcluster (pink) that nicely overlays with the matrix cluster (cluster 9) from adult skin. Considering an ~1:1 distribution of prenatal placode and matrix cells, why	To better separate Placode and Matrix, which was difficult due to low cell numbers of keratinocytes in prenatal skin in our original submission, we generated new data from three additional human prenatal skin samples from 14-17 PCW when hair follicle is actively forming (as described in point 1.14). This added a further 200,000 additional cells to the overall dataset (now 433,961 cells). As we enriched for keratinocytes (strategy detailed in point 1.14), we have now captured more rare cells and are able to clearly distinguish Placode from Matrix cells in human prenatal skin. We now present separate clusters for Placode and Matrix, that both align with their adult counterparts as seen in extended figure 3d and shown below:

is the correlation with adult matrix cells rather low (Extended Data Fig. 3c)?



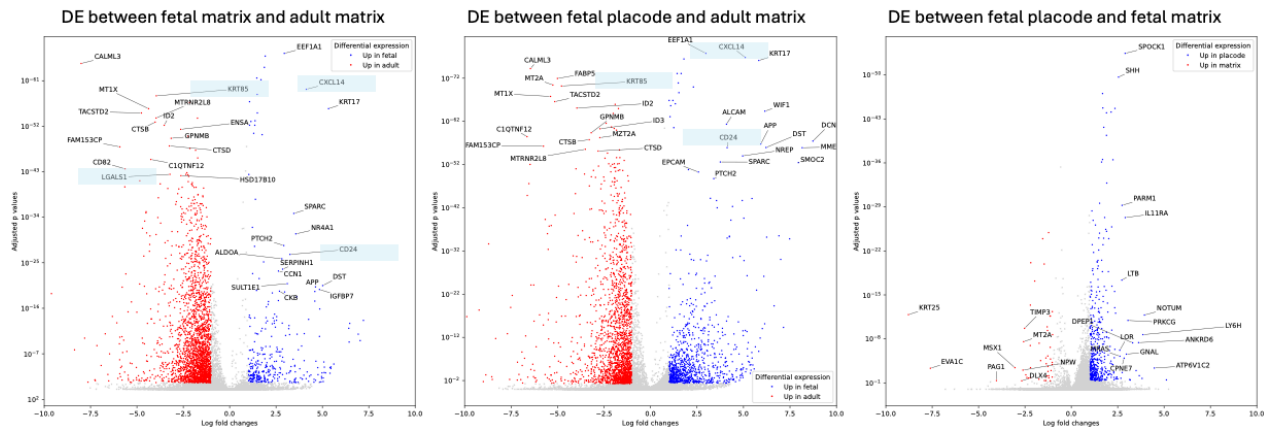
Extended data Fig. 3c (left): Heatmap showing the correspondence (measured by Jaccard index) between prenatal skin/skin organoid (y-axis) and adult (x-axis) epidermal and hair follicle cell states from a logistic regression model trained on adult hair follicle data, projected onto integrated prenatal skin/skin organoid data.

Extended Data Fig. 3d (right): UMAPs showing clustered cell states in integrated data from adult hair follicles and prenatal/skin organoid, coloured by prenatal skin/skin organoid cell types (left) and adult cell types (right).

The enhancement in resolution to distinguish placode and matrix has significantly contributed to a more accurate correlation assessment with adult hair follicle cell types. This improvement is evident in Extended Data Fig. 3c (see above), where we observe a stronger correlation between prenatal Matrix cells and adult Matrix cells compared to the mixed Placode/matrix population used previously.

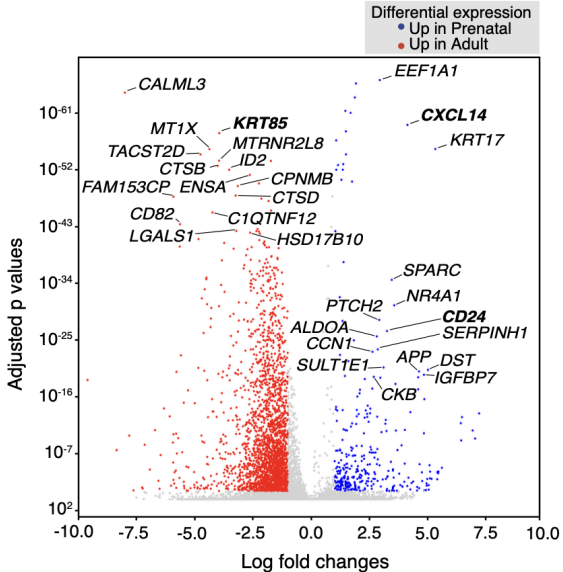
Moreover, with the improved resolution, we can now identify differentially expressed genes (DEGs) between prenatal Placode and prenatal Matrix cells separately from adult Matrix cells. Our analysis reveals a high expression of *LGALS1* and *KRT85* in adult cells compared to prenatal Placode and Matrix cells, alongside elevated expression levels of *CXCL14* and *CD24* in prenatal Placode and Matrix cells, as was previously described in the original submission of our manuscript. Additionally, we now see that fetal Placode and Matrix cells exhibit differential

expression patterns of *SHH*, *SPOCK1*, *KRT25*, and *MSX1* (see Reviewer Figure 1.15 below).



Reviewer Figure 1.15: Volcano plot showing differentially expressed genes between prenatal matrix cells and adult matrix cells, prenatal Placode cells and adult Matrix cells and prenatal Placode and prenatal Matrix cells.

We show the comparison of prenatal Matrix and adult Matrix in **Extended Data 3e** (shown below):

		e  Extended Data Fig. 3e: Volcano plot showing differentially expressed genes between prenatal matrix cells and adult matrix cells.
1.16	To study the dermal trajectories the authors relied solely on prenatal data. For consistency the authors should here also use the organoid data and show the prenatal or integrated prenatal/organoid dataset in the extended data.	We have conducted inferred pseudotime trajectory analysis on fibroblasts in the combined data object of prenatal human skin and skin organoid. This analysis reaffirms our previous findings from the human prenatal skin dataset. Specifically, we observe two trajectories in keeping with our original findings: 1. A hair follicle-related trajectory originating from <i>HOXC5</i>⁺ early fibroblasts, progressing towards the dermal papilla via dermal condensate. 2. A dermal fibroblast trajectory originating from <i>HOXC5</i>⁺ early fibroblasts to Myofibroblasts and <i>PEAR1</i>⁺ fibroblasts.

We show these findings in **Fig. 2f** and also below:

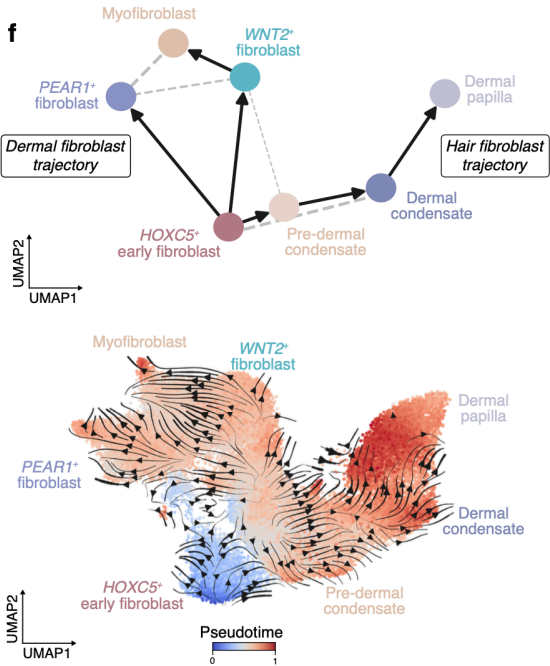
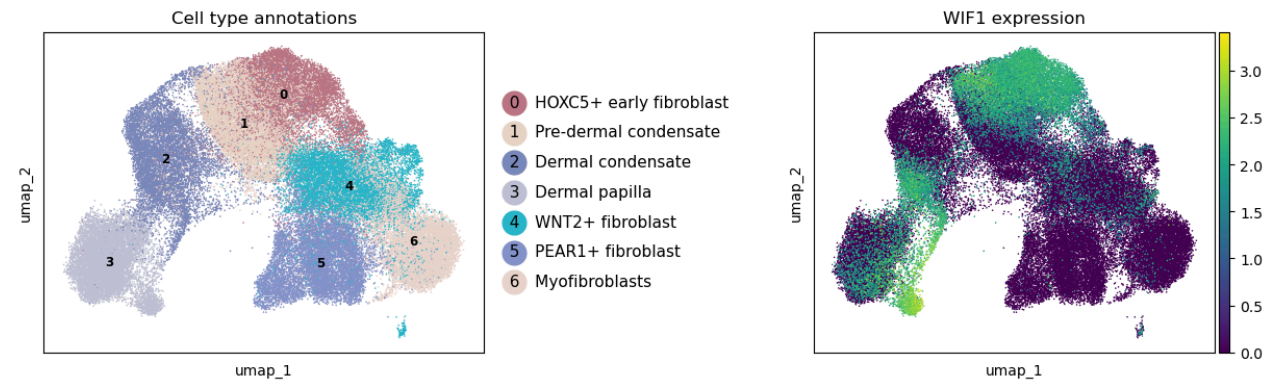


Fig. 2f: *Inferred pseudotime trajectory of prenatal and skin organoid fibroblasts differentiating along the ‘hair’ and ‘dermal’ fibroblast trajectories. UMAP overlaid with PAGA and coloured by cell state (top) and overlaid with cell directions (shown in arrows) inferred over the cell-to-cell transition probability matrix from running CellRank, and coloured by pseudotime (bottom).*

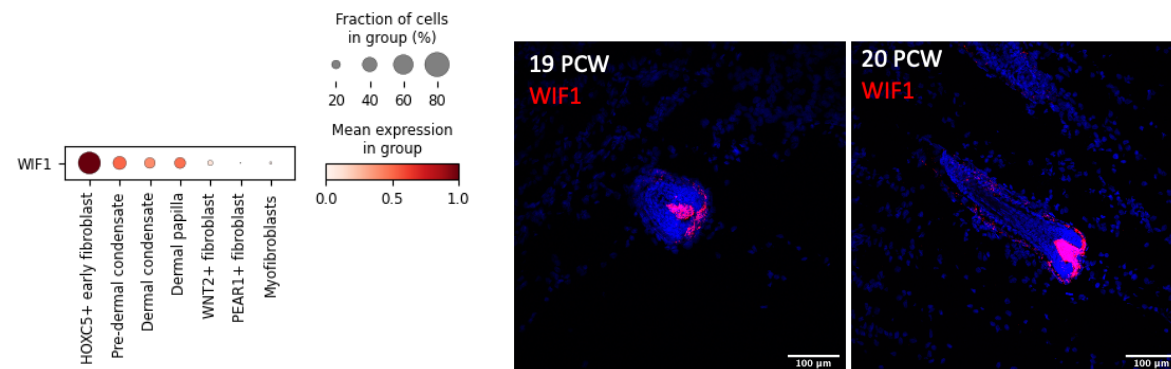
1.17 From comparing the PAGA plot (Fig 2f, top) with the CellRank UMAP (Fig 2f bottom), it appears that one would draw the trajectories differently as

Please refer to **point 1.16**. In the new analysis incorporating the additional newly generated data for the revision, the inferred trajectory analysis shows clear arrows (by RNA velocity analysis) for the trajectory from *HOXC5*⁺ fibroblasts towards pre-dermal condensate (pre-DC).

	there are no direct arrows from HOXC5+ fibroblasts to Pre-DC. How the authors explain that the Dermal Rank plot would not show clear directions towards the Pre-DC leaving the HOXC5 state?	
1.18	WIF1 is only expressed in early HOXC5 fibroblasts (Extended Data Fig 4c), but it is also a DP signature gene. Why is it not expressed in the DP?	<i>WIF1</i> is indeed a known marker of the adult human hair follicle dermal papilla (Dp) as previously reported using microarray analyses (PMID: 22623722; PMID: 24145441). However, <i>WIF1</i> was not identified as a signature gene in a recent study of human fetal scalp skin using bulk RNA sequencing where 121 marker genes for human prenatal Dp were reported (PMID: 33324639). Our scRNA-seq data analysis and new <i>in situ</i> validation data using RNAscope performed during revision clarifies the above observations. Our findings collectively show that <i>WIF1</i> is highly expressed by all fibroblast clusters differentiating towards the Dp in prenatal skin, but in the adult is restricted to the Dp, and as such is an adult Dp signature gene. We show <i>WIF1</i> in all cell types along the hair fibroblast trajectory including in <i>HOXC5</i>⁺ early fibroblasts, pre-Dc, Dc and Dp (Reviewer Fig. 1.18a below). However, expression in prenatal Dp is lower compared to earlier cell types such as <i>HOXC5</i>⁺ early fibroblast (highest expression) (Reviewer Fig. 1.8b below). This is also in keeping with <i>WIF1</i> being differentially expressed in early pseudotime of the hair fibroblast trajectory rather than late pseudotime (Extended Data Fig. 4c and below). In addition, we show the expression of <i>WIF1</i> in the prenatal skin Dp by RNAscope analysis in 19-20 PCW prenatal skin (Reviewer Fig. 1.18b below).



Reviewer Fig. 1.18a: *WIF1* expression shown along the inferred pseudotime trajectory of prenatal skin fibroblasts differentiating along the ‘hair’ and ‘dermal’ trajectories with UMAPs coloured by celltype (left) and log-transformed *WIF1* expression (right).

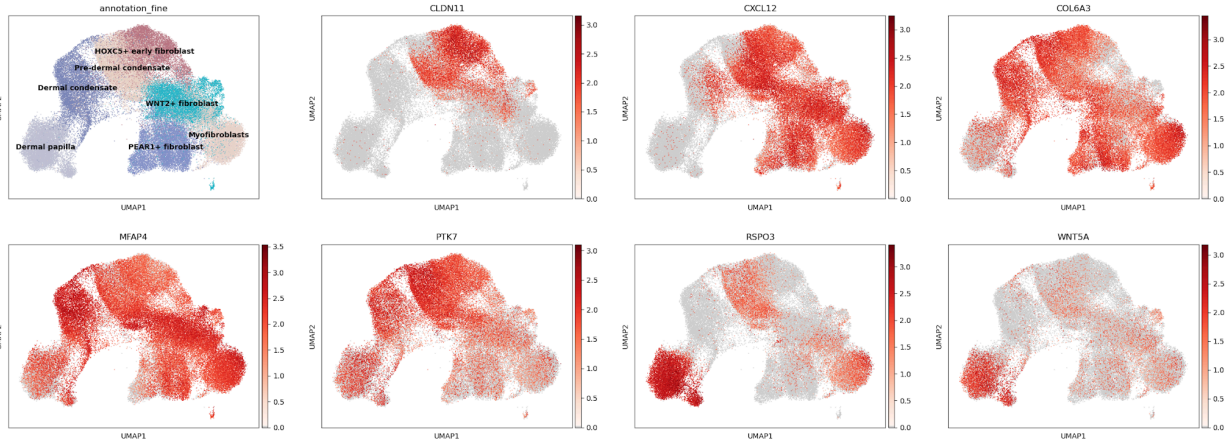


Reviewer Fig. 1.18b: *Dot plot showing variance-scaled, mean expression (dot colour) of WIF1 and percent of expressing cells (dot size) in prenatal skin fibroblasts (left) and RNAscope images of prenatal skin (representative 19 and 20 PCW samples) demonstrating WIF1 expression in the dermal papilla at the base of hair follicles (right).*

		Heatmap showing differentially expressed genes across pseudotime along the 'hair fibroblast trajectory'. The heatmap displays gene expression (color scale 0.0 to 2.5) and pseudotime (color scale 0.0 to 1.0). Genes are listed on the right, including <i>WIF1</i>, <i>CLDN11</i>, <i>GPC6</i>, <i>ADAMTS1</i>, <i>GRP</i>, <i>CXCL12</i>, <i>VIM</i>, <i>PTK7</i>, <i>CD9</i>, <i>TWIST2</i>, <i>COL3A1</i>, <i>COL1A1</i>, <i>LGALS1</i>, <i>COL6A3</i>, <i>MEF2C</i>, <i>MFAP4</i>, <i>RSPO4</i>, <i>SLC26A7</i>, <i>DLL1</i>, <i>SPON1</i>, <i>NDP</i>, <i>PAPPA2</i>, <i>BMP7</i>, <i>CKB</i>, <i>CXCR4</i>, <i>TRPS1</i> (highlighted in grey), <i>WNT5A</i>, <i>CYTL1</i>, <i>CRYM</i>, and <i>RSPO3</i>. A legend at the bottom identifies cell types: <i>HOXC5</i>⁺ early fibroblast (red), Pre-dermal condensate (orange), Dermal condensate (blue), and Dermal papilla (purple). A scale for PCW (Prenatal Cutaneous Wound) is also shown, ranging from 7 to 16. A color scale for gene expression (0.0 to 2.5) and pseudotime (0.0 to 1.0) is provided. Extended Data Fig.4c: Heatmap showing differentially expressed genes across pseudotime along the 'hair fibroblast trajectory'. Gene associated with genetic hair disorders is highlighted in grey.
1.19	Inspection of the prenatal skin UMAP on the web portal appears to show a huge difference between male and female. In particular, the entire FRZB⁺ fibroblast cluster is just from male samples. Can the authors please explain how and	FRZB⁺ fibroblasts are found primarily during early development in prenatal skin and as such the earliest CS18 sample (male) is the largest contributor to this population. The male: female percentage ratio in our entire prenatal skin dataset is 82:18. However, we do not think this is due to a sex effect as our developmental datasets from multiple organs in our previous publication (PMID: 35549310) shows that the male: female percentage ratio for FRZB^{hi} fibroblasts is 32:68 overall across gestation, which we show below (Reviewer Fig. 1.19)

	whether this could be an issue that needs to be addressed experimentally?	Percentage of FRZB fibroblasts <table><thead><tr><th>Sex</th><th>Percentage</th></tr></thead><tbody><tr><td>female</td><td>~68%</td></tr><tr><td>male</td><td>~32%</td></tr></tbody></table> Reviewer Fig.1.19: Barplot showing percentage of FRZB expressing fibroblasts by sex across all samples in PMID: 35549310. Nevertheless, to mitigate any concerns regarding the contribution of this population from primarily one sample in our prenatal skin dataset, we excluded it from our dermal trajectory analysis, as highlighted in our manuscript in the results section and stated here: Lines 281-287: <i>To infer the origin of the pre-Dc, Dc and Dp in human prenatal skin, we performed trajectory analysis (CellRank) of the different fibroblast clusters (Fig. 2f, Extended Data Fig. 4a, Supplementary Table 6). FRZB⁺ fibroblasts were excluded from this analysis as they are primarily observed in one sample from the earliest gestation stage (7 PCW) (Extended Data Fig. 3b). Although rare in prenatal skin, FRZB-expressing fibroblasts are present in several other developing organs, including the yolk sac, spleen, and gut (Extended Data Fig. 4b).</i>	Sex	Percentage	female	~68%	male	~32%
Sex	Percentage							
female	~68%							
male	~32%							
	Reviewer 1 comments - minor	Response to reviewer						
1.20	Genes of interest. A general improvement would be if the authors could highlight in the Figures the genes of interest	We thank the reviewer for their suggestion. We have highlighted the genes of interest in bold in the relevant figures (Fig. 3b, c, e ; Fig. 4f ; Extended Data Fig. 3e, Extended Data Fig. 4h, i, j; Extended Data Fig. 6d, f, e ; Extended Data Fig. 8a; Extended Data Fig. 9b, c; Extended Data Fig. 10a. We show one representative panel (Fig. 3c) below:						

	that are mentioned in the text, thereby guiding and helping the reader to find the important factors within the long lists of genes.	Fig. 3c: Matrix plot showing variance-scaled, mean expression (colour) of Milo-generated DEGs by gestational age (grouped by PCW) in WNT2⁺ fibroblast population. DEGs are grouped by function.
1.21	It would be great if the authors could also improve the web portal such that they offer only the latest annotations. Alternatively, they could provide description of the various refinements so it can be followed better. Please highlight the annotations used in manuscript.	The web portal has now been updated to include various levels of annotations that a user may prefer. These include ‘very broad’ (Fig. 1f), ‘broad’ (Fig. 1b) and ‘fine’ cell annotations referred to in the manuscript. The other data categories in the web portal have also been streamlined and reordered to help navigate and interrogate the datasets. Please use the link to access our webportal: https://developmental.cellatlas.io/fetal-skin (password: fs2023).
1.22	It would improve the validity of some discussed key genes if the authors would present feature plots in addition to dot plots. Feature plots would provide a great visual way to demonstrate expression levels by specific cell types compared to others.	We opted for dot plots due to the number of figure panels and space constraints but enabled feature plots to be readily visualised through our web portal (https://developmental.cellatlas.io/fetal-skin) (password: fs2023). In addition to the feature plots we provide in the manuscript for genes expressed along the inner and outer root sheath differentiation trajectories (shown in point 1.14), we provide feature plots below for the key genes (<i>CLDN11</i>, <i>CXCL12</i>, <i>COL6A3</i>, <i>MFAP4</i>, <i>PTK7</i>, <i>RSPO3</i>, <i>WNT5A</i>) discussed in relation to the hair fibroblast trajectory (Reviewer Fig. 1.22).

	In the manuscript, feature plots were only used once in Extended Data Fig 3g.	 Reviewer Fig. 1.22: Feature plots showing expression of key genes along the hair fibroblasts differentiation trajectory.
1.23	Along the same lines, when outlining hair follicle development in prenatal skin, it would be helpful to also show the feature plots of the key markers (i.e. SHH, SLC26A7, POSTN...) used for identifying the various cell types in the prenatal epidermis and dermis.	We are showing some feature plots and dot plots showing relevant markers in hair follicle development in the manuscript in Extended Data Fig. 3a, i, j. However, due to space constraints we could not include feature plots for each and every population. Instead we have opted to provide comprehensive tables including DEGs between populations (Supplementary Table 3) and describe key markers in the manuscript. We have also uploaded an extensive Github project folder containing manual annotation strategies for each lineage (https://github.com/haniffalab/FCA_fetal_skin/tree/main/analysis/scrna-seq/1_Prenatalskin_Initial_QC_processing_and_annotation). We also have made the data available for easy query online via our webatlas (https://developmental.cellatlas.io/fetal-skin) (password: fs2023). In addition to the feature plots we provide in the manuscript for genes expressed, we provide dotplots below (Reviewer Fig. 1.23a, Reviewer Fig. 1.23b) for the key markers used for identifying cell types in the prenatal epidermis and for the dermis (see also point 1.22).



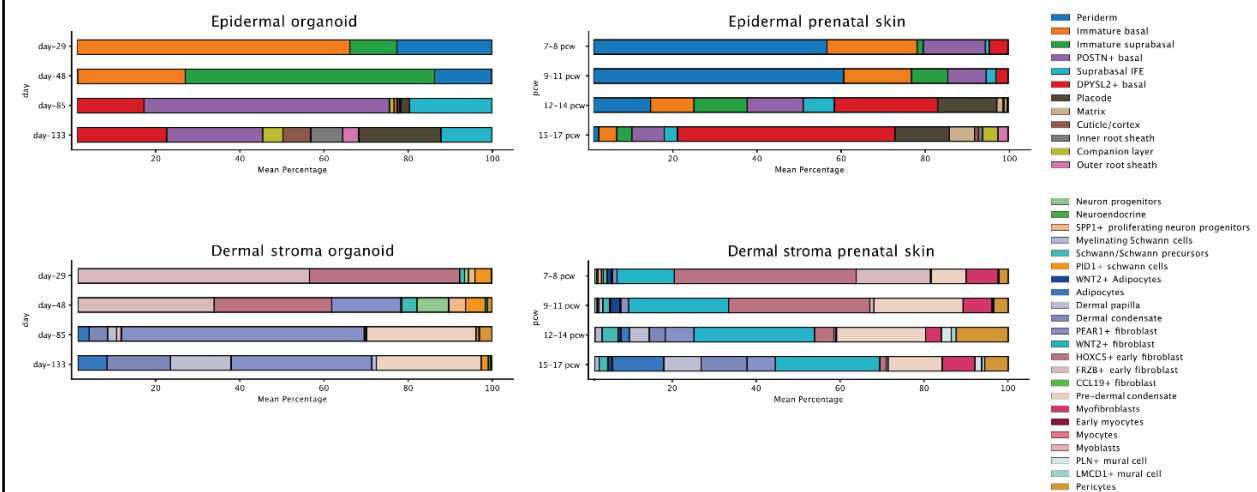
Reviewer Fig. 1.23b: Feature plots showing expression of some of the key marker genes in epidermal cell types used for annotation.

1.24

It would be very useful if the authors could show the cell composition of the skin organoid model for the corresponding timepoint equivalent to prenatal skin. Unless missed, it appears that only the cell composition chart derived from UMAPs of prenatal skin is presented in Extended Data Fig. 2b.

We have decided against including bar plots illustrating the cell composition of the skin organoid data. This choice was made to manage the number of figure panels and address space constraints. Additionally, part of the organoid scRNA Seq data has been published and described extensively here (PMID: 32494013).

When comparing cell composition of prenatal skin with skin organoid, we observe a higher percentage of immature cells at the earliest time point of the skin organoid (day 29) compared to the earliest prenatal time point (pwc 7) (see **Reviewer Figure 1.24 below**).



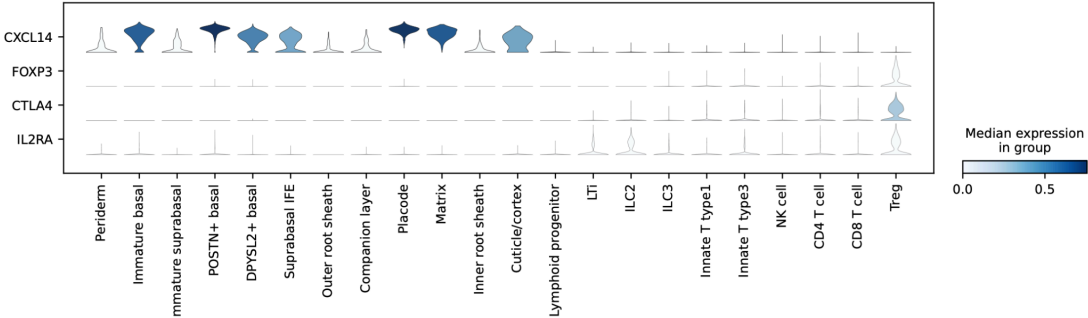
Reviewer Fig. 1.24: Bar plot showing average proportions of epidermal cell states and dermal stroma cell states across time in culture in skin organoid and across developmental time in prenatal skin. Bar colours represent cell states.

Moreover, we conducted data integration, gene expression and logistic regression analyses to evaluate how well the skin organoid recapitulates human skin, at transcriptional level, beyond comparison of cellular proportions (**Fig. 1 f**, **Extended Data Fig. 2 a-d**, **Extended data Fig. 5 c, d, f**). Our findings indicate differences in the tempo of differentiation between prenatal skin and

		skin organoid despite the overall conservation of cell states and similar expression profiles of disease-causing genes. Additionally, we aligned transcription factor (TF) regulation between prenatal skin and skin organoid during hair fibroblast differentiation using genes2genes (please see reviewer point 4.11). This analysis shows comparative frequency distribution of hair fibroblasts along matched pseudotime and close matching of TF dynamics between prenatal skin and skin organoid (Fig. 2k in point 4.11). Together, these analyses rigorously benchmark human skin organoid against prenatal skin.
1.25	The Figure legend 2 has multiple (c) labeling.	Thank you for noticing this; the legend for Figure 2 has now been corrected as shown below: (b) Schematic of stages of hair follicle formation. (c) Bar plot showing average proportions of epidermal cell states across gestational age in prenatal skin. Bar colours represent cell states. (d) Large-area (left) and magnified peri-follicular (top right) and inter-follicular (bottom right) RNAscope images of prenatal skin (representative 15 PCW sample) demonstrating ORS (<i>SLC26A7</i>), matrix (<i>SHH</i>), dermal papilla (<i>NDP</i>) with Tregs (<i>FOXP3</i>) localising around the hair follicle. Scale bars=100µm and 20µm. (e) Inferred pseudotime trajectory of prenatal skin and skin organoid epidermal cell states differentiating along the ‘ORS/CL’ and ‘IRS’ trajectories; UMAP overlaid with PAGA and coloured by cell state (top) and overlaid with the cell directionality (shown in arrows) as inferred over the cell-to-cell transition probability matrix from running CellRank and coloured by pseudotime (bottom). Shown are connectivities (dashed) and transitions (solid/arrows). Threshold for Cellrank PAGA was kept at default.
1.26	As the authors identified Treg markers related to the placode and concluded a role of Tregs in placode differentiation it	We provide violin plots for the expression of <i>CXCL14</i>, <i>FOXP3</i> as well as <i>CTLA4</i> and <i>IL2RA</i> as additional Treg markers across the keratinocyte and T-lymphoid populations (Reviewer Fig. 1.26a below). This shows high expression of <i>CXCL14</i> in placode, matrix and <i>POSTN</i>⁺ basal cells. Among the T cells, Tregs have the highest expression of <i>FOXP3</i>, <i>CTLA4</i> and <i>IL2RA</i>.

would be great if the authors could provide their expression heatmaps or violin plots (CXCL14 and FOXP3), especially as the detection of Tregs using RNAscope appears rather low (Fig. 2d).

How do the authors explain the large differences in the timing of Treg detection (23 PCW vs 15 PCW)?



Reviewer Fig. 1.26a: Stacked violin plot showing variance-scaled, mean expression (colour) of CXCL14 and Treg marker genes in prenatal skin epithelial and immune cells.

We now provide the RNAscope validation images at higher magnification to better demonstrate the presence of *FOXP3* staining around the hair follicle (**Fig. 2d** shown below). To further support a role for Treg in placode differentiation and hair follicle formation, we have now also quantified the relative abundance of *FOXP3*⁺ cells in the peri-follicular and inter-follicular areas of skin (please see point 3.7)

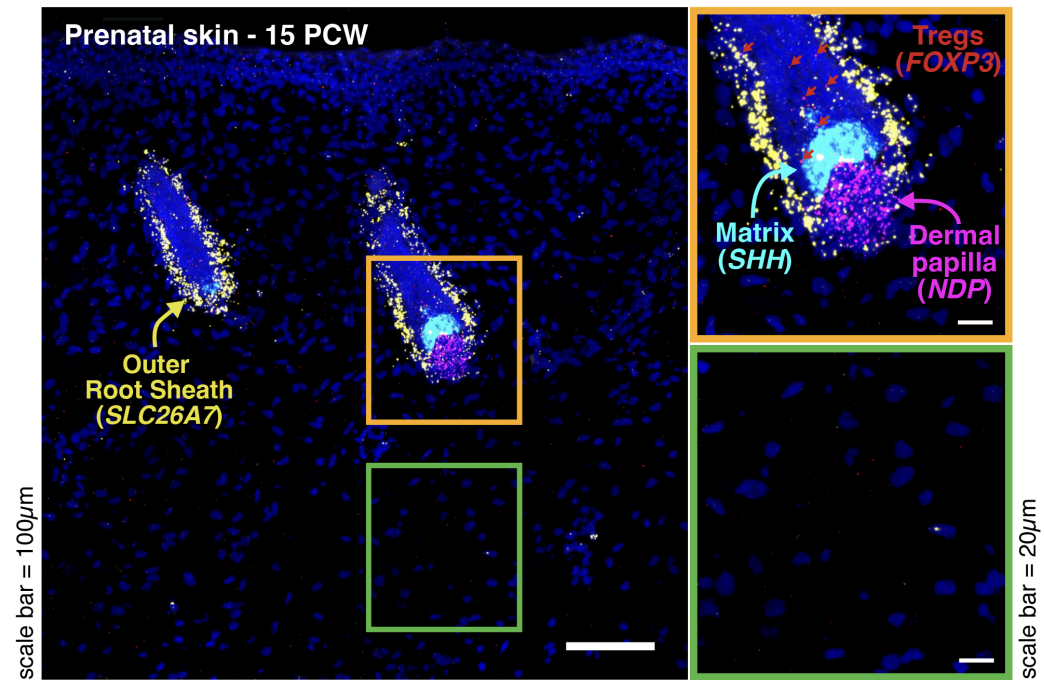


Fig 2d: Large-area (left) and magnified peri-follicular (top right) and inter-follicular (bottom right) RNAscope images of prenatal skin (representative 15 PCW sample) demonstrating ORS (SLC26A7), matrix (SHH), dermal papilla (NDP) with Tregs (FOXP3) localising around the hair follicle. Scale bars=100µm and 20µm.

The difference in the gestational stage for Treg presence between our study and that of Dhariwala et al. (PMID: 33294857) is due to the gestational stages profiled. Dhariwala et al. focused their analysis on prenatal skin samples from second trimester (17-23 weeks) and assessed the presence of Tregs using immunohistochemistry in developed hair follicles at 23 gestational weeks. Tregs were also detected in the skin by flow cytometry from 17-20 gestational weeks in their study, and increased in percentage with gestational stage. Of note, Dhariwala et al., used gestational weeks rather than post-conception weeks, which we have as the recommended term for human

developmental studies (gestational weeks = post-conception weeks + 2 weeks). To improve clarity of our comparison we have cited Dhariwala et al. in the results section and used the term post-conception weeks, and as such their report would suggest the presence of Tregs at 21 PCW. We have amended the text accordingly in the results section and shown here:

Lines 246-247: *Tregs have been reported to localise around the hair follicle in late second trimester (~21 PCW) and in postnatal skin.*

1.27

Fig. 2h and others. Highlighting in the CellPhoneDB heatmaps the ligand-receptor pairs that are discussed in the manuscript would be beneficial.

Relevant ligand-receptor pairs are now highlighted in figure panels for **Fig. 2h, Extended data Fig. 4g** and shown below:

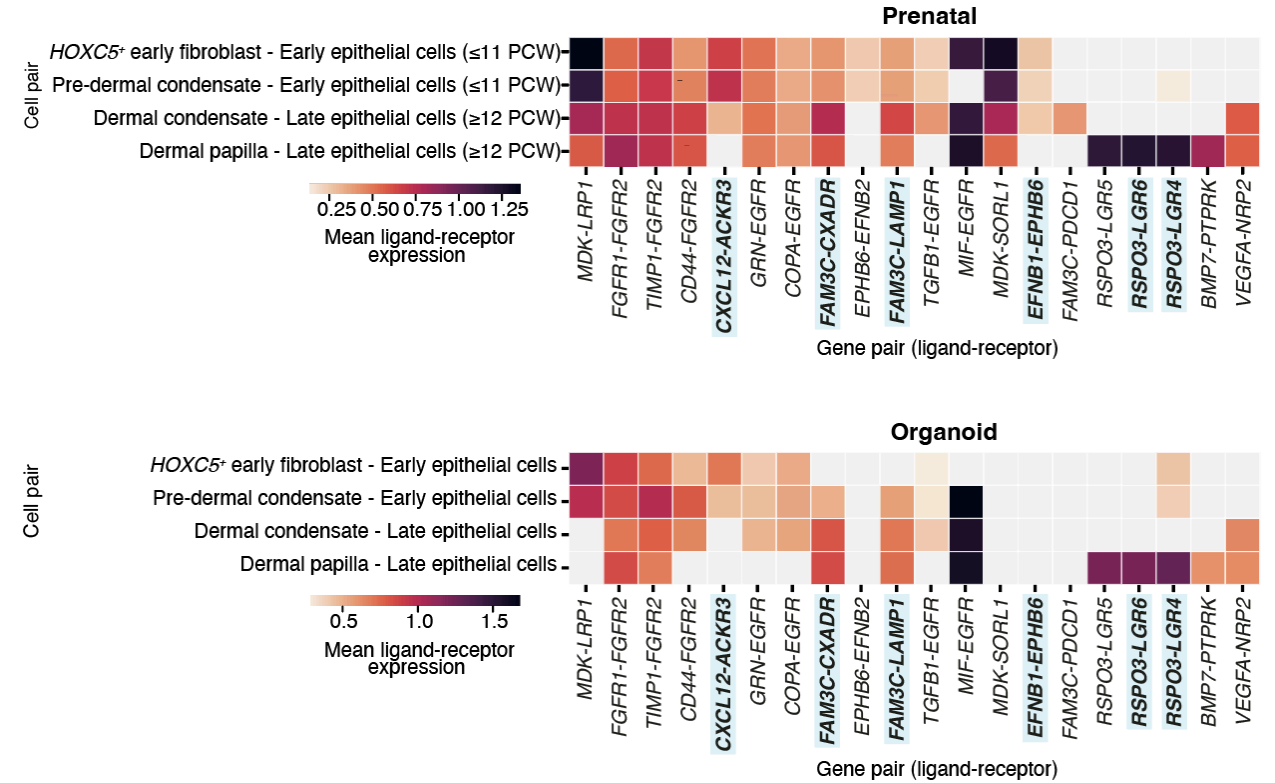
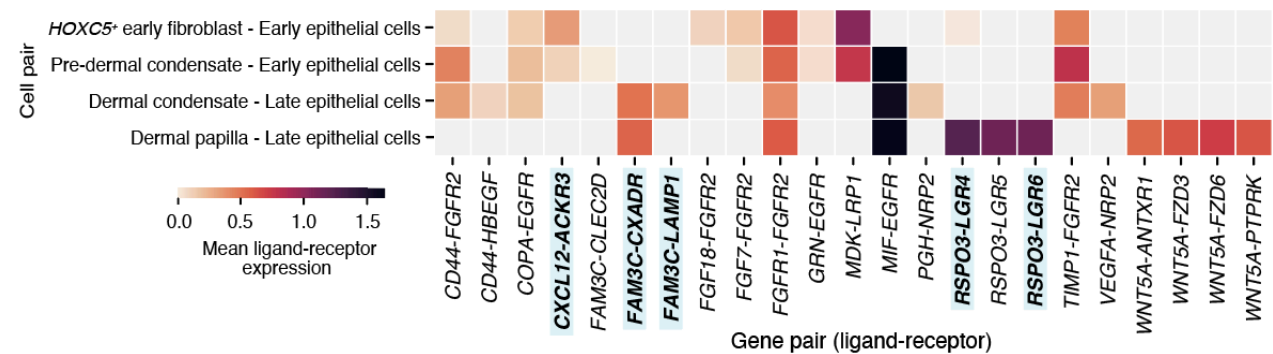


Fig. 2h: Heatmap showing significant (adjusted p -value <0.05) CellphoneDB-predicted interactions between prenatal skin hair follicle mesenchymal cells and epithelial cells (early: Immature basal; late: DPYSL2⁺ basal, POSTN⁺ basal, Placode, Matrix, ORS, CL, IRS, Cuticle/cortex). Top 10 interactions per cell pair in prenatal skin are shown (top), with the same interactions plotted from the skin organoid CellphoneDB results (bottom). Colour scale represents the mean expression values of each ligand-receptor pair in corresponding cell pairs. Ligand-receptor pairs of interest are highlighted.



Extended Data Fig. 4g: Heatmap showing significant (adjusted p -value <0.05) predicted interactions between hair mesenchymal cells and epithelial cells (early: Immature basal; late: DPYSL2⁺ basal, POSTN⁺ basal, Placode/matrix, ORS, CL, IRS, Cuticle/cortex) in skin organoid. Top 10 interactions per cell pair are shown. Colour scale represents the mean expression values of each ligand-receptor pair in corresponding cell pairs. Ligand-receptor pairs of interest are highlighted.

1.28

Why do POSTN expression levels show a strikingly different result in the Milo plot (Fig 3c, consistent up) compared to the pseudotime heatmap (Fig.3b, down up down)?

Genes labelled on the heatmap (**Fig. 3b, shown below**) consist of a selection of genes from all those plotted on the heatmap. The labelled genes therefore do not necessarily sit right next to its expression profile but rather is coloured by the population in which it is differentially expressed, in this case *WNT2*⁺ fibroblast. We have amended all heatmaps showing DEGs from trajectory analysis to reflect this better (eg. Fig. 3b, Extended Data Fig. 5a, b and Extended Data Fig. 9b, c). In the heatmap in question, Fig. 3b we show similar progressive increase in *POSTN* expression by pseudotime in *WNT2*⁺ fibroblast, shown below:

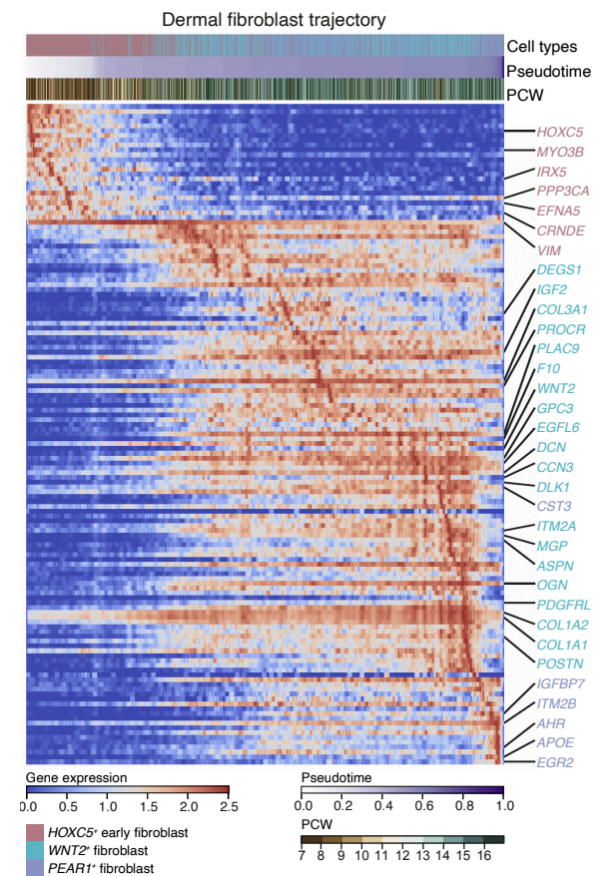


Fig. 3b: Heat map showing differentially expressed genes across pseudotime along the 'Dermal fibroblast trajectory'.

1.29	Why is the DP not represented in any the microenvironments in Fig 1d?	The human prenatal skin samples used for spatial profiling were from 6, 8 and 10 PCW as shown in Fig. 1a. Cell2location was therefore performed using only cell types present before 10 PCW (celltype with >20 cells before 10 PCW). Dermal papilla (Dp) cells were present from 12 PCW with only 10 cells before 10 PCW and were therefore not included in the Cell2location deconvolution analysis.
1.30	A few suggested corrections for nomenclature and used terms: Line 74. It should be “epithelial hair sheath”. The hair is not “epidermis”. Lines 137/138. It should be “suprabasal epidermal and hair follicle epithelial cells...” Line 146. Better call it “maturation” and not “evolution”?	Thank you for the suggestions, these changes have now been incorporated into the manuscript and shown below: Lines 82-83: <i>Subsequently, the keratinised hair shaft forms within the centre of the hair follicle, which is surrounded by an epithelial outer root sheath and a dermal sheath.</i> Lines 146-148: <i>Amongst ectoderm-derived cells, neural cells and periderm were enriched in early gestation whilst suprabasal epidermal and hair follicle epithelial cells were mainly observed in later gestation.</i> Lines 154-156: <i>Some subsets of macrophages, innate lymphoid cells (ILCs) and fibroblasts exhibited distinct gene expression profiles between early and late gestation, suggesting functional maturation during development or dual waves of production.</i>
	Reviewer 2 comments	Response to reviewer
2.1	Gopee et al. present a study on prenatal human skin, focusing on macrophages and their potential role in angiogenesis. The team has created a comprehensive atlas of prenatal human skin using various data sources,	We thank the reviewer for their enthusiasm and positive comments, particularly emphasising our study as thoroughly done and as a fantastic resource for many fields. We have undertaken additional experiments including validation of major findings in situ at protein level to strengthen and expand the organoid findings as suggested by the reviewer.

	including single-cell and spatial transcriptomics. Their findings suggest that interactions between immune and non-immune cells are crucial for proper skin development. To validate the findings, a skin organoid model was compared to prenatal and adult skin. Additionally, the research employs the human prenatal skin cell atlas to gain insights into the pathogenesis of genetic hair and skin disorders. The data from the skin atlas indicated that macrophages and the growth factors they produce play a significant role in prenatal endothelial cell development. As proof of principle, a co-culture model and the organoid model were taken to show that macrophages indeed enhance angiogenesis. In summary, the study strengthens the long-standing fact (mainly observed in mice) that particularly macrophages have additional roles in tissue development beyond their	
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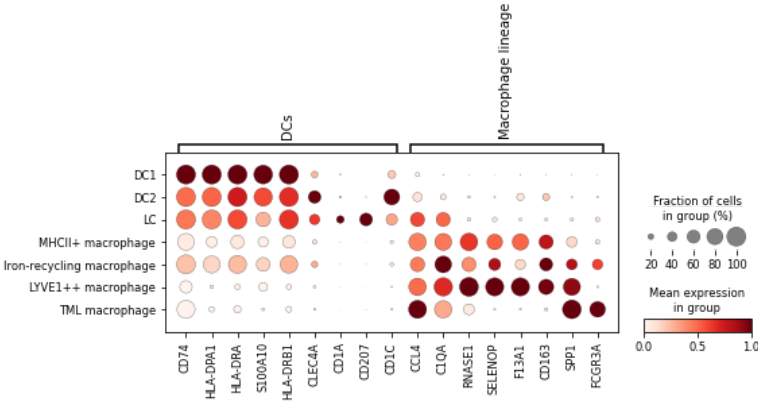
	traditional immune functions. The study is thoroughly done and will be a fantastic resource for many fields (e.g. developmental biology, macrophage biology). The bioinformatics analysis is easy to follow, despite its complexity. A major strength is the observation and validation of macrophage function in human tissue and a human organoid model. However, validation of the major findings on protein level and in situ is required to strengthen the statements that completely rely on transcriptome data so far. Additionally, the organoid model dataset should be expanded to strengthen the manuscript, which will certainly revolutionize the view of many scientists working on skin development and related pathophysiologies.	
2.2	The authors group Langerhans cells into DCs. However, it is well-accepted that these cells are macrophages.	Although Langerhans cells share similar origins with macrophages in early embryogenesis and are capable of self-renewal, they perform many of the functions associated with dendritic cells (DCs), including the canonical migratory function from skin into draining lymph nodes via lymphatics and the ability to activate naive T cells. Human skin LC precursors in fact acquire the canonical

It may well be that the MHCII+ macrophage fraction belongs together with LCs, especially considering that the authors used 1 single gene (MMP19) to designate this cell population in their Excel sheet (why is the dot plot for macrophages included so late during the study? One wonders about the specific genes for all the populations already in Figure 1). Further, only 2 genes are shown in the dotplot in Extended Data Fig. 1c to indicate the expression of specific genes in all cell types. The study completely relies on a proper cell population designation. Thus, the genes used for the 'manual designation' of cell types should be shown for all subpopulations or a better overview as Excel table should be provided.

skin cDC2 marker CD1c prior to CD1a and CD207 in prenatal skin (PMID: 19139172, PMID: 28720426, PMID: 29379502)

MHCII⁺ macrophages cluster distinctly from LCs in our data and express typical macrophage markers such as *F13A1*, *CD163* and *SPP1*. These macrophages lack the defining LC markers *CD207* and *CD1A* as well as DC markers such as *CD1C* (**Reviewer Fig.2.2a below**), indicating that they are macrophages rather than LCs. Additionally, while they do express several HLA-genes, their expression is much lower than that of actual DCs and in keeping with previous reports comparing dermal macrophages and LCs/DCs in adult human skin (PMID: 19171766).

Our cell type assignment is based on unsupervised clustering which reveals LCs, macrophages and DCs as distinct clusters. Based on the functional properties of LCs that are closer to DCs than macrophages and the expression of markers shared with DCs, we have elected to group LCs with DCs.



Reviewer Fig.2.2a: Dot plot showing variance-scaled, mean expression (dot colour) and percent of expressing cells (dot size) of DC and macrophage lineage marker genes in prenatal macrophages and LC.

For the macrophage subpopulations, we have now included more genes (6 per subpopulation) in **Extended Data Fig. 6e** and **Reviewer Fig. 2.2b** shown below. We previously reported these

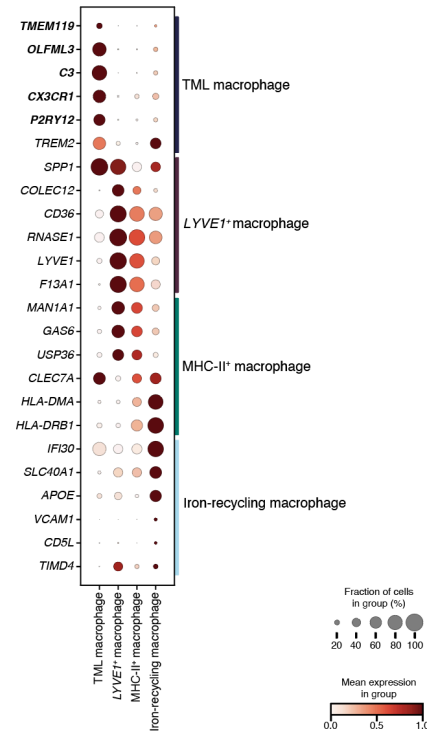
macrophage populations in a multi-organ developmental dataset (PMID: 35549310). This publication included some of the same prenatal skin samples as the ones we have used in this manuscript and reported the following marker genes for macrophages that are found consistently in all of the developmental organs studied:

MHCII^{hi} expressing: *CLEC7A*, *HLA-DPA1*, *HLA-DRA*

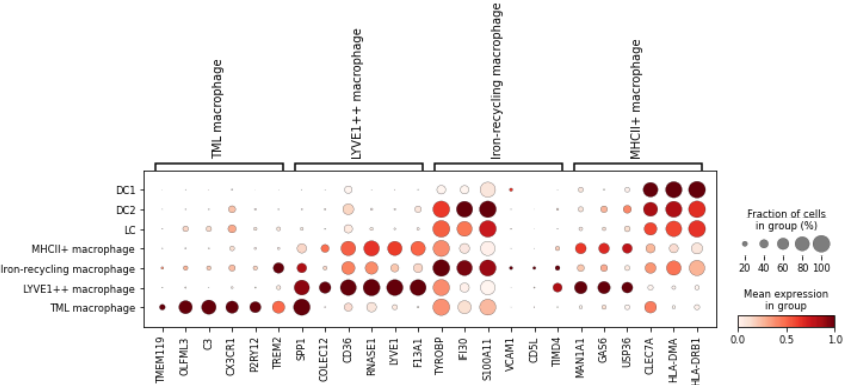
Iron-recycling macrophages: *CD5L*, *APOE*, *VCAM*, *TIMD4*, *SLC40A1*

LYVE1^{hi} macrophages: *LYVE1*, *SPP1*, *F13A1*

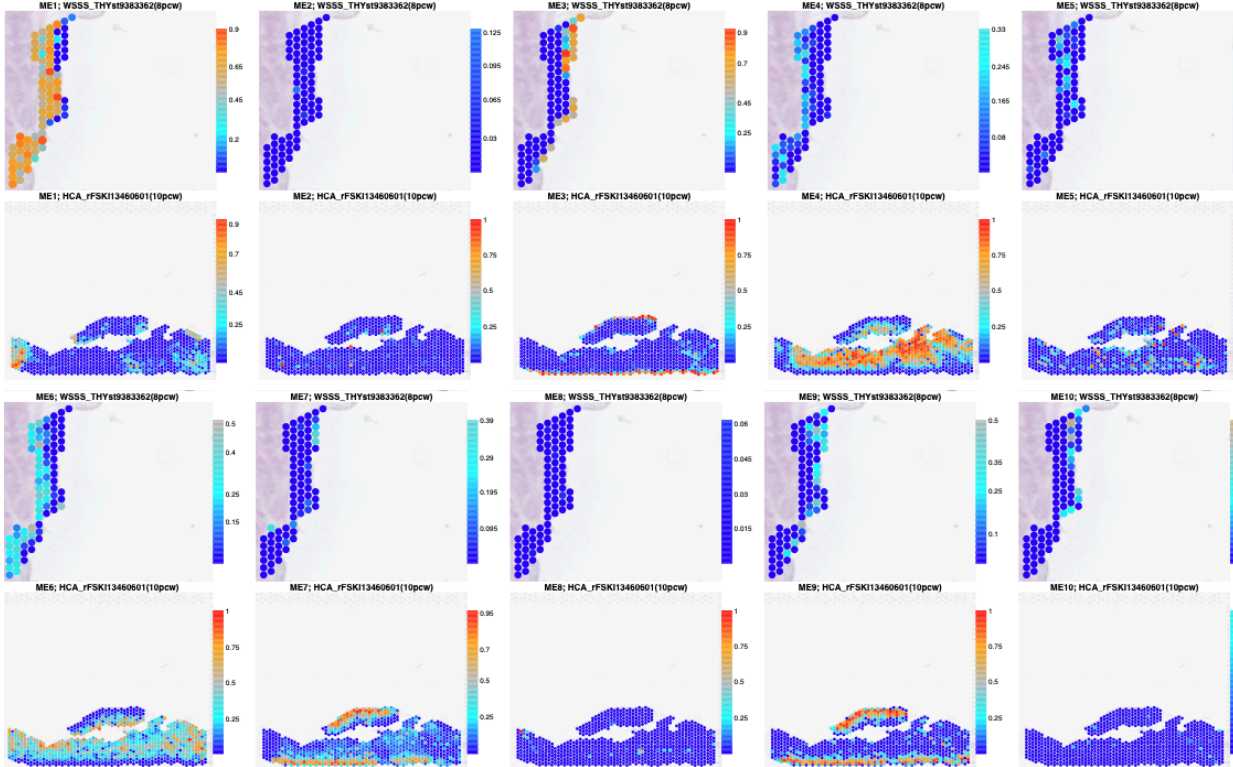
TREM2⁺ macrophages: *TREM2*, *P2RY12*



Extended Data Fig.6e: Dot plot showing variance-scaled, mean expression (dot colour) and percent of expressing cells (dot size) of marker genes used to annotate macrophage subsets in prenatal skin.

		 Reviewer Fig. 2.2b: Dot plot showing variance-scaled, mean expression (dot colour) and percent of expressing cells (dot size) of marker genes used to annotate macrophage subsets in macrophage, LCs and DC sub-populations. The list of differentially expressed genes used for manual annotations of each population is referred to early in the manuscript in Supplementary Table 3. We have amended this list to a table format to provide a better overview as suggested by the reviewer. Additionally, we provide all data interactively in the webatlas (https://developmental.cellatlas.io/fetal-skin, password: fs2023), to enable gene and cell type query online beyond the manuscript print format.
2.3	In Figure 1d/e the authors use spatial transcriptomics data published in a preprint to show neighbourhoods. The authors should show actual data to show the reader where (anatomically) in the tissue they see these neighbourhoods. This is especially true for macrophage neighbourhoods	We thank the reviewer for this comment. We used both published spatial transcriptomic data from embryonic limb skin as well as Visium data that we newly generated for our study from human embryonic facial and abdominal skin to assess neighbourhoods of cells (microenvironments). To show the anatomical distribution of these microenvironments, we have now visualised them on the histological images, with the same two representative tissue sections shown for each microenvironment (Reviewer Fig. 2.3). We also provide these images in higher resolution together with microenvironment spatial distribution across all Visium sections on our web portal (https://developmental.cellatlas.io/fetal-skin, password fs2023) for further exploration.

that become important later in the study.



Reviewer Fig. 2.3: Spatial distribution of individual microenvironments in prenatal skin shown on two representative samples. Colour scale represents the distribution density of each microenvironment with the same scale used across all samples.

2.4

ACKR3 is an atypical receptor that does not signal the same way as CXCR4, the receptor that has been implicated in skin development. If the authors would want to keep the claim

To validate in situ the predicted interactions between pre-Dc and epithelial cells, we initially attempted to perform immunofluorescence antibody labelling with anti-ACKR3 and anti-CXCL12 during the revision. However, the antibodies did not perform well. We therefore undertook RNAscope validation to increase the sensitivity of detecting specific target genes of interest and allow clear demonstration of ligand-receptor interactions. Our results demonstrate the expression of *ACKR3* in basal epidermal cells (*SERPINB7*⁺) adjacent to pre-Dc cells (*PDGFD*⁺) expressing

that the interaction of CXCL12 with ACKR3 mediates migration of pre-Dc (lines 295-299), they would need to prove this experimentally. In general, pure gene expression without validation on protein level and/or experimental data does not provide the basis for any claims. The authors should be very cautious in phrasing any ‘conclusions’ when only transcriptome data is the basis for their results.

CXCL12, as well as co-expression of *ACKR3* and *CXCL12* (white arrows in bottom right panel of **Fig. 2i**) at the junction between the epidermis and dermis, supporting these predicted interactions in early prenatal skin (**Fig. 2i shown below**). We however recognise that protein-level validation and functional assays are required to conclusively establish the roles of these interactions in skin development. We have amended our manuscript to acknowledge this and relevant sections are copied below.

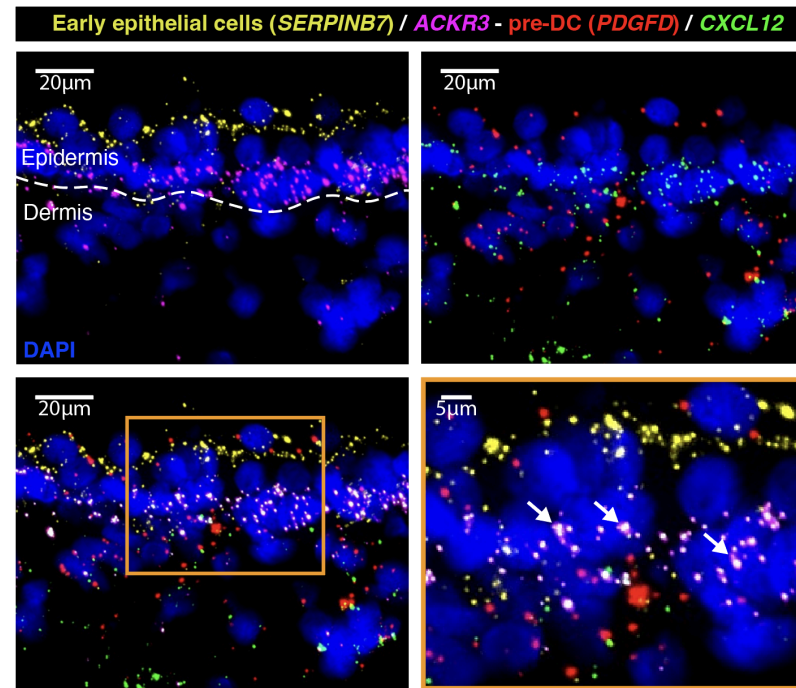


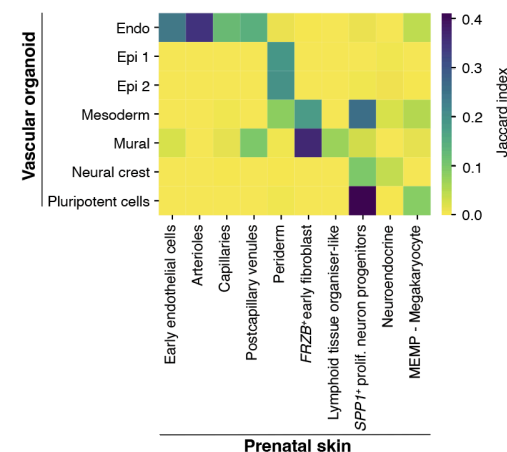
Fig. 2i: RNAscope images (representative 10 PCW prenatal skin sample) showing expression of *ACKR3* in early epithelial cells (*SERPINB7*⁺) adjacent to pre-Dc cells (*PDGFD*⁺) expressing *CXCL12* (top left and right panels, scale bars = 20µm). Bottom images demonstrate co-expression of *ACKR3* and *CXCL12* (white arrows on magnified right panel) at the junction between the epidermis and dermis (scale bar = 5µm).

		Lines 308-312: <i>We validated the co-localisation of CXCL12 from pre-Dc cells (PDGFD⁺) and ACKR3 in early epithelial cells (SERPINB7⁺) by RNAscope analysis (Fig. 2g). This co-localisation and close proximity of pre-Dc to early epithelial cells suggests that CXCL12 interacts with ACKR3 on epithelial cells to mediate migration of pre-Dc to form the Dc.</i> Lines 317-318: <i>Additional experiments are required to conclusively establish the roles of these interactions in prenatal hair formation.</i>
2.5	The RNAScope data is not of such a quality that would allow a co-localization study of single macrophage subpopulations with endothelial cells. The authors have identified 4 different macrophage populations and before claiming that all of them are in close proximity with endothelial cells, they could do (at least) a 4 colour IF staining where one can appreciate the cell borders and the actual interaction with endothelial cells. Multiplex proteomics (e.g. CODEX) are nowadays broadly available and should be used to validate the most important findings claimed in this study.	As suggested by the reviewer, we have performed new imaging analysis (whole-mount and tissue cryosections of prenatal skin) to demonstrate the interactions between macrophages and endothelial cells (Fig. 4a center and right panels and Supplementary video S1). Our scRNA-seq data suggests that the majority (up to 70%) of macrophages in prenatal skin are <i>LYVE1⁺</i>. We therefore used a panel of antibodies against LYVE1, CD45 and CD31 to label <i>LYVE1⁺</i> macrophages and endothelial cells in prenatal skin cryosections. In addition, we used the antibody combination of anti-LYVE1 and anti-CD31 in whole-mount imaging of prenatal skin. We see multiple interactions and close proximity of macrophages using both approaches on 10 PCW and 15 PCW prenatal skin, which we have now included in our revised manuscript Fig. 4a shown below and Supplementary video S1.

		Prenatal skin - 8 PCW TML macrophages Endothellum DAPI - CD68 - P2RY12 - CDH5 Scale bars = 100µm Prenatal skin - 10 PCW LYVE1⁺ macrophage Endothellal cell DAPI - CD45 - LYVE1 - CD31 Scale bars = 10µm Prenatal skin - 15 PCW Endothellum Macrophages Putative contact point LYVE1 - CD31 Scale bar = 80µm Scale bar = 5µm Fig. 4a: (Left panel) RNAscope images of prenatal skin cryosections (representative 8 PCW sample) demonstrating close proximity of endothelium (CDH5) with TML macrophage (P2RY12) and macrophages (CD68). Scale bars=100µm. (Center panel) Immunofluorescence images of prenatal skin cryosections (representative 10 PCW sample) demonstrating close proximity of LYVE1⁺ macrophages (LYVE1⁺ CD45⁺) with endothelial cells (CD31⁺). Scale bars = 10µm. (Right panel) 3D rendering of co-localised areas (magenta) of endothelial cells (CD31⁺) and LYVE1⁺ macrophages (LYVE1⁺) from whole-mount immunostaining of a 15 PCW prenatal skin sample. Scale bar = 80µm (top), 5µm (bottom).
2.6	Lines 486-488: Just because the lack of macrophages in an organoid model does not result in proper angiogenesis, does not mean that macrophages are actually responsible for this	We performed both cryosection and whole-mount imaging analysis during revision to provide more evidence of macrophage-endothelial cell interactions and have added these findings in the revised manuscript. We agree that the vascularisation of tissue organoids to achieve vascular complexity remains a challenging area (PMID: 36716724; PMID: 36829618), especially considering that even vascular

biological process. Many organoid models where macrophages are being introduced still lack endothelial cells. Nevertheless, the hypothesis is obvious and the data at the end of the manuscript support this point. However, the authors should use cryosections in addition to the whole mounts shown to assess macrophage-endothelial cell interaction and maybe use also Iba1 as macrophage marker since the CD206 antibody seems unspecific or rather staining endothelial cells that are negative for CD31.

organoids do not recapitulate the heterogeneity observed in vivo as shown in our analysis (**Extended Data Fig. 9d**, shown below). The addition of macrophages to organoid models does not automatically result in endothelial cell formation (PMID: 38150367), especially if the differentiation protocol does not support vasculature generation like in the skin organoids. However, this method is recognised as a possible avenue for improved vascularisation (PMID: 36716724; PMID: 36829618). A few existing models in neural and tumour organoids have shown improved angiogenesis and vascular network organisation in the presence of macrophages, either by direct addition of macrophages or incorporation of mesodermal progenitor cells which internally generate macrophages (PMID: 16404363; PMID: 31666641). These models further demonstrated the close association between macrophages and endothelial cells in the neural organoids, supporting a role for macrophages in vascular network formation (PMID: 31666641).



Extended Data Fig. 9d: Heatmap showing the correspondence (measured by Jaccard index) between prenatal skin (x-axis) and blood vessel organoid cell states (y-axis) for a logistic regression model trained on prenatal skin data. The top 10 predicted prenatal cell states were retained for visualisation.

In terms of immunofluorescent markers, we previously selected CD206 to stain macrophages based on reports that showed that CD206 (MRC1) was a valid marker to isolate pro-angiogenic macrophages and their progenitors in the skin (PMID: 37703875). We have now carried out

immunofluorescence on the cryosections obtained from our skin organoids co-cultured with macrophages as suggested, using CD45 as a broad macrophage marker to show the close interaction of endothelial cells (CD31) with co-cultured macrophages (CD45) (**Fig. 4j**, shown below). Additionally, we have further characterised our macrophages in the skin organoid demonstrating that a small subset of macrophages are CD206⁺ or HLA-DR⁺ (please refer to **Reviewer Fig. 1.7**).

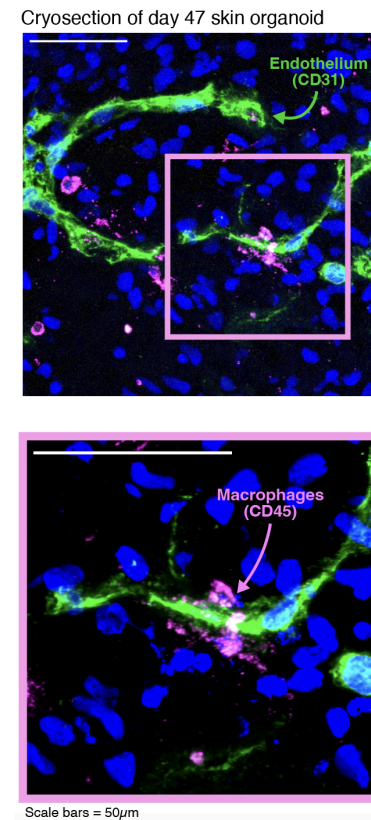
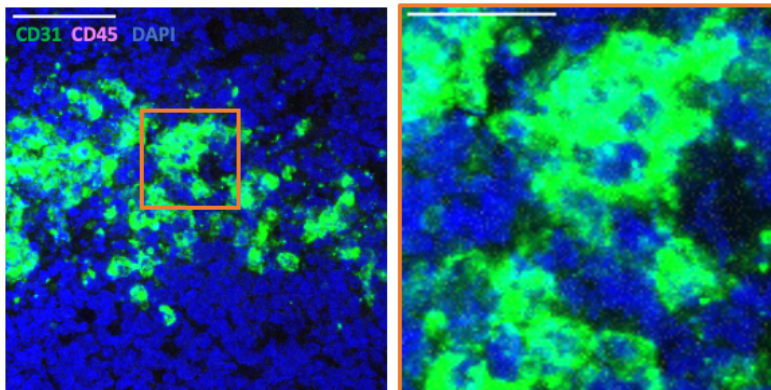
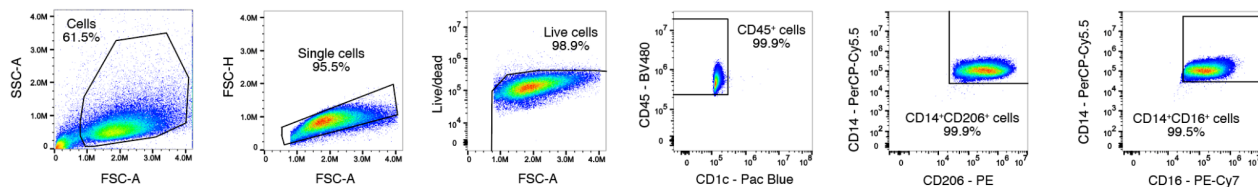


Fig. 4j: Z-projection immunofluorescence of cryosections of day 47 skin organoid co-cultured with macrophages (day 35 of co-culture) demonstrating close interactions of macrophages (CD45, in magenta) and endothelium (CD31, green). Scale bar=50μm.

		In keeping with our observation in the 3D whole-mount immunofluorescence analysis, skin organoids cultured without macrophages showed a lack of vessel structure with individual endothelial cells failing to assemble in an organised network (Reviewer Fig. 2.6, shown below). Skin organoids co-cultured with macrophages displayed a refined and organised network of endothelial cells (Fig. 4j, shown above and please see reviewer point 1.6).  Reviewer Fig. 2.6: Large area (left, scale bar=50µm) and magnified (right, scale bar=20µm) immunofluorescence of cryosections of day 47 skin organoid without macrophages demonstrating endothelium (CD31, green) and lack of macrophages (CD45, in magenta).
2.7	Further, the addition of macrophages to organoids is very variable and n = 3 with only 1 iPCS line is not the standard procedure in the organoid field. For some experiments, the figure legends say even only n=1 or n=2, which is not acceptable for in vitro experiments. Please include the same set of	We acknowledge the utility of using several iPS cell lines to demonstrate the robustness of organoid differentiation protocols. In this instance however, it was not possible to obtain a new iPS cell line, check the skin organoid differentiation protocol (which needs at least 3 months differentiation to check efficiency) and carry out our specific organoid experiments within the revision timeframe. We wish to highlight that the lab of Karl Koehler published the skin organoid protocol validated on several cell lines (Lee et al, 2022 Nature Protocols) and several other research groups have since published original research articles utilising the skin organoid model with different iPS cell lines (PMID: 36193049, PMID: 38044307, PMID: 37828248). We have repeated the 3D skin organoid co-culture in three batches.

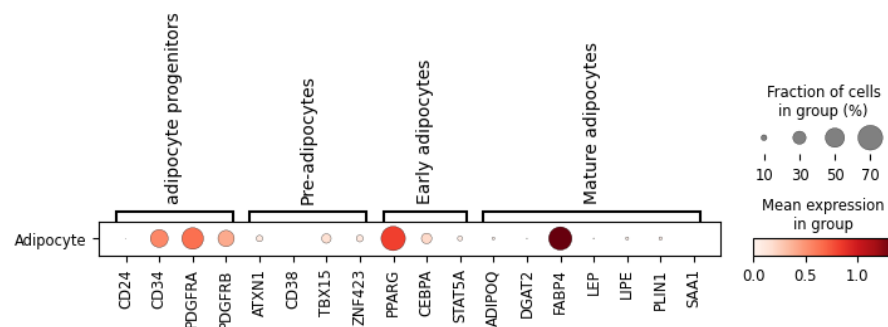
	experiments with at least one more iPSC line and add more numbers showing in how many % of the cases you end up with more vasculature.	
2.8	Finally, perform flow cytometry on the organoids with and without macrophages using the same flow cytometry markers as for macrophages before adding them to the organoids to obtain the ratio of macrophages within each organoid. Only this will show the reproducibility and consistency of the results.	We undertook the suggested flow cytometry experiments to characterise the iPSC-derived macrophages before co-culture and dissociated the skin organoid cultured with and without macrophages. iPS-derived macrophages on the day of co-culture expressed CD45, CD14, CD16, CD206 and were negative for CD1c, in keeping with a macrophage identity as shown in Extended Data Fig. 10f, also shown below:  Extended Data Fig. 10f: <i>Gating strategy used on iPSC-derived macrophages before co-culture with skin organoids (n=3 batches) to isolate single live cells and analyse expression of macrophage markers (CD45, CD14, CD16 and CD206) and exclude dendritic cells (CD1c).</i> However, we were unable to successfully retrieve macrophages by tissue dissociation for flow cytometry experiments after they were co-cultured with skin organoids. Nevertheless, we were able to visualise macrophages co-cultured in skin organoids using whole-mount and cryosection immunofluorescence microscopy analysis using the following markers: CD45, CD206 and HLA-DR/DP/DQ. We found that all macrophages cultured in skin organoids are CD45⁺, and a small subset are also CD206⁺ or HLA-DR⁺ (please see reviewer comment 1.7, 2.6 and Reviewer Fig. 1.7).

2.9

Adipocytes typically become too big for scRNAseq and are prone to cell death after enzymatic digestion and are simply not found in the cell pellet after centrifugation. The data should be checked for previously described pre-adipocyte populations, which may be the actual predominant cell type in the data set.

We thank the reviewer for their comment and suggestion. The adipocyte population was annotated based on differential expression of genes such as LPL present in adipose tissue (PMID: 35296864, PMID: 22140268). As suggested, we have checked this population for expression of marker genes from published literature (PMID: 35296864, PMID: 22140268, PMID: 24591537, PMID: 37119138) for adipocyte progenitors, pre-adipocytes, early and mature adipocytes. These cells expressed to varying degrees genes which are upregulated in adipocyte progenitors (*CD34*, *PDGFRA*, *PDGFRB*) and in committed pre-adipocytes and early adipogenesis (*PPARG*, *ATXN1*, *TBX15*, *ZNF423*, *CEBPA*). They also expressed *FABP4*, a marker of mature adipocytes, but lacked other mature adipocyte markers such as *ADIPOQ*, *PLIN1* and *SAA1* and the stem cell marker *CD24* (**Reviewer Fig. 2.9a**)

Of note, we have now added 3 additional donors to our dataset as detailed in points 1.2 and 1.13 to improve resolution and detection of rarer cell types in human prenatal skin. As a result, we identify an additional adipocytic population with higher expression of adipocyte progenitors/pre-adipocytes marker genes and lower expression of early adipogenesis and *FABP4*. We have amended our annotations and labelled these clusters as ‘Pre-adipocytes’ and ‘Early adipocytes’. The gene expression profile of pre-adipocytes and early adipocytes are shown in **Reviewer Fig.2.9b** below:

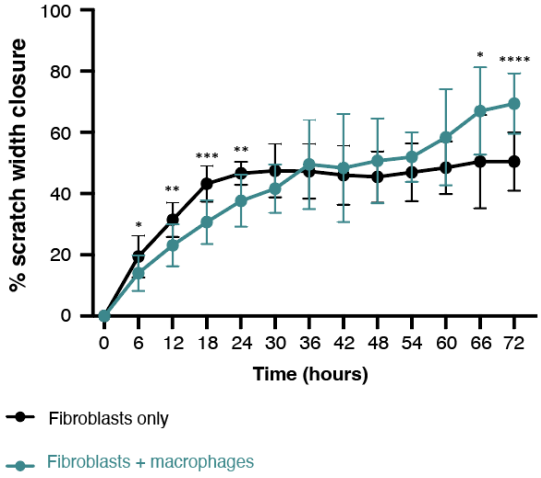


Reviewer Fig. 2.9a: Dot plot showing mean expression (dot colour) and percent of expressing cells (dot size) of genes expressed during adipocyte differentiation and maturation in our original population of adipocytes.

		Reviewer Fig. 2.9b: Dot plot showing mean expression (dot colour) and percent of expressing cells (dot size) of genes expressed during adipocyte differentiation and maturation in adipocytic sub-populations (expanded prenatal skin data).
2.10	Why are B progenitors and HSCs in the skin? This is likely a contamination with fetal liver or bone marrow, which are the only organs that harbour these precursor and progenitor cells.	Our prenatal skin samples were dissected by the trained staff at the Human Developmental Biology Resource (HDBR) based in Newcastle and London, UK. HDBR dissects requested embryonic and fetal tissues and distributes it to researchers who have requested the material in the UK and globally. Dissection by expertly trained staff ensures that there is no/minimal contamination that may affect analysis. We have previously analysed multiple prenatal organs and described the presence of haematopoietic stem cell progenitors and B cell progenitors outside of the bone marrow including non-lymphoid tissue such as fetal skin and gut. We validated the findings in situ with RNAscope analysis of fetal gut (PMID: 35549310).
	Reviewer 3 comments - major	Response to reviewer
3.1	Gopee, Huan, Admane, et al. report a novel and interesting	We thank the reviewer for their enthusiasm and positive comments specifically highlighting the rich resource of the prenatal skin data which will impact the field as well as the compelling data

investigation into the single-cell and spatial transcriptome of fetal skin development. As a product of this work, the investigators present a rich resource consisting of fetal human skin tissue spanning multiple timepoints and compelling data from a skin organoid model. This work constitutes the earliest and most complete atlas of human fetal skin at single cell resolution. The authors demonstrate important advancements in understanding the differentiation of cell types that contribute to hair follicle formation and demonstrate an unconventional role for innate immune cells in promoting angiogenesis. This resource will undoubtedly impact the field by driving hypothesis development and motivating many follow-up analyses. However, there are a few areas where the authors can meaningfully improve the	from skin organoid.
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	manuscript and increase readability.	
3.2	Many data panels are presented in extended data figures; the authors should consider moving more panels to the main figures to decrease the amount of cross-referencing that is currently required of the reader.	We have incorporated as many panels as we can to the main figures including data from the additional experiments we performed during revision while adhering to the journal guide to authors.
3.3	The authors should also carefully consider conjectures vs experimentally-derived conclusions based on their data particularly in regards to the analysis of fibroblasts and macrophages in promoting scarless healing. Additional specific suggestions are outlined below.	We thank the reviewer for the suggestion made and have taken steps to demonstrate what is experimentally confirmed versus hypothesised. Please see reviewer point 1.9 for a detailed analysis of the new experimental data (in vitro scratch assay, presented in Extended Data Fig. 7g and shown below) and computational analysis (comparison of human prenatal skin macrophages and fibroblasts with reindeer antler skin and back skin macrophages and fibroblasts) to provide more evidence in support of embryonic macrophages contributing to scarless healing.

		 Extended Data Fig. 7g: <i>Percentage scratch width closure (y-axis) quantified over time (x-axis) for fibroblasts cultured with macrophages (green) or in isolation (black).</i> <table border="1"> <caption>Estimated data for Extended Data Fig. 7g</caption> <thead> <tr> <th>Time (hours)</th> <th>Fibroblasts only (%)</th> <th>Fibroblasts + macrophages (%)</th> </tr> </thead> <tbody> <tr><td>0</td><td>0</td><td>0</td></tr> <tr><td>6</td><td>20</td><td>15</td></tr> <tr><td>12</td><td>30</td><td>25</td></tr> <tr><td>18</td><td>45</td><td>35</td></tr> <tr><td>24</td><td>48</td><td>40</td></tr> <tr><td>30</td><td>48</td><td>45</td></tr> <tr><td>36</td><td>48</td><td>48</td></tr> <tr><td>42</td><td>45</td><td>48</td></tr> <tr><td>48</td><td>45</td><td>50</td></tr> <tr><td>54</td><td>48</td><td>52</td></tr> <tr><td>60</td><td>48</td><td>58</td></tr> <tr><td>66</td><td>50</td><td>68</td></tr> <tr><td>72</td><td>50</td><td>70</td></tr> </tbody> </table>	Time (hours)	Fibroblasts only (%)	Fibroblasts + macrophages (%)	0	0	0	6	20	15	12	30	25	18	45	35	24	48	40	30	48	45	36	48	48	42	45	48	48	45	50	54	48	52	60	48	58	66	50	68	72	50	70
Time (hours)	Fibroblasts only (%)	Fibroblasts + macrophages (%)																																										
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54	48	52																																										
60	48	58																																										
66	50	68																																										
72	50	70																																										
3.4	It is not always clear which datasets are new and which are from a previous study. To infer the novelty of this dataset, it will be useful to have a table indicating the source of each dataset.	We have added a supplementary table (Supplementary Table 2) to clarify the sources of different datasets used in our study.																																										
3.5	Could the authors clarify concerns of maternal cell contamination? Only 49 cells seems surprising – Perhaps this could be rectified with more	The analysis to detect maternal cell contamination was run using Souporecell (methods described below) across all samples from different prenatal organs published in PMID: 35549310, which included the prenatal skin samples in our original dataset. This identified a total of 2468 maternal cells in fetal skin samples, 312 cells from liver samples and 935 cells from yolk sac prior to data pre-processing (~0.4% across organs overall and ~1.0% from prenatal skin only).																																										

	details on sample preparation techniques that were applied to decrease contamination and specific details on implementation of souporecell.	The barcodes of the identified maternal cells were used to manually remove maternal cells from our original dataset, of which 49 (out of 2468) were still present following data pre-processing (QC). In our additional three samples sequenced for the revision, a further 69 maternal cells were identified. In terms of sample preparation, to reduce maternal contamination, prenatal skin samples were washed in PBS following removal from transport medium. We have added the following details to our methods section: The implementation of Souporecell for detection of maternal cell contamination was performed as we previously described (PMID: 35549310, PMID: 37590359). Samples were pooled on a per-donor basis and processed with souporecell (v.2.4.0). The common GRCh38 variants file (SNPs with $\geq 2\%$ frequency from 1k genomes) from souporecell authors was used. The pipeline was run twice, with genotype clusters set to 1 and 2 to obtain models for no maternal contamination and possible maternal contamination. The better model was identified via BIC (Bayesian Information Criterion), calculated using the formula $BIC = kn \log(m) - 2l$, where k is the number of genotype clusters set for each souporecell run, n denotes the number of loci used for genotype deconvolution, m is the cell count for a given donor, and l is the log likelihood obtained after running the pipeline with each k. The cells with the minor genotype were identified as possible maternal contaminants where identified.
3.6	Annotations could use more explanation, especially when marker genes that have not previously been referred to in the literature were chosen (particularly: fibroblast subpopulations (WNT2, PEAR1) and macrophages)	Please see point 1.2. Our annotation strategy was grounded in the leiden clustering of integrated data objects. Differentially Expressed Genes (DEGs) between leiden clusters served as a foundational component for manual annotations. These DEGs were further complemented by leveraging known marker genes associated with individual populations. To further enhance transparency and facilitate reproducibility of our cell annotation process, we have taken the following steps: 1. We provide a comprehensive Supplementary Table 3 detailing the DEGs specific to each

		individual population. The detailed annotation of each subpopulation is available on GitHub (https://github.com/haniffalab/FCA_fetal_skin/tree/main/analysis/scrna-seq/1_Prenatalskin_InitialQC_processing_and_annotation). This resource serves as a valuable reference for researchers, enabling reproducible manual annotation. 2. For subpopulations that have not been previously described e.g. fibroblast clusters, we used the distinctive marker genes for the specific cell state e.g. <i>WNT2</i> for <i>WNT2</i>⁺ fibroblasts and <i>PEAR1</i> for <i>PEAR1</i>⁺ fibroblasts. The annotations were derived based on the leiden clustering of integrated data objects. Macrophages and other immune cells were annotated as previously described (PMID: 35549310). We have clarified the annotation process, reference to the Supplementary Table and availability of the codes used in our analysis in the MS in the results/methods section, shown below: Lines 141-143: <i>Further dimensionality reduction and clustering was performed on the broad cell clusters prior to fine-grained manual annotation of cell states based on differentially expressed genes (Supplementary Table 3).</i> Lines 1281-1285 (Methods): <i>Leiden clusters at the highest resolution were annotated manually using marker genes identified through literature search and their expression of distinctive differentially expressed genes (DEGs) specific to each cluster, such as WNT2 expression in WNT2⁺ fibroblasts. The full list of DEGs for each cluster is provided in Supplementary Table 3.</i> Lines 1762-1763: <i>Python/R code and notebooks for reproducing these analyses are publicly available at https://doi.org/10.5281/zenodo.8164271</i>
3.7	While Fig. 2d does show red FOXP3 ⁺ dots, no quantification is performed and there is no comparison to a control region further away from the hair	Please see point 1.26 above. We have now taken more representative images to demonstrate the expression of <i>FOXP3</i> in hair follicle (HF) regions and a control region of skin tissue away from the HF. We have also performed quantification using the QuPath image analysis software (PMID: 29203879). The coverage of <i>FOXP3</i> expression in HF regions and non-HF regions across the whole skin section (normalised to the skin area) was quantified from five different prenatal skin

follicles. Please perform a quantitative analysis showing preferred localization of FOXP3+ cells near the hair follicles or edit your statement “We identified FOXP3+ Tregs primarily within and around hair follicles” to be more inclusive of other regions of the skin.

samples. Our quantification confirms a significantly higher percentage of *FOXP3* coverage in HF regions versus all other regions of the skin (paired t-test, $p=0.0131$).

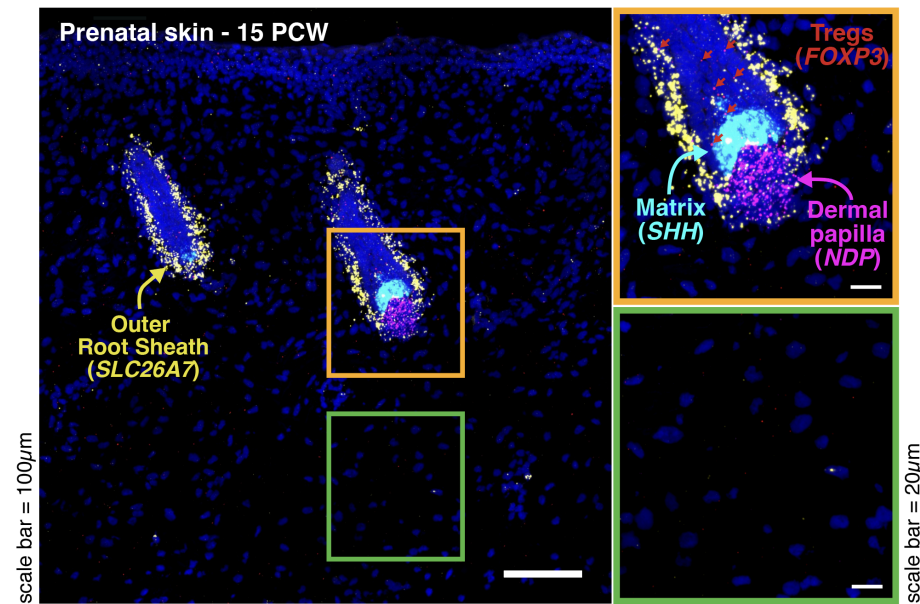
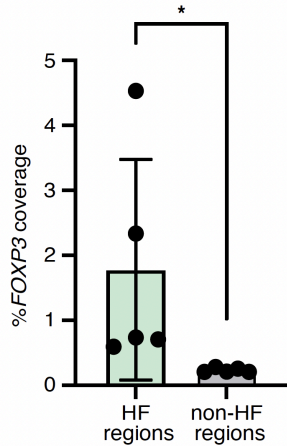
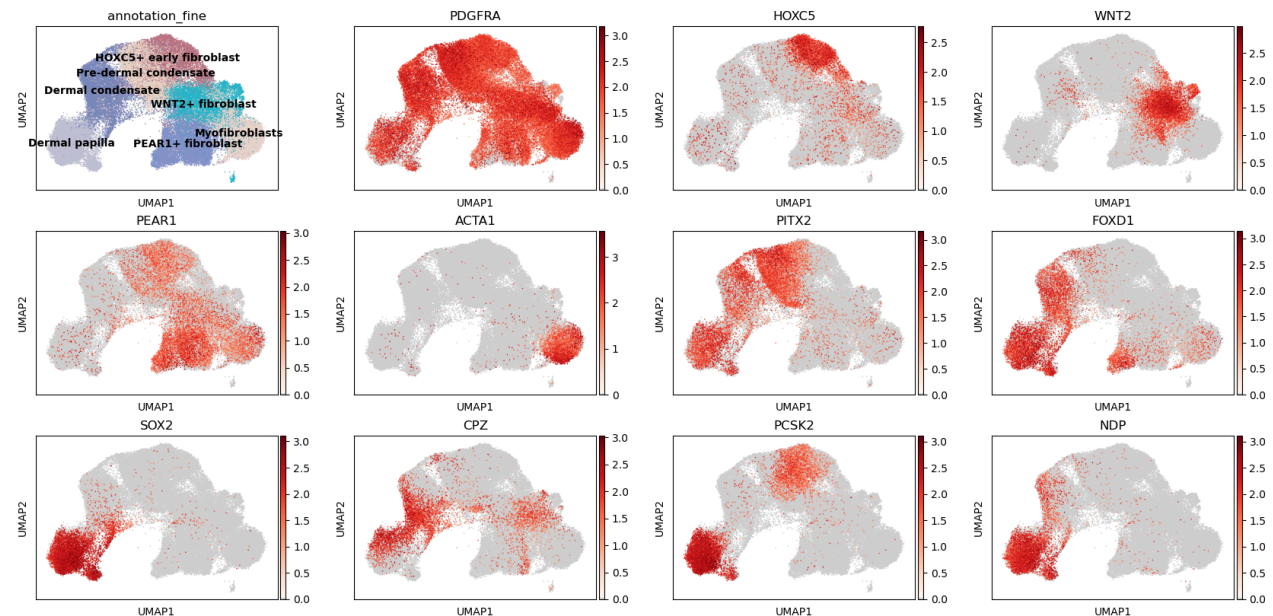


Fig. 2d: Large-area (left) and magnified peri-follicular (top right) and inter-follicular (bottom right) RNAscope images of prenatal skin (representative 15 PCW sample) demonstrating ORS (SLC26A7), matrix (SHH), dermal papilla (NDP) with Tregs (FOXP3) localising around the hair follicle.

		 Extended Data Fig. 3f: Percentage of FOXP3 coverage in hair follicle (HF) regions and non-HF regions across five prenatal skin samples. Data are mean $\pm$ SD and statistics generated with an unpaired t-test.
3.8	It would help to increase readability if the authors could clarify details about how they determined pre-dc and dc using information from prior work in murine skin. Did the authors explicitly compare pre-Dc and/or Dc between fetal skin and published mouse data? Because the authors claim this is the first time that Dc has been identified in human skin, an explicit analysis (Harmony integration, UMAP	Here we provide details on how we annotated pre-DC and DC in our scRNA-seq data and performed new analysis to align mouse and human pre-DC and DC as suggested by the reviewer. Annotation of human pre-DC and DC. Among the fibroblast populations, we identified three distinct clusters which downregulated fibroblast cluster markers (<i>WNT2</i>⁺, <i>HOXC5</i>⁺, <i>PEAR1</i>⁺) and myofibroblast marker (<i>ACTA1</i>), and upregulated Dc genes (<i>SOX2</i>, <i>FOXD1</i>) (Reviewer Fig. 3.8a below). <i>PDGFRA</i>⁺<i>FOXD1</i>⁺<i>SOX2</i>^{low} cells were annotated as pre-dermal condensate (pre-DC), followed by dermal condensate (Dc) and dermal papilla (Dp) respectively with increasing levels of <i>FOXD1</i> and <i>SOX2</i> expression as shown in the feature plots below. This is in keeping with markers used to annotate murine skin pre-DC, Dc and Dp (PMID: 30595537).

co-embedding) addressing discernable differences (if any) between human Dc and previously described murine Dc.



Reviewer Fig. 3.8a: *UMAPs of prenatal skin fibroblast sub-populations coloured by gene expression.*

We have amended the relevant section of the manuscript to add this:

Lines 265-273: *Next, we delineated the dermal cell types that are involved in crosstalk with epidermal cells during early hair follicle development. We captured the mesenchymal cells of the developing hair follicle and identified for the first time the precursors of the human Dc (**Extended Data Fig. 2b, 3b**). In mice, a transitional fibroblast state comprising $\text{PDGFRA}^+\text{FOXD1}^+\text{SOX2}^{\text{low}}$ cells termed pre-Dc aggregate to form the Dc ($\text{FOXD1}^+\text{SOX2}^+$) which abuts the epithelial hair placode during embryonic hair follicle formation⁴². Following invagination of the hair follicle, the Dc becomes encapsulated at its base as the Dp (NDP^+ , SOX2^+)^{21,42} (**Fig. 2d, Extended Data Fig. 3g**). Using orthologous marker genes, we annotated the pre-Dc, Dc and Dp in human prenatal skin⁴²⁻⁴⁴.*

2. Mouse and human pre-Dc and Dc comparison.

We used an embryonic murine skin scRNA-seq dataset generated using the 10x platform (from E12.5, E13.5 and E14.5) (PMID: 37591247). This included progenitor fibroblasts (FIB Origin) which differentiate into muscle-supporting fibroblasts (FIB Muscle), fascia fibroblasts (FIB Inter), and fibroblasts of the lower (FIB Lower) and upper (FIB Upper) dermis, with the latter subsequently giving rise to the pre-dermal condensate (FIB EarlyDC) and the dermal condensate (FIB LateDC) (**Reviewer Fig. 3.8d**). Addition of another murine skin dataset (PMID: 32685006) which captures the dermal papilla only added a small number of cells (<100 cells) and integrated poorly with the rest of the mouse data, and was therefore not included in further analyses.

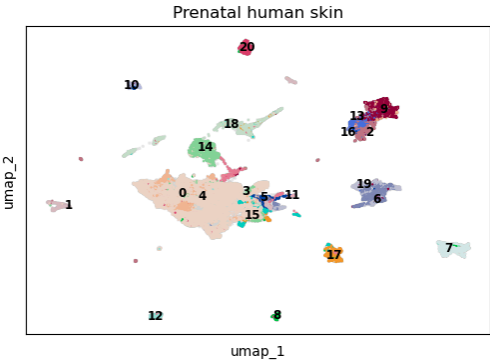
We employed a number of different approaches for comparison:

- a) a dedicated method for homologous cell type detection between species (SAMap) which integrates cross-species data symmetrically to create a co-embedding (PMID: 33944782) (**Reviewer Fig. 3.8b**)
- b) integration using a conditional variational autoencoder (scVI) (PMID: 35132262) with logistic regression analyses (**Extended Data Fig. 4k**)
- c) asymmetric data integration using the scanpy tool ingest (sc.tl.ingest) to map labels from human prenatal skin fibroblasts onto murine embryonic fibroblasts (**Reviewer Fig. 3.8d**).

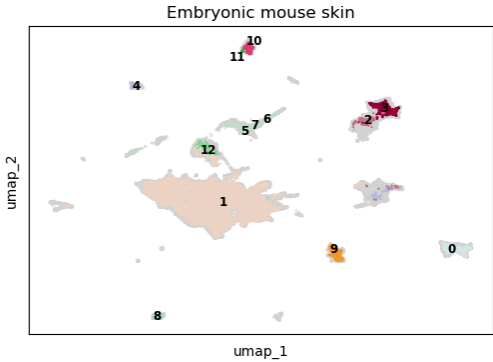
Consistently, we found that whilst overall there is a high level of correspondence for the broad lineages in human and murine skin, these distinctions are less clear when comparing sub-populations within a lineage, such as pre-Dc and Dc. This could be due to residual heterogeneity within the cell states and limitations of gene expression-based cross-species comparison methods (PMID: 37838716), particularly if there are divergent gene expression profiles between species.

- a) Using SAMap co-embedding of human and mouse skin, we found a high level of correspondence observed for cells of the same broad lineages but less clear distinctions

within fibroblast sub-populations. Results shown below **Reviewer Figure 3.8b, c.**

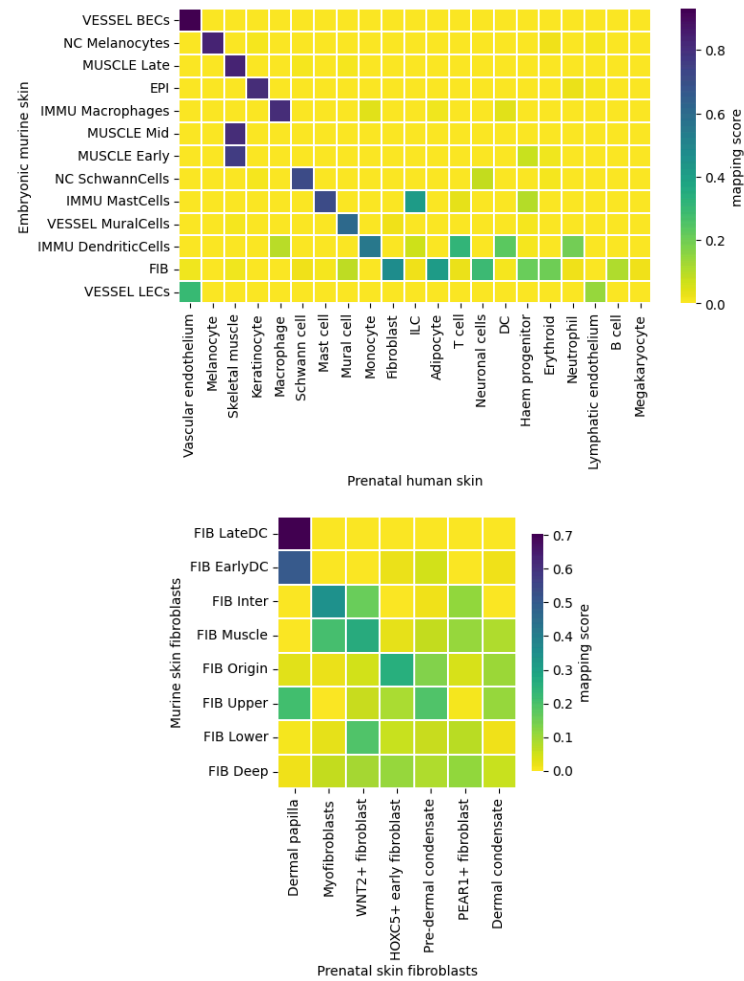


- | | | |
|-------------------|-------------------------|-------------------------|
| 0 Adipocyte | 7 Keratinocyte | 14 Mural cell |
| 1 B cell | 8 Lymphatic endothelium | 15 Neuronal cells |
| 2 DC | 9 Macrophage | 16 Neutrophil |
| 3 Erythroid | 10 Mast cell | 17 Schwann cell |
| 4 Fibroblast | 11 Megakaryocyte | 18 Skeletal muscle |
| 5 Haem progenitor | 12 Melanocyte | 19 T cell |
| 6 ILC | 13 Monocyte | 20 Vascular endothelium |



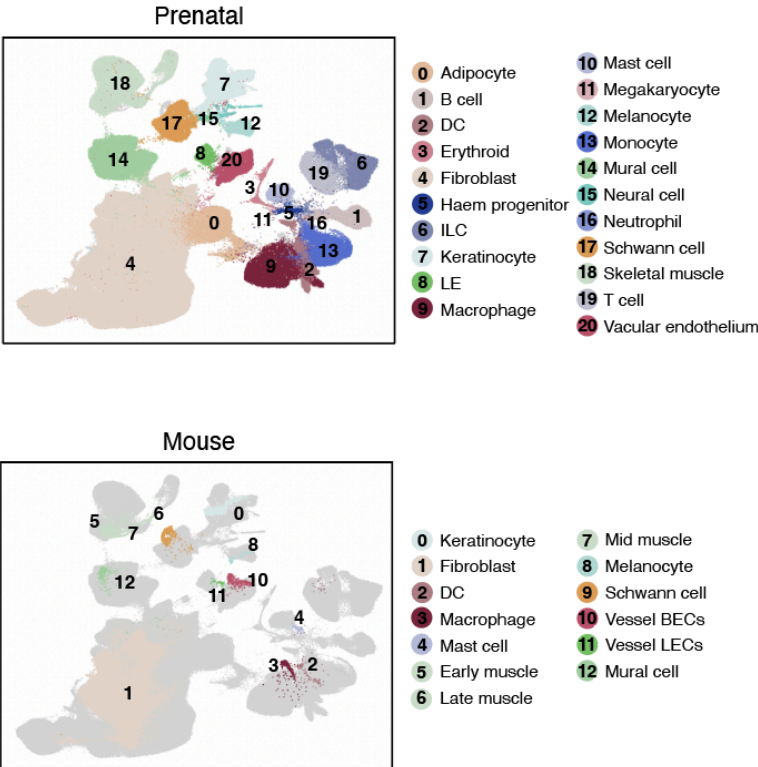
- | | |
|-----------------------|----------------------|
| 0 EPI | 7 MUSCLE Mid |
| 1 FIB | 8 NC Melanocytes |
| 2 IMMU DendriticCells | 9 NC SchwannCells |
| 3 IMMU Macrophages | 10 VESSEL BECs |
| 4 IMMU MastCells | 11 VESSEL LECs |
| 5 MUSCLE Early | 12 VESSEL MuralCells |
| 6 MUSCLE Late | |

Reviewer Figure 3.8b: *UMAP co-embedding of human (left) and murine (right) prenatal skin coloured by broad cell cluster annotations.*

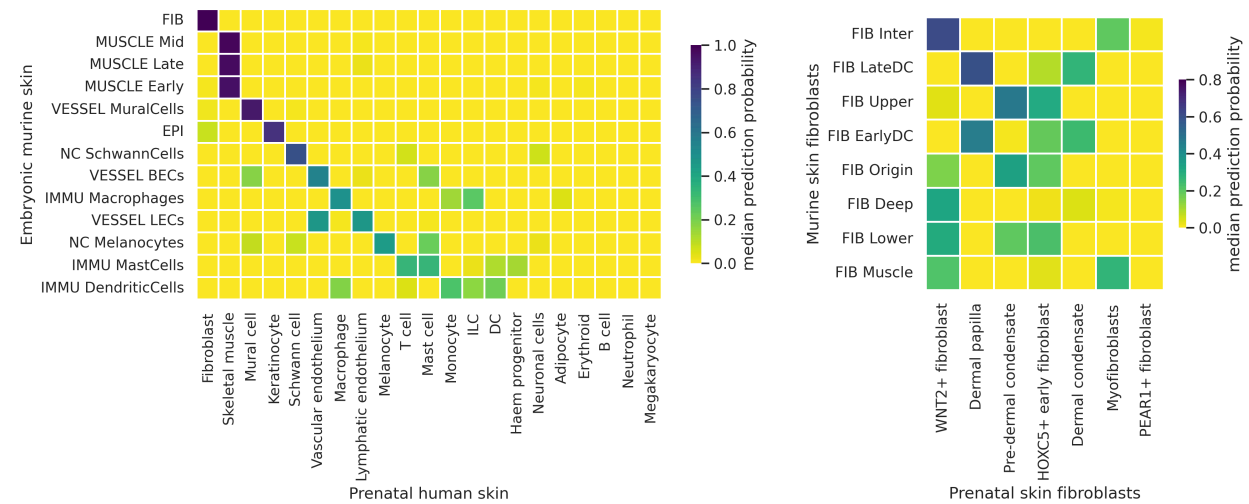


Reviewer Figure 3.8c: Heatmap showing the mapping scores (as calculated in SAMap package) between human prenatal skin (x-axis) and murine embryonic skin (y-axis) for broad cell groupings (top) and refined cell clusters (bottom, only fibroblast sub-populations shown from all cell types) .

b) scVI co-embedding of human and mouse skin and logistic regression: similar to SAMap where only broad lineages correlate across species (**results shown below Extended Data Fig. 4k-m**)

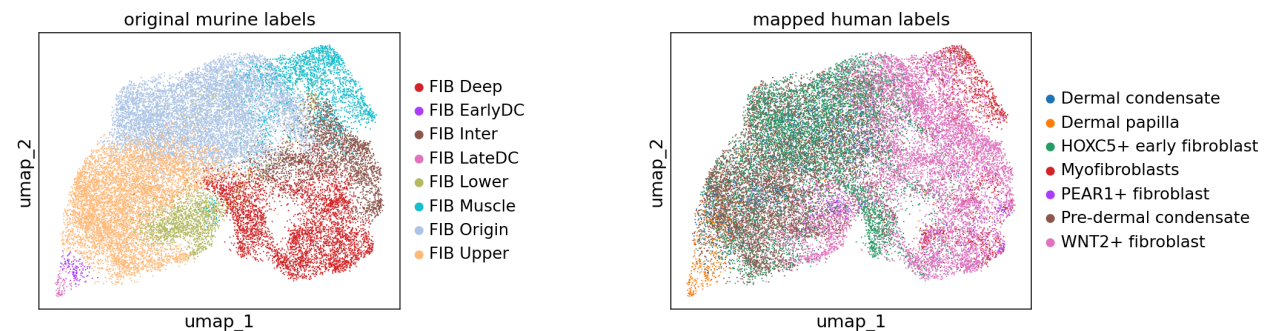


Extended Data Fig. 4k: *UMAP co-embedding of human (top) and murine (bottom) prenatal skin coloured by broad cell cluster annotations.*

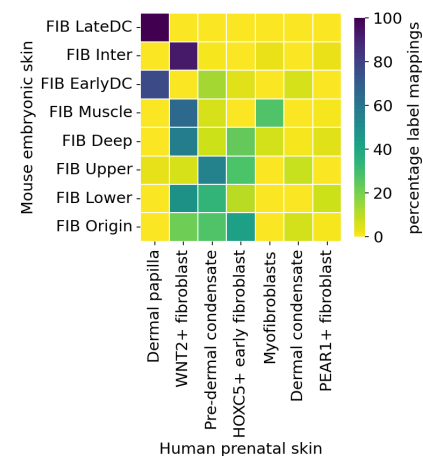


Extended Data Fig.4l, m: Heatmaps showing prediction probabilities from the LR model trained on human prenatal skin (x-axis), projected onto murine embryonic skin (y-axis) for broad cell groupings (left) and refined cell clusters (right, only fibroblast sub-populations shown from all cell types). Colour scale indicates median prediction probabilities.

- c) Asymmetric data integration using scanpy tool ingest: Mapping of human prenatal skin fibroblasts labels onto mouse embryonic fibroblasts. Results shown below **Reviewer Figure 3.8d, e.**



Reviewer Figure 3.8d: *UMAPs of murine embryonic fibroblasts coloured by original labels (left) and mapped labels from human prenatal skin fibroblasts (right).*

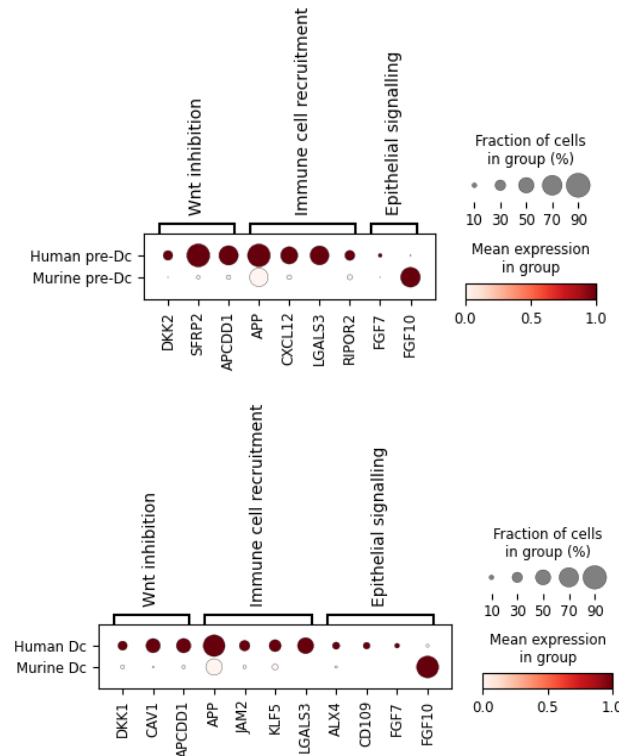


Reviewer Figure 3.8e: *Heatmap showing the percentage of mapped human fibroblast labels (x-axis) in each murine fibroblast cluster (y-axis) for a model fitted on human prenatal skin fibroblasts.*

The above analyses highlight notable differences between human and mouse fibroblasts:

1. Some fibroblast populations in human prenatal skin such as *PEAR1*⁺ fibroblasts have no

		counterparts in embryonic mouse skin, potentially due to the limited gestational ages profiled in mice or species specific evolution. 2. Critically, for corresponding cell types in HF development, mouse HF fibroblasts appear to be in a more differentiated state compared to their human counterparts. The human Dc aligns both to mouse Dc as well as the earlier stage pre-Dc. Similarly, the human pre-Dc aligns to mouse pre-Dc and the earlier fibroblast stages in hair development (FIB upper, FIB origin). We have added the following text to describe our results: Lines 353-360: <i>However, cross-species data analysis between human and mouse hair follicle mesenchymal cells using a scRNA-seq dataset of murine embryonic skin (E12.5, E13.5 and E14.5)⁶⁴, revealed that the human Dc aligns both to mouse Dc as well as to the earlier stage pre-Dc (Extended Data Fig. 4k-m, Supplementary Tables 10, 11). Similarly, the human pre-Dc aligns to earlier fibroblast stages in mouse hair development (FIB upper and FIB origin) which precede the pre-Dc (Extended Data Fig. 4k-m, Supplementary Tables 10, 11). This suggests that for corresponding cell types in hair follicle development, mouse hair follicle fibroblasts are in a more differentiated state compared to their human counterparts.</i> As the human and mouse pre-Dc and Dc did not align in a robust manner, we have opted not to show the results of additional comparisons of differential gene expression (from the scVI integrated object) between murine and human pre-Dc and Dc in the current manuscript. Nevertheless, we provide these results for the reviewer below (Reviewer Fig. 3.8f) and added the following text to acknowledge the need for future studies for cross-species comparison: Lines 650-652: <i>Future studies are required to systematically compare human and mouse prenatal skin to fully delineate the specific features that distinguish human skin development.</i>
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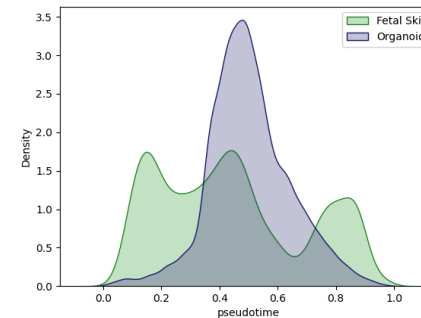
Reviewer Fig. 3.8f: Dot plot showing mean expression (dot colour) and percent of expressing cells (dot size) of genes differentially expressed between human and murine pre-Dc (top) and Dc (bottom) during hair fibroblast development.

The Wnt inhibitors (*DKK1*, *CAV1*) are expressed in human Dc but in the mouse, these genes are downregulated when pre-Dc transitions into Dc (PMID: 37591247). Likewise, *DKK2*, which is expressed in mouse progenitor fibroblasts (FIB origin) but is permanently downregulated as they transit into hair-committed fibroblasts (PMID: 37591247), is still retained in the human pre-Dc. In addition, several genes involved in mesenchymal-epithelial signalling in hair development (*ALX4*, *CD109*, *FGF7/10*) (DOI: 10.1016/j.jia.2023.10.012, PMID: 22846721, PMID: 36964149, PMID:

		9786416) are differentially expressed between human and mouse pre-Dc and Dc, including the pre-Dc marker <i>FGF10</i>. This highlights the existence of transcriptional differences in key hair follicle development genes between human and mouse during embryogenesis.
3.9	Receptor-ligand interaction analyses could benefit from increased detail in methods. a. The authors note that they down-sampled to 200 cells – why this threshold and what about cell types with fewer than 200 cells? b. Were the results validated in any way? The down-sampling approach seems really amenable to bootstrapping.	Thank you for your comments regarding the receptor-ligand interaction analyses in our manuscript. We appreciate the opportunity to provide further clarity on our methods and validation procedures. a. Regarding the threshold of down-sampling to 200 cells, we selected this threshold as a balance between computational efficiency and maintaining representation of cell populations and we only included annotated populations with at least 10 cells in our analysis. Downsampling is recommended by the developers of the package to ensure that the null distribution represents all the cell types evenly and is not biased towards cell types with high numbers (PMID: 34857954). Cells with fewer than 200 cells were included in the analysis without down-sampling to ensure all cell types were accounted for. We acknowledge that this threshold may raise questions about the representation of cell types with fewer than 200 cells, and we have clarified that all cells from populations with under 200 cells were used in the revised manuscript. b. We agree that bootstrapping is a good approach for additional validation and ensuring the predictions are robust. We undertook bootstrapping by random downsampling to 200 cells per population and running the analysis three times independently. In addition, we also utilised the updated CellPhoneDB4, which efficiently handles memory constraints, to perform an additional subsampling with 5000 cells per population. Only interactions that appeared in each and every independent bootstrapped CELLPhoneDB analyses were included in the final results. We believe that these computational steps and analysis visualisation strengthen the reliability of

		our findings and have incorporated this information into the manuscript in the methods section: Lines 1482-1495: <i>CellPhoneDB (v3.0.0) package¹⁶⁰ was used to infer cell-cell interactions within the prenatal skin scRNA-seq dataset overall and in early/late gestation and within the skin organoid scRNA-seq dataset overall. In the overall analysis, we randomly subsampled each cell type into no more than 200 cells 3 separate times, using all cells if a population contained less than 200 cells and excluding populations with less than 10 cells. The subsampled datasets were analysed separately using the permutation-based method to establish statistical significance (p-value cut-off = 0.05). For the analysis by early/late gestation, the prenatal skin scRNA-seq dataset was first split into early (≤ 11 PCW) and late (≥ 12 PCW) gestation datasets which were then subsampled (no more than 200 cells per cell type) and analysed individually (p-value cut-off = 0.05). A summary output file was created for each analysis run, compiling the interactions for each cell pair ($p < 0.05$) and adjusting p-values for multiple testing (FDR set at 0.05) (Supplementary Tables 8, 28). Circos plots (Circlize package (v 0.4.15)¹⁶¹) were used for downstream visualisations of selected significant (adjusted p-value < 0.05) interactions between co-locating cell types for interactions found in all subsampled analyses.</i> We have further emphasised in the manuscript that cellphoneDB delivers predictions that need to be validated. We also validated using RNAscope the CellPhoneDB predicted interactions between <i>CXCL12</i> and <i>ACKR3</i> which are expressed in pre-dermal condensate and epithelial cells respectively. Please see reviewer point 2.4. Our results demonstrate the expression of <i>ACKR3</i> in basal epidermal cells (<i>SERPINB7</i>⁺) adjacent to pre-Dc cells (<i>PDGFD</i>⁺) expressing <i>CXCL12</i> as well as co-staining of <i>ACKR3</i> and <i>CXCL12</i> (white arrows in top right panel of Fig. 2i) at the junction between the epidermis and dermis to support these predicted interactions in early prenatal skin (Fig. 2i).
3.10	Genes2Genes Analysis/Figure 2J – Please expand on the methodology used.	We have now expanded our description of Genes2Genes analysis in the <i>In vivo-in vitro trajectory alignment analysis</i> section of methods (copied below). For details on the methods underlying the Genes2Genes alignment framework, please refer to the paper:

a. I am confused on how the authors can have an “Average 100% alignment” at the cell level (especially with 95% at the TF level). b. For pseudotime trajectory, how were pseudotime bins picked? Do each of the bins have (roughly) equivalent numbers of cells? c. A y-axis should be added to the barplots in the axes in 2.	https://doi.org/10.1101/2023.03.08.531713 which is currently in press at Nature Methods. We address the reviewers query on a) average 100% alignment, b) pseudotime trajectory bins and c) y-axis as follows: a. The average alignment reflects whether time points are matched or mismatched on average across all TF-level alignments. If there is a higher proportion of TFs for which a specific reference-organoid time point pair is matched, then the average alignment includes them as a match. Therefore, an average alignment of 100% match does not necessarily mean that all TFs are matching. The overall distribution of TF-level alignments gives different clusters of alignment patterns, where different groups of genes have matches and mismatches in different ways. This is why the average alignment similarity percentage (a statistic computed across all TF-level alignments) is different from the similarity percentage calculated using the average alignment – it is the mean of the proportion of matched time points along pseudotime in a TF alignment on average). While we reported 100% average alignment with 95% mean TF-alignment similarity in our previous submission, in the revised manuscript, we report different statistics, as we have now performed the TF-alignment using an updated version of G2G and an interpolation structure of equal-length intervals (in contrast to the optimal irregular structure used for our previous findings). Accordingly, we now get an average alignment of early matches and late mismatches, with a mean TF-alignment similarity of 58.07%. This agrees with what we see from the pseudotime estimates distributions, as organoid cell populations are more dense towards the middle of the pseudotime range (Reviewer Fig. 3.10). This middle range is fully matched between reference and organoid on average (accounting for 50% in the overall average alignment) across the TFs. Our previously reported alignment of 100% average match based on an optimal discretisation structure is also consistent with this observation (as it only compared irregular discrete time points where there is a high cell density around). Therefore we can conclude that the majority of the TFs between the two systems follow similar dynamics.
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Reviewer Fig. 3.10: Cell population density across pseudotime coloured by query (skin organoid) and reference (prenatal skin) datasets.

- b. The number of bins were picked based on the *OptBinning* package that optimises a binning structure of the pseudotime axis based on the cell density across pseudotime. The bins only define the interpolation time points. At each interpolation time point, the gene expression distribution is estimated based on all cells in the trajectory, weighted by a Gaussian kernel. Therefore it is not a hard binning in terms of the cells (i.e. each cell contributes to the estimation of expression distribution at each interpolation point based on its distance to it). We now use the term ‘discrete time points’ instead of ‘bins’ to avoid confusion with the commonly-used hard binning strategy. As mentioned in response 3.10a, we have now run this using equal-length intervals of interpolation time points instead of an irregular structure for consistency in comparison.
- c. We have now modified Fig. 2k to improve clarity including adding a y-axis to the bar plots as shown in review point 4.11.

3.11

The Figure 3A main caption claims are not supported by experimental evidence.

We have amended the main caption for Fig. 3 and the title for the corresponding results section in the manuscript to the following instead:
 Lines 399 and 1832: *Early dermal fibroblasts and macrophages potentially contribute to scarless skin healing*

3.12	The analysis in Figure 3A analysis feels like a bit of a stretch and is at risk of identifying technical rather than biological differences due to batch/study biases. What steps did the authors take to mitigate this concern? The differences presented between fetal and adult fibroblast populations are interesting, but this analysis oversimplifies established adult fibroblastic heterogeneity.	To address concerns related to batch/study biases, we integrated individual studies before analysis, effectively mitigating potential technical variations. This integration process, which involved methods such as harmony and scVI, was instrumental in removing technical differences. For robustness and reproducibility, we systematically tested different integration methods, notably BBKNN, Harmony and scVI. Each approach consistently yielded similar overall results. We acknowledge the complexity of adult fibroblast heterogeneity and have performed new analysis of adult fibroblast scRNA-seq datasets to harmonise adult fibroblast populations and annotation in point 3.13 below to validate findings from Fig. 3a.
3.13	Several recent studies (Sole-Boldo et al, Reynolds et al, Ascension et al) of single cell dermal fibroblasts have identified multiple fibroblast subpopulations, including the published data used by the authors in this analysis. a. It would be worthwhile for the authors to identify the fibroblast populations that are most similar between the fetal and adult human dermal populations. b. Consider adding in an analysis like Ext. Data Figs 3C or 7B and a coembedded UMAP.	[REDACTED]

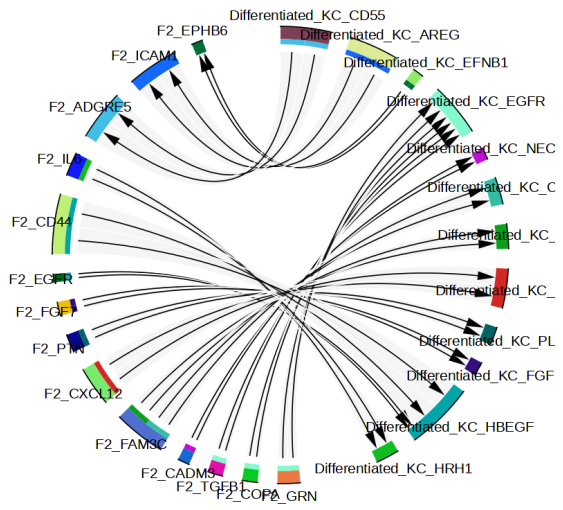
		[REDACTED]
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		[REDACTED]
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		[REDACTED]
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		[REDACTED]
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		[REDACTED]
3.14	In Figure 3, are the predicted cell interactions between fibroblast populations and macrophages conserved	As the reviewer suggested, we have now run CellphoneDB3 adult datasets to test for interactions of macrophages and the distinct adult fibroblast subsets F1, F2 and F3. The analysis showed no interaction between adult fibroblast subtypes F1, F2 and F3 with any of the adult macrophage subtypes. This indicates the specificity of interactions between prenatal fibroblasts and

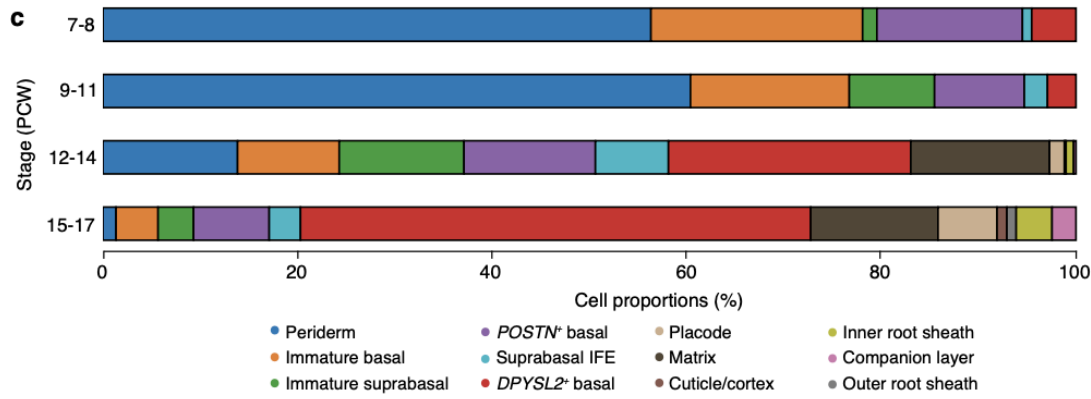
	between fetal and adult samples? The authors should speculate on whether a loss of a specific fibroblast population (WNT2+) or the changes in cell signaling/interactions play a role in loss of scarless healing.	macrophages. We speculate therefore that the loss of prenatal fibroblast populations rather than fibroblast interactions may be underpinning the explanation for scarless healing observed in prenatal skin Adult fibroblasts show interactions with differentiated keratinocytes and with DC1/2 (see figure, example F2 Differentiated KC, significant interactions shown).  Reviewer Fig. 3.14: <i>Circos plot showing significant (adjusted p-value<0.05) predicted interactions between adult fibroblast F2 and differentiated keratinocytes in adult skin. Arrows represent directionality of interactions (ligand to receptor); connection width is proportional to the CellphoneDB mean value for each ligand receptor pair.</i>
3.15	The concluding paragraph from lines 444-448 is not supported	We have amended the concluding paragraph to remove the statement regarding tissue remodelling and highlight that we speculate a potential role of TML macrophages in scarless healing which

by the evidence. The manuscript shows good evidence that the prenatal skin fibroblasts are not yet expressing genes required for ECM formation and collagen deposition, but doesn't show their involvement in tissue remodeling specifically. The fibroblasts themselves may just be developing without actively performing remodeling activities in the tissue. Additionally, while colocalization and likely signaling between TMLM cells and WNT2+ fibroblasts is established by the data, a clear role of this interaction in scarless healing remains conjecture based on data from other tissues. This should be made more clear. If Schwann cells are also co-localizing with TMLMs (and WNT2+ fibroblasts, by extension), were the cell-cell interactions investigated to see if there could be a role for Schwann cells in promoting scarless healing? This was not	remains to be fully established in future studies. <i>Lines 484-490: Collectively, our findings suggest that prenatal skin fibroblasts in early gestation downregulate genes involved in extracellular matrix formation, collagen deposition and inflammation which may favour tissue regeneration over scarring. Based on our data and from previous studies, we also posit a potential role for early skin macrophages in conferring the unique property of scarless healing in early prenatal skin. However, further studies are required to fully elucidate the interactions between macrophages and fibroblasts in human prenatal skin and conclusively establish their role in scarless healing.</i> [REDACTED]
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	addressed.	
3.16	The gene modules discussed in relation to Extended Data Fig. 7c don't line up with what is displayed by the figure. Gene module scoring – this approach has a lot of benefits for normalizing scores; but I am curious about controlling for sample variation. Was the same reference gene list used for all comparisons? Were any genes included in both the reference gene list and the gene list of interest?	Thank you for highlighting this. The angiogenesis gene programmes described were identified by gene set enrichment analysis with the full results provided in Supplementary Tables 23-26 as cited in the manuscript. Extended Data Fig. 7C only displayed the top 10 results for each macrophage subtype, which for some macrophages did not include angiogenic programmes. We have removed the reference to Extended Data Fig. 7c at this point in the manuscript and only retained Supplementary Tables reference to avoid confusion as shown below: Lines 514-518: <i>The four macrophage subsets (LYVE1⁺, MHC-II⁺, TML and iron-recycling) expressed gene programmes driving angiogenesis by gene ontology analysis (Supplementary Tables 23-26). We sought to investigate if these macrophage subsets contribute to distinct angiogenic processes using module scoring of reference angiogenesis gene sets^{17,118} and cell-cell interaction analysis (Extended Data Fig. 7e, 8a, Supplementary Tables 27, 28).</i> We used the gene module scoring package in scanpy (scanpy.tools.score_genes) and defined the gene pool (all genes passing QC in our dataset), the number of expression bins and the number of reference genes per bin which were consistent for all the angiogenesis gene modules. The reference gene list for each module is randomly selected in this package from the provided gene pool and is therefore expected to vary between the different gene modules. For each angiogenesis gene module, no gene from the gene list of interest (provided in supplementary tables 27) were included as part of the reference gene list as per the source code for scanpy.tools.score_genes.
3.17	The images in Fig 4j are quite striking. Please add information about replicates and a quantification of increased endothelium.	We have addressed this comment and also added more replicates and quantification. Please see more information in reviewer point 1.6 and Fig4. h-i.
	Reviewer 3 comments - minor	Response to reviewer

3.18	For figures 1d-e, when discussing the microenvironments, please discuss whether these microenvironments have been previously observed through other methodologies such as histology or if they are novel.	The microenvironments are predicted groupings of cell types that have a high probability of co-localising as described in point 1.10. As such, they provide more detailed information than what can be observed histologically. We have added the following discussion point to our manuscript to highlight the novelty of this method applied to prenatal skin analysis. We have also validated some of these predicted microenvironments identified by demonstrating in situ co-localisation macrophages with fibroblasts and endothelial cells (please see points 1.9 and 2.5). Lines 165-169: <i>NMF can predict significant cellular co-locations that are not readily evident on conventional histopathology analysis^{9,19}. It has previously been used to identify a supportive role for ILC3, NK cells and macrophages in B-cell lymphopoiesis in developing organs including liver, spleen and thymus but prenatal skin has not been similarly studied⁹.</i>
3.19	Extended Data Fig. 2a is hard to interpret, please include facets for each sample in which only the cells from each individual sample are highlighted/colored.	Separate plots for each of the different datasets (prenatal, adult and organoid) have been added in place of current Fig 2a as shown below, highlighting cells from each group.

		Extended Data Fig. 2a: <i>UMAP visualisations of the integrated prenatal skin, adult skin and skin organoid scRNA-seq datasets, coloured by broad cell types.</i>
3.20	For Extended Data Fig. 3e, does GO term enrichment back up the claims about differences in function between the adult and prenatal/organoid matrix cells? Specifically the authors should carefully consider and better explain the their reasoning behind their heavy	a) We have further quantified the expression of <i>CXCL14</i> - please see 1.26 showing high expression of <i>CXCL14</i> in placode, matrix and <i>POSTN</i>⁺ basal cells. We have additionally quantified the amount of <i>FOXP3</i>⁺ cells in close proximity of hair follicle (see point 3.7) and thereby provide corroborating evidence for our claims of the potential role of Treg accumulation in the early stages of placode/matrix differentiation. Additionally, separating prenatal skin Placode/matrix population into Placode and Matrix and rerunning the analysis with larger cell numbers reproduced the previous findings, and <i>CXCL14</i> and <i>CD24</i> again came up as DEGs between prenatal and adult Matrix (please see point 1.15).

	reliance on the use of the following genes: a. CXCL14 – implicating Treg recruitment and immune protection (CXCL14 has been implicated in many biological activities) b. LGALS1 indicating cell proliferation – consider evaluating MKI67, TOP2A, etc to back up this claim	b) We have now removed mention of LGALS1 as indicating proliferation as we did not observe expression of MK167 or TOP2A to support this claim.
3.21	Figure 2C – How many embryos were used in this analysis? Do the cell proportion percentages represent an average?	The bar plot (shown below) has now been updated following the inclusion of three additional prenatal skin samples (please see point 1.2) we included in the revised manuscript. The bar plots show data from 18 samples: n=5 for 7-8 PCW, n=4 for 9-11 PCW, n=4 for 12-14 PCW and n=5 for 15-17 PCW. We have updated the figure legend to include that this represents average proportions.  Fig. 2c: Bar plot showing average proportions of epidermal cell states across gestational age in

		<i>prenatal skin. Bar colours represent cell states.</i>
3.22	Figure 2E – What do the different lines indicate? (color, thickness)	Lines refer to the overlaid PAGA on the trajectory analyses for epithelial/fibroblast. Solid black lines/arrows indicate transitions between connected nodes and dotted grey lines connectivities that have not reached transition threshold. The threshold was kept at default (PMID: 30890159). We have included this information in the revised figure legends (example shown below): <i>Lines 1794-1799: Fig 2. e: Inferred pseudotime trajectory of skin organoid epidermal cell states differentiating along the ‘ORS/CL’ and ‘IRS’ trajectories; UMAP overlaid with PAGA and coloured by cell state (top) and overlaid with the cell directionality (shown in arrows) as inferred over the cell-to-cell transition probability matrix from running CellRankCellRank state transition matrix inferred arrows and coloured by pseudotime (bottom). Shown are connectivities (dashed) and transitions (solid/arrows). Threshold for Cellrank PAGA was kept at default.</i>
3.23	Ext. Data Fig 3g,h – How were genes identified from the trajectory analysis? If same as Ext Data Fig 4c, the authors should consider adding this type of figure.	Yes, the genes were identified in the same fashion for Extended Data Fig. 3 g, h as for the fibroblasts in Extended Data Fig. 4c. The corresponding heatmap to that analysis can be found in Extended Data Fig. 5 a, b.
3.24	Were both prenatal skin and organoid cells used to infer fibroblast differentiation trajectory? Is the decreasing proportion of HOXC5+ fibroblasts a consistent finding between prenatal and organoid datasets?	Please see response to point 1.16.

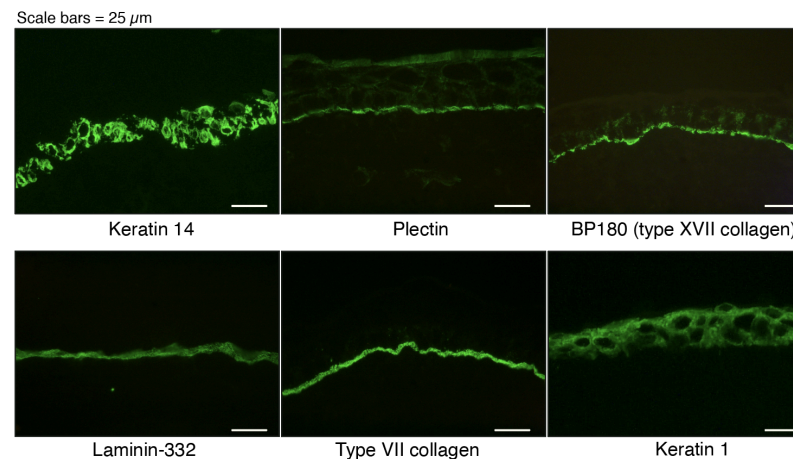
3.25

When discussing the presence of genes involved in hair/skin disease, the statement “suggesting that these disorders result from dysfunctional hair follicle development” is not supported. The presence of a gene(s) by RNA-seq does not always mean that it is functional/required at that specific space and time. You also don’t discuss if these genes are found elsewhere in the dataset or are exclusive to these pseudotime trajectories.

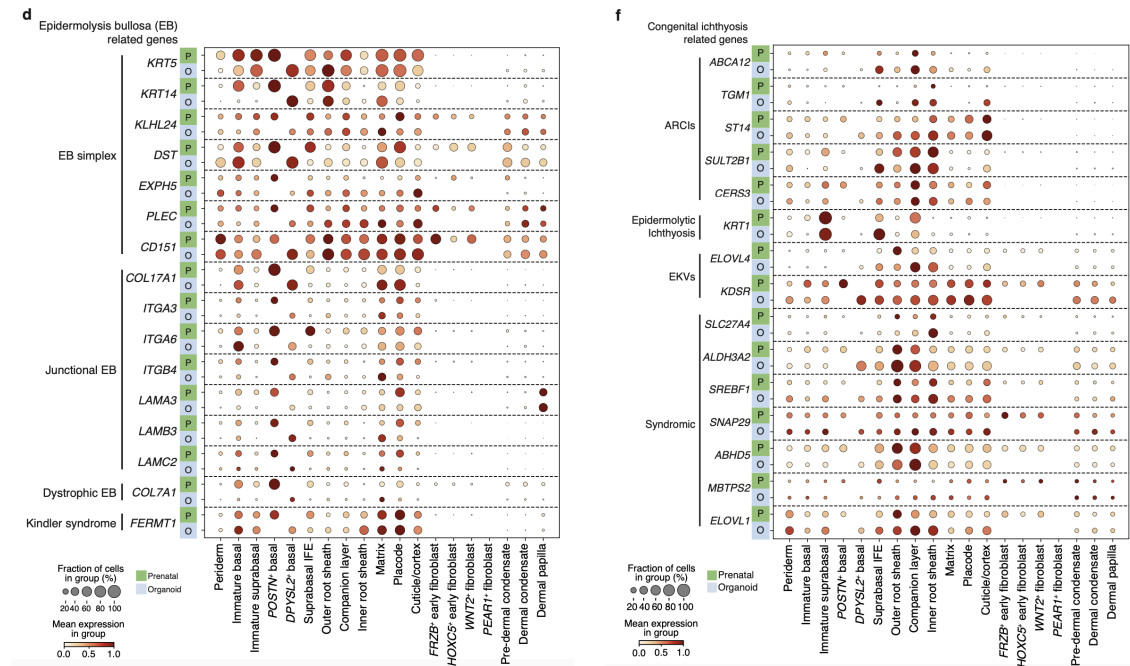
We have performed new experiments and data analysis to provide the following evidence to support the findings from our scRNA-seq data analysis:

1. Protein level spatial validation on prenatal skin for congenital epidermolytic and ichthyotic disorders

We performed new immunofluorescence microscopy analysis to demonstrate protein-level expression in prenatal skin (15-17 PCW) of a number of genes we highlighted in genetic skin disorders. Namely, we validate the expression of several proteins identified from our scRNA-seq analysis. These include keratins 1 and 14 (*KRT1*, *KRT14*) throughout the epidermis (**Extended data Fig. 5d, e, f shown below**), plectin (PLEC), type XVII collagen (*COL17A1/BP180*), type VII collagen (*COL7A1*) and laminin-332 (*LAMA3*, *LAMB3*, *LAMC2*), all of which are primarily present at the dermo-epidermal junction in keeping with highest expression in basal epidermal cells (**Extended data Fig. 5d, e shown below**).



Extended Data Fig. 5e: Indirect immunofluorescence of 15-17 PCW prenatal skin with antibodies against keratin 14, plectin, BP180 (type XVII collagen), laminin-332, type VII collagen, keratin 1. Scale bars = 25 μ m.



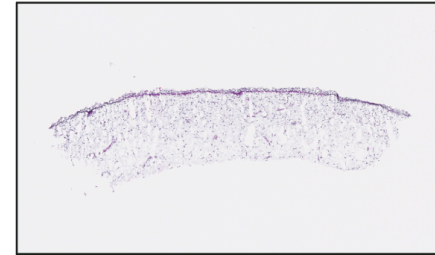
Extended Data Fig. 5d, f: Dot plot showing variance-scaled mean expression (dot colour) and percent of expressing cells (dot size) of genes causing Epidermolysis Bullosa (left) and congenital ichthyoses (right) in prenatal skin and skin organoid.

2. Demonstrate the alignment of the in situ protein expression with our spatial transcriptomics data for the above disorders.

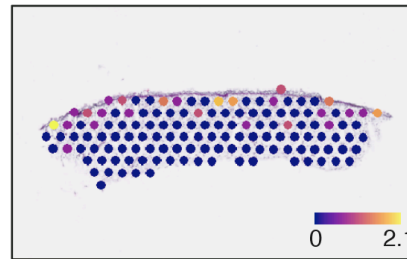
The protein level validation also corresponds to the expression of these genes queried from our prenatal skin Visium data (available on our webportal:

<https://developmental.cellatlas.io/fetal-skin>, password: fs2023) (Reviewer Fig. 3.25a).

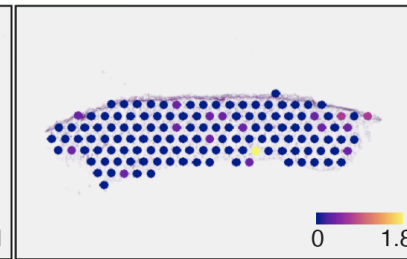
This shows expression spatially restricted to epidermal/basal epidermal cells further demonstrating close correlation between spatial transcriptomic and protein expression.



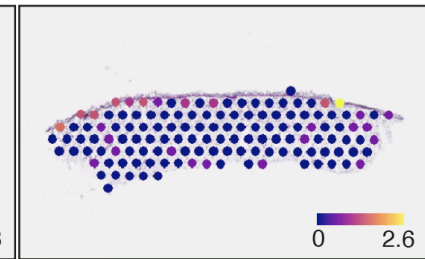
H & E



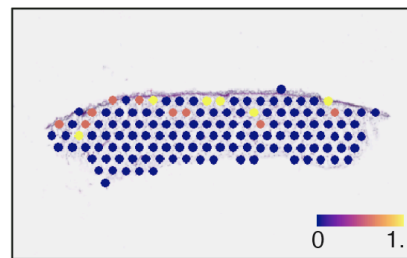
KRT14



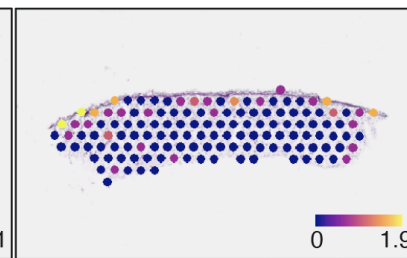
PLEC



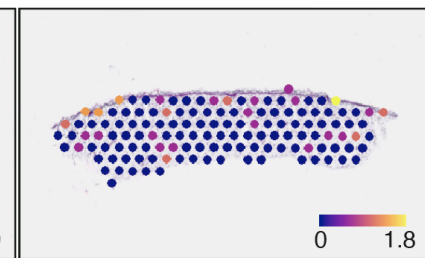
COL17A1



LAMA3



COL7A1



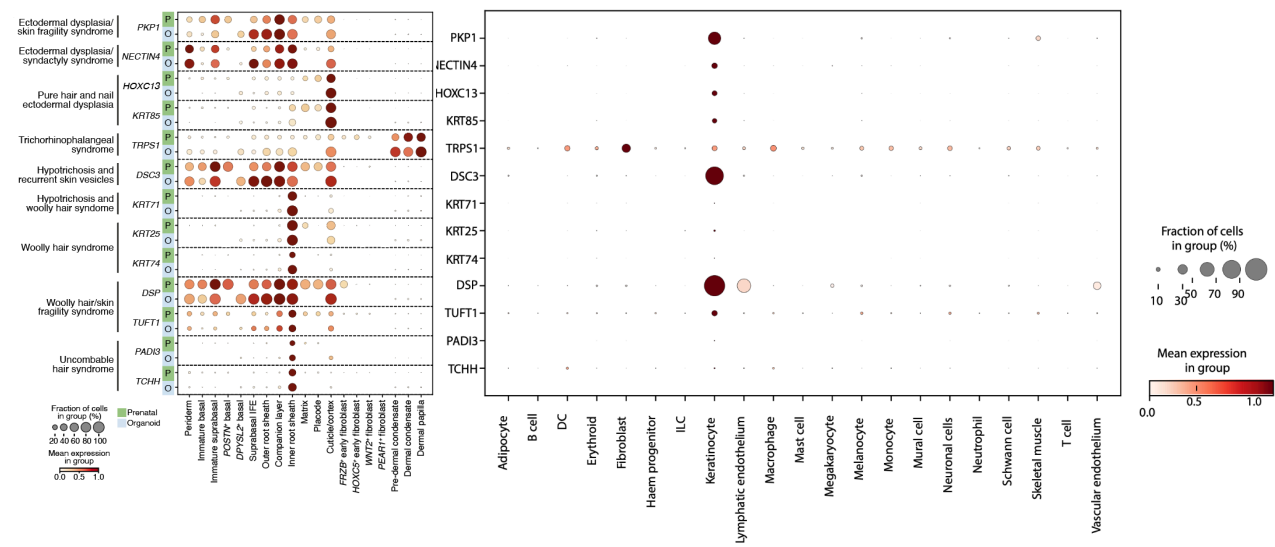
KRT1

Scale bars = Counts (log1p) per spot

Reviewer Fig. 3.25a: *Spatial expression of genes implicated in genetic skin and hair diseases (representative sample shown) queried using our portal.*

3. Show the specificity of the gene expression to hair follicle inferred trajectory

In terms of genes implicated in genetic hair disorders that we identify along the inner and outer root sheath pseudotime trajectories, these are primarily expressed in epithelial cell states as seen in Extended data Fig. 5c (shown below). The accompanying dot plot (Reviewer Fig. 3.25b below) further demonstrates their overall restricted expression in keratinocytes compared to all other broad cell lineages, in keeping with their implied roles in hair follicle disorders and ectodermal dysplasias.

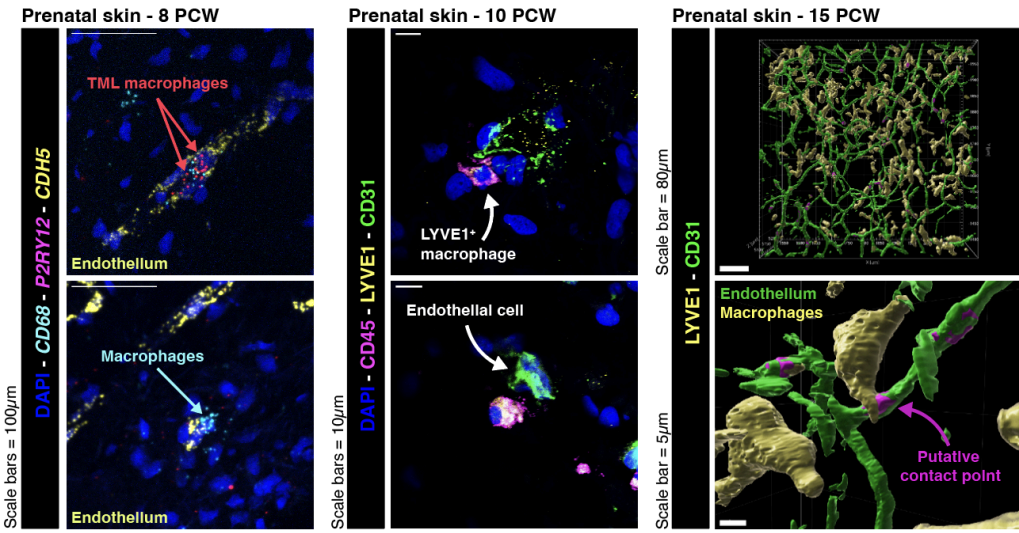


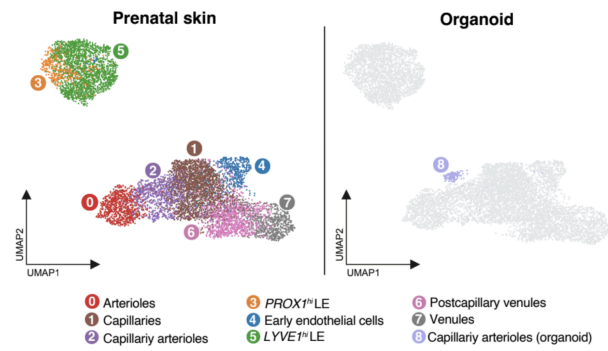
Extended data Fig. 5c (left): Dot plot showing variance-scaled, mean expression (dot colour) and percent of expressing cells (dot size) of genes implicated in genetic hair diseases in prenatal skin and skin organoid.

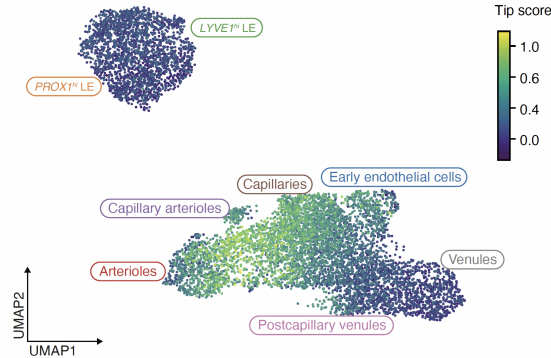
Reviewer Fig. 3.25b (right): Dot plot showing variance-scaled, mean expression (dot colour) and percent of expressing cells (dot size) of genes implicated in genetic hair diseases across broad cell groups in prenatal skin.

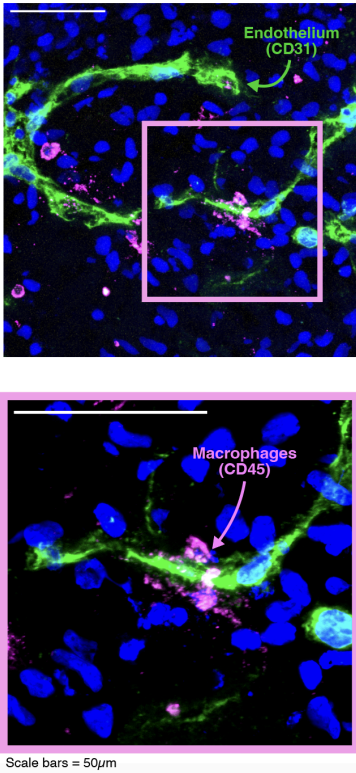
		In summary, we validated the protein-level expression of genes involved in epidermolytic and ichthyotic skin diseases, which correspond to the correct in situ location by spatial transcriptomic. These, together with the clinical age of onset of epidermolytic and ichthyotic skin diseases at birth or early infancy supports their functional requirement in skin development. We also show the specificity of hair follicle disorders gene expression to the relevant cell states. The single cell and spatial transcriptomic level information we provide in our study, which correlates with protein expression, is the first demonstration of such information and provides a valuable resource for the community through our freely accessible web portal to inform future studies and potential therapeutic interventions of skin disorders.
3.26	When discussing the differences in immune gene expression between the prenatal and adult fibroblasts, only cherry picked genes are included. Please perform GO term enrichment or other similar analysis to show the unbiased detection of differentially expressed genes that fall into these categories.	Since adding new scRNA-seq data in the revised manuscript, we have performed gene set enrichment analysis (Enrichr workflow from the python package GSEAPy) for genes differentially expressed between prenatal and adult skin fibroblasts. This produced consistent results to our previous analysis including terms relating to pro-inflammatory activity (<i>‘Cytokine-mediated signaling pathway’</i>, <i>‘Positive regulation of cytokine production’</i> and <i>‘Cellular response to cytokine stimulus’</i>) being significantly enriched in adult fibroblasts. The analysis also produced similar DEGS to our previous analysis (<i>C1R</i>, <i>C1S</i>, <i>CD55</i>, <i>CDKN1A</i>, <i>HLA-B</i>, <i>IFITM1</i>, <i>IL6</i>, <i>IL32</i>, <i>IL15RA</i>, <i>PTGES</i>, <i>SQSTM1</i>) (Fig. 3a shown below, Supplementary Tables 13-15). We have amended the genes upregulated by prenatal fibroblasts based on anti-inflammatory and immune-regulatory pathways identified in the updated gene set enrichment analysis (Fig. 3a below, Supplementary Tables 13-15). The updated figures and corresponding text sections are copied below.

		Fig. 3a: Dot plot showing variance-scaled, mean expression (dot colour) and percent of expressing cells (dot size) of genes differentially expressed by prenatal (‘anti-inflammatory & immune suppression’) and adult skin fibroblasts (‘pro-inflammatory & immune activation’) Lines 407-414: All adult fibroblast subsets expressed higher levels of inflammatory cytokines (IL-6, IL32) and receptors (IL1R1, IL15RA), interferon-induced genes (IFITM1) and genes involved in antigen presentation (HLA-A, HLA-B), complement pathway and innate immune response (C1R, C1S, CD55), inflammatory response (CD44, PTGES, SQSTM1) and cellular senescence (CDKN1A) (Fig. 3a, Supplementary Tables 13-15). In contrast, prenatal skin fibroblasts upregulated genes involved in immune suppression (CD200), regulation of inflammation (RAMP2, PLD3, TMSB4X) and tissue regeneration (MDK) (Fig. 3a, Supplementary Tables 13-15).
3.27	The statement “WNT2+ fibroblasts downregulated IL6 expression compared to adult fibroblasts” should be edited to “WNT2+ fibroblasts had downregulated IL6 expression compared to adult fibroblasts” or similar. The current version	This sentence has now been modified to: <i>TML macrophages were predicted to co-locate with WNT2+ fibroblasts in early prenatal skin (6-8 PCW) (Fig. 3d) and WNT2+ fibroblasts had downregulated IL6 expression compared to adult fibroblasts (Fig. 3a). [Lines 458-460]</i>

	implicates that the WNT2+ fibroblasts are a causative factor in downregulating IL6 expression broadly which you are not showing here.	
3.28	Figure 3D,F would benefit from titles explaining what the correlation coefficient is measuring.	The label of the x-axis has been amended to the following for greater clarity: ‘<i>Correlation of cell pair abundance per sample (Pearson coefficient)</i>’. We have also modified the figure legend to include information that pearson correlation coefficients were “<i>calculated between per-spot normalised cell type abundances.</i>”
3.29	In Figure 4a, the dots are quite small/hard to see. Please make two different insets (one for each macrophage type) from smaller regions of the whole field.	As suggested by the reviewer, we have now modified our Fig. 4a (shown below) which includes magnified regions of the whole field of RNAscope. This modification is demonstrated in the left panel of Fig. 4a.  Fig. 4a: (Left panel) Magnified RNAscope images of prenatal skin cryosections (representative 8 PCW sample) demonstrating close proximity of endothellum (CDH5) with TML macrophage

		(P2RY12) and macrophages (CD68). Scale bars=100μm (Center panel) Immunofluorescence images of prenatal skin cryosections (representative 10 PCW sample) demonstrating close proximity of LYVE1⁺ macrophages (LYVE1⁺ CD45⁺) with endothelial cells (CD31⁺). Scale bars = 10μm. (Right panel) 3D rendering of co-localised areas (magenta) of endothelial cells (CD31⁺) and LYVE1⁺ macrophages (LYVE1⁺) from whole-mount immunostaining of a 15 PCW prenatal skin sample. Scale bar = 80μm (top), 5μm (bottom).
3.30	For Extended Data Fig. h, please reference the UMAP panel which cell types are defined (Fig 4b) in the figure legend.	All endothelial cell types present in Fig. 4b (new figure copied below) were included in Extended Data Fig. 4h (amended figure below). Cell type labels have now been added to the updated UMAP panel and list of cell types defined added to the figure legend for clarity.  Prenatal skin Organoid UMAP2 UMAP1 UMAP2 UMAP1 0 Arterioles 1 Capillaries 2 Capillary arterioles 3 PROX1^{hi}LE 4 Early endothelial cells 5 LYVE1^{hi}LE 6 Postcapillary venules 7 Venules 8 Capillary arterioles (organoid)

		 Fig. 4b (top): UMAP visualisation of endothelial cell states present in prenatal skin and skin organoid. Extended Data Fig. 9h (bottom): UMAP visualisation of the 'Tip' cell module score in prenatal skin (arterioles, capillaries, capillary arterioles, $PROX1^{hi}$ LE, early endothelial cells, $LYVE1^{hi}$ LE, postcapillary venules, venules) and skin organoid endothelial cells (capillary arterioles).
3.31	The legend for Extended Data Fig. 10g does not match the data. Please update this legend.	Extended Data Fig. 10g has now changed during the revision process and been replaced by the figure panel shown below as Fig. 4j instead. This panel demonstrates immunofluorescence of cryosections of skin organoid co-culture with macrophages showing $CD31^{+}$ endothelium in green, $CD45^{+}$ macrophages in magenta and DAPI nuclei stain in blue.

		Cryosection of day 47 skin organoid  Fig. 4j: Z-projection immunofluorescence of cryosections of day 47 skin organoid co-cultured with macrophages (day 35 of co-culture) demonstrating close interactions of macrophages (CD45, in magenta) and endothelium (CD31, green). Scale bar = 50 μm.
3.32	In Figure 2, what should be panels b-e are all labeled as c.	The legend labels for Figure 2 have now been amended as below: (b) Schematic of stages of hair follicle formation.

		(c) Bar plot showing average proportions of epidermal cell states across gestational age in prenatal skin. Bar colours represent cell states. (d) Large-area (left) and magnified peri-follicular (top right) and inter-follicular (bottom right) RNAscope images of prenatal skin (representative 15 PCW sample) demonstrating ORS (<i>SLC26A7</i>), matrix (<i>SHH</i>), dermal papilla (<i>NDP</i>) with Tregs (<i>FOXP3</i>) localising around the hair follicle. Scale bars=100µm and 20µm. (e) Inferred pseudotime trajectory of prenatal skin and skin organoid epidermal cell states differentiating along the ‘ORS/CL’ and ‘IRS’ trajectories; UMAP overlaid with PAGA and coloured by cell state (top) and overlaid with the cell directionality (shown in arrows) as inferred over the cell-to-cell transition probability matrix from running CellRank and coloured by pseudotime (bottom). Shown are connectivities (dashed) and transitions (solid/arrows). Threshold for Cellrank PAGA was kept at default.
3.33	Fig 4I - How were branches determined/quantitated?	We have now reanalysed the 2D angiogenesis assay using the “Angiogenesis Analyzer” plugin for ImageJ. Please see reviewer point 1.3-1.4 for more information and quantification methods.
3.34	Did the authors perform co-localization analysis for all pairs of cell types? The authors should consider showing the full analysis and summarize the major patterns.	We used two complementary approaches to analyse cell type colocalization. First we used non-negative matrix factorisation (NMF) (PMID: 20598126) of the abundance estimates of all cell types in tissue spots across all Visium samples to identify microenvironments (MEs) of co-localized cell types in an unbiased manner. The summarised results of this analysis are shown in Fig. 1d (shown below).

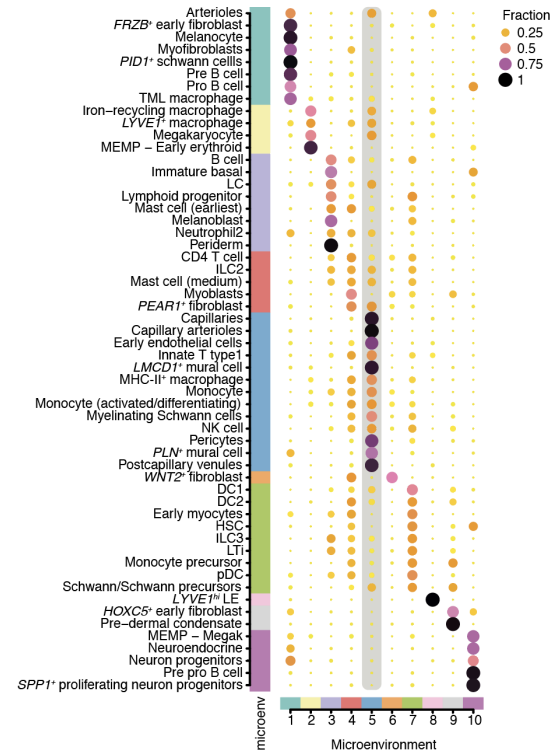


Fig. 1d: Dotplot showing spatial microenvironments. Cell type to microenvironment coefficients are normalised by cell type sums; cell type to microenvironment assignment is shown by colour. Microenvironment 5, which shows co-locating macrophages and endothelial cells, is highlighted.

We then supplemented NMF by per-sample correlation analyses (**Fig. 1e, 3d, 3f** and **Extended Data Fig. 1e**, shown below). This was performed for all pairs of cell types but only shown for selected pairs. The selection was based on the biological focus of our study relating to immune and non-immune cells co-localization and was informed from the initial NMF results where this pattern was originally identified.

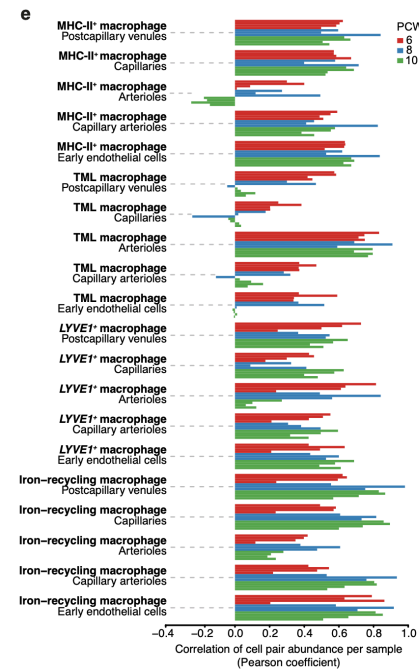


Fig. 1e: Bar plot showing cell type co-location, indicated by positive Pearson correlation coefficients calculated between per-spot normalised cell type abundances, for selected cell type pairs (macrophage and endothelial cells). Pearson correlation coefficients were calculated across all skin-covered spots of Visium samples; each sample is shown by an individual bar.

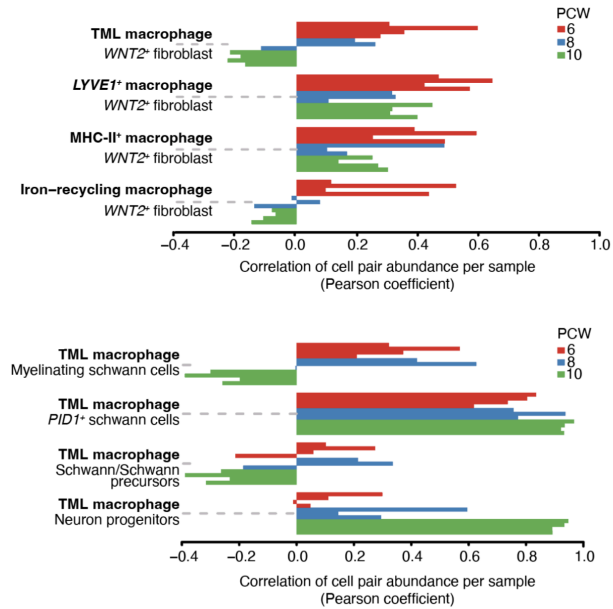
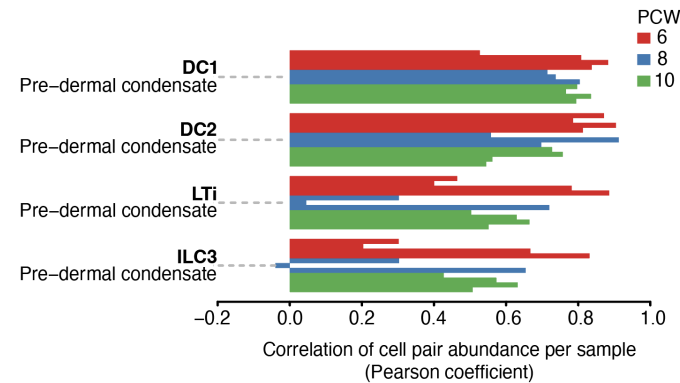
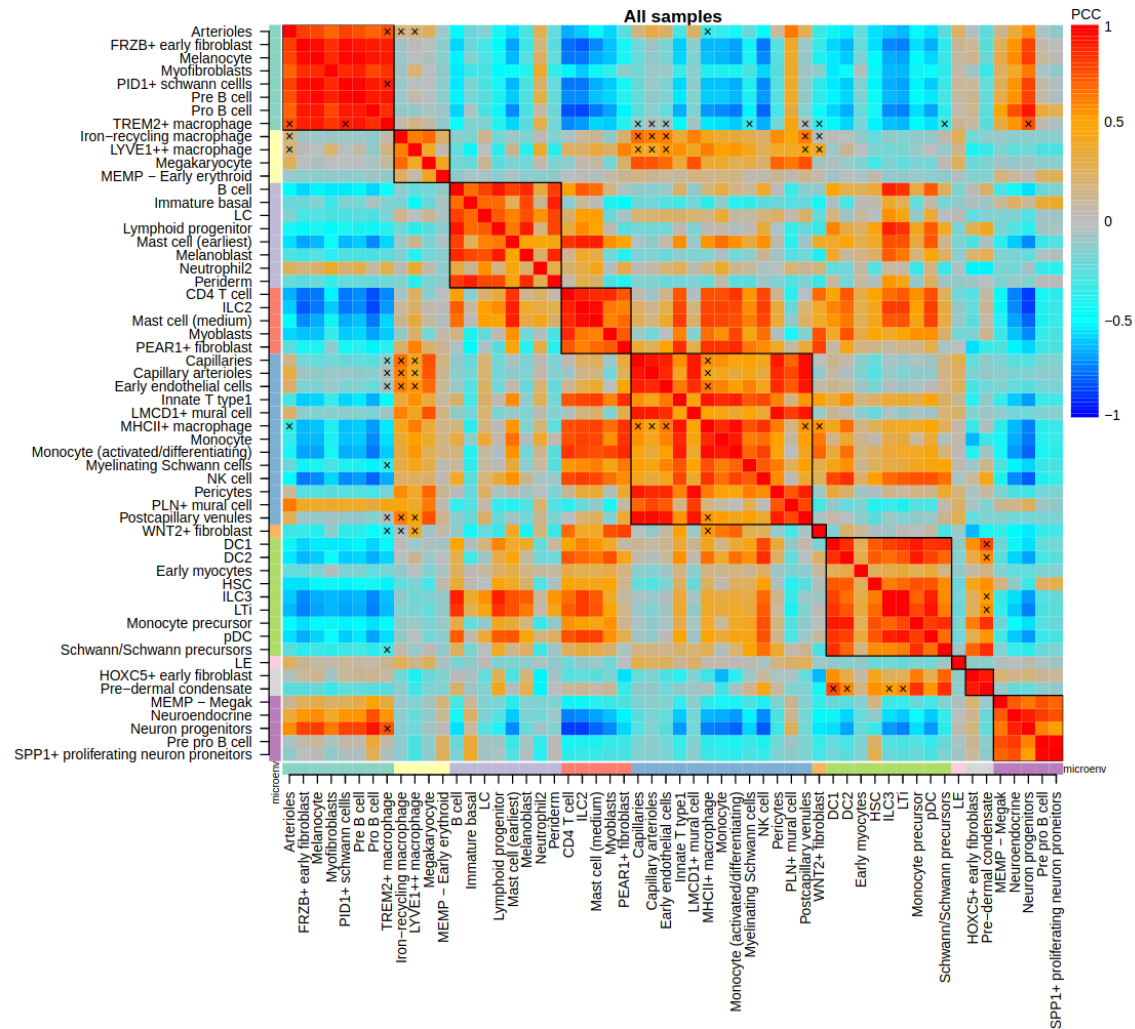


Fig. 3d (top), h (bottom): Bar plot showing cell type co-location, indicated by positive Pearson correlation coefficients calculated between per-spot normalised cell type abundances, for selected cell type pairs (macrophages and WNT2⁺ fibroblasts (top) and TML macrophage and neuronal cells (bottom). Pearson correlation coefficients were calculated across all skin-covered spots of Visium samples; each sample is shown by an individual bar.



Extended Data Fig. 1e: Bar plot showing cell type co-location, indicated by positive Pearson correlation coefficients calculated between per-spot normalised cell type abundances, for selected cell type pairs (pre-dermal condensate and immune cells: DC1, DC2, LTi and ILC3). Pearson correlation coefficients were calculated across all skin-covered spots of Visium samples; each sample is shown by an individual bar.

As showing the results of per-sample correlation analyses for all cell pairs is not practical (56*56 cell pairs for 11 samples would amount to 34496 values), we have calculated the correlation matrix using all visium samples combined together. The matrix is shown in the **reviewer Fig. 3.34 below**, and in heatmaps with pairwise comparisons shown in Fig 1e, 3d, 3f and Extended Data Fig 1e marked by crosses. Overall, the pattern of highly correlated cell pairs in the correlation matrix supports the NMF findings which we described in the manuscript.



Reviewer Fig. 3.34: *Pearson correlation coefficients between all pairs of celltype calculated using cell2location prediction across all Visium samples. Cells are grouped by microenvironments, correlation coefficients for pairs within the same microenvironment are*

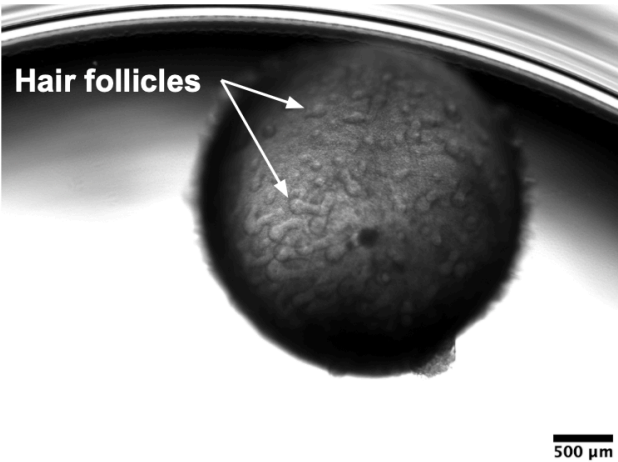
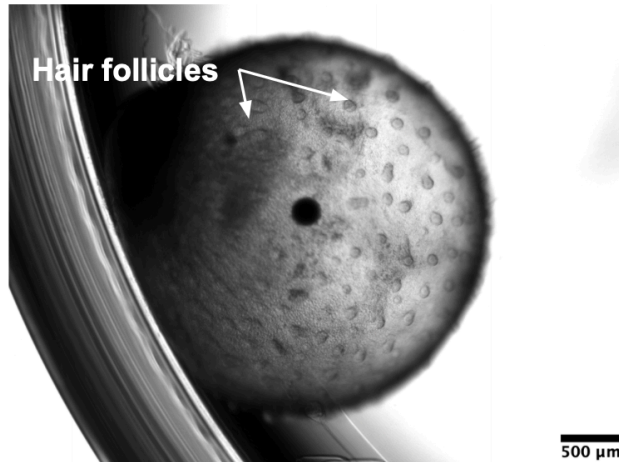
		<i>outlined in black. Pairs shown on main figures and discussed in the text are marked by crosses.</i>
3.35	Please include a brief discussion of other organoid systems in which co-culturing of immune cells has been successful. (E.g. Huang et al 2022 Arthritis Rheumatology (DOI: 10.1002/art.42097) in the skin and the use of microglia in brain organoids reviewed by Zhang et al. 2023 Mol Psychiatry (DOI: https://doi.org/10.1038/s41380-022-01892-1 [doi.org])	We thank the reviewer for the suggestion and have included a discussion of other organoid systems co-cultured with immune cells in the manuscript. Lines 625-634: <i>Successful co-culture with immune cells has been demonstrated in various organoid systems^{134,135}. A neural organoid demonstrated improved blood vessel organisation and maturation in the presence of macrophages, with close association observed between macrophages and endothelial cells¹³⁶. Our findings further demonstrate that the addition of macrophages presents a possible avenue to improve vascularisation of tissue organoids, which remains a key challenge^{137,138}. Notably, iPS-derived macrophages survived at least five weeks in the skin organoid, in contrast to 1-2 weeks in liquid culture supplemented with macrophage colony-stimulating factor (M-CSF)¹³⁹. Increased viability of macrophages was previously noted in co-culture with self-assembling skin equivalents¹³⁵, highlighting the importance of tissue environment in sustaining macrophage residency and longevity.</i>
	Reviewer 4 comments - major	Response to reviewer
4.1	The manuscript authored by Gopee et al. presents a study focusing on the role of immune cells in skin morphogenesis. To the best of my knowledge, this represents the first single-cell analysis of human prenatal skin, revealing intricate interplay among epithelial cells, dermal fibroblasts, and immune cells. The authors	We thank the reviewer for their positive comments and for highlighting the comprehensive nature of our prenatal skin cell atlas.

	created a comprehensive atlas of prenatal human skin utilizing advanced methods and tried to demonstrate that communication between immune and non-immune cells is crucial for the formation of hair follicles, scarless wound healing, and skin angiogenesis. In addition, they introduced a skin organoid model that mimics hair follicle development and provides insights into skin angiogenesis in the absence of immune cells by comparing it with prenatal skin. However, it seems highly descriptive without conceptual advancement in skin morphogenesis.	
4.2	Most of the data heavily relies on transcriptomic analysis, which may not adequately elucidate the intricate mechanodynamic processes underlying skin development, particularly in scarring. Transcriptional regulation of scarring is complex and subject to influence from the surrounding microenvironment.	We agree with the reviewer that the outcome of skin wounding as either scarless regeneration or scar formation results from an intricate and complex interplay of multiple factors including inflammatory response, fibroblast and extracellular matrix composition, growth factor profiles as well as mechanical forces and mechanosignaling (doi: 10.1007/978-3-030-44766-3_1, PMID: 21253544, PMID: 33888614). However, the interconnected processes leading to scarless healing have not been fully elucidated and have mainly been investigated in animal models (doi: 10.1007/978-3-030-44766-3_1). Our study aims to assess some of the mechanisms underpinning scarless repair in the human context and provides new insights into the potential roles of embryonic macrophages, early gestation-specific fibroblasts and differential inflammatory-prone priming of adult skin fibroblasts. Although mechanodynamic processes are important to address, investigating this in the context of human prenatal skin is extremely challenging.

	For instance, Mascharak et al. (Science 2021;372(6540)) demonstrated the interplay between mechanical forces, fibroblast subsets, and transcriptional regulation in tissue regeneration and scarring. A simplistic comparison between prenatal and adult skin, along with two-dimensional transcriptomic analysis, may prove insufficient in unraveling the underlying mechanisms and yielding novel insights into the scarless healing observed in prenatal skin.	In our revised manuscript we have performed new analyses by comparing human prenatal skin macrophages and fibroblasts with reindeer skin (antler skin with scarless healing capacity and back skin that lacks this capacity) as well as in vitro functional experiment (scratch assay) (please see point 1.9 for details). The findings from these analyses and experiments provide additional evidence for the role of prenatal fibroblasts and macrophages in potentially conferring scarless healing properties of human prenatal skin. Nevertheless, we acknowledge the limitations of our study particularly in not assessing mechanodynamic functions. We have toned down the claims regarding scarless healing throughout the manuscript as well as explicitly mentioned the need for further studies to fully unravel the multifaceted process of scarless healing in human prenatal skin in our discussion. The revised statements are as below: <i>Lines 653-661: A combination of fibroblast and macrophage associated molecular features, including the presence of fibroblast progenitors, a down-regulated immune milieu and reduced collagen expression potentially contribute to the ability of prenatal skin to heal without scarring. However, although skin fibroblast differentiation is incomplete by 16 PCW, progressive ‘ageing’ is already evident with gradual acquisition of the pro-inflammatory phenotype, characteristic of adult fibroblasts, beginning in utero from as early as 9 PCW. Future studies that align human fibroblast subsets across the human lifespan are required to investigate the dynamics of scarless healing and the roles of mechanical forces, microbiota and environmental exposure on fibroblast functions.</i>
4.3	In terms of hair biology, while the manuscript offers abundant and valuable information on the early development of human skin, the scientific breakthrough is minimal. The authors discuss the formation of dermal papillae, hair placode	We thank the reviewer for their comments. We would like to highlight that a lot of what is known about hair follicle (HF) development in humans has been inferred from mouse studies with a notable gap in research on primary human tissue which this study addresses. Our use of the established murine literature to identify similarities between human and mouse presents important advances to the field, and provides valuable information as noted by the reviewer, especially given that human development does not necessarily always recapitulate mouse development as exemplified in other organ systems such as immune and brain development (PMID: 14978070, PMID: 23583307, PMID: 35947979, PMID: 38020907).

induction, and their interplay during human skin development. However, these descriptions largely overlap with and are partly dependent on previous findings that have been extensively studied in a mouse model. The new atlas study merely confirms that similar processes take place in humans as well. It would be great if the study could identify human-specific features of embryonic skin development. As noted by the authors in the introduction, human skin is distinct from mouse skin in several ways, with differences in the coexistence of hair follicles and sweat glands, as well as regional variations in hair follicles (i.e., scalp vs. body). However, the current human embryonic skin atlas study fails to answer key questions in human skin biology, such as how hair follicles and sweat glands develop in the same location, how the regionally heterogeneous pattern of hair	To further identify human-specific features, we have conducted a detailed comparison of human and mouse skin fibroblasts (please see point 3.8 for details of analysis and results), demonstrating significant differences between human and mouse fibroblasts in their expression of key genes involved in HF development such as Wnt inhibition, epithelial signalling and immune cell recruitment. Overall, we find that for corresponding cell types in HF development, mouse HF fibroblasts are in a more advanced stage of differentiation compared to their human counterparts. Notably, this is in keeping with what we also observed for dermal fibroblast differentiation in human prenatal human skin compared to mice, likely attributable to differences in gestation lengths and tempo of differentiation, and highlights the limitations of mice as models for human skin development. We have expanded our comparison of human and mouse skin in the manuscript to include these findings (Lines 353-371, also copied in point 3.8). Although our study is the first to chart prenatal human skin and HF development using a multi-omic approach and identify important similarities and differences as above, we acknowledge that there are questions in HF and skin biology that remain to be explored, in part limited by the availability of human prenatal tissue. In the UK, it is not ethically possible to obtain human fetal samples over 20 PCW and later stage samples are scarce, making investigations about sebaceous glands difficult as they only begin to develop around 16 PCW and several samples from later gestation stages (over 18 PCW) would be required to provide enough cells for analysis. We already noted in our manuscript that sebaceous and apocrine gland cells are not detected in our data and have updated our discussion to include the need for future studies to address outstanding questions in skin and HF development. Lines 648-652: <i>Although we also note similarities between human and mouse in the signalling pathways co-ordinating hair follicle formation, our study further reveals key cross-species differences in the differentiation tempo of hair follicle mesenchymal cells. Future studies are required to systematically compare human and mouse prenatal skin to fully delineate the specific features that distinguish human skin development.</i>
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	follicles is established, and how human-specific features can be explained from an evolutionary perspective.	
4.4	A significant finding of the study is that macrophages play a crucial role in skin angiogenesis. However, as the authors have pointed out, macrophages have been implicated in various other contexts, such as embryonic tissues and adult wound healing and cancer, in their pro-angiogenic roles. Therefore, the impact of this finding is not surprising. The authors used a human skin organoid model that contained developing hair follicles to validate the pro-angiogenic roles of macrophages. However, the results presented in the figure (Fig 4j) were not quantified, which makes the argument invalid. In addition, the authors used an antibody (against CD206) to detect macrophages in skin organoids. However, the staining pattern does not look like immune	In our manuscript, we have investigated and demonstrated the pro-angiogenic role of macrophages in the human prenatal skin, particularly in the context of the physiological events associated with prenatal skin development. Previously reported pro-angiogenic roles of macrophages have been primarily focused on animal models, as the reviewer highlights, in various contexts such as embryonic tissue formation, wound repair and cancer. It is therefore important to identify if similar functions are indeed relevant in a human setting to guide the utility of animal models and how relevant these models are to human biology. The demonstration of a role for macrophages in supporting the development of human prenatal skin demonstrates the importance of macrophage functions during human development per se and distinct from their well characterised role in antigen presentation and immunity. In addition to describing the human prenatal skin development using cutting edge single-cell and spatial genomics approaches, we functionally validated the pro-angiogenic role of macrophages using a 3D iPSC-derived skin organoid model to mimic prenatal skin. In response to the reviewer's valid suggestions, we have now incorporated a thorough quantification of the vasculature in our skin organoid model cultured with and without macrophages (as demonstrated in reviewer point 1.6/Fig. 4h, i). To confirm the presence of macrophages after co-culture with the skin organoid, we performed immunofluorescence microscopy analysis with CD45 antibody in both whole-mount and cryosections of the skin organoid co-cultured with macrophages and the relevant control staining without macrophages (please see point 2.6) . We also characterised the phenotype of the macrophages we added to the culture by multi-parameter flow cytometry which shows the macrophages to be CD45⁺, CD14⁺, CD16⁺, CD206⁺ but CD1c⁻ (please see reviewer point 2.8).

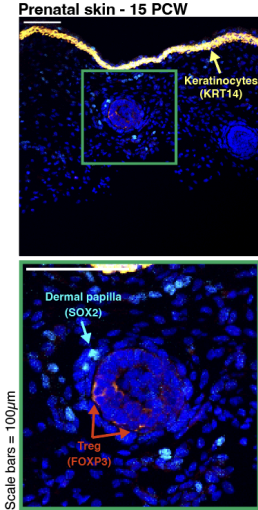
	cells, but fibroblasts or neurons. The existence of macrophages should be checked with other antibodies such as CD45, CD11b, LYVE-1, etc.	
4.5	Furthermore, the data regarding the effect of co-culture with macrophages on the formation of hair follicles should be presented properly. Even though the authors mentioned in the text that co-culture with macrophages did not affect the formation of hair follicles, no hair follicles were presented in Fig 4j and Extended Fig 10g.	We observed hair follicles consistently in the skin organoids co-cultured with macrophages and cultured without macrophages showing that the addition of macrophages did not affect hair follicle formation. We show the presence of hair follicles in the skin organoid without macrophages and co-cultured with macrophages in Reviewer Fig. 4.5 (below). Skin organoids - Day 95  With macrophages  Without macrophages Reviewer Fig. 4.5: Skin organoids cultured with (left) and without (right) macrophages. Hair pegs start appearing around day 70 of skin organoid differentiation and at day 95 (day 83 of co-culture), hair follicles are similarly present in both culture conditions.

4.6	The authors propose that the expression of 'immune activation' signatures in adult fibroblasts contributes to scarring, while 'immune suppression' profiles are associated with scarless healing. They further highlight that TMLM (TREM2+ macrophages) in prenatal skin express genes related to immunomodulation, suggesting that the interaction between fibroblasts and these immunosuppressive TMLM cells may contribute to scarless healing. However, it's important to note that the impact of inflammation on scarring and tissue remodeling is dual-sided. While inflammation stimulates the initial stages of scar formation, anti-inflammatory cytokines and the anti-inflammatory properties of macrophages play a role in fibrotic scar development in the later stages of scarring. Although TMLM and other subsets of macrophages may differ from those in adult skin,	We thank the reviewer for their comments. Indeed a dual role for macrophages has been described in wound healing, including pro-inflammatory functions during the initial inflammatory phase of wound healing followed by anti-inflammatory roles to promote tissue repair which eventually results in generation of a wound healed by scar formation (PMID: 21253544, PMID: 26635814, PMID: 26982353). However, it is worth highlighting that these processes pertain to postnatal wound healing. Wound healing in prenatal mammalian skin, in contrast, is consistently characterised by minimal inflammatory response as observed in animal models and in limited human studies (PMID: 155931, doi: 10.1007/978-3-030-44766-3_1, PMID: 21253544). Using our comprehensive atlas of human prenatal skin, we observed that prenatal skin fibroblasts downregulate inflammatory genes (such as <i>IL6</i>, <i>IL32</i>, <i>IFITM1</i>) compared to adult skin fibroblasts. Prenatal skin fibroblasts interact with early macrophages expressing immunomodulatory gene programmes and as such potentially contribute to the reduced inflammatory milieu in prenatal skin. To further substantiate our claims, we have compared prenatal human skin to an in-vivo model of reindeer skin where antler velvet skin heals without scarring whilst back skin scars (PMID: 36493752) (please see point 1.9 for details of the analysis and results). We demonstrate that early gestation fibroblasts in human prenatal skin resemble pro-regenerative reindeer fibroblasts whilst later gestation fibroblasts resemble pro-fibrotic fibroblasts. Critically, we show that macrophages in human prenatal skin align to 'early macrophages' in reindeer skin that interact with velvet skin fibroblasts to confer non-scarring competence and not to the macrophage clusters which populate reindeer back skin and contribute to scarring, lending further support to the role of macrophages in scarless healing in prenatal skin. We also assessed whether macrophages can enhance healing by performing an in vitro scratch assay of fibroblasts cultured with and without iPSC-derived macrophages, the results of which are detailed in point 1.9. Nevertheless, we acknowledge that further studies are still required to fully elucidate the role of macrophages in scarless healing. We have toned down the claims in the manuscript to reflect this
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	the evidence presented in the manuscript is insufficient to conclusively establish the role of macrophages in scarless healing in prenatal skin.	and explicitly mentioned the need for future studies. <i>Lines 484-490: Collectively, our findings suggest that prenatal skin fibroblasts in early gestation downregulate genes involved in extracellular matrix formation, collagen deposition and inflammation which may favour tissue regeneration over scarring. Based on our data and previous studies, we also posit a potential role for early skin macrophages in conferring the unique property of scarless healing in early prenatal skin. However, further studies are required to fully elucidate the interactions between macrophages and fibroblasts in human prenatal skin and conclusively establish their role in scarless healing.</i>
4.7	While the authors aimed to illuminate novel roles of immune cells in skin morphogenesis and scarring, the available data may not be robust enough to substantiate these claims. Besides transcriptomic analysis, there is a paucity of evidence to corroborate their conclusion. The imaging data, including RNAScope, reveals a scarcity of immune cells, raising questions about the potential impact of such a small population on the regulation of skin morphogenesis. To strengthen their argument, it would be advisable for the authors to employ organoid co-culture systems, akin to the	Immune cells have been described primarily in the context of immunity. Our previous work showed the early emergence of innate immune cells, primarily macrophages, during human embryonic development in the sterile in utero environment (PMID: 35549310). In the context of macrophages, the general view of these cells within the scientific community are as antigen presenting cells. However, our earlier studies showed that while the MHC II gene programme is upregulated at the end of the first trimester, these cells express many cytokines and chemokines from early gestation. In this manuscript, our unbiased exploration of scRNA-seq and spatial transcriptomics datasets from human prenatal skin provided evidence that innate immune cells, particularly macrophages, play a role in prenatal skin angiogenesis to support local tissue morphogenesis. To support our study findings we have performed orthogonal computational approaches and undertaken a significant amount of in situ spatial validation including at protein level (please see points 1.9, 2.5 and 3.25), in vitro assays (please see points 1.3, 1.9) and an iPS-derived skin organoid model (please see points 1.6 and 1.7), as well as comparisons between human prenatal skin cells with with populations from reindeer skin and mouse skin (please see points 1.9 and 3.8). The fact that immune cells in the early embryonic skin are rare relative to non-immune cells does not preclude an important role for these cells. There are many examples where rare cell types are critical for life, for example the rare haematopoietic stem cells that continue to replenish the human blood and immune cell repertoire for the entire human lifespan. Similarly, rare immune

	angiogenesis assay, in order to provide further support for the involvement of immune cells in skin development.	subsets are known to play a critical role in immunity. For example the rare conventional dendritic cells (cDC1), that account for 0.1-1% of all leukocytes in adult human blood and skin, are essential for cross-priming and cross-presentation to cytotoxic T cells, a process critical for immune response against intracellular pathogens and cancer. These rare cells are valuable therapeutic targets that are currently exploited in clinical settings. It is beyond the scope of this study to perform functional validations of all immune cell types in the human prenatal skin. This is further compounded by the limitations of human prenatal skin as a research tissue source, the difficulties of isolating primary immune cells in sufficient numbers directly from prenatal skin for functional studies, and the lack of established culture systems to readily generate prenatal immune cells in vitro apart from macrophages. To undertake the analysis of all immune cell types will require many years of research and cannot be performed in the timeline given to us to revise our manuscript. We have included statements that our human prenatal skin datasets and study findings will provide a rich resource for the research community to facilitate future research into the developing human skin and more broadly on human skin in health and disease.
4.8	The study provides correlative and suggestive evidence. The authors rely solely on single-cell RNA-seq data for their explanations and descriptions, without proper validation. To confirm their findings, the expression of key factors that they newly discovered and described should be tested using in-situ hybridization and/or immunostaining. It would be helpful to perform protein	We have performed several additional in situ validation experiments and included these findings in the revised manuscript as suggested by the reviewer. These include: 1. Close localisation of macrophages with fibroblasts in early prenatal skin by RNAscope analysis and IF (please see point 1.9) in support of their potential role in fibroblast maintenance and contribution to scarless healing in prenatal skin. 2. Similarly, in addition to previous RNAscope analysis included in the manuscript showing co-location of TML macrophages with endothelial cells (higher magnification images now included in Fig. 4a, please see point 3.29), we further show by whole-mount (with 3D rendering and image segmentation) and cross section immunofluorescence imaging of prenatal skin the close proximity of <i>LYVE1</i>⁺ macrophages to endothelial cells (please see

	expression analyses since RNA expression and protein expression do not always match. Furthermore, the authors inferred molecular interactions among cell populations using computational prediction tools, but it would be better if they functionally tested some of the interactions, particularly since a skin organoid system is available.	point 2.5 and Fig 4a). 3. We further confirm the protein level expression in prenatal skin (please see point 3.25) of key genes implicated in genetic skin disorders corresponding to expected expression pattern identified in scRNA-seq analysis including <i>KRT14</i> and <i>KRT1</i> across epidermis in prenatal skin whilst plectin (PLEC), type XVII collagen (<i>COL17A1/BP180</i>), type VII collagen (<i>COL7A1</i>) and laminin-332 (<i>LAMA3</i>, <i>LAMB3</i>, <i>LAMC2</i>) were mostly restricted to the basal layer in keeping with highest expression in basal epidermal cells (Extended data Fig. 5c, d, e). 4. The predicted ligand-receptors interactions between pre-dermal condensate and epidermal cells were validated by RNAscope (please see point 2.4). With respect to functional validation: 5. We expanded our skin organoid co-culture assay to further confirm the role of macrophages in angiogenesis (please see reviewer point 1.6 including Fig 4h). The analysis also demonstrates macrophages interacting with endothelial cells to enhance vascular network remodelling (please see reviewer point 1.6 including Fig 4i). Additional images of immunofluorescence of cryosectioned skin organoid co-culture demonstrate macrophages interacting with endothelial cells (please see reviewer point 2.6 including Fig. 4j) 6. We performed scratch assay on cultured iPSC-derived skin organoid fibroblasts - which we previously showed to resemble early prenatal skin fibroblasts (Extended Data Fig. 2d) - to assess healing with and without matched iPSC-derived macrophages. We observed improved wound closure in the presence of macrophages (Extended Data Fig. 7g, please see reviewer points 1.9 and 3.3 for more details).
	Reviewer 4 comments - minor	Response to reviewer

4.9	In line 74, "an epidermal hair sheath and a dermal root sheath" is corrected to "an outer root sheath and a dermal sheath".	This has now been amended in the manuscript text as below: Lines 82-83: <i>Subsequently, the keratinised hair shaft forms within the centre of the hair follicle, which is surrounded by an epithelial outer root sheath and a dermal sheath.</i>
4.10	In Fig 2d, Foxp3+ Treg cells are not visible (maybe just one dot?). To make the analysis convincing, it is highly recommended to use convincing immunofluorescence staining (IFS) to display intact tissue structure. Good IFS antibodies against FOXP3 (Tregs), SOX9 or Krt14 (outer root sheath), LEF1 or SOX2 (dermal papilla) are readily available.	We have performed immunofluorescence staining in the same fetal skin tissue at 15 PCW used for the RNAscope presented in Fig. 2d using antibodies for FOXP3, KRT14 and SOX2. The immunofluorescence stain demonstrates there are FOXP3⁺ cells (green, indicated by white arrows) close to the hair follicle structures in the skin as demarcated by the SOX2⁺ dermal papilla (yellow) and KRT14⁺ epithelial cells and outer root sheath (magenta), now included in Extended Data Fig. 3g (shown below). This is in keeping with our RNAscope analysis for <i>FOXP3</i>, <i>NDP</i> and <i>SLC26A7</i> hybridisation (see reviewer point 3.7).  Extended data fig. 3g. Immunostained human prenatal skin at 15PCW for Tregs with FOXP3 (red; red arrows), epithelial keratinocytes with Keratin 14 (yellow) and dermal papilla with SOX2 (cyan; cyan arrows). Scale bar 100µm.

4.11	To ensure easy comprehension by skin biologists and general readers, clear presentation of figures is crucial. For instance, the message conveyed by Fig 2j is difficult to comprehend.	We have modified Fig. 2k (shown below) by adding details for more clarity. We explain that the alignment of pseudotime trajectories between skin organoid and prenatal skin was inferred across 1369 transcription factors (TFs) by considering 15 equispaced (discrete) pseudotime points. These pseudotime points are plotted along the x- and y-axes of the heatmap for prenatal skin and skin organoid respectively, together with corresponding cell count composition (bar plots top and left). For each pair of skin organoid-prenatal skin pseudotime points, the total number of matching transcription factors is visualised by the colour scale: the highest number of matched TFs occur between prenatal skin pseudotime point 10 and skin organoid pseudotime point 12. The average mapping between pseudotime points is represented by the white line: the diagonal portion indicates 1-1 matches and the vertical (early pseudotime) and horizontal (late pseudotime) represent mismatches. This is now also illustrated in the right panel which shows the average alignment mapping across the 15 individual pseudotime points between prenatal skin and skin organoid with early and late mismatches. For details of methods underlying this trajectory alignment we performed using the Genes2Genes framework, please refer to https://doi.org/10.1101/2023.03.08.531713 (currently in press at <i>Nature Methods</i>). Overall, these results indicate that there are a large number of matching TFs between prenatal skin and skin organoid as the hair mesenchyme differentiates, although this process is initially delayed in pseudotime for the skin organoid. The observed early and late mismatches are largely driven by differences in HOX versus craniofacial development genes differentially expressed in prenatal skin and skin organoid, and are due to the different origins of their respective dermal cells as we discuss in our manuscript (below).
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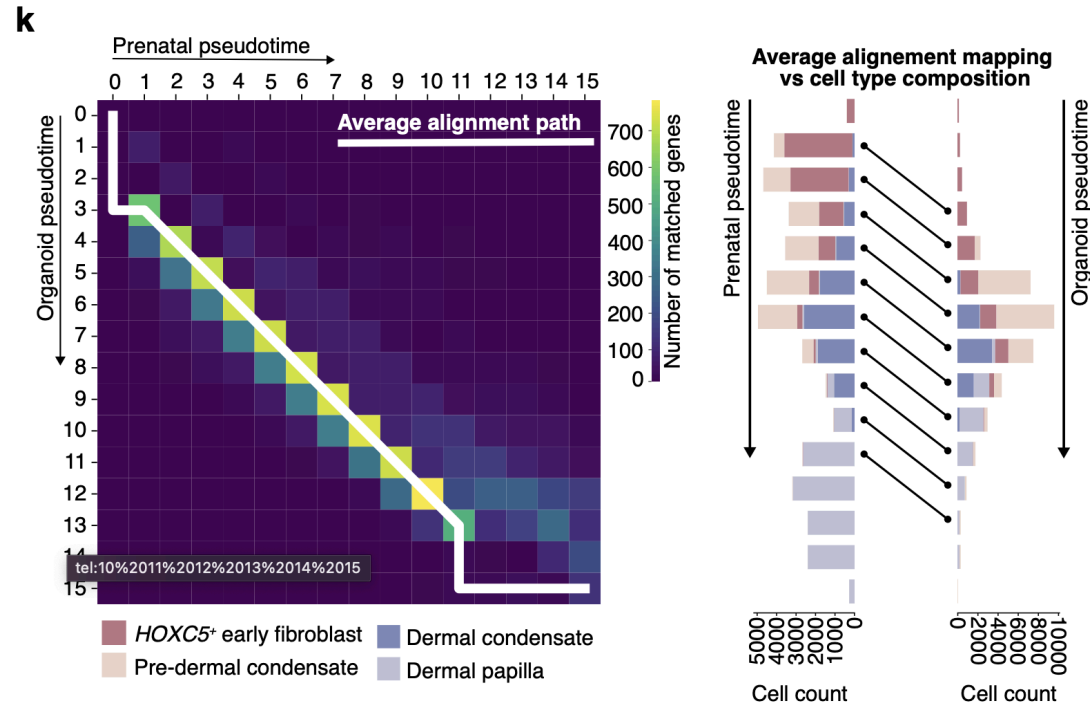


Fig. 2k: Alignment of pseudotime trajectories across 1369 TFs between in vitro skin organoid and in vivo prenatal skin data. Alignment was computed over 15 equispaced pseudotime points. The heatmap (left) represents the number of matching transcription factors (TFs) (colour scale) for each pair of organoid-prenatal pseudotime points along their trajectories. The overall alignment path (white line) represents the average mapping between skin organoid and prenatal skin across all the TFs along pseudotime: the best matched path (diagonal) accounts for matched pseudotime point pairs (37% to 57% of TFs); the vertical and horizontal lines represent mismatches. This is further reflected in the average alignment mapping visualised against the cell type composition in each pseudotime point (right). Bar plots represent the cell counts at each pseudotime point coloured by cell type.

		Lines 329-342: <i>We further evaluated the differentiation trajectory alignment between prenatal skin and skin organoid using the Genes2Genes analysis framework⁶³ to compare the expression of transcription factors (TFs) along the ‘hair fibroblast’ trajectory (HOXC5⁺ early fibroblast to dermal papilla). Overall, we observed a high number of matching TFs across pseudotime, signifying similar activated gene regulatory programs between prenatal skin and skin organoid during hair follicle fibroblast differentiation (Fig. 2k, Supplementary Table 9). TFs which were mismatched across pseudotime and which drove misalignment in early and late pseudotime were largely attributable to the different origins of dermal cells between prenatal skin and skin organoid. Mammalian patterning homeobox (HOX) genes governing trunk and limb development (HOXC6, HOXA7, HOXA10, HOXC11) were upregulated in prenatal skin⁶⁴⁻⁶⁶ (Extended Data Fig. 4h, i, Supplementary Table 9). In contrast, neuronal and craniofacial development genes (HMX1, BARX1, DLX1) were upregulated in skin organoids, in which dermal cells were derived from cranial neural crest differentiation¹⁵ (Extended Data Fig. 4h, i, Supplementary Table 9).</i>
4.12	There are multiple typos in figure numbering within the figure legend (e.g., Fig.2, where there are four (c)s in the legend, as indicated in Line 1564).	The legend labels for Figure 2 have now been amended as below: (b) Schematic of stages of hair follicle formation. (c) Bar plot showing average proportions of epidermal cell states across gestational age in prenatal skin. Bar colours represent cell states. (d) Large-area (left) and magnified peri-follicular (top right) and inter-follicular (bottom right) RNAscope images of prenatal skin (representative 15 PCW sample) demonstrating ORS (<i>SLC26A7</i>), matrix (<i>SHH</i>), dermal papilla (<i>NDP</i>) with Tregs (<i>FOXP3</i>) localising around the hair follicle. Scale bars=100µm and 20µm. (e) Inferred pseudotime trajectory of prenatal skin and skin organoid epidermal cell states differentiating along the ‘ORS/CL’ and ‘IRS’ trajectories; UMAP overlaid with PAGA and coloured by cell state (top) and overlaid with the cell directionality (shown in arrows) as inferred over the cell-to-cell transition probability matrix from running CellRank and coloured by

		pseudotime (bottom). Shown are connectivities (dashed) and transitions (solid/arrows). Threshold for Cellrank PAGA was kept at default.
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Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

In this revised manuscript, Gopee et al., have strengthened their conclusions by several new experiments including new scRNAseq datasets, improved marker stainings and repeat angiogenesis assay, and by additional deeper correlation analysis of their human embryonic skin and organoid data, improved figures, improved web platform, and clarifications on all our concerns with meaningful interpretations.

The new quantification of the 3D vascular networks in the skin organoids is strong. A concern here could be that CD45 is a pan immune marker and not macrophage specific, but the data now are convincing in terms of staining quality and immune cell detection in relationship to blood vessels.

The new analysis of co-localization of macrophages with fibroblasts in prenatal skin is also a major improvement.

The new trajectory analysis on the now integrated prenatal and skin organoid scRNA-seq is another strong improvement.

Overall, the authors have done a great job in this revised manuscript to address all my concerns in a rigorous way.

On a final note, many data I found useful were presented as REVIEWER FIGURES only and as such may as well be shown to the readers if the space permits in supplement.

Referee #2 (Remarks to the Author):

The majority of the points raised by the reviewer have been addressed. However, regarding point 2.7.: What was meant is the addition of macrophages and quantification of endothelial cells using different cell lines, not necessarily the skin organoid per se. If only one iPSc line is used and reproducibility cannot be shown in this manuscript with another donor, I would ask to remove statements like "benchmarked a skin organoid model", as stated in the abstract and the manuscript.

Furthermore, in the meantime, another human cell atlas was published (Wang et al. (2023), Cell 186, 4454–4471), showing that CD206/MRC1+ proangiogenic macrophages exist during human development in several tissues and that they become highly enriched for the expression of genes such as VEGFA, TNF, and CXCL8, genes associated with positive regulation of angiogenesis and vasculature development. Further, conditioned medium collected from cultured MRC1+CD38+ macrophages sorted from different tissues led to increased tube formation of endothelial cells in an in vitro assay.

These findings overlap with those in the present manuscript. Therefore, the authors should at least refer to the already published data in Wang et al. in the introduction and/or discussion.

Referee #3 (Remarks to the Author):

Major comment:

The authors have sufficiently responded to all major comments.

Minor comment:

Re: 3.9 - The authors note in the rebuttal that they down-sample to 200 cell per population and then also 5000 cells per population. The analyses using 200 cells per population are well described, but I cannot locate details describing why or how down-sampling to 5000 cells was performed. Please remove or clarify.

Referee #4 (Remarks to the Author):

Because the authors' responses are insufficient, the revised manuscript still has questions that have not been fully answered.

Comment on the response 4-2. Even with experimental challenges, it is difficult to justify not considering mechanodynamic conditions to confirm the scarless healing feature of prenatal skin. It is well known that skin in areas subjected to much tension creates abnormal hypertrophic scars. Moreover, mechanical stretching forces also influence macrophage phenotype, as already published in many papers. It is questionable how the authors can exclude the possibility that the scarless wound healing feature of the prenatal skin does not result from intrinsic characteristics mediated by the interaction between fibroblasts and macrophages but rather due to the extrinsic condition of the fetus not being exposed to mechanical forces. Moreover, experimental approaches involving the application of mechanical stretching force to organoid culture systems are feasible in the intestinal organoid system.

Comment on the response 4-3. By analyzing cross-species differences in human and murine prenatal skin development, the authors showed that pre-DC and DC cells exhibit differential gene expression patterns such as WNT inhibitors, immune cell recruitment, and epithelial signaling between two species. Particularly, the high expression of WNT inhibitors such as DKK1/2, SFRP2, CAV1, APCDD1 in human prenatal pre-DC and DC is quite surprising and eye-catching because the activation of WNT pathway is critical to induce hair follicle morphogenesis. The functional importance of WNT pathway in hair follicle morphogenesis and regeneration has been studied by many studies. For example, in a murine model, the overexpression of DKK1 or the deletion of beta-catenin in embryonic skin completely inhibited the initiation of hair follicle development by disconnecting interactions between DC and epithelium (PMID: 12015971, PMID: 11371349). The high expression levels of DKK1/2 and SFRP2 in human pre-DC and DC presented in this manuscript would propose an interesting potential mechanism of WNT-independent hair follicle development in human prenatal skin. As this finding is unexpected and has a potentially major impact, the authors should carry out validation experiments using ISH tests and a human skin organoid model.

Comment on responses 4.4 & 4.5. The authors showed that using human skin organoid culture, the

addition of macrophages promotes the maturation of vasculatures, confirming the previously known pro-angiogenic functions of macrophages. Macrophages are known to regulate sprouting angiogenesis, lumen formation, and proper vascular patterning, and the manuscript confirms this in the context of a human skin organoid model. In addition, given that hair follicle development is not affected by the absence and presence of macrophages and vascular maturation in the human skin organoid model, the impact of the study is marginal.

Author Rebuttals to First Revision:

We thank the reviewers for their positive and constructive feedback.

We provide our response which addresses every point raised by the reviewers (table below).

	Reviewer 1 comments	Response to reviewer
1.1	In this revised manuscript, Gopee et al., have strengthened their conclusions by several new experiments including new scRNAseq datasets, improved marker stainings and repeat angiogenesis assay, and by additional deeper correlation analysis of their human embryonic skin and organoid data, improved figures, improved web platform, and clarifications on all our concerns with meaningful interpretations. The new analysis of co-localization of macrophages with fibroblasts in prenatal skin is also a major improvement. The new trajectory analysis on the now integrated prenatal and skin organoid scRNA-seq is another strong improvement. Overall, the authors have done a great job in this revised manuscript to address all my concerns in a rigorous way.	We thank the reviewer for their positive comments and highlighting the improvements on the previous manuscript to significantly strengthen the findings and conclusions of our study. We have addressed the outstanding comments below.
1.2	The new quantification of the 3D vascular networks in the skin organoids is strong. A	We thank the reviewer for stating that our data on the 3D vascular network and macrophage staining is convincing. We performed extensive staining of skin organoids with CD45 to identify

	concern here could be that CD45 is a pan immune marker and not macrophage specific, but the data now are convincing in terms of staining quality and immune cell detection in relationship to blood vessels.	macrophages, as the only immune cell population present in the skin organoid. We have also analysed the skin organoid extensively via single cell sequencing across 3 iPSC lines and never found de novo formation of immune cells in the skin organoid.
1.6	On a final note, many data I found useful were presented as REVIEWER FIGURES only and as such may as well be shown to the readers if the space permits in supplement.	We will endeavour to include as many panels as possible within the permitted space. Additionally, we plan to submit our rebuttal to the reviewers' comments as a supplementary file as per the journal transparent peer review option which will include the reviewer figures as well.
	Reviewer 2 comments	Response to reviewer
2.1	The majority of the points raised by the reviewer have been addressed. However, regarding point 2.7.: What was meant is the addition of macrophages and quantification of endothelial cells using different cell lines, not necessarily the skin organoid per se. If only one iPSc line is used and reproducibility cannot be shown in this manuscript with another donor, I would ask to remove statements like "benchmarked a skin organoid model", as stated in the abstract and the manuscript.	The skin organoid data used for comparison and benchmarking against prenatal skin was derived from two different cell lines (WA25 human embryonic stem cell line and desmoplakin-mEGFP human induced pluripotent stem cell line) (PMID: 32494013). We have removed the term 'benchmarked' from the summary and manuscript as requested.
2.2	Furthermore, in the meantime, another human cell atlas was published (Wang et al. (2023), Cell 186, 4454–4471), showing that CD206/MRC1+ proangiogenic macrophages exist during human development in several tissues and that they become highly enriched	We have updated the manuscript to refer to the publication from Wang et al (PMID: 37703875). Lines 390-392: <i>Furthermore, macrophages expressing proangiogenic genes have been observed in diverse tissues during human development (PMID: 30455424, PMID: 37703875).</i>

	for the expression of genes such as VEGFA, TNF, and CXCL8, genes associated with positive regulation of angiogenesis and vasculature development. Further, conditioned medium collected from cultured MRC1+CD38+ macrophages sorted from different tissues led to increased tube formation of endothelial cells in an in vitro assay. These findings overlap with those in the present manuscript. Therefore, the authors should at least refer to the already published data in Wang et al. in the introduction and/or discussion.	Lines 459-466: <i>In this study, we characterise the dynamic composition of human prenatal skin during the early stages of de novo HF formation and highlight the critical skin immune and non-immune crosstalk contributing to skin morphogenesis, in line with emerging evidence in animal and human studies (PMID: 36922638, PMID: 37703875). Our atlas indicated that macrophages contribute to scarless skin repair, fibroblast homeostasis and neurovascular development. This is in part contributed to by yolk-sac derived TML macrophages which seed prenatal skin and gonads, suggesting broader functions for these cells outside of the central nervous system in early gestation. The presence of TML macrophages have been previously identified in several prenatal organs (PMID: 37590359, PMID: 37703875, PMID: 35794482).</i>
	Reviewer 3 comments - minor	Response to reviewer
3.1	Re: 3.9 - The authors note in the rebuttal that they down-sample to 200 cell per population and then also 5000 cells per population. The analyses using 200 cells per population are well described, but I cannot locate details describing why or how down-sampling to 5000 cells was performed. Please remove or clarify.	For the previous reviewer comment, we performed bootstrapping of CellphoneDB analysis with downsampling 3 individual times. It was also questioned if the downsampled cells were an adequate representation of the total cell populations. While rare populations in prenatal skin are likely adequately represented with 200 cells per population, we investigated whether downsampling to 5,000 cells per population would improve the reproducibility of more frequent cell populations in response to the reviewer's comment. Our analysis showed that the same relevant interactions were identified with downsampling to 200 or 5,000 cells per population respectively for the rare and frequent populations. Therefore, we assessed that downsampling 3 times to 200 cells per population adequately represents even the more frequent cell types (Methods section, lines 1283-1297).
	Reviewer 4 comments	Response to reviewer

4.1	Because the authors' responses are insufficient, the revised manuscript still has questions that have not been fully answered.	
4.2	Comment on the response 4-2. Even with experimental challenges, it is difficult to justify not considering mechanodynamic conditions to confirm the scarless healing feature of prenatal skin. It is well known that skin in areas subjected to much tension creates abnormal hypertrophic scars. Moreover, mechanical stretching forces also influence macrophage phenotype, as already published in many papers. It is questionable how the authors can exclude the possibility that the scarless wound healing feature of the prenatal skin does not result from intrinsic characteristics mediated by the interaction between fibroblasts and macrophages but rather due to the extrinsic condition of the fetus not being exposed to mechanical forces. Moreover, experimental approaches involving the application of mechanical stretching force to organoid culture systems are feasible in the intestinal organoid system.	We do not disregard the role of external mechanical forces to scar formation or lack thereof as one of the mechanisms contributing to scarless healing in prenatal skin. This has already been shown in previous studies in human and other mammalian skin (PMID: 6611757, PMID: 17504973, PMID: 21606834, PMID: 21776006, PMID: 24804638) and thought to be primarily driven by fibroblast-mediated mechanisms rather than macrophage-related stretching (PMID: 21776006). Our study electively focuses on the intrinsic aspects of prenatal skin fibroblasts and their immune interactions which may confer scarless healing potential, based on findings of co-location of prenatal skin fibroblasts and early macrophages. However, we fully acknowledge, in the discussion, that external factors such as mechanical forces and environment need to be considered. Furthermore, prenatal skin changes from a pro-regenerative to reparative, pro-fibrotic phenotype whilst in utero itself without exposure to external mechanical forces as mentioned by the reviewer. This underscores the contribution of intrinsic features to scarless repair irrespective of external mechanical stress.
4.3	Comment on the response 4-3. By analyzing cross-species differences in human and murine prenatal skin development, the authors showed that pre-DC and DC cells exhibit differential gene expression patterns such as WNT inhibitors, immune cell recruitment, and epithelial signaling between	As discussed in our previous response to points 4.3 and 3.8, the differences observed between human and mouse pre-Dc and Dc requires further exploration and validation which is beyond the scope of this study focused primarily on prenatal human skin. We have noted these limitations and need for future studies in our manuscript (Lines 475-478). The validation experiments suggested by the reviewer are indeed interesting but would be challenging to complete within a short time frame. We believe that these experiments are more suited for future studies, where a dedicated effort can be made to thoroughly investigate the

	two species. Particularly, the high expression of WNT inhibitors such as DKK1/2, SFRP2, CAV1, APCDD1 in human prenatal pre-DC and DC is quite surprising and eye-catching because the activation of WNT pathway is critical to induce hair follicle morphogenesis. The functional importance of WNT pathway in hair follicle morphogenesis and regeneration has been studied by many studies. For example, in a murine model, the overexpression of DKK1 or the deletion of beta-catenin in embryonic skin completely inhibited the initiation of hair follicle development by disconnecting interactions between DC and epithelium (PMID: 12015971, PMID: 11371349). The high expression levels of DKK1/2 and SFRP2 in human pre-DC and DC presented in this manuscript would propose an interesting potential mechanism of WNT-independent hair follicle development in human prenatal skin. As this finding is unexpected and has a potentially major impact, the authors should carry out validation experiments using ISH tests and a human skin organoid model.	observed differences between murine and human hair follicle development and the relevance of the expression of DKK1/2 and SFRP2 in human pre-DC and DC.
4.4	Comment on responses 4.4 & 4.5. The authors showed that using human skin organoid culture, the addition of macrophages promotes the maturation of vasculatures, confirming the previously known pro-angiogenic functions of macrophages. Macrophages are known to	In the previous reviewer response 4.4, we discussed the significance of our study to extend knowledge through its focus on human prenatal skin, and additionally assessing the distinct pro-angiogenic roles of different macrophage subsets. In terms of the organoid literature, the implications of our study are even more noteworthy, given that successful co-culture of organoids with immune cells have only been shown in very few models so far and vascularisation of tissue organoids remains challenging as previously discussed. Our successful co-cultures demonstrate persistence of macrophages for at least 5

	regulate sprouting angiogenesis, lumen formation, and proper vascular patterning, and the manuscript confirms this in the context of a human skin organoid model. In addition, given that hair follicle development is not affected by the absence and presence of macrophages and vascular maturation in the human skin organoid model, the impact of the study is marginal.	weeks and their contribution to vascular network formation in a skin organoid model. This provides a major step forward to producing skin organoids incorporating immune cells as a valuable experimental model for researchers.
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