Single-cell RNA sequencing of human nail unit defines RSPO4 onychofibroblasts and SPINK6 nail epithelium

Hyun Je Kim, Joon Ho Shim, Ji-Hye Park, Hyun Tae Shin, Jong Sup Shim, Kee-Taek Jang, Woong-Yang Park, Kyung-Hoon Lee, Eun Ji Kwon, Hyung-Suk Jang, Hanseul Yang, Jong Hee Lee, Jun-Mo Yang & Dongyoun Lee

Research on human nail tissue has been limited by the restricted access to fresh specimen. Here, we studied transcriptome profiles of human nail units using polydactyly specimens. Single-cell RNAseq with 11,541 cells from 4 extra digits revealed nail-specific mesenchymal and epithelial cell populations, characterized by RSPO4 (major gene in congenital anonychia) and SPINK6, respectively. In situ RNA hybridization demonstrated the localization of RSPO4, MSX1 and WIFI in onychofibroblasts suggesting the activation of WNT signaling. BMP-5 was also expressed in onychofibroblasts implicating the contribution of BMP signaling. SPINK6 expression distinguished the nail-specific keratinocytes from epidermal keratinocytes. RSPO4+ onychofibroblasts were distributed at close proximity with LGR6+ nail matrix, leading to WNT/β-catenin activation. In addition, we demonstrated RSPO4 was overexpressed in the fibroblasts of onychomatricoma and LGR6 was highly expressed at the basal layer of the overlying epithelial component, suggesting that onychofibroblasts may play an important role in the pathogenesis of onychomatricoma.
The nail unit is one of the major skin appendages. It is comprised of epithelial and subjacent mesenchymal elements. The epithelial components include the nail matrix, nail bed, and nail plate. The nail plate is composed by maturation and keratinization of the cells from the nail matrix and attached to the nail bed, which may also contribute to the nail formation.

The nail and hair units are both hard keratin generating organs that share many commonalities with respect to their embryogenesis, anatomy, and co-involvement in various diseases. The hair unit has been more extensively studied due to easier access to research materials compared to the nail unit, and it produces hair via coordinated interactions between specialized mesenchyme cells known as dermal papilla and epithelial stem cells. We have been studying the nail unit to identify analogous mesenchymal-epithelial interactions, specifically investigating the specialized mesenchymal portion of the nail unit which we propose are responsible for communicating with nail stem cells.

In line with this endeavor, we identified a CD10-positive mesenchymal cell population underneath the nail matrix and nail bed and proposed to name these specialized mesenchymal cells as onychoblasts. We also documented the presence of a specialized onychoblast-containing mesenchyme that demonstrates histologic and immunohistologic features that are distinguishable from another part of the nail unit. Based on these findings, we hypothesized that onychoblasts may play an important role in nail formation and growth through epithelial-mesenchymal interactions.

In an attempt to obtain a holistic view of the potential functions of onychoblasts, we profiled transcriptomes of nail units from four fresh human polydactyly specimens using single-cell RNAseq (scRNAseq). We also investigated the gene expressions in nail-specific epithelial and mesenchymal cells.

**Results**

**Single-cell RNAseq of human nail units.** We strategically enriched single cells from the nail matrix and portions of the nail unit containing onychoblasts by removing volar skin, distal phalangeal bone and nail plate (Fig. 1a) since single cells from the non-nail unit could weaken the nail-specific signatures in the dataset. A total of 11,541 cells with a median of 2354 genes per cell from 4 polydactyly samples (515–4023 cells/sample; Supplementary Fig. 1a, b; Supplementary Table 1) were profiled. None of the polydactylous patients reported other phenotypes or mutations. Uniform manifold approximation and projection (UMAP) plot reflected 18 conserved clusters (Fig. 1b, c; Supplementary Fig. 1c). Canonical markers clearly demarcated each cell cluster (Fig. 1d): KRT1 or KRT5 for keratinocytes; COL1A1 for fibroblasts; RSGB5 for myofibroblast-like cells; VWF or endothelial cells; LYVE-1 for lymphatic endothelial cells; PTPRC for lymphocytes; AIF1 for macrophage/dendritic cells; TPSB2 for mast cells; SGC1B2P for eccrine gland cells; DEFB1 for eccrine duct cells; COL9A2 for chondrocytes; NRRX1 for neural cells, and PAMEL for melanocytes.

The full list of differently expressed genes associated with each cell-type cluster is presented in Supplementary Data 1 and Supplementary Fig. 1d. Three to five supervised gene sets representing each cluster deduced from literature search are summarized in Supplementary Fig. 2.

**Subclustering of keratinocytes identifies the nail-specific cell population (onychofibroblasts).** To further analyze the heterogeneity of nail unit fibroblasts, we extracted the fibroblast cluster and performed sub clustering analysis (Fig. 2a). Based on our previous observation of positive MME (CD10) expression on the onychofibroblasts within the onychodermis, we investigated the expression of MME amongst the different fibroblasts clusters. Violin and feature plot demonstrated a distinct cluster of fibroblasts (Fibroblast 4 cluster) demonstrating a high MME expression level. Hence, we defined this cluster as onychoblasts. Next, we sought to investigate the expression of RSPO4, a member of the R-spondin family of a secreted protein involved in WNT signaling as it is implicated in congenital anonychia. We observed that the onychoblast cluster also demonstrated a high expression of RSPO4 (Fig. 2b). We also found additional genes that were strongly and specifically expressed in the onychoblast cluster including MSXI, TWIST1, CRABP1, WIFI1, and BMP5 (Fig. 2c). Gene Ontology enrichment analyses showed that genes involved in skeletal system development, ossification, and extracellular structure organization are significantly enriched in the onychoblast (fibroblast 4 clusters) compared to fibroblast 1–3 clusters (Fig. 2d).

**In situ RNA hybridization confirms gene expression patterns of onychoblasts revealed by scRNAseq.** To confirm the expression of RSPO4 in the nail unit, we performed in situ RNA hybridization (ISH) on polydactyly samples. ISH demonstrated RSPO4 mRNA expression restricted to mesenchymal cells below the nail matrix and nail bed with no expression in dermal fibroblasts elsewhere around the nail unit (Fig. 2e). Adult nail biopsy also showed onychoblasts expressing RSPO4 mRNA within the onychodermis (Fig. 2f).

**Subclustering of keratinocytes identifies the nail-specific cell populations.** Next, we focused on the epithelial cell populations to define nail-specific keratinocytes responsible for nail formation. A total of six epithelial subpopulations were identified from polydactyly specimens (Fig. 3a). Basal keratinocytes were defined by the expression of KRT5 and KRT14, suprabasal keratinocytes by KRT1 and KRT10 expression, proliferating keratinocytes by MKI67 expression, and cornified cells by SPRR1B expression.

We further identified two keratinocyte subpopulations in the polydactyly specimens demonstrating high expression of SPINK6 (Supplementary Fig. 5a) and WNT6 (Fig. 3b). To address whether these keratinocyte subpopulations are nail-specific, we obtained transcriptome data for 2758 single keratinocytes from a published cohort of skin samples and integrated it with a polydactyly dataset (Fig. 3b; Supplementary Fig. 6a, c; Supplementary Table 2). As an expression of these latter two genes was rarely observed in the keratinocytes derived from the skin samples, we thought these keratinocyte subpopulations are nail-specific (Fig. 3b; Supplementary Fig. 5a). SPINK6+ keratinocytes mainly expressed KRT16 suggesting that they might be derived primarily from nail bed. These major cell types of the nail unit including onychoblasts were also represented in the individual analysis (Supplementary Fig. 7).
**Fig. 1** scRNAseq of human nail unit reveals conserved cell populations. 

- **a** An overview. Four extra-digits were dissociated into the single-cell suspension ($n = 11,541$ cells).
- **b** A UMAP plot demonstrated 18 clusters.
- **c** Relative proportions of each cell-type color-coded by cell-type and annotated for clusters as in Fig. 1b.
- **d** Cluster annotation. The violin plot represents an expression of canonical markers of each cluster.
Immunostaining and ISH confirm gene expression patterns of nail-specific keratinocytes identified by scRNAseq. To validate the localization of SPINK6 positive nail keratinocytes, immunohistochemistry (IHC) was done on the sections of normal nail units. IHC showed SPINK6 expression in the nail epithelium while its expression was not found in the non-nail unit epidermis (Fig. 3c). Considering the SPINK family’s roles as a protease inhibitor in keratinocyte differentiation, SPINK6 might have a role in maintaining epithelial barrier in the nail unit. Since SPINK6 was reported to be expressed on normal human skin, we conducted Microarray and qPCR analyses to investigate whether nail epithelium expresses a much higher level of SPINK6 compared to normal skin. As scRNAseq data suggested, we confirmed a specifically higher expression of SPINK6 in nail
epithelium than normal skin epithelium (Supplementary Fig. 5b, c). We initially thought that WNT6 expression is specific to the nail keratinocyte of polydactyl since structural anomaly of WNT6 gene is linked to polydactyly syndrome. However, WNT6 was expressed in both the polydactyly samples and adult nail matrix suggesting WNT6 expression on keratinocytes is nail-specific.

**LGR6 demarcated nail-specific stem cells.** Next, we tried to investigate the clusters harboring nail stem cells. We explored LGR6 expression on nail-specific keratinocytes since LGR6 has demonstrated to be a marker for nail stem cells in mice. By comparing LGR6 and WNT6 expressions amongst the two nail-specific keratinocyte subpopulations, we found that they were limited to the Keratinocyte-1 cluster, implying that it was mainly isolated from nail matrix (Fig. 3d). Also, ISH studies showed LGR6 was mainly expressed in the basal layer of nail matrix just above the RSPO4 expressing mesenchyme (Fig. 3e–g). LGR6 expression was strong in the basal layer of the proximal nail matrix. WNT6 expression extended into higher levels of the nail matrix epithelium (Fig. 3h).

**Network analysis predicted interactions between onychofibroblasts and basal layer of nail matrix epithelium.** Localization of LGR6 expression prompted us to analyze whether interactions between LGR6 expressing basal keratinocytes and RSPO4 positive onychofibroblasts in the subjacent mesenchyme exist. To assess the potential roles of LGR6 and RSPO4 in the epithelial–mesenchymal interactions of the nail unit, we used NichNet to predict interactions between various receptors and ligands hypothesized in having roles in modulating nail development and regeneration. NichNet predicted significant interactions between the RSPO4 onychofibroblasts and LGR6 at the basal layer of nail matrix epithelium (Fig. 4a). The interactions were further supported by a ligand-target link between RSPO4 and the LEF1 gene, a downstream target of RSPO4 (Fig. 4b). To validate whether RSPO4 and LGR6 expression correlate with the activation of Wnt/β-catenin signaling, we performed IHC for β-catenin and LEF1 (Fig. 4c, d). We observed an increase of nuclear localization β-catenin in the nail matrix epithelium, indicating the activation of β-catenin signaling (Fig. 4e). This was also highly expressed in the nucleus of lower layers of the nail matrix (Fig. 4d).

RSPO4-positive cells were observed in a zone of connective tissue immediately beneath the nail matrix. Considering the findings of our previous studies as well as this study, a zone of connective tissue containing RSPO4-positive cells right below the nail matrix should be included in the nail-specific dermis. Thus, we propose to expand the definition of the onychodermis containing onychofibroblasts to include this latter area, as delineated in Fig. 4e.

**Clinicopathologic and molecular characteristic of onychomatricoma.** Next, we searched for pathologic conditions arising from the specialized nail tissue we defined. Onychomatricoma is a tumor arising within or in the vicinity of the nail matrix (Fig. 5a–c). We hypothesized that onychomatricoma is arising from onychofibroblasts and evaluated CD10 expression in onychomatricoma (Fig. 5f, h). We found that CD10 was strongly expressed in the mesenchymal part of the tumor. In addition, RSPO4 was highly expressed in the fibroblast of onychomatricoma and an increased density of these fibroblasts compared to the normal nail unit, suggesting WNT signaling contributes to onychomatricoma histogenesis (Fig. 5g). To further investigate the involvement of WNT signaling in the pathogenesis of onychomatricoma, we tested the expression patterns of other WNT-related molecules. We found that LGR6 was highly expressed at the basal layer of the overlying epithelial component (Fig. 5d). Also observed was WNT6 expression on the epithelial component of the onychomatricoma (Fig. 5e). BMP5, MSX1, and WIF1 were expressed on the fibroblasts of onychomatricoma (Fig. 5j). Furthermore, IHC analyses for β-catenin and LEF1 showed strong nuclear β-catenin and LEF1 expressions in the epithelial cells of onychomatricoma (Fig. 5i). Together, our histological analyses suggest that WNT signaling is a key molecular characteristic of onychomatricoma.

**The functional importance of onychofibroblasts in the nail epithelial-mesenchymal interaction.** To understand the importance of RSPO4 in the nail matrix keratinocytes (NMKs), we sought to characterize transcriptomic changes induced by the RSPO4 treatment in cultured NMKs using RNAseq analysis. Bioinformatic analyses showed that in the comparison with control NMKs, 26 differentially expressed genes (DEGs) were identified in the NMKs treated with RSPO4 1000 ng/ml, with 18 upregulated and 8 downregulated genes (P value < 0.05 and absolute fold change > 2; Fig. 6a; Supplementary Table 3; Supplementary Data 2). The six replicates of RSPO4 1000 ng/ml treated, RSPO4 200 ng/ml treated, and control NMKs were analyzed by a hierarchical clustering and we found that RSPO4 treated groups showed the upregulation of WNT signaling associated genes including LGR6 and FOXQ1 (Fig. 6b, c). By comparing the transcriptomes of onychomatricoma with the nail matrix biopsy sample, we observed similar trends of the changes of WNT signaling associated genes, LGR6 and FOXQ1. These results suggest that epithelial hyperplasia in onychomatricoma...
was potentially induced by overexpression of \textit{RSPO4} in its mesenchyme.

In addition, we treated cultured NMKs with BMP-5 or WIF-1, which are highly expressed by onychofibroblasts (Supplementary Fig. 8). RNAseq analysis identified 181 DEGs for BMP5 100 ng/ml treated NMKs versus control NMKs, and 153 DEGs for WIF-1 1000 ng/ml treated NMKs versus control NMKs (Supplementary Fig. 8). There was no significant difference in \textit{LGR6} expression.

Comparison of human nail unit with human hair follicle. To gain a deeper understanding of nail biology, we compared our
Fig. 3 Subclustering of keratinocytes derived from polydactyly identifies the nail-specific cell populations. a UMAP of keratinocyte reveals six distinct clusters. b The violin plot shows an expression of nail-specific markers (SPINK6 and WNT6) in keratinocytes from the publicly available skin dataset and polydactyly dataset. c Immunohistochemical staining reveals a strong expression of SPINK6 in the nail epithelium. Scale bar = 50 μm. d Feature and violin plot represent KRT16 expression on nail keratinocyte-2 (nail bed) and, WNT6 and LGR6 on nail keratinocyte-1 (nail matrix). eISH of LGR6 on polydactyly tissue (with a magnified view of nail matrix indicated by dashed-black box on the right). Scale bar = 100 μm. f Additional ISH of boxed area in e (inset 2) using mRNA probes for RSPO4. RSPO4 expression is subjacent to LGR6+ basal layer of nail matrix epithelium. Scale bar = 20 μm. gh no-probe control. Scale bar = 100 μm. IISH of WNT6 on adult nail matrix tissue and polydactyly tissue. Scale bar = 20 μm. ISH in situ RNA hybridization.

Discussion

In this study, we revealed transcriptome profiles of human nail units by polydactyly samples at a single-cell resolution. By sub clustering fibroblasts and keratinocytes respectively, we identified one nail-specific mesenchymal cell population and two nail-specific epithelial cell populations. 

Nail-specific mesenchymal cell population (onychofibroblast cluster) which were characterized by MM1 (CD10) expression demonstrated high expression of RSPO4. The expression of RSPO4 in the mesenchymal cells below the nail matrix and nail bed in polydactyly and adult nail unit was confirmed by ISH. RSPO4 is a member of the R-spondin family of secreted protein which is involved in Wnt/β-catenin signaling.25 Previously, RSPO4 expression was localized to developing mouse nail mesenchyme, suggesting a crucial role in nail morphogenesis.26 In addition, a gene encoding RSPO4 in humans is mutated in inherited anonychia or hyponychia.27 Thus, our data demonstrate that RSPO4 expression in onychofibroblasts is crucial for human nail development and maintenance. We also found additional genes that were strongly and specifically expressed in the onychofibroblast cluster including MSX1, TWIST1, CRABPI, WIFI, and BMP5 (Fig. 2c). Mice with a homozygous deletion of MSX1 exhibited a complete cleft palate and failure of tooth development.28 In humans, MSX1 mutation is associated with orofacial clefting and tooth agenesis.29 A nonsense mutation in MSX1 causes Witkop syndrome, also known as tooth and nail syndrome.30 MSX1 participates in the regulation of WNT signaling of induction of WNT antagonist DKK1, DKK2, DKK3, and SFRP1.31 We also observed the expression of DKK1 in chondrocytes (Supplementary Fig. 2). TWIST1 is recently spotlighted as a regulator of cancer-associated fibroblast.32 This basic helix-loop-helix transcription factor is also essential for the development of mesodermally derived tissues including muscle and bone. TWIST1 mediated fibroblast activation implicates that onychofibroblasts are metabolically active. CRABP1 is known to be expressed on the dermal papilla of developing hair.33 Murine scRNAseq results dissecting fibroblast heterogeneity in skin demonstrated CRABP1+ fibroblasts were enriched subjacent to the epidermis, while CRABP− fibroblasts were primarily localized to the deeper dermis.34 These reports are comparable with our findings in the nail unit, with positive CRABP1 gene expression in onychofibroblasts and our localization of onychofibroblasts also in the juxta-epithelial areas. WIFI is known as WNT signaling regulator and a potential cause of a Nail-Patella-like disorder.35 And BMP5 is reported to regulate murine keratinocyte stem cells.36 Taken together, high expression of RSPO4 and expression of other genes related to nail abnormalities in onychofibroblasts suggest that onychofibroblasts play an important role in nail formation and growth through epithelial-mesenchymal interactions.

Two nail-specific epithelial cell populations demonstrated high expression of SPINK6 and WNT6. The expression of SPINK6 and WNT6 in the nail-specific epithelial cells was confirmed respectively by IHC and ISH. Previously, SPINK6 was found to be expressed in the stratum granulosum of human skin and in the skin appendages, and it was identified as a selective inhibitor of human Kallikrein-related peptidases in human skin.17,37 In addition, SPINK6 expression was found in the claw region of mouse.38 However, in quantitative RT-PCR analysis of our study SPINK6 expression in the epidermis was too low compared with the nail matrix. In addition, by IHC SPINK6 expression was not found in the epidermis. Considering SPINK family’s roles as a protease inhibitor, SPINK6 might have a role in maintaining epithelial barrier in the nail unit. In the past, Wnt6 expression was reported in the developing mouse skin.39 This is the first report of WNT6 expression in the nail matrix and nail bed of the nail unit. In our study, we found that LGR6 was mainly expressed in the nail matrix that overlies the onychofibroblasts. LGR6 was strongly expressed in the basal layer of proximal nail matrix. Previously, it was reported that nail stem cells reside in the proximal nail matrix of mice.40 In addition, LGR6 were expressed in the nail matrix and demonstrated to be a marker for nail stem cells in mice.19

Further network analysis using NicheNet predicted significant interactions between RSPO4 secreted by onychofibroblasts with LGR6 positive nail matrix epithelium. A link was also found between RSPO4 and the LEF1 gene, a downstream target of RSPO4. These findings suggest interactions between RSPO4 secreted by onychofibroblasts with LGR6 in the nail matrix, possibly resulting in activation of the Wnt/β-catenin signaling. Indeed, functional activation of β-catenin-LEF1 was confirmed in the nail matrix just above the RSPO4 expressing mesenchyme (Fig. 4c, d). These results are consistent with findings in previous murine studies evaluating mechanisms underlying epithelial-mesenchymal interactions in the nail unit.41 To identify genes regulated by onychofibroblasts in nail matrix epithelium, we profiled the gene expression changes following RSPO4, WIFI, and BMP5 treatment in cultured NMKs. We hypothesized that RSPO4 protein might induce activation of LGR6 positive nail matrix epithelium. Strikingly, RSPO4 treatment induced the increase of LGR6 expression, whereas LGR6 expression was not influenced by BMP5 or WIFI treatment. Notably, we also
observed overexpression of \textit{FOXQ1} which is considered as the marker for WNT signaling activation in solid tumors\textsuperscript{23}. These results highlight the potential role of \textit{RSPO4} in the nail development through WNT signaling.

Onychomatricoma is a rare benign tumor of the nail unit, usually located at or around the nail matrix\textsuperscript{42}. Although its name implies a tumor of the nail matrix epithelium, it is a fibroepithelial tumor with both epithelial and mesenchymal elements (Fig. 5), and thus the concept of epithelial onychogenic tumor with onychogenic mesenchyme was introduced\textsuperscript{43}. Indeed, we observed very strong expression of \textit{RSPO4} and \textit{LGR6} in the mesenchymal cells and epithelial components of the tumor,
**Fig. 4** Network analysis predicted interactions between onychofibroblasts and basal layer of nail matrix epithelium. Schematic diagram of human nail unit was described. 

**a** NicheNet analysis between ligands in fibroblast clusters and receptors in the basal layer of nail matrix epithelium. Greenline represents the connections involving onychofibroblasts and non-onychofibroblasts. Blue line represents the connection specifically involving onychofibroblasts. RSPO4 in onychofibroblasts specifically connected to LGR6. 

**b** Network analysis reveals interaction between ligands in onychofibroblast clusters and WNT pathway target genes. 

**c, d** IHC showing expression of β-catenin and LEF1. Cells with strong nuclear β-catenin staining can be observed in the suprabasal layers of proximal nail matrix epithelium (arrow). 

**e** Schematic outline illustrating the location of RSPO4⁺ onychofibroblasts in the onychodermis and LGR6⁺ keratinocytes in the human nail unit. Scale bar = 100 μm. ISH in situ RNA hybridization, IHC immunohistochemistry.

---

**Fig. 5** Clinico-histopathologic findings of onychomatricoma. 

**a** Clinical images of onychomatricoma. 

**b** Surgical pictures of onychomatricoma. 

**c** Representative image of H&E from surgical resection. 

**d, e** ISH of LGR6 and WNT6 on onychomatricoma. 

**f** IHC of CD10. 

**g** ISH of RSPO4. 

**h** Dual RSPO4 ISH and CD10 IHC. 

**i** Representative IHC showing expression of β-catenin (left) and LEF1 (right), markers for Wnt/β-catenin signaling. Cells with nuclear β-catenin staining are found in the epithelial region of onychomatricoma. 

**j** ISH of WIF1, MSX1, and BMP5 on onychomatricoma. Scale bar = 100 μm. ISH in situ RNA hybridization, IHC immunohistochemistry.
respectively. Of note, we also observed strong nuclear β-catenin staining, implying translocation of cytosolic-β-catenin into the nucleus, and high expression of LEF1, a transcriptional coactivator of β-catenin, in the epithelial cells of onychomatricoma44. Previously, we suggested that onychofibroblasts may be involved in the pathogenesis of the onychomatricoma22. Interestingly, we observed a significant upregulation of LGR6 in the RSPO4 treated NMKs (Fig. 6c). The expression pattern observed in this study suggests a possible link between RSPO positive onychofibroblasts and overexpression of LGR6, which could lead to dysregulation of Wnt/β-catenin signaling in onychomatricoma. These findings are in line with previous studies that RSPO proteins could lead to a significant epithelial proliferation in the gastrointestinal tract45,46. Collectively, we suggest onychomatricoma is
a nail-specific tumor with mesenchymal origin from onychoblasts, rather than a nail matrix epithelium-limited process, and propose an alternate term onychoblastoma for this entity.

As the nail unit and hair follicle share numerous similarities, we hypothesized that the onychoblasts could be the nail counterpart of follicular dermal papilla. We demonstrated that the follicular dermal papilla cells showed high expression of RSP04 suggesting the possibility they may be counterpart of onychoblasts. However, there appears to be a spatial gap between cells expressing RSP04 and cells harboring its putative receptors (LGR4–6). Additional studies are needed to elucidate whether and how RSP04 secreted by dermal papilla interact with ligands in the hair epithelium.

In summary, through integrated analysis of molecular data and IHC staining, we demonstrated nail-specific mesenchymal and epithelial cell populations, which are characterized by RSP04 and SPINK6, respectively in human nail unit. We identified that RSP04+ onychoblasts are situated at close proximity with the LG6+ nail matrix, which is linked to WNT/β-catenin activation. We demonstrated evidence of analogous findings in the mesenchymal and epithelial components of onychomatrichoma, a pathologic hyperproliferative state involving the nail matrix, and we conclude that onychomatrichoma is a tumor which derives substantial portion of its origination from onychoblasts. Altogether, this study highlights the importance of the interaction between onychoblasts and LG6+ nail matrix epithelium, which contribute the nail formation and growth via WNT/β-catenin signaling pathway. Although further analysis will be needed to identify RSP04-mediated downstream signaling responsible for the precise controlling of the nail homeostasis, our findings shed light on molecular basis of the nail biology.

Methods

Patient cohort(s) and sample preparation. All samples were collected under Institutional Review Board (IRB)-approved consent (IRB number: SMC 2017-10-137). All patients provided written informed consent. Extra-digits from 4 patients (6–12 months) were delivered to the dermatologic clinic immediately after the digit removal. All samples containing nail mesenchyme and epithelium were dissected, minced, and suspended within 3 h of digit removal. Specimens were transferred to freshly dissociated solution composed of 120 μL of Liberase TL (2 mg/mL; Sigma Aldrich) and 840 μL phosphate-buffered saline (PBS) and incubated at 37 °C for 45 min. The cells were collected through 70-μm cell strainer (#352340, Corning) and stored on ice. The tissue was transferred into dissociation solution for a second round of dissociation as mentioned above followed by dissociation in Tryspin solution (350 μL PBS, 50 μL 0.25% Trypsin). Cells were washed once and re-suspended in 50–200 μL of freshly prepared PBS–bovine serum albumin (BSA) (1× PBS and 0.04% BSA). Cell viability was assessed using LUNA-FL dual fluorescence cell counter (Logos biosystemics).

For the onychomatricoma samples, tissues were obtained from two patients after surgical resections. The diagnosis was confirmed on the basis of pathologic review and clinical examination.

scRNAseq data for the skin and hair dataset were obtained from two independent published cohort of five human skin samples32 and two human scalp samples33. Additional details regarding these patient cohorts are presented in Supplementary Table 2.

Single-cell capture, library preparation, and sequencing. Single-cell dissociates were loaded into the Chromium system (10x Genomics) targeting 5000 cells. The Chromium Single Cell 3′ Reagent Kit V2 (10x Genomics) was used to generate scRNAseq libraries, according to the manufacturer’s instructions. Barcoded sequencing libraries were generated using Chromium Single Cell 3′ v2 Reagent Kits and sequenced across by a HiSeq 4000 platform targeting 25,000 reads per cell.

scRNAseq data align and quality control filtering. Sequencing data were aligned to the human reference genome (GRCh38) and processed using the CellRanger 3.1.0 pipeline (10x Genomics). The raw gene expression matrix from the CellRanger pipeline was filtered, normalized using the Seurat package version 3.1.1 in R version 3.6.0 software (R Foundation for Statistical Computing, Vienna, Austria) and selected according to the following criteria: cells with >500 unique molecular identifier (UMI) counts; and <6500 genes; and <30% of mitochondrial gene expression in UMI counts. In order to integrate multiple single-cell datasets and correct for batch effects, we performed the standard integration protocol described in Seurat v3. Briefly, we performed standard preprocessing (log-normalization) and integration using FindIntegrationAnchors() implemented in Seurat v3 and 40 canonical correlation analysis dimensions to identify the integration anchors between our datasets. A total of 11,541 remaining cells were enrolled in the final analysis.

Clustering and single-cell RNA sequencing analysis. The integrated data were analyzed with a graph-based clustering and visualization with Seurat R package. The 2000 most variable genes of the integrated dataset were used as input. ScaleData() function and RunPCA() function were used for scaling and principal component analysis (PCA), respectively. We clustered the cells with the FindNeighbors() and FindClusters() functions. The resolution and PCA dimensions were selected variably per sample origin; resolution 0.6 and ‘dims’ = 140 for polydactyly samples, resolution 0.7 and ‘dims’ = 120 for polydactyly and hair and LG6 samples, resolution 0.4 and ‘dims’ = 120 for public skin samples. After unsupervised clustering, we ran RunUMAP() with 40 PCA dimensions to visualize a multi-dimensional dataset. Differentially expressed genes (DEGs) for each cluster were identified using FindAllMarkers() function with default parameters. To determine cell types, we compared top DEGs between each cluster using cell type-specific markers known from a literature review (Supplementary Fig. 2).

For the second-level clustering, we isolated the keratinocyte cluster and fibroblast cluster in the broad cell type UMAP, respectively. We further subclustered these cell types based on the expression of RSP04+ and FindClusters() functions at resolution 0.6 with 20 PCA dimensions. Average log-normalized expression profiles for keratinocyte-associated genes were calculated using the AverageExpression() implemented in Seurat packages. For the gene ontology (GO) analyses, lists of genes differentially expressed between fibroblast (1–3) and onychomatricoma clusters were calculated using FindMarkers () with default parameters. These genes were further selected with a P value < 0.05 and average log (fold change) > 0.4. GO analysis was performed with Metascape web tool (www.metascape.org)37.

Ligand-receptor interaction analysis. To investigate the epithelial-mesenchymal interactions potentially contributing to nail development and regeneration, we employed the ligand–receptor interaction tool NicheNet20. LG6+ nail matrix cells (basal layer of the nail matrix) were isolated from the nail keratinocyte-1 (nail matrix) cluster using LG6x expression level. To determine genes expressed in each cluster, we used the definition used by of Puram et al.40. We defined the gene set of interest as the genes differentially expressed within interacting cell population. FindAllMarkers() function with default parameters was used to calculate the DEGs and these genes were further selected with a P value < 0.01 and average log (fold change) > 0.4. Ligand activities were predicted using the predict_ligand_activities() function implemented in NicheNet, and the top 30 ligands were selected by Pearson correlation coefficient. The upper 20% of the ligand-target links according to the regulatory potential scores were selected to be visualized in circos plot to avoid making a circus plot with too many ligand-target links.

RNA in situ hybridization (ISH). Total of six independent polydactyly, five nail biopsy, one onychomatricoma and two hair bearing scalp samples were included in this study. ISH for mRNA expression was performed on three micrometers-thick formalin-fixed paraffin-embedded of tissue samples. Staining was performed using the Leica Bond Rx autostainer with probes for RSP04 (NM_01029871.3), MSX1 (NM_002448.3), BMP5 (NM_021073.2), LGR6 (NM_001017404.1), and LGR5 (NM_003667.2). All probes used for ISH were obtained from Advanced Cell Diagnostics (Newark, CA). Probes were detected using the RNAscope 2.5 LS Reagent Brown Kit (cat#32100; Advanced Cell Diagnostics).

Immunohistochemical staining. Samples were sectioned longitudinally parallel to the long axis of the nail plate or transversely. Hematoxylin- and eosin- (H&E) stained slides of the patients were reviewed to determine the most comprehensive section. Immunohistochemical staining was done using a monoclonal antibody against CD10 (clone 56C6; Novocastra, Newcastle, UK), SPINK6 (ab110850; Abcam, Cambridge, UK), β-catenin (1:4; Cell Marque, CA) or LGR5 (ab121732; Abcam, Cambridge, UK). For IHC, tissue sections were deparaffinized with xylene and rehydrated with ethanol. To block endogenous peroxidase in the tissue sections, the slides were treated with hydrogen peroxide in methanol. Sections were incubated in a blocking solution (Protein block, DAKO) to prevent nonspecific antibody binding. The sections were then incubated with primary antibody for a few hours at room temperature. The slides were washed in phosphate-buffered saline, followed by antigen detection using DAKO EnVision System and diaminobenzidine or Fast Red for visualization. The slides were counterstained with hematoxylin solution.

Microarray. Two polydactyly samples were used for this experiment. Epithelial tissue was separated from dermal tissue after disperse treatment. Then nail epithelium and epidermis were obtained respectively. RNA microarray analysis was performed to measure gene expression levels. Expression profiling analysis was performed using Agilent Oligo Microarray Kit 8x60K according to the Agilent
One-Color Microarray-based Gene Expression Analysis Protocol (Agilent Technologies). Statistical significance of the expression data were determined using LPE test and fold change in which the null hypothesis was that no difference exists among groups. False discovery rate was controlled by adjusting p value using Benjamini–Hochberg algorithm.

Quantitative real-time RT-PCR. Four polydactyly and two cadaveric samples were used in this study. Nail epithelium and epidermis were obtained respectively. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). SPINK6 gene-specific PCR primers (forward primer, 5′-ACC TCA GCT GGA CAA AGC AG-3′; and reverse primer, 5′-TGG CAA GTG ACC AAG AAA CA-3′) were designed following the previous study published17. The expression levels of AG-3 among groups. False discovery rate was controlled by adjusting p value using FDR.

For the Seurat computational pipeline, we used seurat clustering standard work (Illumina Technologies). Statistical significance of the expression data were determined using LPE test and paired Wilcoxon test was used for comparison of differences in means values were two-sided. As stated above, IHC experiments and RNA ISH were performed in 1 passage five or six were plated in triplicate in six-well plates. Recombiant human RSPO4, BMP-5, or WIFI-1 (R&D systems) were treated, respectively in KGM without rhEGF. After 48 h of culture, NKM was treated with 1 mL of Trizol to isolate RNA for further bulk RNAseq experiments.

RNA isolation and bulk RNA-seq analysis. Total RNA was extracted and the libraries were prepared using the TruSeq Stranded Total RNA Sample Prep Kit with Ribo-Zero H/M/R (Illumina Technologies, San Diego, CA, USA). The concentration and integrity of extracted RNA was evaluated by Nanodrop 8000 UV-Vis spectrometer (Nanodrop Technologies Inc., Wilmington, DE, USA). Total 1 μg of RNA molecules were pooled for cDNA synthesis and these cDNA libraries were qualified with the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). After cluster amplification of denatured template, the sequencing of each library was performed as150-bp paired-end using Illumina Novaseq6000 (Illumina Technologies, San Diego, CA, USA).

The RNA-sequencing reads were mapped to the human reference genome (hg19) with STAR and gene expression level was quantified using RNA-seq by expectation–maximization10. Normalized gene counts were transformed to log2 counts per million (logCPM) with the R package, edgeR31. DEGs were identified using the R package, edgeR53.

Statistics and reproducibility. All statistical analyses were performed using R3.6.0 software (R Foundation for Statistical Computing, Vienna, Austria). Statistical analyses of the scRNA-seq data (n = 4, see Supplementary Table 1) were performed using the CellRanger and Seurat packages in R. The Mann–Whitney U test and paired Wilcoxon test was used for comparison of differences in means using R. A P value less than 0.05 was considered to indicate a statistically significant difference for all comparisons. All reported P values were two-sided. As stated above, IHC experiments and RNA ISH were performed in 1–6 replicates per genes.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The scRNAseq data that support the findings of this study has been deposited in the Gene Expression Omnibus (GEO) under accession code GSE158970. The scRNA-seq data from previously published cohorts are available at the GEO under the following accession number: GSE12611 and GSE130973. Any other data are available from the corresponding author upon reasonable request.

Code availability
For the Seurat computational pipeline, we used seurat clustering standard work flow (https://satijalab.org/seurat/vignettes.html). The codes generated during this study are available at Github repository (https://github.com/SMC-Derma/scRNAseq_NailMatrix).
34. Guerrero-Juarez, C. F. et al. Single-cell analysis reveals fibroblast heterogeneity and myoid-derived adipocyte progenitors in murine skin wounds. Nat. Commun. 10, 1–17 (2019).
35. Jones, M. C. et al. Mutation of WiFi: a potential novel cause of a Nail-Patella-like disorder. Genet. Med. 19, 1179–1183 (2017).
36. Kangsamanik, T. & Morris, R. J. Bone morphogenetic protein 5 regulates the number of keratinocyte stem cells from the skin of mice. J. Investig. Dermatol. 131, 580–585 (2011).
37. Kanytka, T. et al. Inhibition of kallikrein-related peptidases by the serine protease inhibitor of Kazal-type 6. Peptides 32, 1187–1192 (2011).
38. Fischer, J. et al. Characterization of Spink6 in mouse skin: the conserved inhibitor of kallikrein-related peptidases is reduced by barrier injury. J. Investig. Dermatol. 134, 1305–1312 (2014).
39. Reddy, S. et al. Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. Mech. Dev. 107, 69–82 (2001).
40. Takeo, M. et al. Wnt activation in nail epithelium couples nail growth to digit regeneration. Nature 499, 228–232 (2013).
41. Saito, M., Ohyama, M. & Amagai, M. Exploring the biology of the nail: an intriguing but less-investigated skin appendage. J. Dermatol. Sci. 79, 187–193 (2015).
42. Baran, R. & Kint, A. O. Filamentous tufted tumour in the matrix of a funnel-shaped nail: a new entity (report of three cases). Br. J. Dermatol. 126, 510–515 (1992).
43. Perrin, C. et al. Onychomatricoma in the light of the microanatomy of the normal nail unit. Am. J. Dermatopathol. 33, 131–139 (2011).
44. Kim, K. A. et al. R-Spondin proteins: a novel link to beta-catenin activation. Cell Cycle 5, 23–26 (2006).
45. Kim, K. A. et al. R-Spondin family members regulate the Wnt pathway by a common mechanism. Mol. Biol. Cell 19, 2588–2596 (2008).
46. Zhou, Y. et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat. Commun. 10, 1–10 (2019).
47. Puram, S. V. et al. Single-cell transcriptomic analysis of primary and metastatic tumor ecosystems in head and neck cancer. Cell 171, 1611–1624. e1624 (2017).
48. Naga, H., Nakamishi, H., Urano, Y. & Arase, S. Serial cultivation of human nail matrix cells under serum-free conditions. J. Dermatol. 22, 560–566 (1995).
49. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21 (2013).
50. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140 (2010).

Acknowledgements
This work was supported by NRF-2018R1D1A1A02086103.

Author contributions
D.L conceived and designed the study; J.S.S., K.-H.L., J.-H.P., and K.-T.J contributed to the sample acquisition and patient recruitment. H.J.K and J.-H.P. processed the samples. J.H.S. and D.L. analyzed and interpreted the data. J.-M.Y., E.J.K., H.T.S., W.-Y.P., H.Y., H.-S.L., and J.H.L. edited the paper. H.J.K., J.H.S., and D.L wrote the paper with the input from other authors. All authors read and approved the final paper.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42003-021-02223-w.

Correspondence and requests for materials should be addressed to D.L.

Peer review information Communications Biology thanks the anonymous reviewers for their contribution to the peer review of this work. Peer Reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.