

Title of Thesis

Mechanisms of Dermal Type I Collagen Formation in the Axolotl Skin

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General introduction

The skin is the outermost layer of the body and the largest organ in humans, playing essential roles in maintaining life, including protection of the internal environment, thermoregulation, secretion, and sensory perception. Structurally, the skin is composed of three layers: the epidermis, dermis, and hypodermis. In mammals, the epidermis consists of four layers from the body surface: the stratum corneum, granular layer, spinous layer, and basal layer. Most of these layers are formed by epidermal keratinocytes, while the basal layer contains pigment cells (melanocytes) and skin stem cells. Epidermal cells are known to maintain the barrier function against the external environment through continuous turnover. This turnover is driven by the proliferation of skin stem cells in the basal layer, which pushes the upper epidermal layers toward the surface. The mammalian dermis is composed of two layers, the papillary dermis and the reticular dermis, which consist of extracellular matrix (ECM) and fibroblasts. The papillary dermis is the site of hair follicle formation and is characterized by relatively low ECM density. In contrast, the reticular dermis is located beneath the hair follicles and exhibits a higher density of ECM. The dermal ECM is composed of collagen fibers (type I collagen), elastic fibers (elastin), and ground substances such as hyaluronic acid, and the properties of these components determine the physical characteristics of the skin. Between the epidermis and dermis lies the basement membrane, a sheet-like structure composed of type IV collagen and laminin. This basement membrane connects the epidermis and dermis and enables communication between them, thereby contributing to the maintenance of skin homeostasis. The hypodermis is located beneath the dermis and contains extensive networks of lymphatic vessels, blood vessels, and nerves, functioning as the structural foundation of the skin. Thus, the skin fulfills indispensable roles in life by the coordinated interaction of multiple tissues. Among these layers, the dermis is primarily responsible for the mechanical strength and elasticity of the skin.

Among the ECM components of the dermis, type I collagen is the most abundant and plays a central role in determining the physical properties of the skin, accounting for approximately 70–80% of the dermal matrix. Type I collagen is a triple-helical molecule composed of two $\alpha 1$ chains and one $\alpha 2$ chain. Each chain is highly conserved across vertebrate species, from fish to birds, and is characterized by a repeating Gly–X–Y amino acid motif unique to collagen. The triple-helical structure is assembled intracellularly and subsequently secreted into the extracellular space, where collagen molecules undergo cross-linking to form fibrils that construct the dermal matrix. Dermal type I collagen exhibits a highly ordered organization across diverse animal species. This highly organized arrangement is thought to determine the physical properties of the skin and generate its characteristic texture.

The ordered structure of dermal type I collagen not only determines mechanical strength and elasticity but is also closely associated with wound healing and regenerative processes. When the skin damaged deep injury, excessive accumulation of type I collagen leads to the formation of scar tissue. Scar tissue exhibits a structure distinct from the highly ordered architecture of normal dermis. Moreover, scar tissue lacks skin appendages such as hair follicles and sweat glands, resulting in loss of skin function and impairment of vital physiological processes. Therefore, regeneration of the skin, particularly the dermal structure, without scar formation is of critical importance. However, because the dermis is located deep within the skin, fluorescence-based observation is technically challenging, and our understanding of dermal structure remains limited. In particular, the mechanisms by which the highly ordered collagen structure of the dermis is formed, as well as how this structure changes during aging, remain unclear. Elucidating these processes is essential not only for understanding skin biology but also for advancing regenerative medicine. In this study, I investigated the formation (development) and aging of dermal structure using the axolotl, which

exhibits high skin transparency, in combination with a novel fluorescent probe that specifically labels type I collagen.

Chapter 1: Keratinocyte driven dermal collagen formation in the axolotl skin

This chapter is based on the following publication: Ohashi et al., 2025, *Nature Communications*. Minor modifications were made for the purpose of the doctoral thesis.

Introduction

The skin is the largest organs of the human body, consisting of two layers: the epidermis and the dermis. The dermis is primarily composed of type I collagen, accounting for 40% of total protein in the skin ¹. In most tetrapods, dermal collagen cannot be restored to its original fine structure once its structure becomes malformed. Such structural changes cause wrinkles and sagging if caused by aging; however, they can lead to excessive collagen secretion and scar formation, resulting in skin dysfunction, if they occur as a result of skin damage ². Therefore, maintaining the normal structure of dermal collagen is important, both cosmetically and medically. Despite the importance of collagen, the process of skin collagen formation in the skin has not been thoroughly elucidated. Clarifying the process of dermal collagen formation is essential for understanding and solving various problems related to skin collagen.

Dermal fibroblasts are considered to play a major role in the formation of extracellular matrix (ECM) by secreting type I collagen, a major component of the dermal ECM structure. The human dermal structure comprises two layers: the papillary dermis and the reticular dermis, and each layer displays specific collagen structures. The papillary dermis has a low-density collagen structure with fragmented fibers arranged in a helical pattern, whereas the reticular dermis has a high-density collagen structure, which fibers woven in a regular and orderly lattice pattern. Maintaining the fine structure in each layer is considered important to keep the skin healthy and beautiful. Although research has been conducted on mature skin ^{3,4}, much remains unknown regarding the construction of dermal collagen.

Axolotls provide an excellent research environment for dermatology, particularly for elucidating the formation of dermal collagen. Much dermatological research has been conducted *in vivo* and *in vitro* ^{5,6,7}. Mature mammalian skin has a thick epidermis and melanocytes, making it difficult to observe the deeper dermis and to detect fluorescent signals in the living state. Human or mouse cultured cells cannot fully mimic dermal collagen structure, despite recent developments in skin-equivalent technology. Axolotl skin has relatively higher transparency, which aids in research involving the detection of fluorescent signals. Thus, the axolotl is an ideal model animal by which to investigate dermal collagen development.

Research in axolotl skin has been conducted in association with skin regeneration ^{8,9,10}.

Like that of mammals, the skin of axolotls is composed of epidermis and dermis ¹¹. The structure of the dermal collagen in axolotls consists of by two layers with different structures: the *stratum spongiosum* (SS) and the *stratum compactum* (SC). The SS consists of columnar collagen fibers that extend in the apical-basal direction, whereas the SC has orthogonal lattice-like collagen fibers. Formation of the SC involves cells with lattice-like filopodia in the creation of this collagen structure ¹². The structures within SS and SC can be reconstructed, even after damage. However, there are limits to their regenerative abilities, and the orthogonal lattice-like collagen fiber structure of the SC cannot be completely regenerated after damage in mature axolotls ¹³. Moreover, regulation of matrix metalloproteinase (MMP) expression by keratinocytes is crucial for the reformation of collagen structure without scar formation ⁹. A study comparing skin regeneration and skin development in the axolotl showed that several similarities exist between regeneration and development ¹¹. Although axolotl skin and its regeneration have been widely studied, little is known about the detailed mechanism by which the dermal collagen structure is formed. This knowledge is essential for understanding the development of dermal collagen structures in tetrapods and the complete skin regeneration mechanism in axolotls.

In this work, we investigate the transition of dermal collagen structure formation in the axolotl skin dermis using fluorescent probes recently reported by Kuroda et al., 2024 ¹⁴. We investigate skin in animals of four different body sizes. We find that keratinocytes secrete collagen and supply it to the dermis, showing a lattice-like collagen structure in smaller animals. In larger animals, lattice-patterned cells and mesenchymal cells participate in formation of the dermis. The lattice-patterned cells contribute to both collagen production and collagen fiber modification. We also find that the invading mesenchymal cells help separate the upper and lower layers of the axolotl dermal collagen, resulting in the establishment of the three-layered axolotl dermal structure. Finally, we investigate whether collagen production by keratinocytes is a conserved mechanism among species. Collagen production by epidermal cells is confirmed not only in axolotls but also in zebrafish, chickens, and mouse embryos. The conservation of collagen expression by epidermal cells across species indicates that collagen production by epidermal cells is key to the formation of lattice-patterned collagen structures.

Results

Axolotl dermal collagen structure

To elucidate the mature dermal collagen structure of axolotl, we first examined the entire dermal collagen structure of axolotls with a body size of 12 cm (Fig. 1). We used the fluorescent collagen probes DAF-FM DA (hereafter DAF) and DAR-4M (hereafter DAR) to visualize axolotl collagen. These fluorescent probes specifically stain type I, II, and IX collagen and elastin¹⁴. However, elastin may not be encoded in the reported axolotl genome, and Victoria blue staining for elastin did not generate significant staining in axolotl skin (Fig. 2a–e). Furthermore, immunofluorescence for COL2 revealed no COL2 production in the dermis (Fig. 2f–h). Because COL9 function to crosslink COL2^{15,16}, it is unlikely that COL9 is produced in the absence of COL2. Additionally, we observed a fine overlapping signal between the DAR signal and the GFP signal from TgScl (mPrrx::GFP-axCOL1a2) transgenic axolotls (Fig. 2i). Therefore, we consider that the signals from DAF and DAR are specific to type I collagen as long as we focused on the axolotl skin. Fig. 1 shows the results for the mature dermis layer. The collagen structure of the axolotl dermis was seen to be composed of three layers: the two layers of collagen structures (SS and SC) that were previously reported^{9,13}, and a newly classified layer of collagen structure, named *stratum baladachinum* (SB) (Fig. 1c, d, d3). The SB is located underneath the epidermis (Fig. 1a–d). The structure of the collagen fibers in SC, SS, and SB is shown in Fig. 1d1–d3. SC has a dense, lattice-patterned collagen structure as previously reported¹² (Fig. 1d1). SS has a low density, and the collagen fibers run in the apical-basal direction (Fig. 1b, d2). SB has a lattice-like structure similar to that of SC (Fig. 1d3). However, the lattice-like structure in SB is less dense and rougher than that of SC. These observations clarify that the axolotl skin structure is composed of three layers—SS, SC, and SB—and that each layer has a characteristic collagen fiber structure.

Development of the dermal collagen structure in axolotls

To investigate the developmental changes of the dermal collagen structure in axolotls, we examined the entire skin collagen fibers using histological staining (Masson's trichrome staining) on limbs of axolotls with a body size ranging from 5 cm to 12 cm. In the 5-cm-long animals (5^{cm} dermis), a thin collagen layer was observed beneath the epidermis (Fig. 3a). In the 8-cm-long animals (8^{cm} dermis), the collagen layer was thicker than that of the 5^{cm} dermis (Fig. 3b), and cells were observed to be invading in the collagen layer (Fig. 3b). In the 10-cm-long animals (10^{cm} dermis), we observed the presence of SS-like structures (Fig. 3c). In the 12-cm-long animals (12^{cm} dermis), mature dermal glands became apparent, and the three-layered structure of SB, SS, and SC could be clearly visualized (Fig. 3d).

Next, we investigated dermal collagen development by immunofluorescent analysis using

anti-type I or anti-type III collagen antibodies (Fig.3e–t). It has been reported that human dermal ECM contains large amounts of type III collagen as well as type I collagen, which plays an important role in maintaining dermis structure¹⁷. However, the changes in COL3 expression during dermal development have not been fully clarified. In the 5^{cm} dermis, we observed a single layer of collagen (Fig.3e, m, q). We named this single collagen layer, which emerges temporarily during dermal collagen development, the *stratum coniunctum*. COL3 signal was observed in the lower basal part of the single collagen layer (Fig.3i, m, q). Progression to 8^{cm} dermis resulted in an increase in collagen thickness and the presence of cells within the *stratum coniunctum* (Fig.3f, n, r). As in the 5^{cm} dermis, COL3 signal was found in the lower basal part of the single collagen layer (Fig.3j, n, r). In 10^{cm} dermis, more cells could be observed inside the collagen layer (Fig.3o, s). At this stage, the separation of the three dermal layers became apparent (Fig.3g, o, s). We observed that COL3 signals were present not only in the lower basal part of the dermis but also within the SS-like structure (Fig.3k, o, s). In 12^{cm} dermis, we observed that the mature dermal collagen structure with fully formed SS, SB, and SC layers (Fig.3h, p, t). COL3 was present in SS and the layer basal part of SC (Fig.3l, p, t). Next, we measured the thickness of the dermal collagen layer (Fig.3u), which confirmed that the thickness of the axolotl dermis increases as the animal develops. These analyses revealed that the axolotl dermal collagen develops from the monolayered structure to the triple-layered structure.

When observing axolotl dermal development, we found that numerous mesenchymal cells were involved in the collagenous structure. We counted the number of nuclei existing in the COL1⁺ collagen layer (Fig.3v). In the *stratum coniunctum* (5^{cm} dermis), there were almost no nuclei but the number of the nucleus was increased as the skin developed. In the electron microscopic observation, we found the cells invading into the dermal collagen layer (Fig.4a), where they appeared to digest collagen fibers to integrate the cell body into the dermal collagen. This suggests that the mesenchymal cell invasion starts from the 8^{cm} dermis and settles at the 10^{cm} dermis stage.

We further confirmed whether the SB was part of the dermal collagen structure or not. We performed double staining for COL1 and COL4, which are components of the basement membrane (Fig.3w, x). The *stratum coniunctum* and the SB are distinct from the COL4⁺ basal membrane existing underneath the epidermis (Fig.3w, w', x, x'). This indicates that the *stratum coniunctum* and the SB are the newly reported dermal collagen layer existing underneath the basement membrane.

Cells in the axolotl dermal collagen

Dermal collagen structures are known to be formed by dermal fibroblasts. From our previous studies, we identified the dermal fibroblasts that produce the lattice-like collagen structure of the axolotl dermis¹². These dermal fibroblasts have a characteristic cell morphology with extended lattice-patterned filopodia and are present in the SC. To clarify the changes in the presence of the

lattice-patterned filopodia during dermal formation, we visualized filopodia and collagen fibers at each dermal growth stage. We used CMV::GFP transgenics for the filopodia visualization and DAR for collagen visualization. The results are shown in Fig.5. One layer of *stratum coniunctum* was visible in 5^{cm} dermis (Fig.5a, a'), and no GFP signals were detected inside the collagen layer (Fig.5b, b'). It is noteworthy that the collagen fiber structure of the *stratum coniunctum* in the 5^{cm} dermis had a lattice structure, even though no lattice-patterned filopodia were found (Fig.5b, b'). Similar to the results shown in Fig.3, we detected penetration of mesenchymal cells into the collagen layer in the 8^{cm} dermis (Fig.5c, c'). The presence of filopodia was evident in the 8^{cm} dermis (Fig.5d, d'). The 10^{cm} dermis comprised pre-mature SC, SS, and SB structures (Fig.5e, e'). Moreover, consistent with the results shown in Fig.3, cell invasion between the SC and SB was seen (Fig.5e, e'). Compared to the mature SB in the 12^{cm} dermis (Fig. 1), the SB in 10^{cm} dermis had a thinner and rougher structure (Fig.5f). In the SS, collagen fibers formed in the apical-basal orientation around the cells (Fig.5g). In the SC of the 10^{cm} dermis, lattice-patterned filopodia were clearly visible (Fig.5h, h'). In 12^{cm} dermis, the three mature layers of SS, SB, and SC were found (Fig.5i, i'), and cells were abundant in the SS (Fig.5k, i'). The SB is lattice-patterned collagen fiber and has a more robust structure compared to the 10^{cm} dermis (Fig.5j). Lattice-patterned filopodia were found in the SC (Fig.5l, l'), and we conducted a quantitative evaluation of their proportion (Fig.5m). A large number of filopodia were quantified in 10^{cm} and 12^{cm} dermis, whereas fewer were observed in the 8^{cm} and 5^{cm} dermis, especially in the latter (Fig.5m). Moreover, we analyzed the directionality of collagen fibers at each stage in the SC and in the *stratum coniunctum* (Fig.5n). Directionality analysis revealed that the collagen structures in both the *stratum coniunctum* and SC exhibited bipolarity (Fig.5n). These observations suggested that lattice-patterned fibroblasts appeared in the 8^{cm} dermis; therefore, despite their absence in the 5^{cm} dermis, the collagen structure is lattice-patterned. This suggests the cells other than lattice-patterned cells are involved in forming the lattice-patterned collagen structure.

We performed a detailed investigation of ultrastructure using electron microscopy (EM) in 8^{cm} dermis and 12^{cm} dermis. (Fig. 6). Using TEM imaging, we confirmed the typical collagen structure as previously reported^{18, 19}. In the 8^{cm} dermis, mesenchymal cells could be observed invading the *stratum coniunctum* with filopodia (Fig. 6a), with orthogonal collagen fibers (Fig. 6b). The filopodia of the cells were seen to be inserted between these longitudinally and transversely orthogonal collagen fibers (Fig. 6b). In 12^{cm} dermis, EM confirmed that the collagen lattice structure in SB and SC was composed of orthogonal collagen fibers (Fig. 6c–e). In addition, the thickness of each fiber in the SB and SC was different (Fig. 6d, e). These findings suggest that the *stratum coniunctum*, SB, and SC have orthogonal fiber structures, but SB and SC have slightly different microfiber structures.

We then focused on cell invasion into the collagen layer. As shown in Fig.3 and 5, invasion

of mesenchymal cells in the dermis appeared to be a key step in SS formation. It was not clear how the cells, whether entering or inserting filopodia, interacted with the densely woven collagen layer to achieve entry and filopodia insertion. We focused on the expression of genes encoding collagen-degrading enzymes, specifically *Mmp 1, 2, 9, 13, and 14*, which have type I collagen-degrading activity. We investigated the expression of these genes (Fig. 7a–d, a'–d') and observed *Mmp2*⁺ cells and *Mmp9*⁺ cells at all stages from 5^{cm} to 12^{cm} dermis, either within the dermis or just beneath it (Fig. 7a2, b2, c2, d2, a3, b3, c3, d3). Additionally, *Mmp13*⁺ cells were found at 12^{cm} dermis (Fig. 7d4). These results suggest that cells invade the collagen layer while degrading type I collagen. Previous studies have shown that inhibition of cell invasion during the skin regeneration process in the axolotl results in inhibition of SS formation¹¹. As the regenerative process mimics the developmental process, it was speculated that cell invasion inside the collagen layer is involved in the formation of SS in the developmental process.

Type I collagen production in keratinocytes

As can be seen in Fig.5, a lattice-patterned collagen structure was identified despite the absence of lattice-patterned fibroblasts in the 5^{cm} dermis. This suggests that cells other than lattice-patterned fibroblasts contribute to the formation of lattice-patterned collagen structures in 5^{cm} dermis. To identify the cells that contribute to dermal collagen formation, we conducted *in situ* hybridization to clarify the *Colla1* expression pattern at each stage (Fig. 8a–d). *Colla1* signals were found not only in the mesenchymal cells but also in the keratin (KRT)-positive epidermal cells in the basal layer of the epidermis in the 5^{cm} and 8^{cm} dermis (Fig. 8 a, a', b, b'). Conversely, in 10^{cm} and 12^{cm} dermis, keratinocytes displayed no strong *Colla1* signals, whereas cells in the SS and SC exhibited intense *Colla1* expression (Fig. 8 c, c', d, d'). In mammals, keratinocytes are not typically associated with the production of type I collagen^{20, 21}. Therefore, we further investigated whether protein synthesis from the *Colla1* mRNA in keratinocytes was inhibited by post-translational regulation. For this purpose, we conducted immunofluorescence staining using pro-type I collagen antibodies (Fig. 8 e–h, e'–h') and IEM (Fig. 8 i–k). Briefly, pro-type I collagen (procollagen) can be detected prior to collagen secretion from a cell and just before fiberization²². By using the anti-procollagen antibody, we could identify the location of the collagen molecules within the cell and the region where new collagen was being fiberized. Procollagen signals were detected in the keratinocytes in the 5^{cm}, 8^{cm}, and 10^{cm} dermis (Fig. 8e–g). In particular, we identified a stronger signal on the apical side of the dermis in the 5^{cm} to 10^{cm} dermis. Fig. 8e'–h' shows a heat map of all stages. A strong signal was observed on the epidermal cell side (Fig. 8e', f'), suggesting that collagen is secreted from epidermal cells. Procollagen expression pattern in the 10^{cm} and 12^{cm} dermis suggest that collagen supply is from cells in the SS and that collagen production rate is decreased by the 12^{cm} dermis stage. In the 12^{cm} dermis, relatively weaker procollagen signals were present in keratinocytes and the apical side

of the SB (Fig. 8h). Furthermore, the integrated heatmap combining multiple images also produced similar findings in 5^{cm} dermis to 12^{cm} dermis (Fig. 9a–d). We further confirmed these observations using in 5^{cm} dermis using IEM (Fig. 8i–k). IEM signals were detected in the basement membrane (*lamina lucida*) (Fig. 8i, i'). IEM signals could also be observed in the mesenchymal cell just below the dermal collagen layer (Fig. 8j, j'). In the absence of primary antibodies (negative control), no IEM signals were observed (Fig. 8k, k'). These results suggest that collagen is secreted from keratinocytes and delivered to the dermis through the basement membrane.

To determine how collagen produced by keratinocytes contributes to the formation of dermal collagen structures, we conducted a pulse-chase experiment using DAR and DAF (Fig. 10a). DAR was injected under the skin on the dorsal side of the forelimb and the injected animals were kept for 5 days. Then, DAF was injected into the area where the red fluorescence from DAR was visible. We collected the skin 1 day after DAF injection. Consequently, most collagen fibers had both red and green fluorescence, whereas green signals were found only in newly synthesized collagens that were made between the day of DAR injection and the day of the DAF injection (within 5 days). The skin was analyzed using a confocal microscope. In the apical side, DAF signals occupied a larger area than DAR signals (Fig. 10b). In the basal side, DAF and DAR signals were almost completely overlapping (Fig. 10c). To visualize the spatial occupancy, we reconstituted the confocal images, resulting in an orthogonal image (Fig. 10d), which clearly demonstrated that the newly synthesized collagen fibers were located at the apical side of *stratum coniunctum*. To visualize this quantitatively, we made a histogram of Fig. 10d and calculated the rate of each color (Fig. 10e, f). Whereas most of the apical side contained new collagen (DAF⁺), the area of DAR and DAF gradually equalized as it shifted toward the basal side. We conducted another analysis to quantify the areas of DAR and DAF in all sections (Fig. 10g). The area of DAF was significantly larger than that of DAR (Fig. 10g). To confirm that the offset of the DAR/DAF labeling is not caused by difference in diffusion properties or tissue specificity, we conducted two control experiments. First, we reversed the injection timing of DAR and DAF in the 5^{cm} dermis and left it for 10 days and performed the same analysis (Fig. 11). As in Fig. 10, only new collagen (DAR⁺) signals were observed on the apical side of the dermis (Fig. 11a–e), indicating that the stacking on the apical side is not affected by the difference in the diffusion properties of the dyes. Next, we performed a pulse-chase experiment on tendons in order to test whether tissue specificity caused the offset of the DAR/DAF labeling (Fig. 11f). As a result, in tendons, new collagen (DAF⁺ fibers) was observed within old collagen (DAR⁺ fibers), and the offset of DAR/DAF was observed among the older collagen fibers (Fig. 11f1–3). This indicates that the pulse chase analysis using DAR and DAF accurately monitors newly synthesized collagen fibers regardless of tissues. The accuracy and the results of the pulse chase analysis in the axolotl skin confirm that the newly synthesized collagen layers are stacked on the epidermal side. Considering that neither mesenchymal cells nor lattice-patterned fibroblasts were

present at the 5^{cm} dermis as shown in Fig.3 and Fig.5, these results suggest that the collagen structure formation at 5^{cm} dermis was due to collagen production by keratinocytes only.

A basement membrane exists between the epidermis and dermis. When considering the supply of collagen from the epidermis, the presence of the basement membrane is notable. The results of immunofluorescence staining for the basement membrane component laminin are shown in Fig. 12. Signals of laminin were observed just beneath the epidermis from 5^{cm} to 12^{cm} dermis (Fig. 12a–d). We investigated the ultrastructure of the basement membrane using an EM (Fig. 12e); hemidesmosomes and basal lamina composed of *lamina densa*, and *lamina lucida* was also observed. Furthermore, we found some areas showing hole-like structures on EM (Fig. 12e). It has been reported that holes exist in the basal membrane in *Drosophila* developmental stages and early mouse embryos^{23, 24}. Our results suggest that holes may also exist in the basement membrane of the developing stage of the axolotls. It has been reported that COL4 in the axolotls is partially absent²⁵. It is also possible that the basement membrane of the axolotls may have a very rough structure compared with that of mammals. These observations support the idea that collagen molecules produced by keratinocytes are transported to the dermis through the basement membrane.

Transition of dermal collagen structure through each developmental stage

Our results demonstrate that keratinocytes, in addition to dermal fibroblasts, are a new type of collagen-producing cell in the early stages of dermal formation (5^{cm} dermis), responsible for creating the initial lattice-patterned collagen structure. Next, to elucidate how collagen production by dermal cells and keratinocytes cooperatively contributes to dermis formation, we conducted a DAR/DAF pulse-chase experiment similar to that depicted in Figure 10a in four phases: from 5^{cm} to 8^{cm} dermis, 8^{cm} to 10^{cm} dermis, 10^{cm} to 12^{cm} dermis, and 5^{cm} to 12^{cm} dermis. In the dermis development from 5^{cm} to 8^{cm} dermis, accumulation of new collagen on the apical side could be observed (Fig. 13a, b). The collagen fibers that were generated at 5^{cm} dermis (DAR⁺ fibers in Fig. 13a) were located basally in the 8^{cm} dermis (Fig. 13b, c1–c3). We also found that there are many pillar-like formations (Fig. 13a', c1') with only a DAF signal, indicating that the pillar formation takes place in stage after 5^{cm} dermis. These pillars, which are formed by fibroblasts having a pillar-like filopodia, have been reported previously¹². It is likely that *Colla1*⁺ cells located on the mesenchymal side, as shown in Fig. 8a, b, may be contributed to the formation of this pillar-like structure. From 8^{cm} to 10^{cm} dermis, the DAF signals were also detected on the apical side (Fig. 13d, e). The SB-like structure had only DAF signal, indicating that the SB was composed of newly synthesized collagen between 8^{cm} and 10^{cm} dermis (Fig. 13d, e). However, older collagens (DAR⁺) could be observed in the SC, indicating that the collagen fibers synthesized at the 8^{cm} dermis contributed to the SC of the 10^{cm} dermis (Fig. 13d, f1–f3). Focusing on the hypodermis side of the SC, the DAF⁺ area seemed to be slightly larger than that the DAR⁺ area (Fig. 13f1–f3), suggesting that new collagen fiberization takes place in the SC.

From 10^{cm} to 12^{cm} dermis, the DAF signal was detected apically (Fig. 13g, h), and many cells were observed within the SB. This suggests that SS and SB are formed by cells that have invaded the collagen layer. In contrast, collagen produced at 10^{cm} dermis (DAF⁺) was present in the SC (Fig. 13g–i). In the lattice structure of the SC were DAF-only collagen fibers (Fig. 13h, i1–i3). This means that new collagen fibers appeared inside the SC. In particular, many filopodia of lattice-patterned cells were observed at 8^{cm} to 12^{cm} dermis, which is due to collagen production by lattice-patterned cells. Finally, changes from 5^{cm} to 12^{cm} dermis are shown in Fig. 13j–l. Structures formed at 5^{cm} dermis (DAF⁺ fibers) were found to form the basal layer (basal side) of the 12^{cm} dermis at the SC (Fig. 13j–l). Additionally, the structure from 5^{cm} dermis collagen fibers (DAF⁺ fibers) appears to be in a low-density state (Fig. 13l1–l3). These results suggest that the lattice-patterned collagen structure initially synthesized by keratinocytes becomes the SC and undergoes fine changes during growth.

Since lattice-patterned cells have been shown to contribute to the formation of lattice collagen in SC ¹², we hypothesized that these cells induce subtle structural changes in SC. In line with previous findings, we observed cells with filopodia within the SC (Fig. 14a, b). Additionally, secreted collagen with a lattice pattern was evident (Fig. 14c, d). We closely examined pulse-chase experiments conducted in the dermis. The results of subtracting the red (older collagen) color from the green (newly synthesized collagen) color are displayed in Fig. 14e, f, confirming the independent existence of green fibers (DAF⁺ fibers). This suggests that the mesenchymal cells, including the lattice patterned cells, contribute to building up the preexisting collagen fibers.

To confirm whether the preexisting fibers became thicker during skin development at the single fiber level, we used EM to investigate changes at the single fiber level in 5^{cm} dermis, in which filopodia are absent, and in 12^{cm} dermis, in which filopodia are present. The entire collagen ultrastructure at 5^{cm} dermis is shown in Fig. 15a. No cells or filopodia were detected inside the collagen layer (Fig. 15a). Fig. 15b shows the structure just below the keratinocytes, and Fig. 15c, d shows the ultrastructure of the 12^{cm} dermis. The SB can be observed just below the keratinocytes, and the single collagen fiber is very thin (Fig. 15c). SC collagen fibers are shown in Fig. 15d; the collagen fibers comprising SC were thicker than those comprising SB. In addition, filopodia of lattice-patterned cells were detected inside the SC as if they were inserted between the fibrous layers (Fig. 15d). We measured the area of the single collagen fiber in the 5^{cm} dermis and the single fiber of SC at the 12^{cm} dermis (Fig. 15e); the single collagen fiber at 12^{cm} dermis was significantly thicker than that in the 5^{cm} dermis. These results suggest that the collagen supplied by lattice-patterned cells thickens the single collagen fiber, forms new fibers, and, as a result, modifies the SC structure.

Transition of collagen fibers in the axolotl dermis

We found that lattice-patterned cells expand filopodia and provide collagen to modify the SC

collagen structure. The previous study reported that the lattice patterned cells secrete collagen around the expanded lattice-patterned filopodia¹². We succeeded in further elucidating the role of the lattice-patterned cells in the axolotl dermis. The lattice patterned filopodia are inserted into the existing collagen layer (Fig. 15d) and collagen molecules are secreting around the filopodia, modifying and building up the collagen fibers (Fig. 15 and Fig. 14e, f). This causes slight differences in the lattice-patterned collagen structure. Such modification may cause transition of the dermal collagen fibers. To test and visualize this transition of the collagen fibers, we performed principal component analysis (PCA). Properties of the fibers involved in lattice-patterned cells were subjected to PCA with features derived using a pre-trained deep-learning model (Fig. 16). We analyzed seven lattice fiber structures (5 cm, 8 cm, 10 cm: SS side of SC (10cm Spo), 10 cm: hypodermis side of SC (10cm hypo), 12 cm: SS side of SC (12cm spo), 12 cm: hypodermis side of SC (12cm hypo)) (Fig. 16a). Feature extraction from our collagen images, we adopted ResNet, which is one of the best tested models for image recognition and that has been used on biological data for feature extraction^{26, 27, 28}. Each fiber group occupied specific domains in the PCA plot (Fig. 16b, c). The one-dimensional PCA scores plot in PC1 suggested a gradual change in collagen fiber structure with growth from *stratum coniunctum* to SC. The scores of the SB of the 12^{cm} dermis are plotted between the *stratum coniunctum* and SC (Fig. 16c). This suggests that the SB in the 12^{cm} dermis receives some modification after lattice-patterned collagen production by keratinocytes. We further investigated structural similarities by measuring the distance between each cluster (Fig. 16d). The seven types of fibers could be classified into three groups: 5cm and 8cm, 10cm and 12cm, and SB (Fig. 16d). Notably, SB exhibited a fiber structure distinct from that of SC and *Stratum coniunctum* (5 cm, 8 cm). However, when examining SB, it shared characteristics with 10 cm SC-Spo and 8cm. Given that SB formation occurs after 10cm, it is likely shaped following modifications by lattice-patterned fibroblasts. Therefore, the strong structural similarities between 10cm SC-Spo and 8cm are logical, and the low similarity with 5 cm, which is solely formed from keratinocytes, is expected. These findings support the idea that the 5cm lattice structure is gradually transformed into a 12cm SC lattice structure by lattice-patterned fibroblasts.

Collagen production by epidermal cells in other species

Finally, to investigate whether collagen production in epidermal cells is a conserved mechanism in other animal species, we first performed pulse-chase experiments in zebrafish (Fig. 17a, b). DAF staining of zebrafish at 12 dpf was performed according to the method described before¹⁴. The DAF-stained zebrafish were then fixed at 17 dpf and stained with CHP-Cy3, thereby conducting a pulse-chase experiment like that shown in Fig. 10. The CHP-Cy3 signal was observed over a broader area than the DAF signal (Fig. 17a1–3). Similar to our findings in axolotls, accumulation of new collagen (CHP-Cy3⁺ fibers) on the apical side was apparent when the reconstituted confocal image

was observed orthogonally (Fig. 17b, b'). The procollagen signals were also observed in epidermal cells, suggesting that the epidermal cells are potential source of the collagen even in zebrafishes (Fig. 18a, a'). The quantification of the image in Fig. 17b is shown in Fig. 17c, confirming that the CHP-Cy3⁺ region is shifted epidermal side. This result confirmed that new collagen is layered on the epidermal side in zebrafishes. Next, we examined the patterned of *Colla2* expression using different developmental stages of chicks and mice. We conducted *in situ* hybridization (ISH) and double staining with immunofluorescent staining for COL4 in chicks at the stage 26 (Hamburger-Hamilton) (Fig. 17d–g). *Colla2* signals were expressed in epidermal cells (Fig. 17d–f). A strong signal of *Colla2* was visible in cells on the mesenchyme side. The COL4 signal could be observed at the mesenchyme–epidermis interface (Fig. 17f2). This could be a signal of COL4 in the basement membrane. The merged image is shown in Fig. 17f3, and control stains are shown in Fig. 17g1, g2. A *Colla2* signal could be observed inside the epidermal cells just above the COL4 signal in the immunofluorescence staining (Fig. 17f1–f3). Similar observations were made in mice (E13.5). In mice, as in chickens, a strong *Colla2* signal could be observed on the mesenchyme (Fig. 17h–j). The results of *Colla2* in epidermal cells and immunofluorescence of COL4 are shown in Fig. 17J1, J2. The control staining is shown in Fig. 17k1, k2. The *Colla2* signal was observed in the epidermal cells just above the basement membrane in Fig. 17j3. The results of immunofluorescence using the procollagen antibody were shown in Fig. 18b and b'. Similar to the ISH results in Fig. 17j, procollagen signal could also be observed within the epidermal cells. These results suggest that epidermal cells are producing collagen even during the fetal stage of mammals. Therefore, these findings suggest that in a various animal species, not only mesenchymal cells but also epidermal cells may produce type I collagen at developmental stage.

Discussion

Keratinocyte-based dermal collagen formation

In this study, we demonstrated that the dermal collagen structure in the axolotl was formed by the cooperative activity of keratinocytes and mesenchymal cells (fibroblasts). Keratinocyte-based dermal collagen production had been speculated in previous studies^{11,29}. In those studies, the collagen production by keratinocytes was assumed based on the observation of collagen production in environments with few or no mesenchymal cells. Our data more directly demonstrated that keratinocytes are the primary source of dermal collagen in the early phase of axolotl skin development. Our result may help change the widely held view that keratinocytes do not produce collagen. Our results clearly indicate that collagens produced by the keratinocytes contribute to the dermis of the axolotl throughout their lifespan.

If the collagen production ability in keratinocytes is focused, it is possible to find that other types of collagen are produced by epithelial cells³⁰. Potentially, no genetic regulatory mechanism is shared. However, given the intracellular trafficking of collagen molecules^{31,32}, it is likely that the trafficking system remains active in keratinocytes. This also supports the finding that keratinocytes of axolotls, zebrafishes, chicken embryos and mouse embryos produce type I collagen.

Dermal collagen formation in axolotls

In the present study, we newly identified two dermal layers in addition to the two layers already determined. The *stratum coniunctum* is a monolayer formed transiently from collagens during axolotl dermal development (Fig.5a, c). The SB is the uppermost layer in the relatively mature axolotl dermis (Fig.5j). Collagens up to the 5^{cm} dermis stage appear to be synthesized mainly by keratinocytes, not dermal fibroblasts. In the 5^{cm} dermis, collagens were stacked apically (Fig. 10d), and filopodia from the fibroblasts (lattice- patterned cells) could not be observed (Fig.5m). In contrast, we also observed *Colla1*⁺ cells underneath the *stratum coniunctum* (Fig. 8a, b). The presence of *Colla1*⁺ cells is thought to contribute to the formation of the pillar-like structures in the *stratum coniunctum* (Fig. 13a'). These pillar-like structures extend from the basal side of the *stratum coniunctum* toward the apical side (Fig. 13a'). At the 5^{cm} dermis stage, these structures are not observed (Fig.5a, a'), and it is thought that the pillar-like structures are formed between the 5^{cm} and 8^{cm} dermis through collagen supply from basal *Colla1*⁺ cells. However, we have not addressed the role of basal *Colla1*⁺ cells at a stage earlier than 5^{cm} dermis. Consequently, the role of fibroblasts in the early phases of development warrants further investigation. In the later stage, the *stratum coniunctum* became thicker and the mesenchymal cells could be observed between the epidermis and the *stratum coniunctum* (8^{cm} dermis) although no mesenchymal cell could be observed there in the earlier stage (5^{cm} dermis) (Fig.3q, r). Given that no major collagen stacking takes place in the hypodermal side in the transition of the dermis from 5^{cm} to 8^{cm}, the mesenchymal cells between the

epidermis and the *stratum coniunctum* likely invade through the *stratum coniunctum*. We found that some of those are *Mmp2*⁺ cells (Fig. 7). This gene expression pattern also supports cell invasion through the collagen layer. This cell emergence may contribute to the separation of the dermal collagen layer into three layers (Fig.3 and Fig.5). Regarding SS formation by migrating cells, the inhibition of fibroblast migration in wounded axolotl skin results in impaired SS thickening¹¹. It is likely that invading cells play a similar role in development of the SS in developing axolotl skin. However, it is still unclear whether cell invagination is the major force separating the SB and SC, because elimination of fibroblasts throughout skin development is currently impossible in axolotls. After the separation of the three layers, it is difficult to imagine that keratinocytes affect the lower layers such as the SC. Given the *Coll1a1* expression pattern, keratinocytes seem to produce less collagen after separation of the three layers (Fig. 8d). Nevertheless, the newly synthesized collagens emerged throughout the dermis (Fig. 13a–l). This suggests that the center of collagen production shifts from the keratinocytes to the mesenchymal cells, and this shift might be conserved among species. We demonstrated the conserved collagen production mechanism by keratinocytes in mouse, chick, and zebrafish embryonic skin (Fig. 17). Generally, it is recognized that keratinocytes in the mature mammalian skin lose their ability to produce collagen and fibroblasts take on the role^{20, 21}. However, our findings suggest that the shift in collagen production from keratinocytes to mesenchymal cells (fibroblasts) appears to be a highly conserved evolutionary mechanism. Consequently, the axolotl dermal collagen system presents a valuable model for studying the developmental mechanisms of dermal collagen production across species.

Ultrastructure of the dermal collagen and the role of fibroblasts

Dermal collagen structures in the axolotl were investigated using EM. The ultrastructure of axolotl dermal collagen fibers appears to have a nested pattern (Fig. 6), which results in the characteristic lattice-patterned collagen network¹². We also clarified the spatial relationship between the basement membrane, collagen fibers, and keratinocytes (Fig. 12). Not much is known about the basement membrane of axolotls. However, we could confirm the presence of the *lamina lucida* and *lamina densa*, suggesting that the basic structure of the basement membrane is conserved. Given the collagen production ability of keratinocytes, it is quite intriguing how collagen molecules go through the basement membrane. We confirmed the presence of laminin and COL4 in the axolotl basement membrane⁹. The basement membrane composed of these commonly conserved molecules may knit a loose network, or specific molecules may help the collagen molecules to pass through, or the orthodox structure of the basement membrane might allow collagen transfer. This is possible because a single unit of collagen is about 300 nm long and 1.5 nm in diameter³³, and it has been shown that albumin, which is about 4 nm in diameter³⁴, can pass through the glomerular basement membrane. Furthermore, we observed the gap like structure in our EM image (Fig. 12e). Such holes

in the basement membrane can be found in mouse and *Drosophila* embryos^{23,24}. It has also been reported that there are holes in the basement membranes of adult mammalian skin, serving as pathways for immunocytes³⁵. These findings suggest that the collagen molecule could potentially go through, and the basement membrane might function to arrange and orient the collagen. We cannot currently visualize collagen molecules passing through the basement membrane, but this should be investigated in the future.

EM revealed the unique relationship between lattice-patterned cells and collagen fibers. Lattice-patterned fibroblasts inserted filopodia just between wrap and weft (Fig. 6), consistent with previous findings that the lattice-patterned filopodia extended from cells and contributed to knitting collagen fibers in the SC¹². Given the collagen production in those cells, it is reasonable that the role of lattice-patterned fibroblasts in the axolotl SC is modification and maintenance of the collagen network originally produced by keratinocytes. Our results suggest that two patterns to contribute to SC modification and maintenance: (1) generation of new fibers, and (2) the thickening of existing fibers. The pulse-chase labeling technique using DAR and DAF revealed the slight shift to newly synthesized collagen fibers from older ones (Fig. 14e, f), such that new fibers were added on the side of older fibers. Regarding the thickening of the existing fibers, EM observations clearly indicated that fibroblasts play a role in the process (Fig. 7). However, the biological and physiological meanings of modification by fibroblasts are still unknown. Furthermore, it is unclear whether fibroblasts can generate lattice-patterned collagen fibers without a lattice-patterned collagen template. Elucidating how existing collagen fibers interact with fibroblasts and the biological significance of this will help to confirm the mechanisms when collagen synthesis by keratinocytes has disappeared.

Lattice-patterned collagen networks without mesenchymal cell (fibroblast) participation

We clarified that the lattice-patterned collagen network was formed without or with minimum influence of mesenchymal cells (Fig. 5a, Fig. 10). The mechanism of how the lattice pattern is generated has been unknown but there are some possibilities: (1) autonomous lattice formation of collagen molecules; (2) physical force by movement; or (3) directional filtration by the basement membrane. Collagens are rod-shaped molecules and can self-assemble. This molecular property may be sufficient to form a lattice pattern. To ensure this, finer design of molecular simulation is necessary. Physical force should be part of the force to make a lattice pattern. An axolotl can move its limbs both intentionally and unintentionally, and such movement should generate a stretch force in the collagen fibers. It is well known that one-directional stretching contributes to aligning fibers. Aligning the collagen molecules via filtering by the basement membrane is another possibility. As mentioned above, collagen molecules may be able to pass through the basement membrane vertically. This selection in molecular orientation may reasonably account for collagen alignment.

However, there is no clear explanation why the collagen fibers make the characteristic lattice pattern in the axolotl dermis. The process by which geometrically ordered forms are created by amorphous cells is a very interesting biological topic. More sophisticated observation methods and simulations will help to solve the problem. Elucidating the process of well-ordered collagen formation may lead to a technique whereby complete regeneration of the skin without scar formation is possible.

Perspectives on Dermal Collagen Formation

Dermal collagen, a crucial component of skin structure, has long been attributed solely to fibroblast production in mammals. However, the *in vivo* dynamics of dermal collagen construction have remained elusive, primarily due to the opacity of mammalian skin. The axolotl, with its remarkable skin transparency, offers an unprecedented opportunity to revise our understanding of dermal collagen architecture. Leveraging the unique transparency of axolotl skin, we conducted an in-depth investigation into the process of dermal collagen formation. Our findings are summarized in Fig. 19, illustrating the detailed process of axolotl dermal collagen formation.

These findings reveal that axolotl dermal collagen is the result of intricate spatiotemporal coordination among diverse cell types, collectively constructing a complex three-dimensional collagen framework. Importantly, our research highlights a paradigm shift: keratinocytes play an important role in the early stage of lattice-patterned collagen structure formation. This paradigm-shifting model offers a new foundation for understanding vertebrate dermal collagen formation and holds significant potential for advancing regenerative medicine and skin bioengineering.

Methods

Animals

Axolotls (*Ambystoma mexicanum*) were procured from the Hiroshima University Amphibian Research Center and kept in aerated water at 22°C. The leucistic strain was used in all experiments. For the surgical experiments conducted in this study, axolotls with nose-to-tail lengths ranging from 5 to 12 cm were used, with no specific bias towards either sex. The care and treatment of the animals in this study was carried out under protocols approved by the Animal Care and Use Committee of Okayama University. All animal experiments adhered strictly to the guidelines provided by the Animal Care and Use Committee of Okayama University. Every possible measure was taken to minimize animal suffering, in line with the NIH Guide for the Care and Use of Laboratory Animals. Zebrafish (*Danio rerio*) were originally obtained from the Zebrafish International Resource Center. The individuals bred in our laboratory were used for the experiments. Regarding to ddY mouse embryo, and chick embryo, the fixed samples were bought from companies (mouse :Tokushima Molecular Pathology Institute ,Inc, chick : alphabio, Inc).

Histology

Harvested limbs were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at room temperature overnight. To prepare frozen sections, fixed samples were decalcified in 10% EDTA at room temperature overnight, equilibrated in 30% sucrose in PBS at room temperature overnight, and frozen in O.C.T. compound (Sakura Finetek). Frozen sections were cut to a thickness of 12 µm using a CM1850 cryostat (Leica) and dried. Collagen fibers were visualized using a Masson's Trichrome Stain Kit (HT15-1KT, Sigma). Masson's trichrome staining was performed as follows. The prepared frozen sections were washed with water and post-fixed in preheated Bouin's solution at 65°C for 15 minutes. After post-fixation, the sections were washed with water and stained with Weigert's hematoxylin solution at room temperature for 15 minutes. Following a water wash, fuchsin solution was applied to the sections, which were stained at room temperature for 3 minutes and washed again with water. Next, phosphomolybdic-phosphotungstic acid solution was applied and reacted at room temperature for 5 minutes, followed by staining with aniline blue at room temperature for 5 minutes. Finally, the sections were washed with 100% ethanol and G-NOX and mounted with a soft mounting solution (Softmount, Fuji Film). Elastic fibers were visualized using a Victoria Blue stain solution (Muto Pure Chemicals Co., Ltd, 40772). Victoria Blue staining was performed as follows. The frozen sections were first washed with water and then with 95% ethanol. The sections were stained with Victoria Blue stain solution at room temperature for 2 minutes, followed by a wash with 70% ethanol and replacement with water. Finally, the sections were mounted in glycerin. Images were acquired using a BX61 microscope (Olympus).

Collagen visualization

To visualize the fibers of type I collagen in forelimb at the dermal skin, we used DAR-4M (SK1005-01, Goryo Chemical) and DAF-FM DA (SK1004-01, Goryo Chemical). Each solution (1-2 μ L) was injected under the skin of an axolotl dorsal limb using a fine glass capillary. One days later, injected skin was harvested and fixed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at room temperature overnight. Fixed skin sample was washed with Tris-buffered saline with Tween 20 (TBST) and stained for nuclei using DAPI solution (19178-91,5 μ g/mL, Nakarai tesque) and tissue clearing using CUBIC-R+(N) (Tokyo Chemical Industry), and the samples were mounted in Mounting Solution (PI 1.520) for CUBIC-R+ (Tokyo Chemical Industry). Images were acquired using a FV3000, and FV4000 confocal laser scanning microscopes (Evident). To visualize the structure of the *stratum baladachinum*, maximum intensity projection was applied to the confocal images using Fiji software. Normalization and adjustments of brightness and contrast were applied to figure images using Fiji software. Three-dimensional construction of collagen and cell structure was performed using Amira software. Deconvolution (Fig. 14e) was performed using cellSense software (Evident).

Measurement of region of interest in Z-stack images

We measured the area of GFP⁺ filopodia in the SC (Fig.5m) using Fiji software. We used the image set in which Type I collagen in the CMV::GFP transgenic axolotls was stained with DAR-4M. To measure the area of filopodia, we isolated the SC region. This extraction was limited to the GFP (green) channel. The green channel (GFP) was then converted to grayscale, and normalization was applied using the Fiji Plugin (Nomalization Local Contrast). Then, we set the threshold for the green channel (GFP) to 40–125, selecting the GFP⁺ area within this range. The GFP⁺ area was further limited to regions containing only lattice-patterned filopodia, and both the selected GFP⁺ area and the total area of the selected region were measured. This method was applied to all Z-stack images of the isolated SC. We analyzed more than 15 images taken from n=3 biologically independent samples, with each point on the box plot representing the result from one image analysis. Similarly, the red and green area measurements (Fig. 10, 13, Fig. 11) were conducted in Fiji. Z-stack image color channels were separated and converted to grayscale. Each channel's Z-stack images were normalized using the previously mentioned Fiji. For the normalized Z-stack images, we set the threshold to 45–255 and selected signals within this range. The selected area was then measured across all Z-stack images to quantify the red and green areas.

Analysis of collagen fiber orientation

To investigate fiber orientation, the directionality plugin of Fiji was used: a Z-stack image of collagen visualized using DAF/DAR was converted to greyscale and a 200-pix-square region was

extracted for analysis. Normalization processing was applied to the extracted images using Fiji, and Fourier components were calculated using the directionality plugin.

Immunofluorescence

The following primary antibodies were used for immunofluorescence staining: rabbit anti-GFP pAb (MBL Cat# 598, 1:500, RRID:AB_591819), mouse anti-collagen type I (#C2456-2 ML, 1:500; Sigma), mouse anti-pan cytokeratin (AE1+AE3) antibody (NBP2-29429, 1:100; Novus Biologicals), mouse anti-procollagen type I aminoterminal extension peptide (SP1.D8, 1:10; DSHB), rabbit anti-Laminin (L9393, 1:100; Sigma), rabbit anti-collagen type III alpha1 (N-terminal) (22734-1-AP, 1:500; Proteintech), rabbit anti-collagen type IV (600-401-106-0.1, 1:100; Rockland), rabbit anti-procollagen type I (orb389309, 1:100; Biorbyt) and mouse anti-collagen Type2 (II-II6B3C, 1:100; DSHB). Antigen retrieval was performed for mouse anti-procollagen type I aminoterminal extension peptide and rabbit anti-procollagen type I antibodies. Antigen retrieval and Proteinase K (25530049, Invitrogen, 5 μ g/ mL) digestion at room temperature for 30 minutes were performed for mouse anti-collagen type I, rabbit anti-laminin, rabbit anti-collagen type IV, and mouse anti-collagen type II antibodies. The secondary antibody was the anti-mouse IgG Alexa 488 conjugated antibody (A11017, 1/1000; Invitrogen), goat anti-rabbit IgG Alexa 594 (A-11012, 1:1000; Invitrogen) and anti-rabbit IgG Alexa 488 (Cat# A-21207, RRID: AB_141637, 1:500, Thermo Fisher). The nuclear staining was performed using Hoechst 33342 (19172-51, 0.125 μ g/ mL, Nacalai tesque). Images were acquired using a BX61 microscope (Olympus) and FV3000 confocal laser scanning microscope (Evident). To visualize the structure of an entire single cell, maximum intensity projection was applied to the confocal images using Fiji software. Adjustments of brightness and contrast were applied to the figure images using Fiji software. The heatmaps (Fig. 8e'–h') were created using Fiji. We converted RGB images to grayscale. The heatmap was created using Fiji's look-up table (16 colors) for a grayscale image. The original images used to create the heatmaps were taken under the same image capturing conditions. The integrated heat map was created by processing the captured images using Python code. Briefly, the images were measured at multiple distances between two points that traverse the collagen layer of the dermis, and the Colla signal intensity between the two points was calculated. Data from the same dermal developmental stage were then standardized to the mean value of the distance between those two points. The mean values of all intensities were rearranged to the mean value of the two conversion distances and displayed as a heat map. The code necessary for a series of integrated analyses has been registered and published on GitHub (<https://doi.org/10.5281/zenodo.14729473>).

Measurement of collagen layer thickness and number of nuclei

To measure the thickness of the collagen layer (Fig.3u), images of sections immunofluorescence

stained with type I collagen were converted to greyscale; normalization was performed using Fiji's Normalization to obtain binarized data. The length (pix) of any 10 points from the binarized data was measured. From each of the $n = 4$ independent samples, two images were acquired, and 10 measurements were taken per image. Each point on the box plot represents all measured data points. To count the number of nuclei (Fig.3v), binarized images of sections immunofluorescence stained for type I collagen were acquired in the same way and superimposed with Hoechst staining. For the independent samples ($n = 4$ for 5 cm; $n = 5$ for 8 cm, 10 cm, and 12 cm), two images were obtained and analyzed per sample. Each point on the box plot represents all measured data points. The number of nuclei present inside the collagen layer was measured using a Fiji cell counter.

***In situ* hybridization**

Axolotl *Mmp2*, axolotl *Mmp9*, axolotl *Mmp14*, chick *Colla2*, and mouse *Colla2* were isolated by reverse transcription polymerase chain reaction (RT-PCR) using the following primers: *Mmp2* forward, AAGAAGCCTCGCTGTGGTAA and *Mmp2* reverse, ATCAGGGACACCAGTCCAAG; *Mmp9* forward, GCGCACGATGTCTTTCTGTA and *Mmp9* reverse CTTCCTCTCTTGCCACTTG; *Mmp14* forward, TGAGACCTATGAGGCCATCC, and *Mmp14* reverse; CAGTCACGCAGCACAGATTT, Chick *Colla2* forward, GAGGTGAACCTGGCAACATT and Chick *Colla2* reverse CTGCATCAAAGCCAACTTCA; mouse *Colla2* forward GACATGCTCAGCTTTGTGGA and reverse, ACCTTTGCCACCTTGAACAC; and axolotl *Colla1* and *Mmp1*, as previously described^{36,37}. The isolated genes were subcloned into the pTAC2 vector and sequenced. Probe synthesis was performed by T7 or SP6 RNA polymerase. Regarding *Mmp13*, the gene could not be isolated by RT-PCR. Thus, the sequence of its open-reading frame region was synthesized (Eurofin). Section *in situ* hybridization was performed as previously described³⁸. Images of axolotl tissues were acquired using a BX61 microscope (Olympus). Images for mouse and chick tissues were taken by confocal microscope (FV3000, Evident). The color was applied after taking pictures by monochrome camera by Fiji or Photoshop CS6.

Immunoelectron microscopy

Sample for immunoelectron microscopy (IEM) samples were immersion-fixed with 4% paraformaldehyde PBS for overnight at room temperature. Preparations were dehydrated through increasing concentrations of methanol, embedded in LR Gold resin (Electron Microscopy Sciences), and polymerized under UV lamps at -20°C for 24 h. Immunoelectron microscopy was performed as described previously³⁹. Briefly, ultrathin sections (70 nm thick) were collected on nickel grids that were coated with a collodion film, rinsed with phosphate buffered saline (PBS) several times, and then incubated with 2% normal goat serum and 2% BSA in 50 mM Tris(hydroxymethyl)-

aminomethane-buffered saline (pH 8.2) for 30 min to block nonspecific binding. To detect the Procollagen signals in tissues, a 1:1 solution of undiluted mouse anti-procollagen antibody (DSHB, Cat# SP1.D8) and Can Get Signal Solution 1 (TOYOBO) was used to incubate the sections for 1 hour at room temperature. This was followed by incubation with a biotinylated goat anti-rabbit IgG antibody, and then with an avidin-biotin-HRP complex using a streptavidin-biotin intensification kit (Nichirei). Finally, a goat anti-HRP antibody conjugated to 12 nm gold particles (Jackson Immuno Research Laboratory, West Grove) was applied. The sections were then viewed using an H-7650 electron microscope (Hitachi).

Epoxy resin was used to observe the tissue structure. The skin samples were immersed in 4% paraformaldehyde and 2% glutaraldehyde in PBS for overnight at room temperature, washed with 0.1 M Phosphate Buffer Solution (PB), then post-fixed with 1% osmium tetroxide in 0.1 M PB for 2 h at room temperature. The skin samples were washed with 0.1 M PB, dehydrated, and embedded in epoxy resin (Quetol-812; Nisshin EM, Tokyo, Japan). Ultrathin sections were prepared as described above.

Thickness measurement of single collagen fibers

Normalization was performed on the images obtained from the electron microscope, and the images were binarized. Median was applied to the binarized images. To separate each fiber, the watershed function of Fiji was applied, and the fiber area was measured using Fiji's "Analyze particle". Three biologically independent samples ($n = 3$) were analyzed, and two images were obtained and analyzed per sample. Each point on the box plot represents all measured data points.

Analysis of lattice-patterned collagen fiber

For low-dimensional visualization of the collagen image dataset, we manually cropped image patches (200×200 pixels) without overlapping from the confocal images of collagen fibers. To ensure focused analysis of the collagen fiber pattern, we excluded regions without collagen due to cell invasion and extracted only areas limited to the lattice structure. This produced 484 grayscale images. For transparency and reproducibility, all images have been uploaded to Drayd (<https://doi.org/10.5061/dryad.6q573n66z>). As the first step of dimension reduction (from 200×200 -pixel intensities to 2048-dimensional feature vector), the images were input into a deep learning model for image recognition, ResNet50 with ImageNet-pretrained weights (ResNet50_Weights.IMAGENET1K_V2 from the torchvision library) for feature extraction. We extracted image features from the last global average pooling layer. Then, linear PCA was applied to the 2048-dimensional feature vectors. We performed no training of the neural network with our data; hence, the feature extraction was agnostic on which developmental stages the images came from. To quantify distances between the clusters on the low-dimensional space, we adopted the

Mahalanobis distance. First, we computed $\mathbf{M}$, where M_{ij} represents the Mahalanobis distance from the mean of i -th cluster to j -th cluster. Then, we symmetrized it as $\mathbf{D} = (\mathbf{M} + \mathbf{M}^T)/2$ (Fig. 16).

Plasmid transfection and transgenic

To visualize the fibers of type I collagen in dermal skin, we designed a GFP-Coll1a2 plasmid vector containing a CMV promoter-secretory signal-(Gly₄Ser)₃ linker-AcGFP-(Gly₄Ser)₃ linker-Coll1a2-SV40 poly A signal based on pCS2 plasmid. For visualization of cell morphology, we used an mCherry plasmid vector containing a CMV promoter-mCherry-SV40 poly A signal based on the pCS2 plasmid. The plasmids were purified by CsCl-EtBr ultracentrifugation and prepared in a 1.5 μg / μL concentration. Plasmid solution (0.4–2 $\mu\text{g}/\mu\text{L}$) was mixed with Fast Green dye for visualization and injected under the skin of an axolotl limb using a fine glass capillary. Immediately after injection, electric pulses were applied (20 V, 50 ms pulse length, 950 ms interval, 10 times).

To create transgenic axolotls, we subcloned GFP-Coll1a2 into the pIsceI vector (a gift from T. Hayashi). The plasmid was purified using cesium chloride gradient centrifugation. The generation of transgenic axolotls followed a previously published method (PMID: 25740493; <https://doi.org/10.1111/dgd.12019>). F1 transgenic animals were used for the experiments.

Pulse-chase observation of the collagen fibers in zebrafish dermis

Living larval zebrafish at 12 days post fertilization (dpf) were incubated in a solution of 5 μM DAF-FM DA (diluted in tank water) at room temperature for 12 hr in dark. After the incubation, the fish were immediately returned to fresh tank water and bred for 5 days. The fish were subsequently anesthetized with tricaine (MS-222) at an optimal concentration and fixed in 4% PFA at 4°C overnight. After the fixation, the samples were washed with PBS and incubated for the denaturation of collagen fibers at 80°C for 5 min. Finally, the samples were stained with 10 μM Collagen Hybridizing Peptide (CHP) -Cy3 (3Helix, FLU60) at 4°C overnight and after subsequent washing with PBS they were observed by a confocal microscope, LSM 780 (Carl Zeiss). The quantification was performed using the Fig. 17b tiff image, with original python code created and executed for the analysis (the original python code is provided in GitHub (<https://doi.org/10.5281/zenodo.14729490>)).

Microscopes and image analysis

Confocal laser microscopy was performed using FV3000, FV4000 (Evident), and LSM 780 (Carl Zeiss). The objective lenses used for FV3000 and FV4000 were UPlanXApo 20x/0.80 and UPlanXApo 60x/1.42 Oil. For LSM 780, Plan-Apochromat 20x/0.8 M27 and C-Apochromat 40x/1.20 W Korr FCS M27 were used. For fluorescence microscopy using the BX61 (Olympus), the objective lenses UPlanFL 10x/0.3, UPlanXApo 20x/0.8, and UPlanXApo 40x/0.95 were used and images were acquired with a color camera and long pass filter. Image analysis was conducted using

Fiji and AMIRA software. Images were processed and arranged into figures using Photoshop CS6 or Affinity Photo (version 1.10.6.1665).

Statistics and reproducibility

We did not employ strict statistical methods to determine the sample size, but given the high reproducibility of the results, we considered these sample sizes to be sufficient. This study includes analysis of multiple skin stages of axolotls. Consistent observations obtained from independent experiments performed at different skin stages support the sufficiency and reproducibility of our results (with sample sizes of at least $n = 3$ skins per experiment). No data were excluded from the analyses. We did not specifically distinguish between the sexes of the animals. This is because there is no evidence of gender-based differences in skin properties. The investigators were not blinded to allocation during the experiments or outcome assessment due to the nature of the sample preparation. However, to ensure the robustness of the statistical analysis, the results were independently verified by a different researcher who conducted the analysis with anonymized sample labels, and the same results were confirmed.

Data Availability

All codes used in this study are publicly available on GitHub. All other relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon request. The quantitative data used in this study are provided in the Source Data file.

Code Availability

All python code related to this manuscript is available on GitHub

(<https://doi.org/10.5281/zenodo.14729473>; <https://doi.org/10.5281/zenodo.14729490>)

Figures and Figure legends.

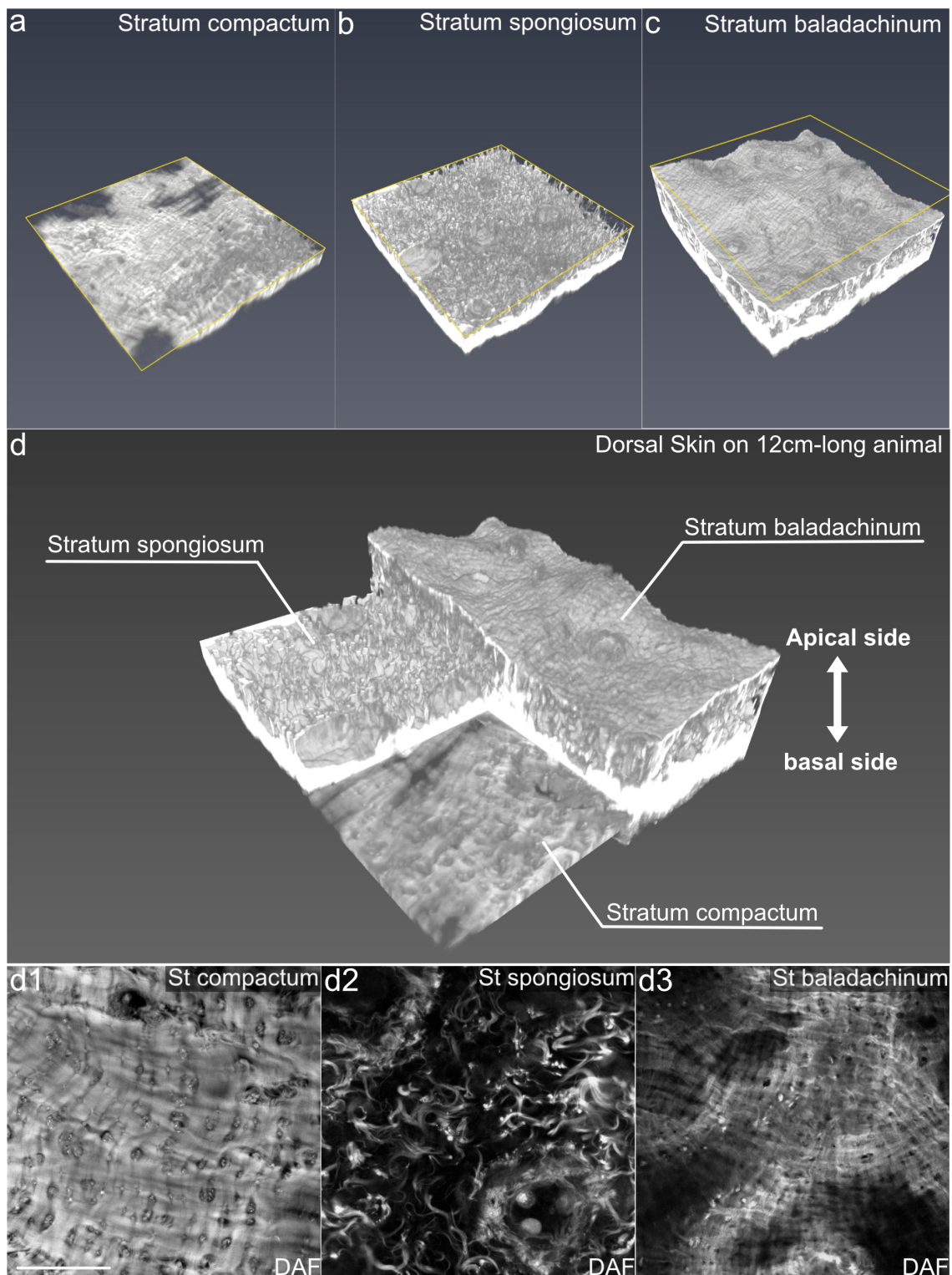


Figure 1: Structure of type I collagen in the 12-cm-long axolotl dorsal skin.

(a–d) DAF was used to label the collagen. The images were taken using a confocal microscope and reconstituted using AMIRA software. Clipping image for visualizing the *stratum compactum* (a),

stratum spongiosum (b), and *stratum baladachinum* (c). (d) The combined image shows all three layers of the dermal collagen structure in the dorsal skin. (d1–d3) Representative images of collagen fibers from the original data taken using confocal microscopy. (d1) *Stratum compactum*, (d2) *stratum spongiosum*, and (d3) *stratum baladachinum*. d1-d3 are the same magnification; the scale bar in d1 is 50 μm . These collagen structures were confirmed $n=10$ biologically independent samples. The representative images are shown.

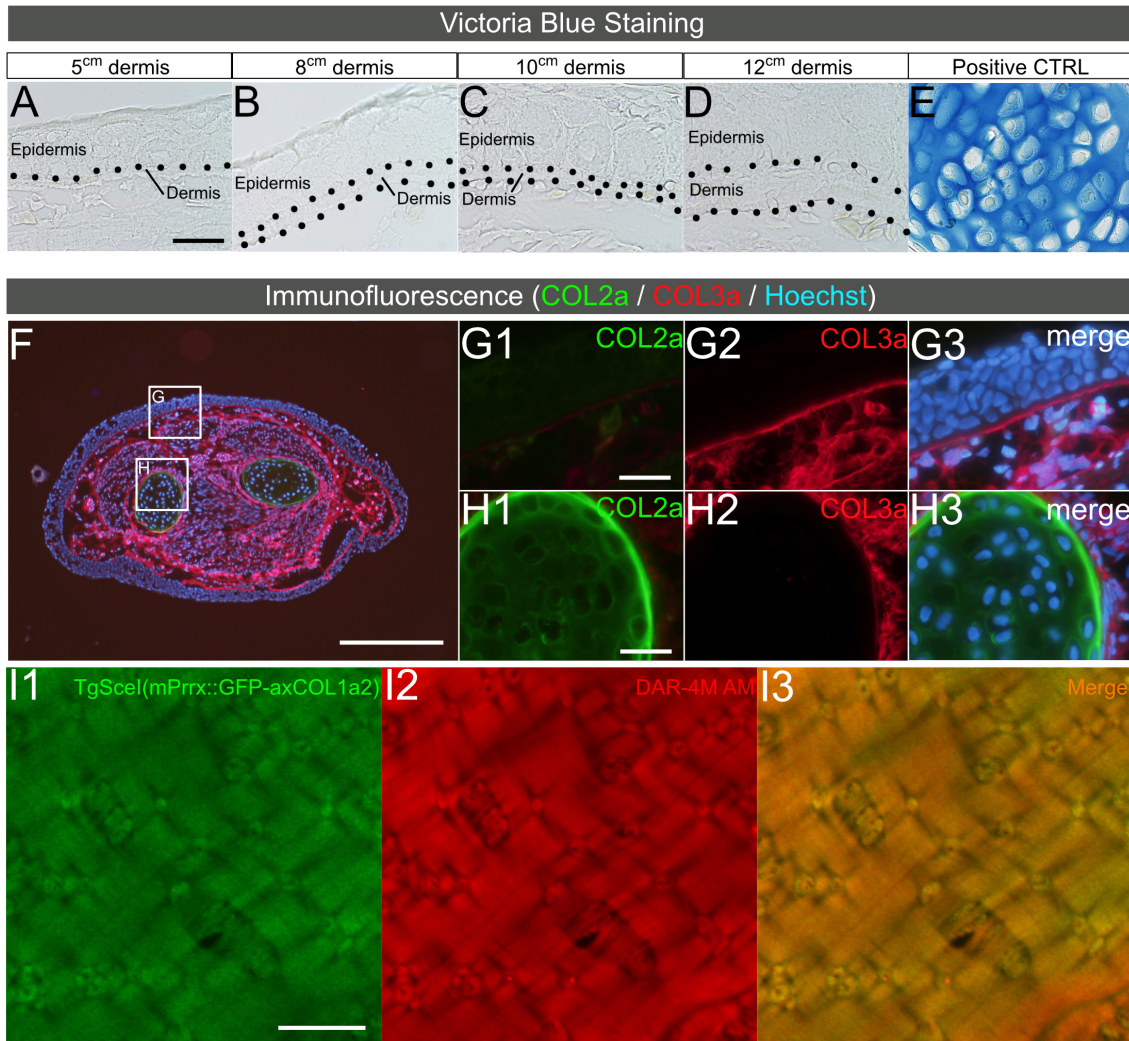


Figure 2: Specificity of the collagen probe for Colla in the axolotl skin

(a–e) Victoria Blue staining for evaluation of the presence of elastin. (e) The positive control in the limb bone of axolotls. No apparent staining could be observed throughout axolotl dermal development. The expression patterns were confirmed in five independent samples at each time point and the representative images are shown. The black dotted lines indicate the border of the dermis. (f–h) immunofluorescence of COL2 (green), COL3 (red) and nuclei (Hoechst 33342; blue). (f) The transverse section of axolotl limb. (g1–g3) High magnification view of dermal region in f. The very faint red/yellow color of g1 is due to the bleed-through of the COL3 signal from g2. (h1–h3) High magnification view of bone region (positive control) in f. The expression patterns were confirmed in three independent samples at each time point and the representative images are shown. (i1–i3) The signals of GFP (mPrrx::GFP-axCOL1a2 transgenic) and DAR. The expression patterns were confirmed in three biologically independent samples at each time point and the representative images are shown. The scale bars in a, f, g1, h1 and i1 indicate 50, 500, 50, 50 and 10 μm respectively. a–e is the same magnification.

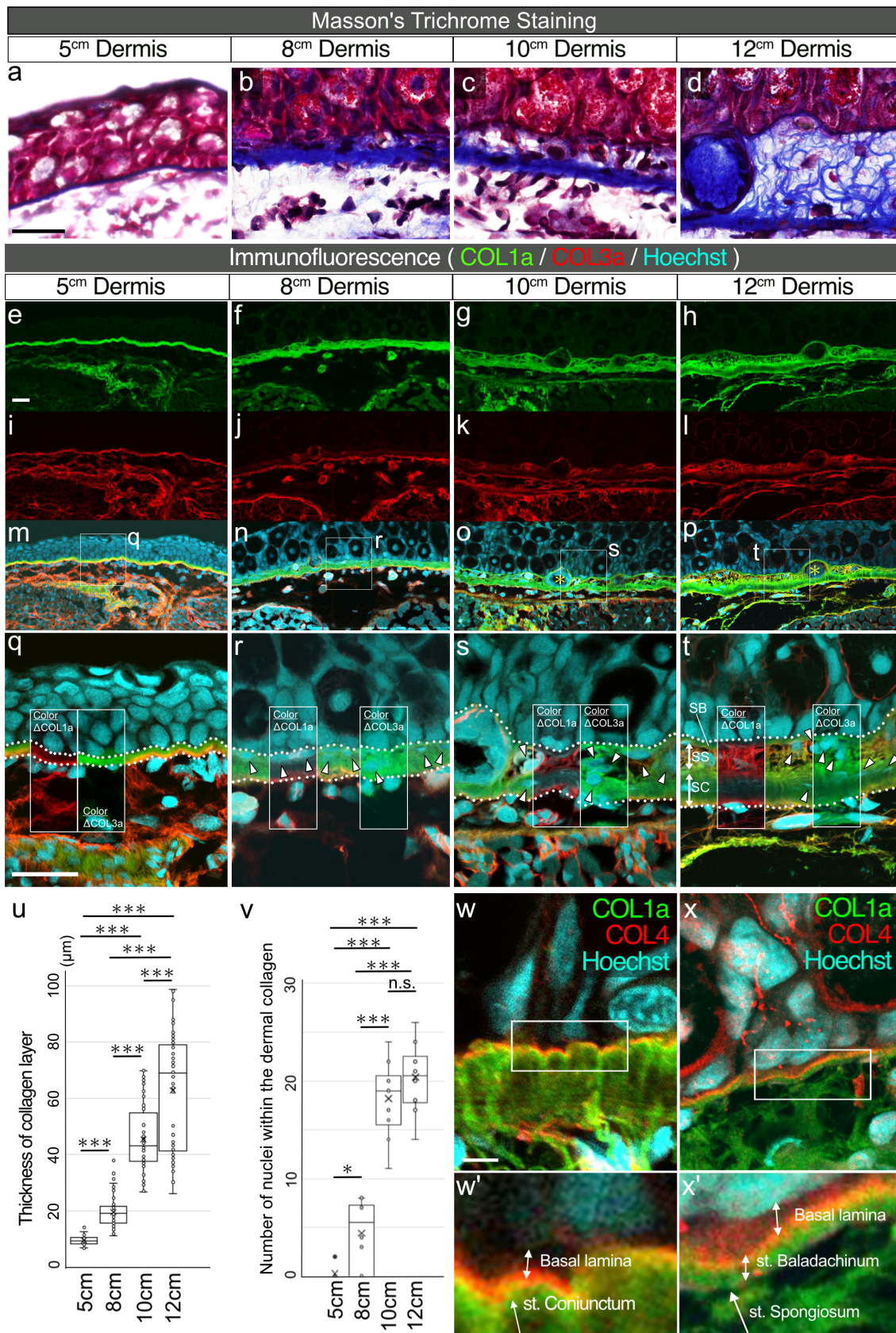


Figure 3: Development of dermal collagen structure during growth.

(a–d) Histological observation of the axolotl dorsal skin. Masson's trichrome staining was performed for 5^{cm} to 12^{cm} dermis. Blue indicates collagen, red indicates cytoplasm, and dark brown color indicates nuclei. (e–t) Immunofluorescence of (e–h) COL1a (green), (i–l) COL3 (red), and nucleus (Hoechst 33342; blue) in the axolotl dermis. (m–p) Merge images of e–l. (q–t) High magnification view of the boxed region in m–p. SB, SS and SC indicate *stratum baladachinum*, *stratum spongiosum* and *Stratum compactum*. The open windows in q–t display views with one color deleted. For example, Δ COL1a indicates that the green signal of COL1a is omitted from the region. The expression patterns were confirmed in five independent samples at each time point and the representative images are shown. (u) Analysis of thickness of collagen layer ($n=4$ biologically independent samples) Each point on the box plot represents all measured data points. p values from up to down: 1.40×10^{-37} , 3.10×10^{-42} , 2.91×10^{-32} , 2.60×10^{-9} , 4.92×10^{-34} , 2.72×10^{-30} . (v) Counting the number of nuclei in the dermis ($n=4$ for 5cm, and $n=5$ for 8cm, 10cm, 12cm biologically independent samples) Each point on the box plot represents all measured data points. p values from up to down: 1.63×10^{-10} , 3.74×10^{-9} , 3.11×10^{-8} , 1.16, 6.28×10^{-7} , 0.025. (w) Immunofluorescence of COL1a (green), COL4 (red) and nucleus (Hoechst 33342; blue) of the *stratum coniunctum*. (w') High magnification view in w. (x) Immunofluorescence of COL1a (green), COL4 (red) and nucleus (Hoechst 33342; blue) of the 12cm. (x') High magnification view in x. The scale bars in a, e, and q are 50 μ m, and that in w is 10 μ m. a–d, e–o, q–t, and w–x are the same magnification. The expression patterns were confirmed in five independent samples at each time point and the representative images are shown. The white dotted line in q–t indicates the dermal collagen layer. The statistical analysis was performed using two-tailed ANOVA, followed by pairwise two-tailed t-tests with Bonferroni correction. $*p < 0.05$, $**p < 0.025$, $***p < 0.01$. n.s. = no significant difference. Each box represents the interquartile range (IQR, the range between the 25th and 75th percentile) with the mid-point of the data, bars indicate the upper and lower value within 1.5 times the IQR. Source data are provided as a Source Data file.

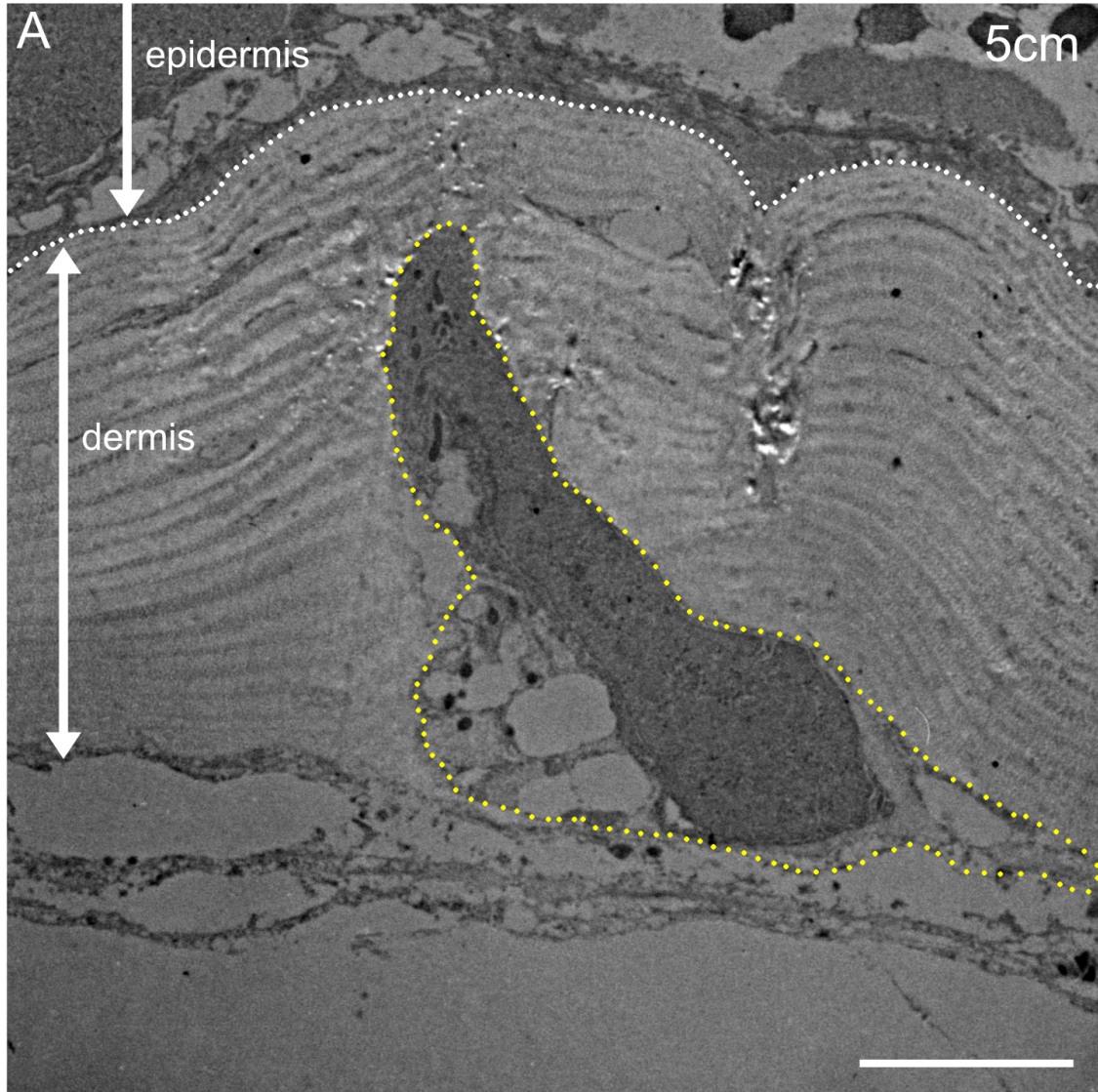


Figure 4: Cell invasion into the dermal collagen layer at the 5cmdermis.

The yellow dotted lines indicate the border of the cell. The white dotted lines indicate the border of the epidermis and dermis. The scale bar is 5μm. Seven images showing cell invasion were obtained from three biologically independent samples. The representative image is shown.

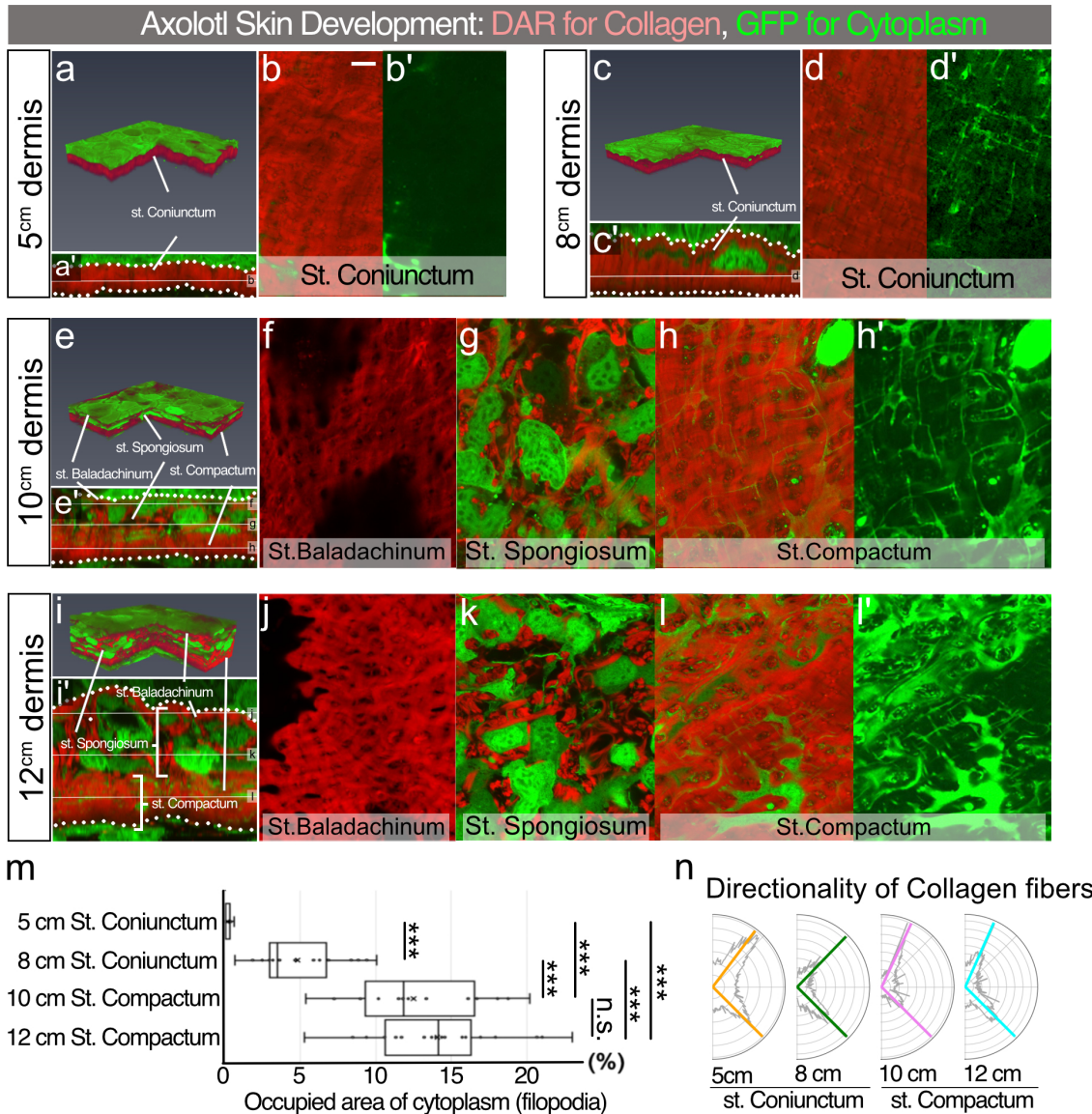


Figure 5: Developmental changes of entire collagen structure and collagen fibers at each stage.

The red color indicates collagen, and the green color is for cytoplasm. (a–f) The structure of collagen fibers and the presentation of cytoplasm on *stratum coniunctum* at 5^{cm} and 8^{cm} dermis. (a, c) 3-dimensional (3D) structure. (a', c') Orthogonal image from the reconstituted images. (b, d) The merge image of collagen fibers and cytoplasm. (b', d') Cytoplasm. (e–l) The structure of collagen fibers and the presentation of cytoplasm on three collagen layers (*stratum baladachinum*, *stratum spongiosum*, *stratum compactum*) at 10^{cm} and 12^{cm} dermis. (e, i) 3D structure (e', i') Orthogonal image from the reconstituted images. (f, j) The structure of collagen fiber in *stratum baladachinum*. These images are the maximum intensity projection. (g, k) The structure of collagen fiber and the location of cytoplasm at the *stratum spongiosum* and (h, l) *stratum compactum*. (h', l') Cytoplasm. The expression patterns were confirmed in five independent samples at each time point and the representative images are shown. (m) Quantification of occupied area of cytoplasm (filopodia) ($n=3$

biologically independent samples.) Each point on the box plot represents all measured data points. p values from left to right: 1.89×10^{-6} , 1.59×10^{-5} , 6.20×10^{-8} , 1.66, 4.17×10^{-9} , 6.36×10^{-12} . (n) Analysis of directionality of collagen fibers. The same analysis was performed at five biologically independent samples and the representative pattern was selected and presented. The scale bars in b is 10 μm . All images are the same magnification. The white line in a', c', e', i' indicate the dermal collagen layer. The statistical analysis was performed using two-tailed ANOVA, followed by pairwise two-tailed t -tests with Bonferroni correction. $*p < 0.05$, $**p < 0.025$, $***p < 0.01$. n.s. = no significant difference. Each box represents the interquartile range (IQR, the range between the 25th and 75th percentile) with the mid-point of the data, bars indicate the upper and lower value within 1.5 times the IQR. Source data are provided as a Source Data file.

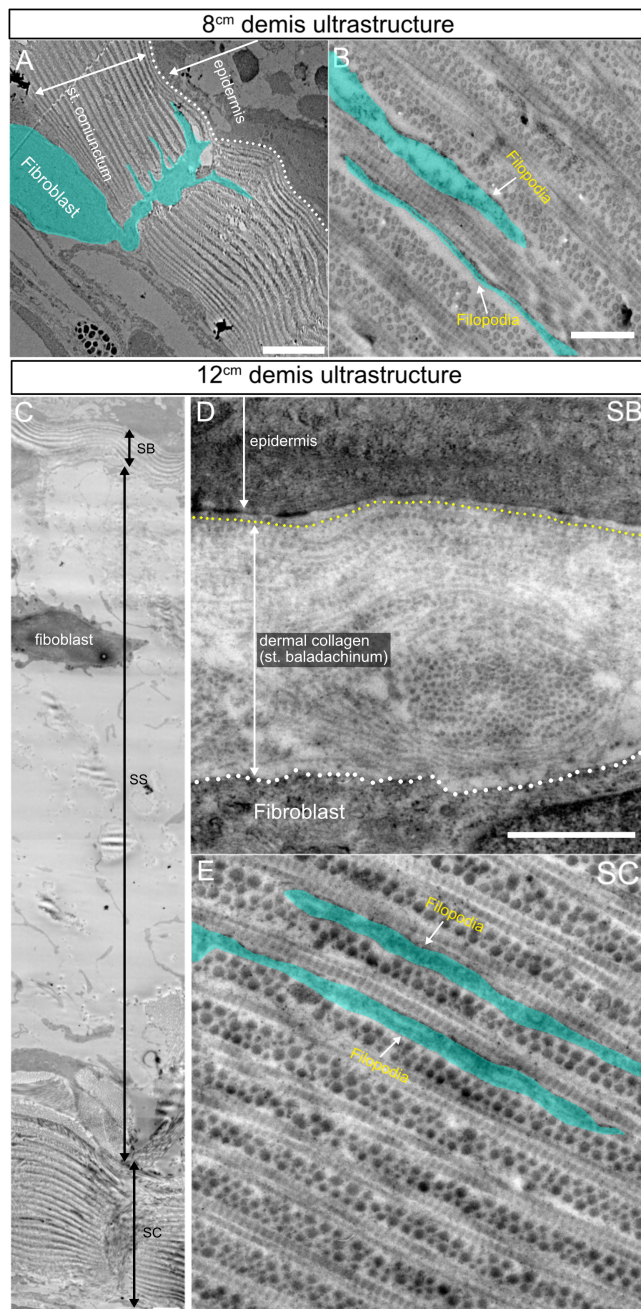


Figure 6: Observation of the dermal collagen structure using electron microscopy (EM). (a, b) The 8cm dermis. (c–e) The 12cm dermis. (c) The entire dermal structure. (d) The stratum baladachinum. (e) The stratum compactum. SB: stratum baladachinum. SS: stratum spongiosum. SC: stratum compactum. The white dotted lines in a and d indicate the boundaries with the cells. The yellow dotted lines in d indicate the basement membrane (lamina densa). The scale bars in a, b, c and d are 5 μm , 50 nm, 2 μm and 1 μm , respectively. The blue in a, b and e indicates lattice patterned cells or filopodia of lattice patterned cells. The representative images were selected from 15 images (3 independent samples).

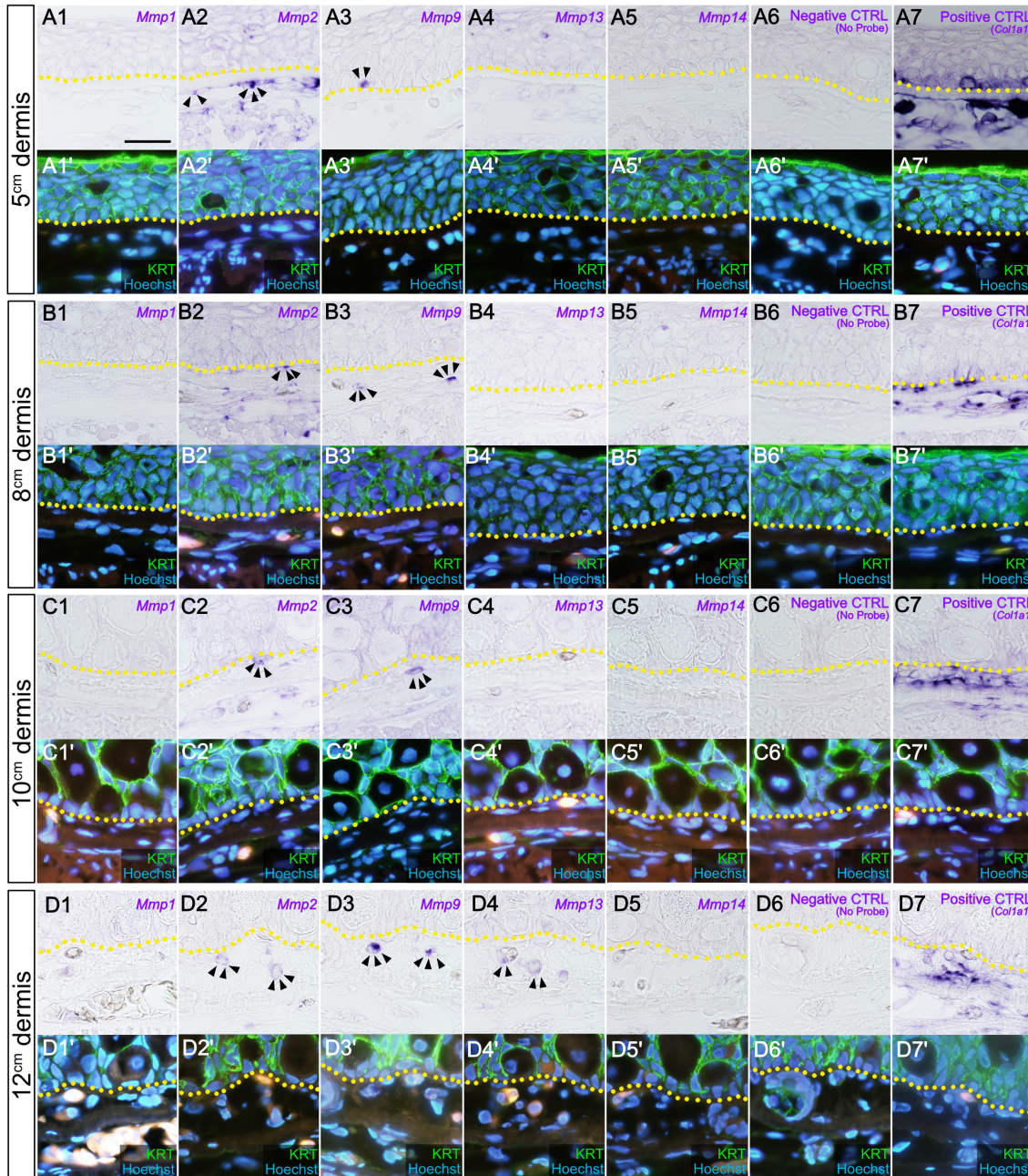


Figure 7: Expression of Mmp1, Mmp2, Mmp9, Mmp13, and Mmp14 in the developing axolotl dermis.

The expression patterns at each stage were revealed by in situ hybridization (ISH). (a) The 5cm dermis. (b) The 8cm dermis. (c) The 10cm dermis. (d) The 12cm dermis. The upper rows in each dermis stage are the result of ISH. The lower rows show the results of the immunofluorescence (KRT, green) and Hoechst33342 staining (blue). The images in the upper and lower images are the identical section. The scale bar in a is 50 μ m. All images are the same magnification. The yellow dotted lines indicate the border of the epidermis and dermis. The arrowheads indicate the ISH signals. The gene expression pattern was confirmed in four independent samples at each time point.

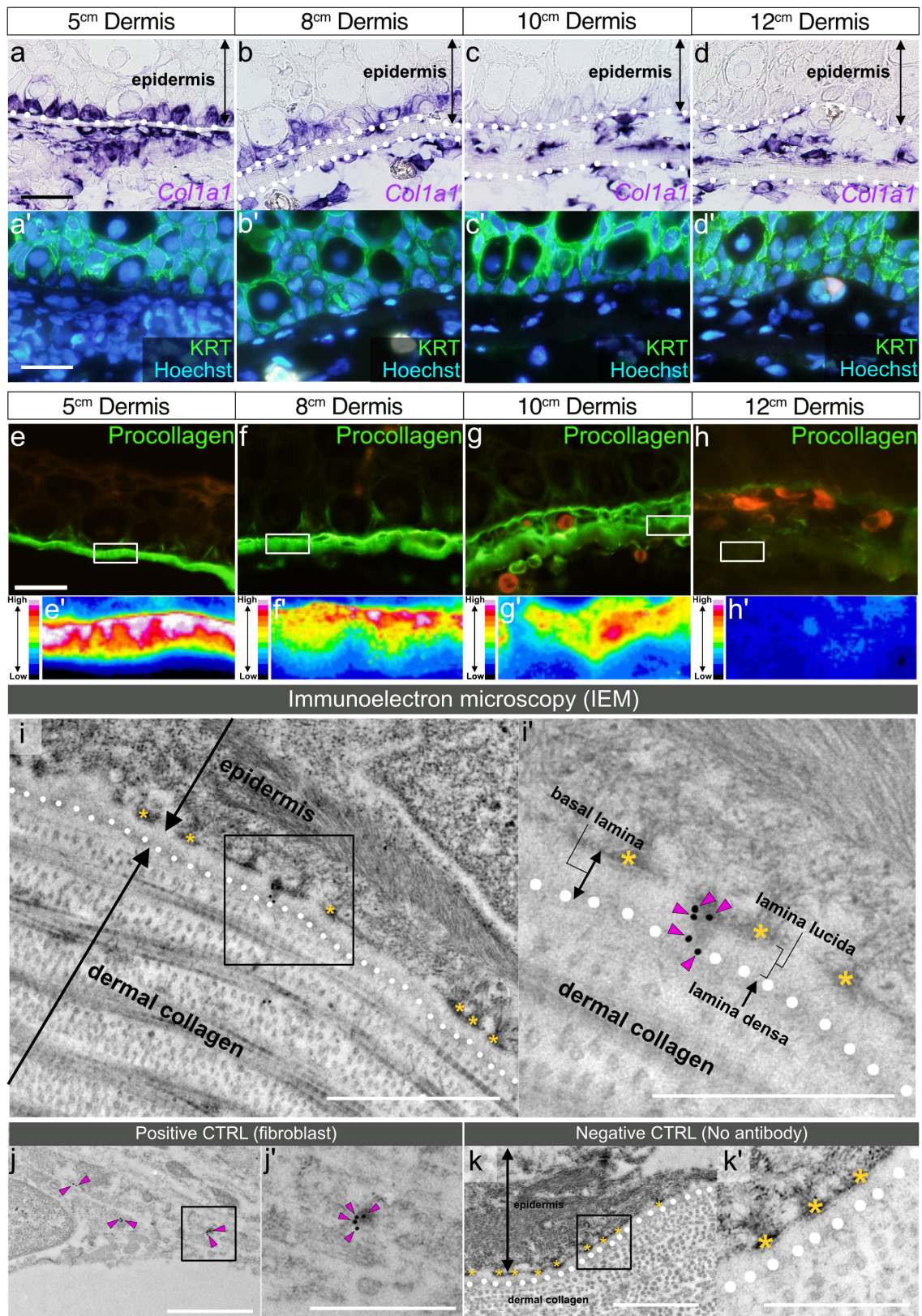


Figure 8: Type I collagen production in keratinocytes.

(a–d) The *Col1a1* expression pattern in the dorsal skin was revealed by *in situ* hybridization. (a'–d')

Immunofluorescence of pan-cytokeratin (KRT) and Hoechst signal (nucleus). (e–h) Procollagen expression from the 5^{cm} dermis to the 12^{cm} dermis. (e'–h') The heat map shows the signal strength of the boxed region in e–h. The expression patterns were confirmed in five independent samples at each time point and the representative images are shown. Heatmap analysis was performed on three selected biologically independent samples from $n=5$, and a representative example is presented. (i–k) Immunoelectron microscopy (IEM) using procollagen antibody. (i') Higher magnification views of the boxed region in i. Arrowheads indicate the signals from in the basement membrane (*lamina lucida*). (j, j') Positive control. Showing collagen production in mesenchymal cells. (j') The high magnification view of the boxed region in j. The pink arrowheads indicate the IEM signals from in the mesenchymal cells. (k, k') Negative control (no antibody). (k') The high magnification view of the boxed region in k. Yellow asterisks indicate the hemidesmosomes. The white dotted line indicates the basement membrane (*lamina densa*). The scale bars in a, a', and e are 50 μm , those in i, j, j', k and k' are 1 μm , and that in i' is 500 nm. More than 20 IEM images were collected from the three biologically independent samples. And the representative images are shown.

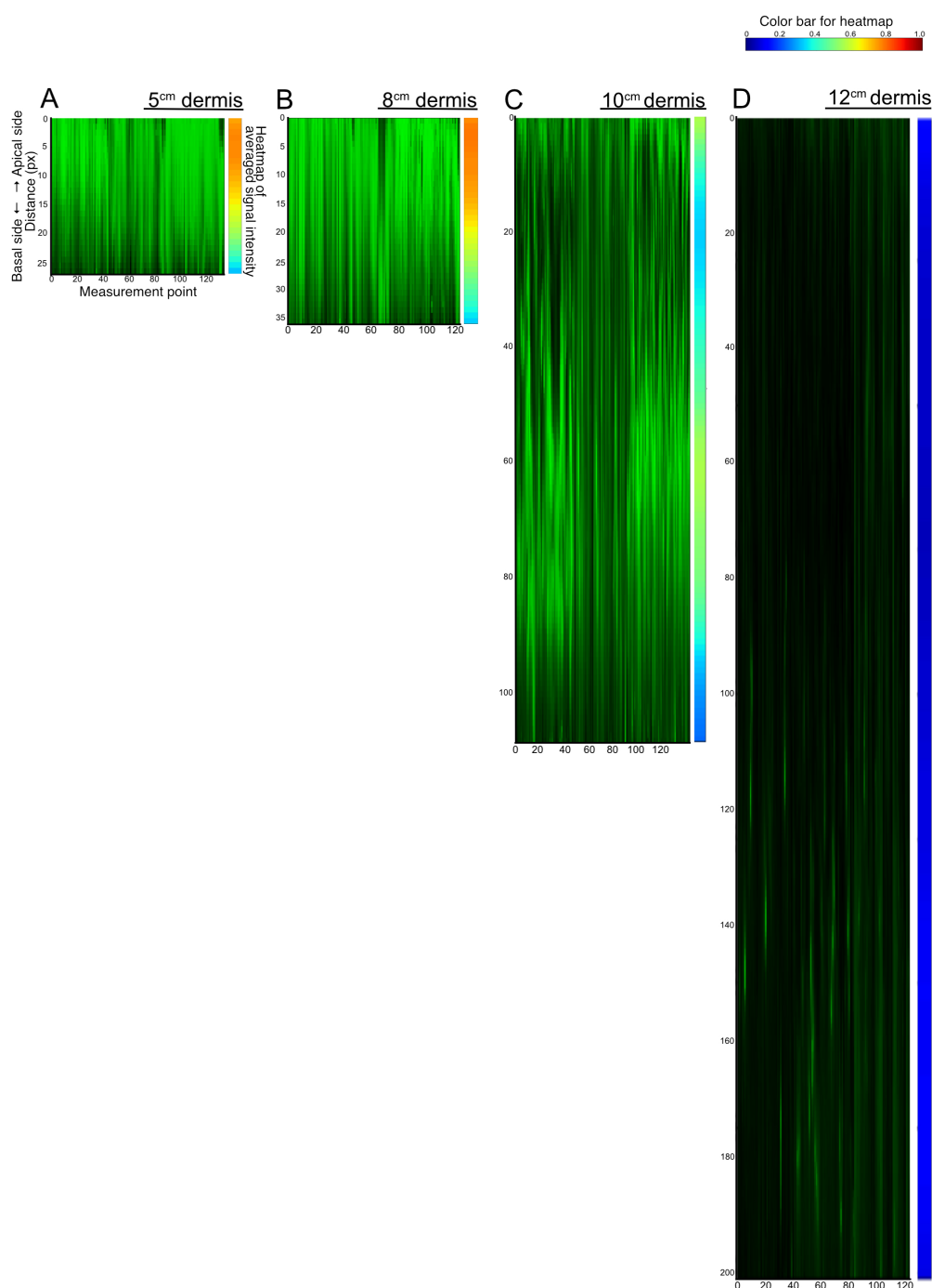


Figure 9: The integrated heatmap of the procollagen signals in the dermis.

(a) The 5 cm dermis, (b) The 8 cm dermis, (c) The 10 cm dermis, and (d) The 12 cm dermis. In each panel, the left side shows the integrated image, and the right side shows its corresponding heatmap. The color bar for the heatmap is shown at the top right. Data from three independent samples were combined and analyzed. Source data are provided as a Source Data file.

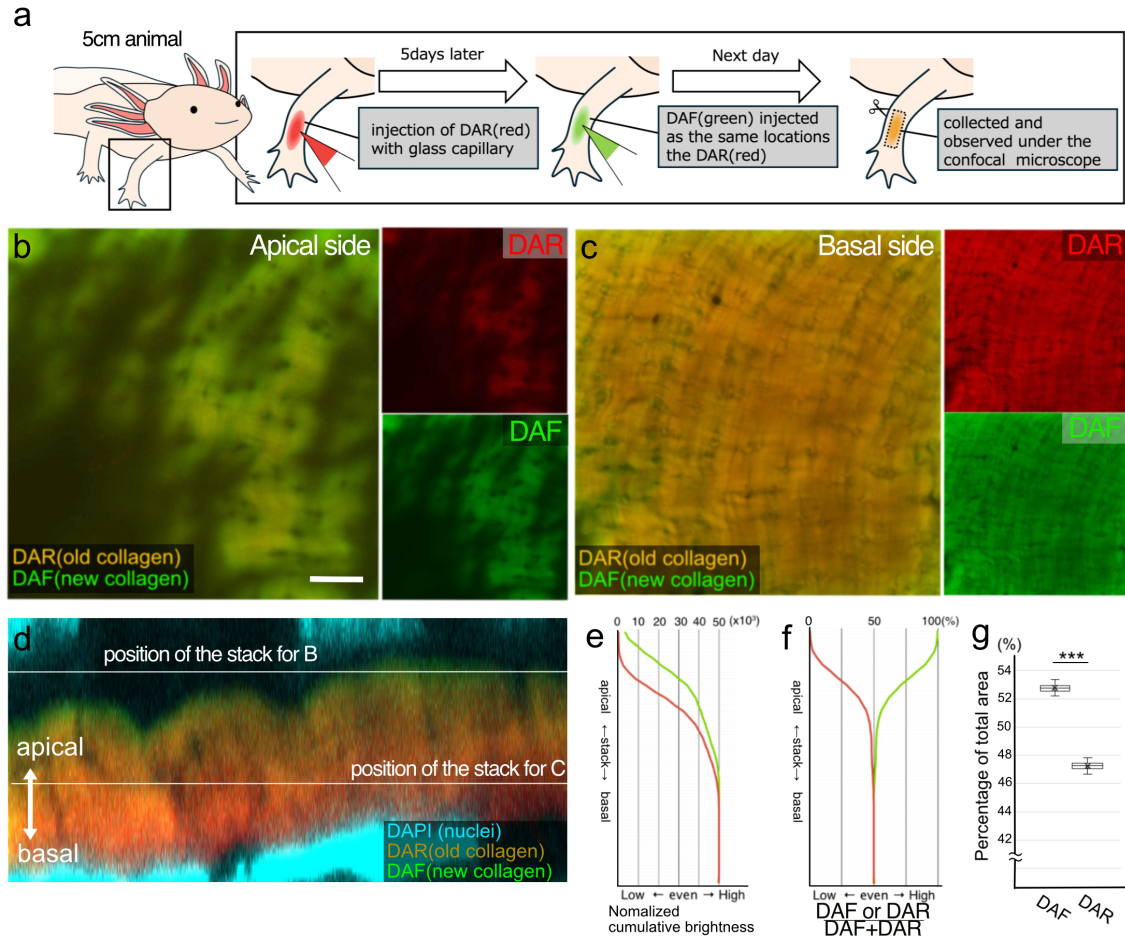


Figure 10: Structural changes of dermal collagen conducted by keratinocytes.

(a) Schematic diagram of the experiment. (1) Injection of DAR under the dorsal skin (Day0). (2) DAF is injected at the same locations as DAR on Day 5. (3) Samples were harvested on Day 6. (b–d) Confocal microscopic images: (b) epidermis side, (c) hypodermis side, (d) orthogonal image from the reconstituted images. The results were confirmed in four independent samples. (e) Histogram of DAR and DAF areas from all stack images. (f) Histogram of the rate of each color. The same analysis was performed on four biologically independent samples and the representative histograms are shown. (g) Percentage of area of DAR and DAF ($n=4$ biologically independent samples). p values = 3.48×10^{-6} . The scale bars in b and c are 10 μm . The statistical analysis was performed using t -tests (two-tailed). *** $p < 0.01$. n.s. = no significant difference. All images are the same magnification. Each box represents the interquartile range (IQR, the range between the 25th and 75th percentile) with the mid-point of the data, bars indicate the upper and lower value within 1.5 times the IQR. Source data are provided as a Source Data file.

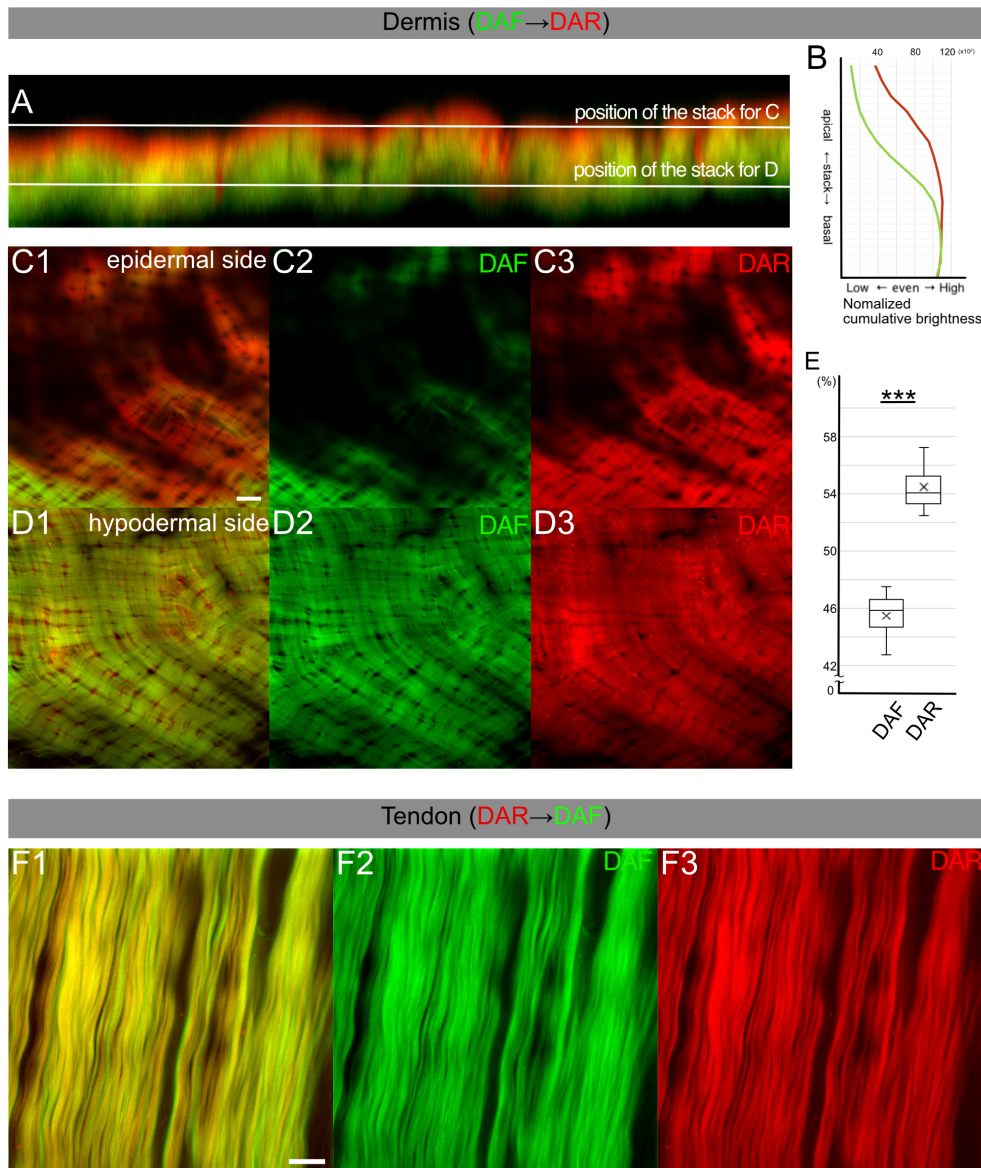


Figure 11: Accumulation of collagen on the epidermal side of the stratum conjunctum. (a) The orthogonal view from the reconstituted confocal microscopic images. (b) The histogram showing the intensity of the fluorescent signal of a. (c) DAR and DAF signals on the epidermal side. (d) The hypodermal side. (e) The percentage of DAR and DAF areas in all stacked images. (n=4 biologically independent samples.) (f) Accumulation of type I collagen in the tendon. The same experiment was performed on all four biologically independent samples and the representative image and histograms are shown. Statistical analysis was conducted using a two-sided t-test. ***p = 0.000768. The scale bar in c and f1 are 10μm. Each box represents the interquartile range (IQR, the range between the 25th and 75th percentile) with the mid-point of the data, bars indicate the upper and lower value within 1.5 times the IQR. Source data are provided as a Source Data file.

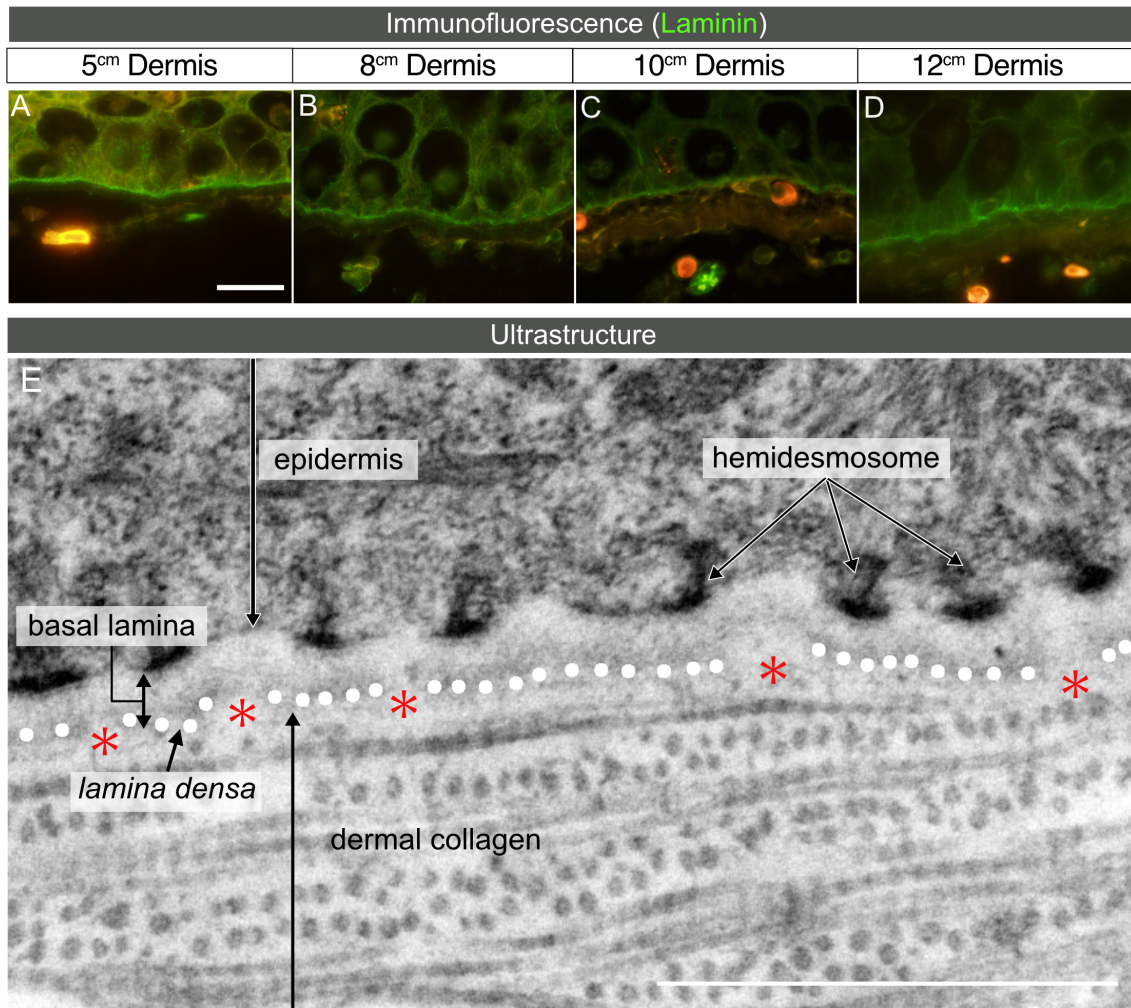


Figure 12: The structure of the basement membrane.

(a–d) Immunofluorescence for Laminin (green). The red-orange color is autofluorescent. (e) The electron microscopy image shows the structure of the basement membrane. Asterisks indicate the gap of the basement membrane, suggesting the holelike structures in the basement membrane. The white dotted lines indicate lamina densa. The scale bars in a and e are 50 μm and 1 μm , respectively. The expression patterns were confirmed in three biologically independent samples and the representative images are shown

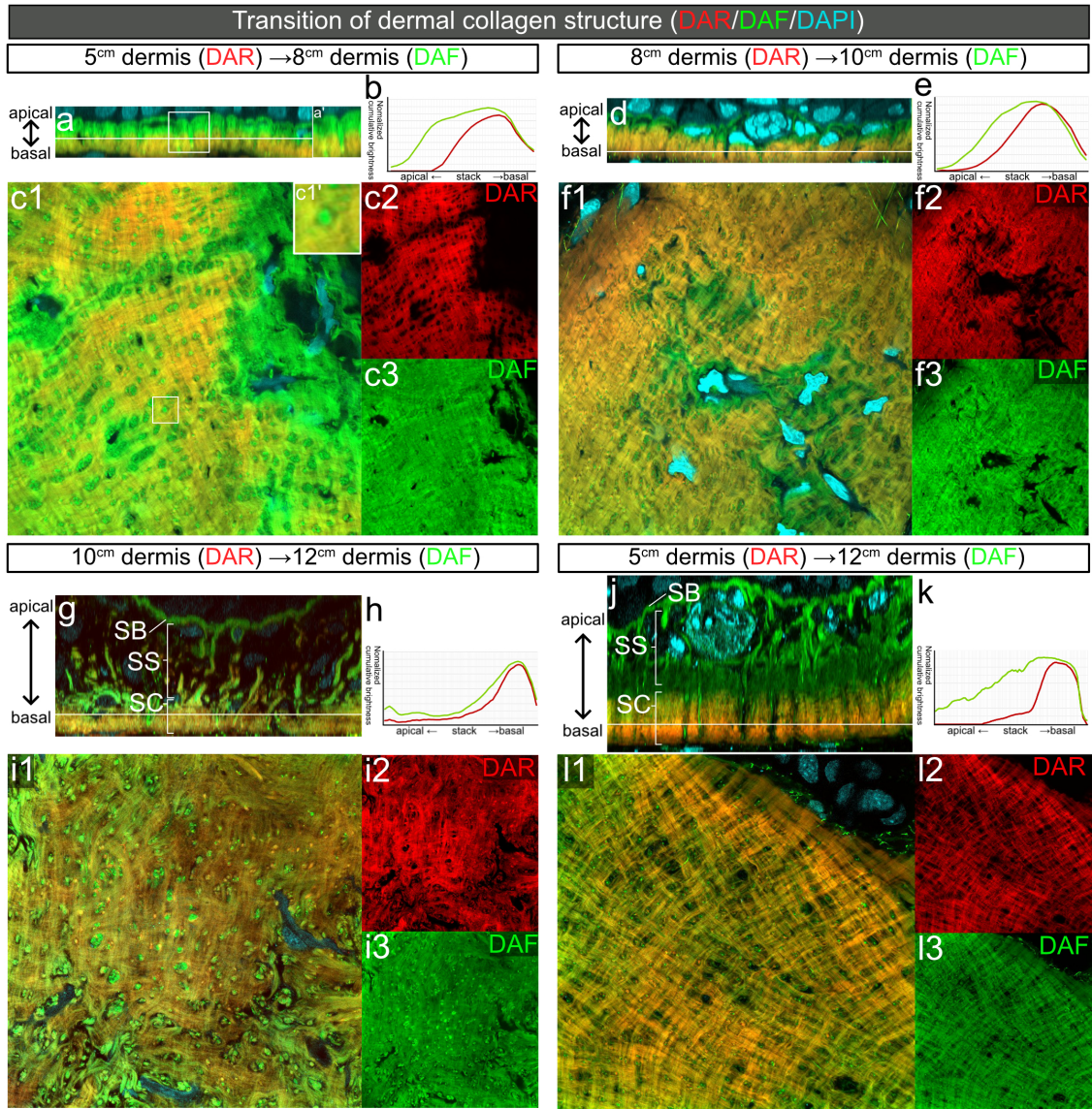


Figure 13: Dynamics of dermal collagen formation during growth.

(a–l) Changes in collagen structure from 5^{cm} to 8^{cm} dermis, 8^{cm} to 10^{cm} dermis, 10^{cm} to 12^{cm} dermis, and 5^{cm} to 12^{cm} dermis. (a, d, g, j) Orthogonal images from the reconstituted confocal images. (b, e, h, k) Histograms of DAR and DAF areas from all stacked images. (c1–c3, f1–f3, i1–i3, l1–l3) Representative fiber images at the basal side of the dermis. The scale bars in c is 20 μ m. All images are the same magnification. SB, SS and SC indicate *stratum baladachinum*, *stratum spongiosum* and *Stratum compactum*. The experiment was confirmed five biologically independent samples at each time point. The representative histograms and images are selected and shown from the five samples. Source data are provided as a Source Data file.

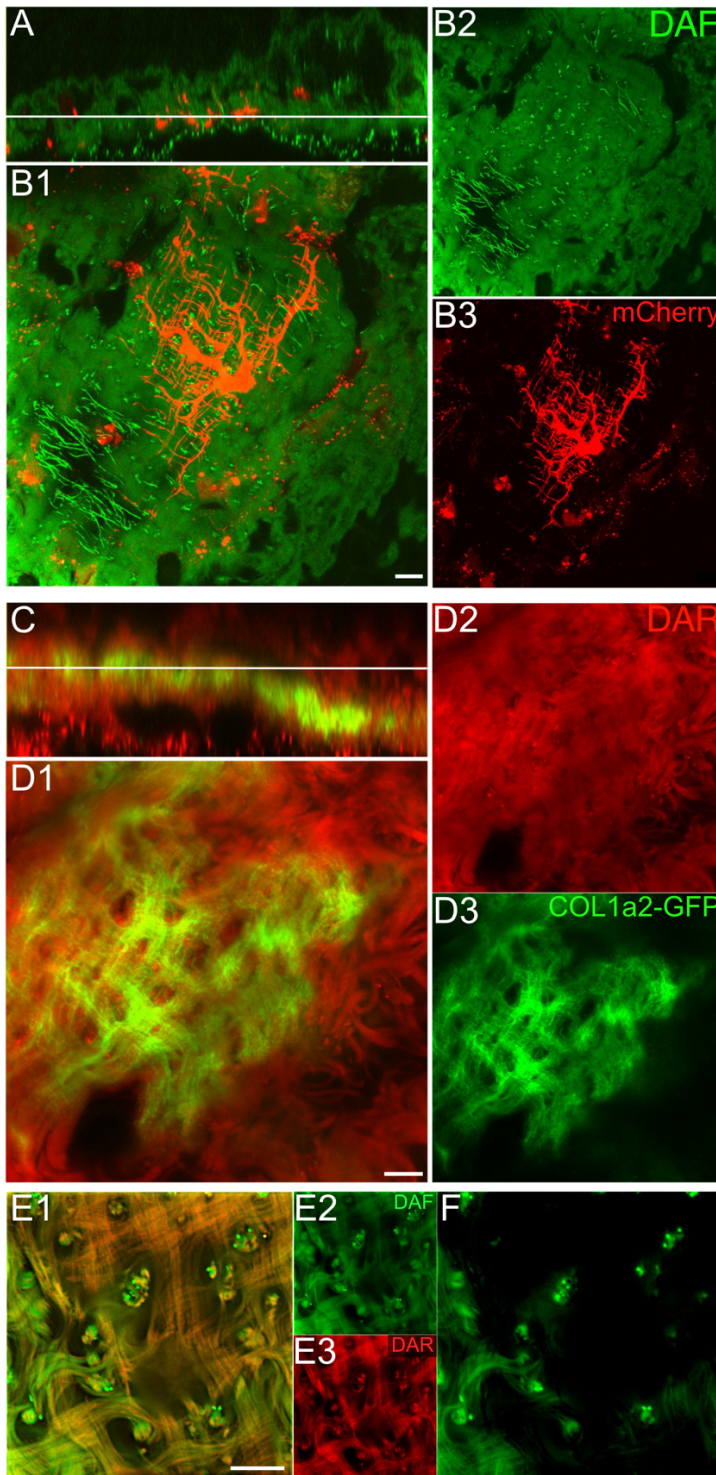


Figure 14: The lattice-patterned fibroblasts in the stratum compactum.

(a, b) The cell morphology was visualized by transfected mCherry (red). The collagen structure was visualized by DAF (green). (a) The orthogonal image from the reconstituted image. (b) The plane image indicated by the white line in a. b1: merged image. b2: DAF (collagen). b3: mCherry (cytoplasm). (c, d) Collagen produced by lattice-patterned cells was visualized by transfection of

COL1a2-GFP vector. The dermal collagen structure was visualized using DAR. (c) The orthogonal image from the reconstituted images. (d) The plane image indicated by the white line in c. c1: merged image. c2: DAR (whole collagen). c3: COL1a2-GFP (collagen secreted from the single transfected cell). (e) The pulse-chase experiments conducted between 10cm dermis and 12cm dermis. e1: merged image. e2: DAF. e3: DAR. (f) The DARpositive regions of f1 were subtracted from the DAF-positive region of e2. The scale bars in b, d, and e are 10 μ m, respectively. The images e1–e3 were processed using deconvolution. The experiment was confirmed in three biologically independent samples and the representative images are shown.

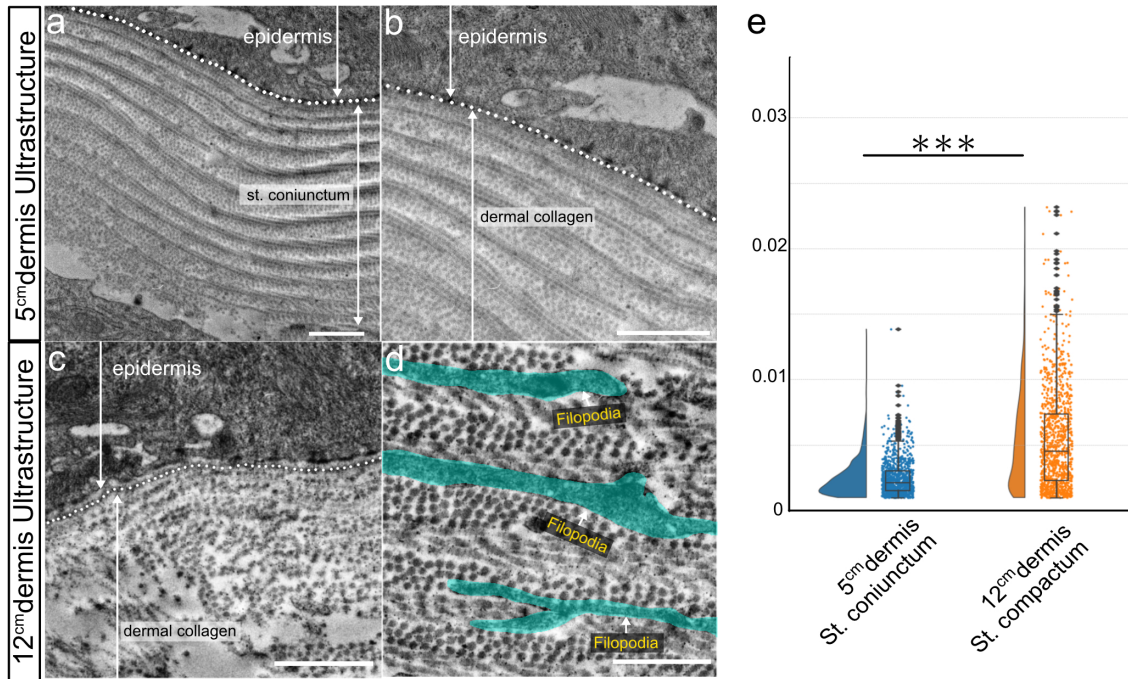


Figure 15: Changes in collagen fiber in the dermis development.

(a, b) Ultrastructure of 12^{cm} dermis. (a) Keratinocyte and collagen fibers in the *stratum baladachinum*. (b) Collagen fibers in the *stratum compactum*. (c, d) Ultrastructure of 5^{cm} dermis. (c) Keratinocyte and collagen fibers in the *stratum coniunctum*. (d) Epidermis side of the *stratum coniunctum*. (e) Analysis of the size of collagen fibrils ($n=3$ biologically independent samples). Each point on the box plot represents all measured data points. p values: 2.83×10^{-99} . The statistical analysis was performed using t -tests (two-tailed). *** $p < 0.01$. The representative images were selected from 15 images made from three independent samples. The scale bars in a, b, c, d are 1 μm . Each box represents the interquartile range (IQR, the range between the 25th and 75th percentile) with the mid-point of the data, bars indicate the upper and lower value within 1.5 times the IQR. Source data are provided as a Source Data file.

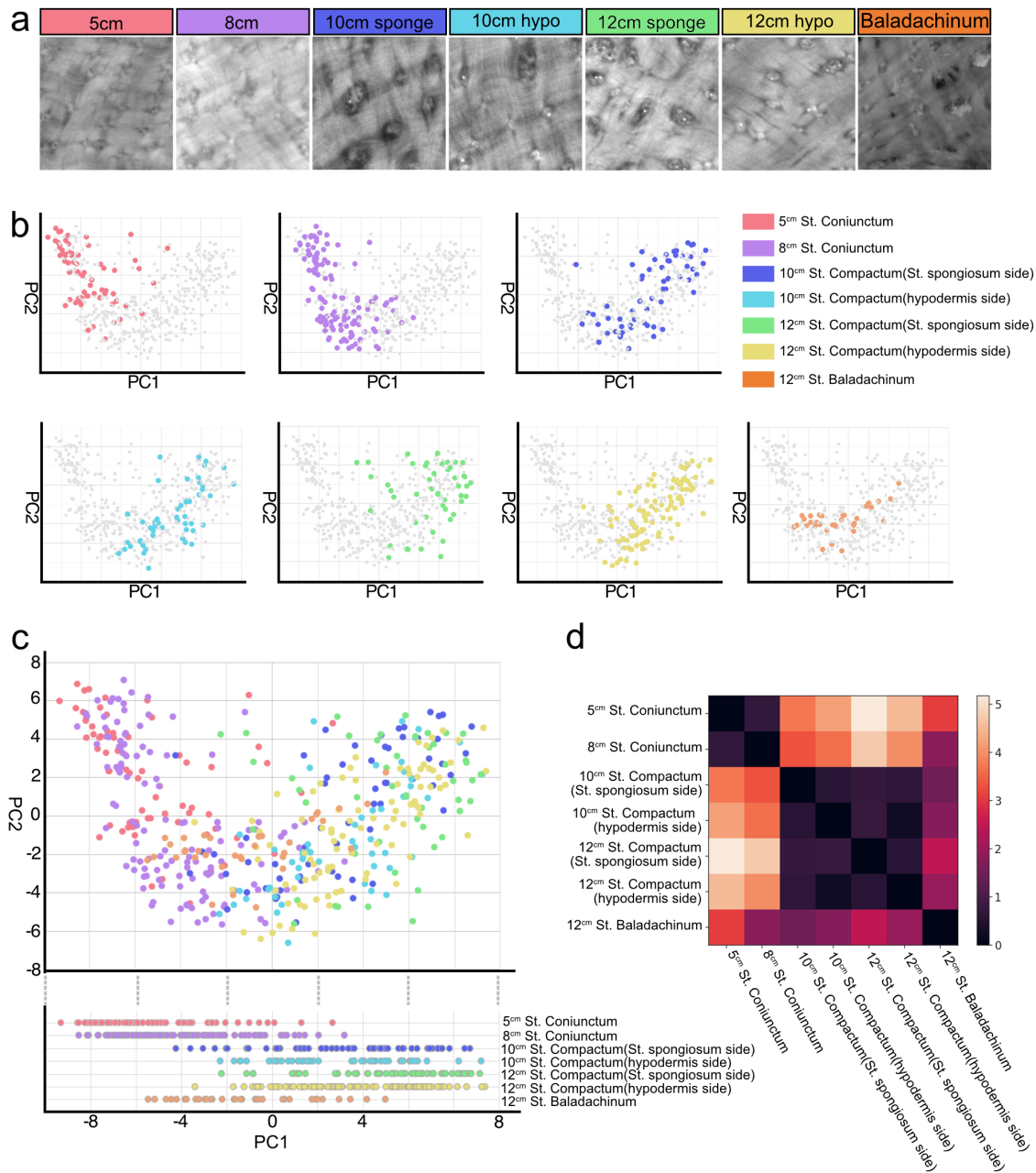


Figure 16: Analysis of structural changes in lattice-like collagen due to the involvement of lattice-patterned fibroblasts.

(a) Representative images used for analysis. All images are the same magnification. The most representative image is shown from more than 30 images obtained from $n=5$ biologically independent samples. (b, c) Principal components analysis (PCA) on features extracted using ResNet50. (b) PCA plot for each group; (c) PCA plot for all groups. (d) Heatmap of the effects of the Mahalanobis distance for seven groups. All images used for this analysis was registered in Dryad. Source data are provided as a Source Data file.

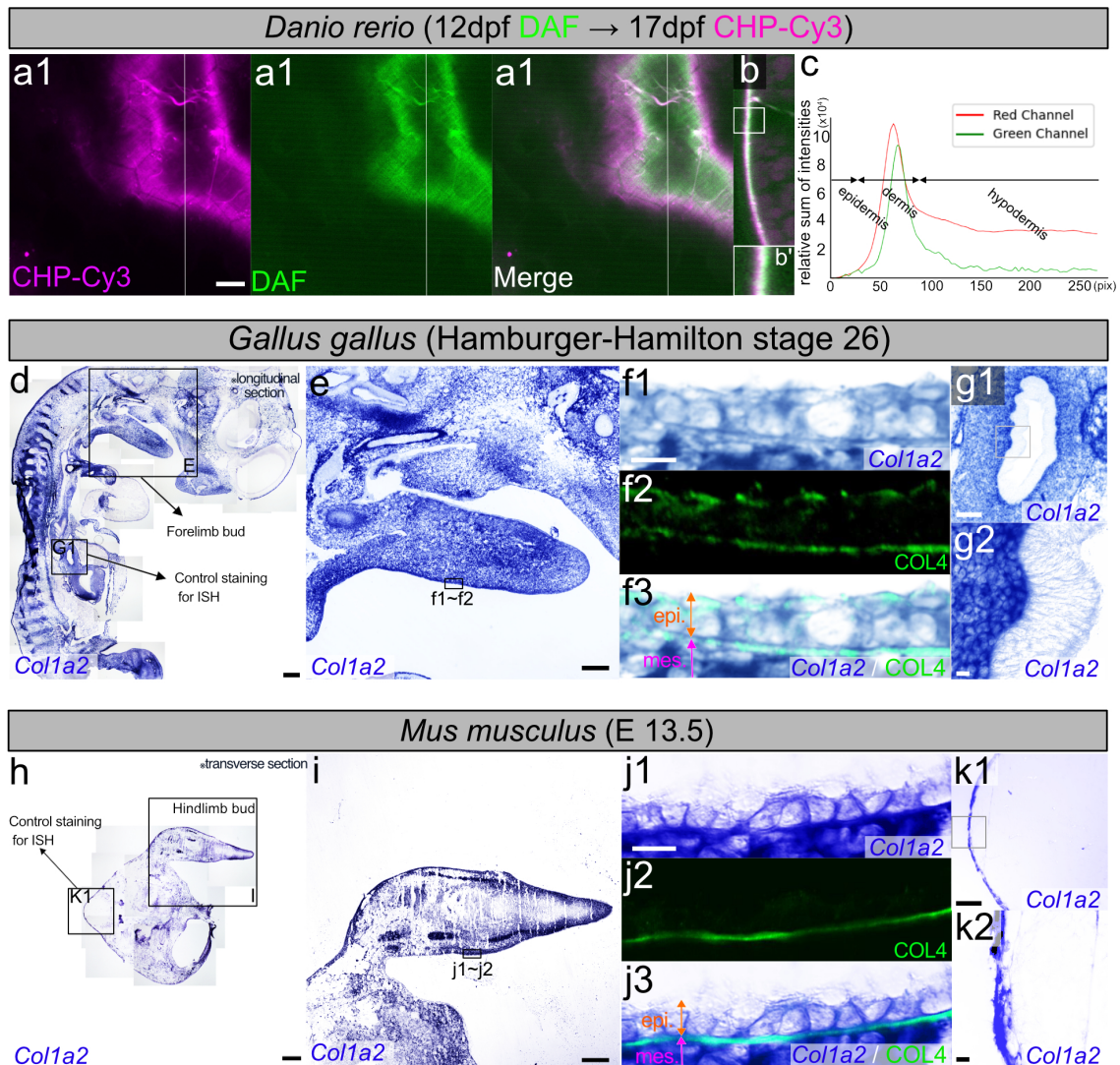


Figure 17: Type I collagen production by epidermal cells by various animal species.

(a–c) Pulse-chase experiments with *Danio rerio*. (a) DAF and CHP-Cy3 signals at the epidermal side. (a1) CHP-Cy3 (a2) DAF (a3) The merge image in a1 and a2. (b, b') Orthogonal view from the reconstituted confocal microscopic images. (c) The histogram of relative sum of brightness. (d–g) Hamburger- Hamilton stage 26 embryo in *Gallus gallus domestics* (d) *Col1a2* gene expression pattern in whole body section, (e) in the forelimb bud. (f) High magnification view in E. (f1) *Col1a2* gene expression pattern. (f2) Immunofluorescence of COL4. (f3) The Merge image of c1 and c2. f1–f3 are the flipped images of the boxed region in e. (g1) The negative control in ectoderm. (g2) High magnification view in d1. (h–k) Stage of E13.5 in *Mus musculus*. (h) *Col1a2* gene expression pattern in lower body section, (i) in the hindlimb bud. (j) High magnification view in f. (j1) *Col1a2* gene expression pattern. (j2) Immunofluorescence of COL4. (j3) Merge image of 1 and 2. j1–j3 are the flipped images of the boxed region in i. (k1) The negative control in back bone. (k2) High magnification view in k1. The d–k (exclude f2, j2) used pseudo-colouring. In f3 and j3, epi. and mes.

indicate epidermis and mesenchyme. The scale bars in a, f1, g2, j1 and k2 are 10µm, d and h are 500 µm, e and i are 100µm, g1 and k1 are 100µm. The results were confirmed in five biologically independent samples and experiments. Source data are provided as a Source Data file.

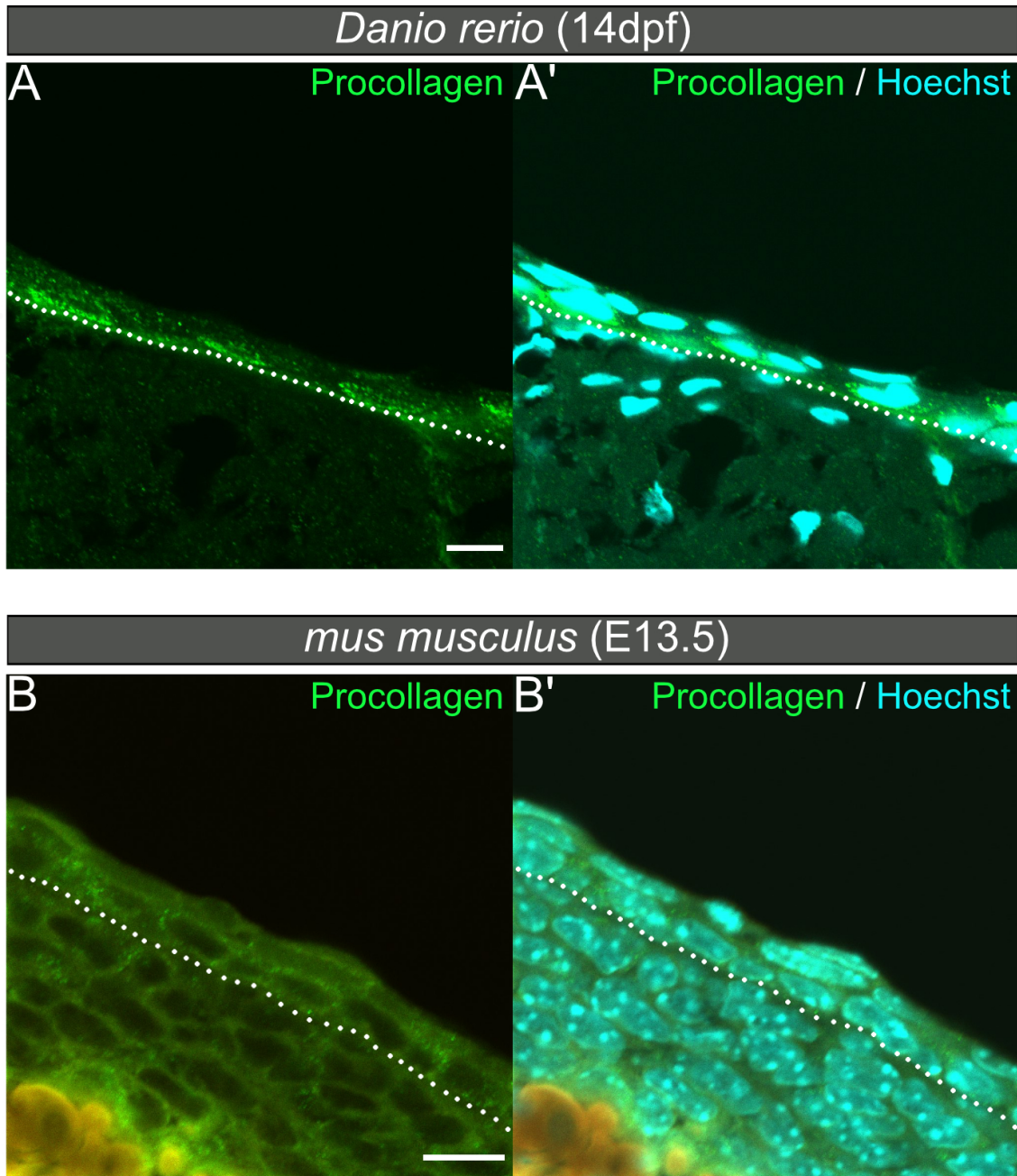


Figure 18: Immunofluorescence of Procollagen (green) in the skin of *Danio rerio* and *Mus musculus*. (a) Procollagen expression (green) in the skin of *Danio rerio* (a') The merged image of Procollagen (green) and nuclei (Hoechst 33342; blue) in the skin of *Danio rerio* (b) Procollagen expression (green) in the skin of *Mus musculus*. (b') The merged image of Procollagen (green) and nuclei (Hoechst 33342; blue) in the skin of *Mus musculus*. The white dotted lines indicate the border of epidermis and dermis. The scale bars in a and b indicate 10 μ m. The representative data are shown from three biologically independent samples.

Development of dermal collagen structure

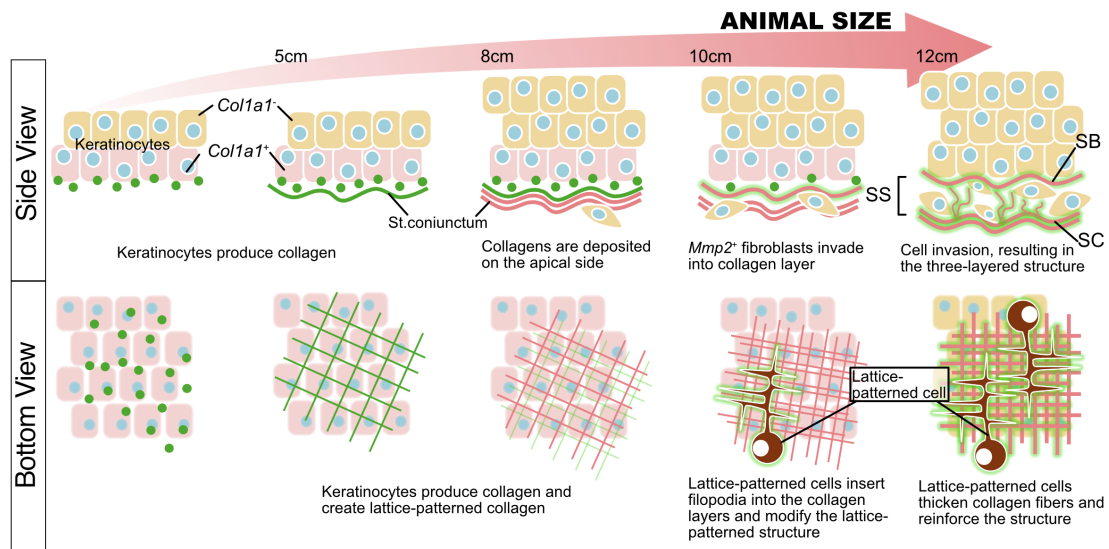


Figure 19: Summary of dermal collagen development in *Ambystoma mexicanum*.

The upper section shows a cross-sectional view of the axolotl dermal collagen formation process.

The lower section shows the same process viewed from a planar perspective.

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Chapter 2: Collagen fiber and cellular dynamics of axolotl skin with aging

This chapter is based on the following publication: Shima and Ohashi et al., 2025, *Development Growth & Differentiation*. Minor modifications were made for the purpose of the doctoral thesis.

Introduction

As skin ages, its texture undergoes significant changes. The cellular and molecular mechanisms driving these changes in skin texture remain unclear. These textural changes, which contribute to wrinkles and sagging, have long been a cosmetic concern that humanity has sought to address. Despite substantial efforts, a fundamental solution has yet to be achieved. The various issues arising from the transformation of skin texture with aging present a significant social demand.

The factors influencing skin aging have been elucidated through experiments in humans and mice. The skin is an organ composed of the epidermis and dermis, with the dermis being rich in collagen. As aging progresses, the dermal collagen undergoes transformations, reflecting age-related changes¹. These dermal changes are often depicted alongside the dynamics of collagen. Collagen is a major component of the dermis, and with aging, the protein fibers of collagen undergo degeneration, the decline in collagen density², conformational change³, and the decrease in collagen production⁴. Such age-related collagen changes are considered irreversible within the organism, and it is generally believed that rejuvenation is not possible. Although the transformations of dermal collagen with aging are well-researched, effective measures to prevent these age-related changes in the skin have not yet been found.

The skin dermal collagens have been considered to be made primarily by fibroblasts. In mammalian skin, LUM+, DCN+, COL1A+, VIM+, and PDGFR+ fibroblasts were described as collagen synthetic fibroblasts^{5,6}. However, recent single-cell RNA-seq analysis reveals that there are some subpopulations within fibroblasts^{7,8,9} and it has not been clarified which is population(s) contributing to collagen braiding in the in vivo dermis. Determination of the collagen braiding cells should be important to pioneer an effective way to solve various types of skin problems, including aging. The lack of determination of the collagen braiding cells is partially because of the skin

opacity in mammalian skin. The tissue opacity has prevented experimental approaches to clarify which fibroblasts play a role in collagen braiding.

Axolotl, a tailed amphibian, has relatively transparent tissues. Its skin transparency is sufficient to see subcutaneous tissues, such as bones, through the skin. Thus, axolotls provide an ideal experimental condition to do skin research. Axolotl skin composed of two tissues, the epidermis and the dermis, which is similar to mammals^{10, 11, 12}. When examining the detailed structure of the dermis, differences in collagen architecture between mammals and axolotls become evident. In mammals, the dermis is known to consist of two layers: the reticular layer and the papillary layer. In contrast, axolotl dermis is composed of a unique three-layered structure¹³. Although the detailed comparison of these structures and the correspondence between the layers in different species remain unclear, similarities can be found in the fact that the dermal collagen in both forms a layered structure. This suggests that axolotls serve as a useful model for studying mammalian skin, leveraging their advantage of high skin transparency as an optimal material for skin research.

The mechanism behind the formation of axolotl skin's three-layered structure has been elucidated only recently¹³. The axolotl dermal collagen forms three layers, stratum baladachinum (st.Bal.), stratum spongiosum (st.Spo.), and stratum compactum (st.Comp.), in a mature state¹³. A recent study revealed that fibroblasts contribute to the st.Comp. and st.Spo. As for the st.Bal., keratinocytes are the major contributor¹³. Keratinocyte contribution to the dermal collagen layers is not axolotl specific rather conserved mechanism^{14, 13}. Collagen braiding fibroblasts contribute to modifying the st.Comp. and forming the st.Spo. The collagen braiding fibroblasts show the characteristic cell morphology¹¹. The collagen braiding cells in the axolotl dermis develop thin and long filopodia. Furthermore, fibroblasts extend their thin filopodia, forming a lattice-patterned cell morphology. It was confirmed that lattice-patterned collagen fibers were braided along with the lattice-patterned filopodia¹¹. Such characteristic cell morphology might be similar to fibripositors reported in mammalian cells¹⁵. The dermis development of axolotls can be considered complete after making the triple-layered structure since no other apparent structures are added after the establishment of the triple collagen layered-structure. Thus, it could be possible to consider that the aging events take place after that. As animals age, keratinocytes stop synthesizing collagen and fibroblasts maintain collagen production and braiding¹³. Although such axolotl skin development was clarified, the dynamics of skin aging are still largely unknown even though the study of aging in

axolotl tissues has just been approached ^{16, 17}.

Here, we investigated the dynamics of the axolotl skin dermal collagen in aged axolotls. As mentioned above, the axolotl dermal collagen is triple-layered. We found that the aging affects cell morphology, cell number, the thickness of each layer and fiber properties. Those insights into the relationship between aging and dermal collagen are valuable for pioneering skin medications and cosmetic treatments.

Results

General Features of Axolotl Skin in Association with Aging

The texture of axolotl skin had not been well focused so far. We first described the texture of axolotl skin across different ages. The animal measuring 8cm-long (snout-to-tail) has relatively transparent skin and is white colored (Fig. 1). As the animal ages, the transparency decreases (Fig. 1A–C). Finally, the aged axolotl skin becomes much opaquer, and the developed dermal glands become much more apparent. Sometimes the skin got a little yellow (Fig. 1C'). To observe the dermal collagens in more detail, we decellularized the skin and observed fibers under a microscope (Fig. 1D–F). The dermal collagens, which were observed from the bottom (muscle-side) showed a lattice-patterned network at all stages. In the histological observation, the dermal collagen development was more apparent (Fig. 1G–I). The Picro-Sirius Red (PSR) staining revealed the presence of collagenous structures in the axolotl skin. As previously reported, the axolotl dermal collagen layer develops as a monolayer (stratum conjunctum), and then it becomes a triple-layered structure (st.Bal., st.Spo., and st.Comp.) as the dermis develops¹³. In larger animals (~22cm-long), the area size of the PSR-positive collagen fibers becomes much wider (Fig. 1I). These data indicate that axolotl skin changes its structure as the animal ages. Thus, axolotl skin provides a useful experimental model for studying skin aging.

Cell Dynamics in the Aged Dermis

We next investigated the cellular dynamics of aged axolotl skin (Fig. 2). First, we focused on cell morphology. As previously reported, the lattice-patterned fibroblasts contribute to building up the lattice-patterned collagen fibers in the bottom layer, the st.Comp., of the mature axolotl skin^{11, 12, 13}. To visualize such lattice-patterned cells in the axolotl dermis, we electroporated pCS2-mCherry for labeling the cytoplasm and pCS2-GFP-Coll1a2 for labeling collagen into the axolotl dermis. We confirmed the lattice patterned cells in the st.Comp. of the dermis of the 8cm-long animals (8cm dermis) (Fig. 2A1–A3). In the 8cm dermis, every electroporation gave rise to labeled lattice-patterned fibroblasts (n=13/13). The labeling rate gradually decreased as the animals aged. In the 13cm dermis, the labeling rate was 33.3% (n=4/12). The observed cell still showed the lattice-pattern filopodia in the 13cm dermis and the lattice patterned collagens were braided around the filopodia (Fig. 2B1–B3). In the 22cm dermis, the labeling rate was much lower (n=1/20). The labeled cells were more disorganized though the filopodia retained the lattice pattern only locally

(Fig. 2C2). The collagens were consistently disorganized (Fig. 2C1, C3). The decreasing labeling rate might be related to the decrease of the fibroblasts in elder animals. To explore the possibility of a decrease in fibroblast numbers, we counted the cells around the st.Comp. (Fig. 3). The collagens in the st.Comp. were visualized by Second Harmonic Generation (SHG) and nuclei were also visualized by Hoechst 33342. The reconstituted images clearly represented the decrease in nucleus in the dermis as the dermis ages. The decrease in the cell number was statistically analyzed and confirmed the statistical differences (Fig. 3D). Those data suggest that aging impacts on cell morphology and the density of cells in the dermis.

The Changes of the Axolotl Dermal Collagen Along Aging

We previously reported on the development of dermal collagen¹³. However, changes in collagen after the establishment of the triple-layered dermis remained unclear. Therefore, we focused on the changes of dermal collagen following the formation of the triple-layered structure. To investigate age-related changes in dermal collagen, we included an additional time point, analyzing the 17cm dermis alongside the 13cm and 22cm dermis. Type I (COL1a) and III (COL3a) collagens in the 13cm, 17cm, and 22cm dermis were investigated since Type I and III are well known as primary components in the dermis (Fig. 4). The COL1a could be found throughout the dermis in all sizes of animals (Fig. 4A, C, D, F, G, I). The COL3a was localized underlying the st.Comp. in the 13cm dermis (Fig. 4B, C). However, such bottom localization of COL3a was lost in the larger samples (Fig. 4E, F, H and I). An apparent difference in the COL3 expression pattern could be found in the st.Spo. The COL3a expression became denser in the st.Spo. of the larger samples (Fig. 4B, C, E, F, H, I). To collect various parameters of the dermis at each age, we focused on the relationship between cells and COL1a, which was stably expressed throughout the dermis at each age. The 13cm, 17cm, and 22cm dermis were labeled by DAR-4M (for COL1a) and cells were GFP-labeled (CMV::GFP transgenic) as previously reported¹³. DAR-4M visualized the triple-layered structure in the dermis (Fig. 5). The rounded dermal glands were observed in all dermal layers. The st.Bal. showed nicely formed lattice patterned fibers at all ages (Fig. 5C1, F1, I1). St.Spo. also showed many bold collagen fibers standing in the apicobasal direction in the 13cm, 17cm and 22cm dermis (Fig. 5C2, F2, I2). Those two layers appear to be stable throughout the stages we investigated. However, st.Comp. showed slight differences (Fig. 5C3, F3, I3). The mesh of the fiber seemed to be more disorganized as the dermis aged (Fig. 5C3, F3, I3). To evaluate the transition in the thickness

of the collagen layers more quantitatively, we measured the captured images. First, the occupation rate of the cells within the dermis was measured by counting GFP+ pixels (Fig. 6A). Consistently, the number of cells in the dermis decreased as the dermis aged. Regarding the thickness of all three layers, the thickness of the dermis increased with age (Fig. 6B). We further measured the thickness of each layer (Fig. 6C–E). The thickness of the st.Bal. was decreased in the 22cm dermis (Fig. 6C). The st.Spo. became thicker, while the st.Comp. tended to decrease in thickness as the dermis aged (Fig. 6D, E). This suggests that the increasing thickness of the whole dermis (Fig. 6B) is primarily caused by the thickening of st.Spo. (Fig. 6D). The histological observation confirmed the massive increase in the width of the st.Spo. (Fig. 1I). We next focused on the quality of the fibers in the st.Comp. As shown in Fig. 1D–F and reported before¹³, the collagen fibers were arranged in a lattice-patterned structure. To assess the orthogonality of the mesh, we evaluated the images using Fourier transformation (Fig. 6F, G). The orthogonality shows how fibers are crossed perpendicularly. The score decreased in the 22cm dermis. Moreover, we also validated the clarity of the collagen fiber mesh by measuring the difference between the peaks and valleys of the bimodal peaks obtained through the Fourier transform (Fig. 6G). This score indicates how clear the mesh pattern is. A higher score suggests that the mesh pattern is more distinct. The results suggest that the properties of the fibers change as an animal ages. To investigate the quality difference of the st.Comp. more comprehensively, we classified the fiber pattern using a conventional neural network. The basic pipeline of the study was described previously¹³. We performed principal component analysis (PCA) on the obtained data and reduced the dimensionality to two dimensions (Fig. 6H, I). The classification result suggests that the aged fibers (22cm dermis) form a different fiber pattern compared to the patterns found in younger dermis (13cm and 17cm dermis). These data apparently describe the dynamics of the dermal collagen fiber with ages.

Discussion

This study has elucidated the age-related changes occurring in the dermis of axolotls. These findings provide not only a foundational understanding for future research on skin aging using axolotls but also offer valuable insights into the broader context of vertebrate skin aging.

A reduction in cell numbers associated with skin aging was observed in axolotls. First, we must acknowledge certain technical limitations. While it is evident that cell numbers decrease with aging, we currently lack the means to determine whether all the reduced cells are involved in collagen weaving. This challenge is shared across all studies related to skin research. Most non-blood cells present in the dermis are COL1A-positive, suggesting that they play at least some role in collagen synthesis. Overcoming this limitation will require the development of technologies that allow simultaneous and comprehensive observation of cell morphology and collagen production capacity in all cells. The reduction in cell numbers was noted not only axolotl skin but also in mammalian skin, indicating that the reduction of dermal cells with aging is a common phenomenon^{18, 19, 20, 7}. Our study revealed that, in addition to this reduction, there are morphological changes in the cells responsible for collagen production (Fig. 2). Previous research has shown that lattice collagen formation requires specific lattice cell morphology^{11, 12}. The disruption of this cell morphology implies that collagen fibers may not form correctly. This disrupted collagen structure is consistent with our observations in Figures 6F and 5. The molecular mechanisms underlying the inability to maintain cell morphology with age remain entirely unknown, as do the mechanisms driving the reduction in cell numbers. By clarifying the cellular events during skin aging in axolotls, our study provides a baseline for evaluating and potentially addressing age-related phenomena at the cellular level.

In this study, we identified changes in collagen associated with aging in axolotls. Although the three-layered collagen structure remained intact, we observed detailed age-related changes. The COL3a expression was already reported¹⁰, and COL3a is localized underlying the st.Comp.¹³. We further clarified that the COL3a expression pattern changes in the aged dermis (Fig. 4E, F, H, I). The COL3a expression became more apparent and looks shifted apically in the aged st.Spo. (Fig. 4 E, F, H, I). Considering the properties of COL3a^{21, 22}, the increase in COL3a presence may contribute to controlling the elasticity of the axolotl dermis. Furthermore, COL3a localization was much more abundant in the surface area of the st.Spo. (Fig. 4E, H). Such surface-biased localization can be

regarded as similar to the expression pattern found in mammalian skin. It has been clarified that COL1a is predominantly found in the deep dermis, contributing to the tensile strength of the tissue and that COL3a is more abundant in the superficial dermis and is responsible for the elasticity of the skin, particularly near the surface²³. Thus, those similarities suggest that the axolotl skin is a good experimental model for general skin research. Regarding COL1a, apparent changes could be found in the st.Spo. and st.Comp., but not in the st.Bal. (Fig. 6). The lack of apparent changes in st.Bal. is likely because, as previous studies have indicated, collagen synthesis in st.Bal. is primarily driven by keratinocytes¹³. Basal layer keratinocytes do not disappear with aging. Additionally, collagen synthesis in keratinocytes of individuals larger than 13 cm significantly decreases, which likely explains the stability of st.Bal. in larger individuals. The significant involvement of fibroblasts in st.Spo. and st.Comp. makes it reasonable to observe age-related changes in these layers. The reduction in the width and changes in the fiber quality of st.Comp. are intriguing. Aging is associated with a decrease in fibroblast numbers (Fig. 3, 5)³⁰ and changes in fibroblast quality (Fig. 2). These changes likely affect the stratification of st.Comp. A decrease in cell numbers may lead to a shift in the balance of synthesis and degradation towards degradation, while changes in cell quality may result in alterations in fiber quality. As for the st.Spo., we acknowledge that it is difficult to collect sufficient data about cell number and others. This is because the target site is deeper than the other two layers. Moreover, in the st.Spo., there are well developed blood vessels and leucocytes. The presence of these components causes a huge background, making the analysis difficult. Thus, the changes in the st.Spo. with aging needs further investigation. While a comprehensive understanding of age-related changes in the axolotl dermis remains elusive, age-related collagen changes are apparently observed in the axolotl skin. Insights have been accumulated in the aged related changed in the mammalian skin¹. In the studies, the thickness and the quality of the collagen fibers were assessed^{24, 25, 26, 3, 27, 4, 2}. Such a body of knowledge about changes in collagen texture in the skin of many animals makes comparative analyses possible. In both mammals and axolotls, the deformation of collagen fibers caused by aging is not fully regenerated¹². Given these similarities, axolotls could serve as a valuable model for studying age-related changes in collagen fibers in mammals.

Previous studies have reported on the regenerative abilities of axolotls. Our study has clarified the changes associated with skin aging. Investigating skin aging with the aim of regeneration is a natural direction for research using the highly regenerative axolotl. Future studies will likely focus on anti-

aging and reverse-aging perspectives. This study provides foundational knowledge for future research on aging in axolotls.

Methods

Animals

Axolotls (*Ambystoma mexicanum*) were procured through the Hiroshima University Amphibian Research Center and kept in aerated water at a temperature of 22 °C. For the surgical experiments conducted in this study, axolotls with nose-to-tail lengths ranging from 8 to 22^{cm} were used, with no specific bias towards any sex. The care and treatment of the animals in this study were executed under protocols approved by the Animal Care and Use Committee of Okayama University. All animals were anesthetized with 0.1% ethyl 3-aminobenzoate-methanesulfonate (SIGMA, MS222), pH 7.0, prior to surgery.

Preparation of Frozen Sections

Samples were fixed in 4% paraformaldehyde for 1 day at room temperature. After fixation, they were decalcified with 10% ethylenediaminetetraacetic acid (EDTA) for 2 days. To prevent tissue damage from ice crystals formed during freezing, the samples were treated with 30% sucrose/PBS for a day. The samples were then embedded in O.C.T compound and frozen at -80°C. Frozen samples were sectioned into 14 µm thickness with a cryostat. The slides were dried using a hair dryer and used for experiments.

Immunofluorescence

To visualization of type I and type III collagen in dermis, we performed immunofluorescence. Procedures for immunofluorescence were also previously described 28. The antibodies we used in the present study were the anti-GFP (Cat#598, 1:500, MBL Life Science the mouse anti-collagen type I (#C2456-2 ML, 1:500; Sigma), and rabbit anti-collagen Type III alpha1 (N-terminal) (22734-1-AP, 1:500; Proteintech). The secondary antibodies were the anti-mouse IgG Alexa 488 conjugated antibody (A11017, 1/1000; Invitrogen) and goat anti-rabbit IgG Alexa 594 (A-11012, 1:1000; Invitrogen). Images were acquired using a BX61 microscope (Olympus). For the GFP immunofluorescence, the samples 5 days after gene transfer were used.

Picrosirius Red staining

Basically, the Picrosirius Red staining was conducted following the attached instructions (Picrosirius Red Stain Kit, Polysciences 24901). Briefly, heat-dried sections were soaked in phosphomolybdic

acid for 2 minutes, then washed with water. Then, the sections were soaked in Picrosirius Red for 1 hour. The sections were placed in hydrochloric acid for 2 minutes. A couple washing steps using Diluted and undiluted ethanol were made. Section were finally washed by G-NOX (GenoStaff) before mounting.

Decellularization

The decellularization method for making ECM from the skin was basically reported before 29. We modified this method on the time for Urea treatment. We took 1 day for the Urea treatment.

Gene transfer (electroporation)

The following two vectors were used; pCS2-GFP-axColla2 and pCS2-mCherry. The condition for the electroporation was as follows; 20 V, 10 times at 950 ms intervals using an electroporator (Neppa gene, NAPA21). The electroporation was performed on lower axolotl limbs (the dorsal skin).

SHG imaging

SHG imaging was performed following the method previously reported by Kashimoto et al. (2022). Collagen structure was detected by SHG imaging using an inverted two-photon microscope TCS SP8 MP (Leica) with 63× magnification with a water immersion lens (Leica, HC PL APO 63×/1,20 W CORR CS2). Fixed skin was mounted in Fluoromount and photographed under the microscope from the dermal side. Using InSight DeepSee femtosecond infra-red laser (Spectra-Physics), 900 nm was chosen as excitation wavelength, and SHG signals and GFP signals amplified with Alexa Fluor 488 were detected through filters of 435 to 485 nm and 500 to 550 nm, respectively. The z-step size was set to 0.8 μm.

Fluorescent probe for dermal collagen visualization

The method was already reported^{31, 13}. DAR-4M (SK1006-01, Goryo Chemical), a fluorescent probe that binds specifically to collagen, was injected into the dorsal skin and collected 24 hours after injection and visualized the signals with a confocal microscope (FV3000, Evident).

Statistical Analysis and Classification Using a Convolutional Neural Network

For the assessment of collagen layer thickness, orthogonal sections were obtained using the orthogonal view function in ImageJ (Fiji). The target areas were isolated, and the area sizes were

calculated using ImageJ's "measure" function. For Fig. 6A, four independent samples at each stage were tested, with three randomly selected orthogonal sections from each sample subjected to measurement. For the data in Fig. 6B–E, three independent samples were analyzed at each time point, and the width of the collagen layer was measured at 10 randomly selected points per sample (3 samples $\times$ 10 points). For the data in Fig. 6F and G, 10, 8, and 9 images were provided for dermis samples measuring 13^{cm}, 17^{cm}, and 22^{cm} in animal length, respectively. The statistical analysis was conducted using a one-way ANOVA. Post-hoc comparisons were performed using Tukey's HSD test. Fourier transform analysis was performed using ImageJ, and the resulting FFT images were further analyzed with a Python script developed using ChatGPT-4o. This script has been deposited on GitHub (<https://github.com/satohlab-py/Fiber-assessment/releases/tag/v1.0>). Image classification was performed using a separate Python code, also available on GitHub (<https://github.com/satohlab-py/Classification-by-CNN/releases/tag/v1.0>).

Figures and Figure legends.

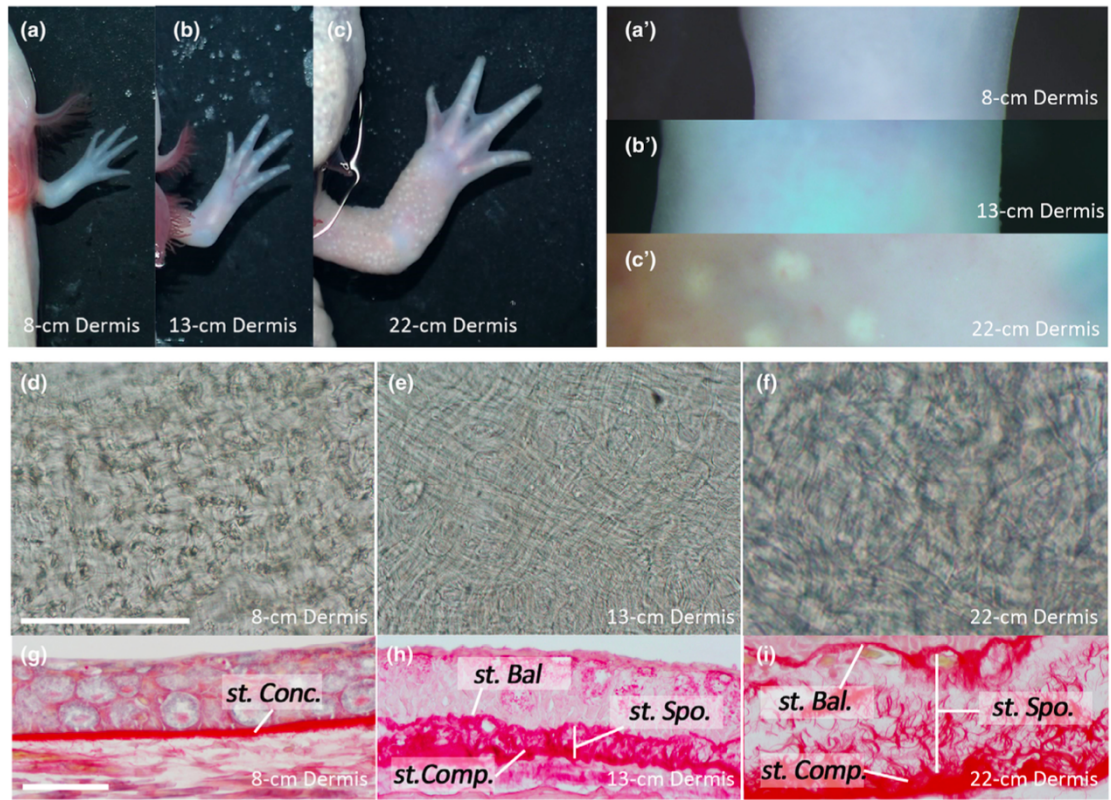


Figure 1: Changes in axolotl skin texture and dermal collagen during dermis development

(A–C) The dorsal views of animals measuring 8^{cm}, 13^{cm}, and 22^{cm} in length; (A'–C') The higher magnification views of A–C. (D–F) The decellularized axolotl dermis. D–F are the same magnification. (G–I) Sections stained with Picrosirius red (pink). G–I are the same magnification. *St. Conc.* = *stratum coniunctum*, *St. Bal.* = *stratum baladachinum*, *St. Spo.* = *stratum spongiosum*, *St. Comp.* = *stratum compactum*. Scale bars in D and G = 0.1mm. The representative images were chosen from five independent samples.

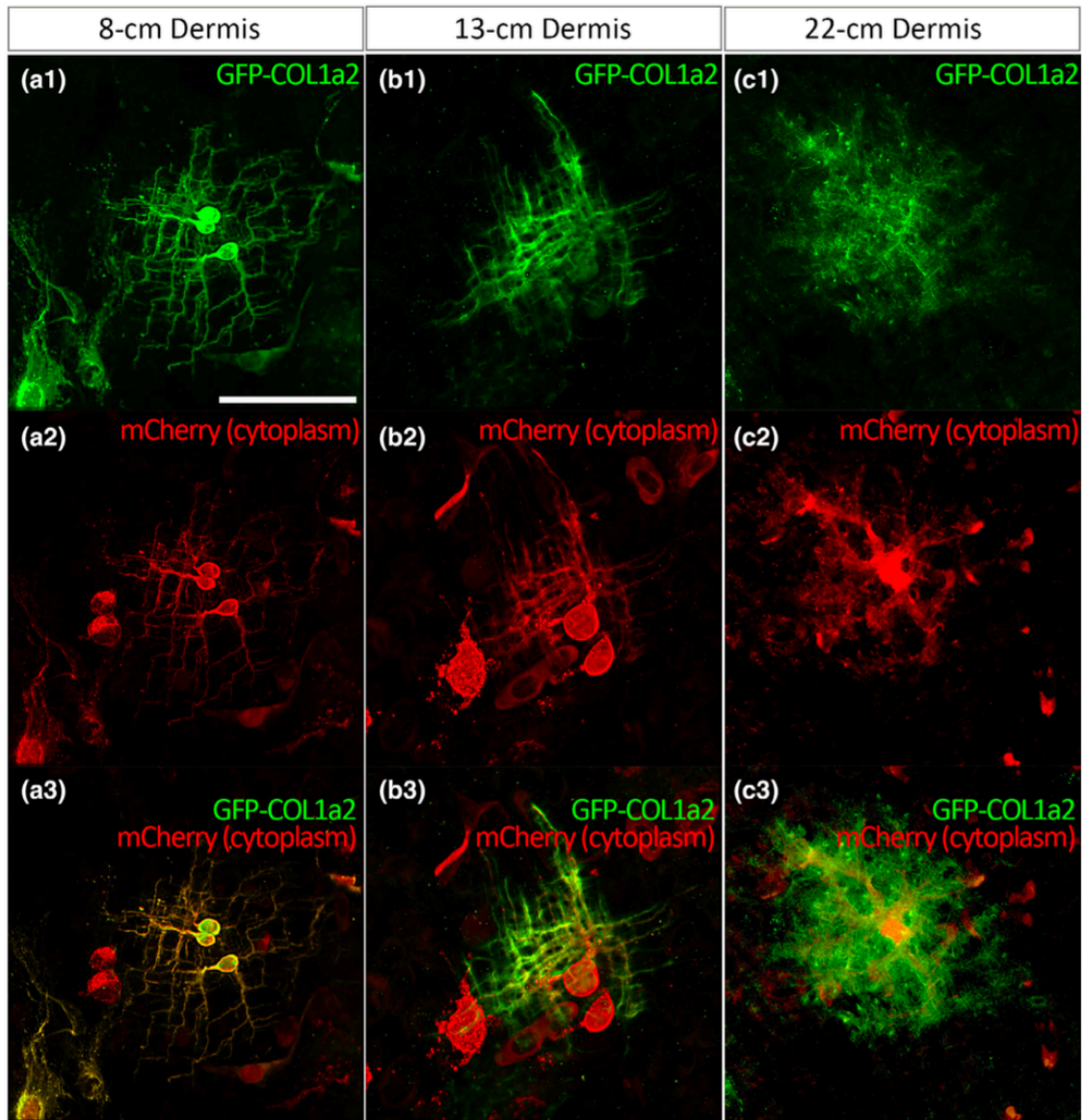


Figure 2: The morphological changes in collagen-producing cells in the *st. Comp.*

(A1–C1) The signals from the introduced pCS2-GFP-axCol1a2. (A2–C2) The signals from the introduced pCS2-mCherry for visualization of cytoplasm. (A3–C3) The merged images of A1–C1 and A2–C2, respectively. The skin was fixed 5 days after the electroporation and the samples were taken from the lower limbs. Scale bar in A1 = 0.05 mm. All images are the same magnification. The representative images were chosen from at least five independent experiments.

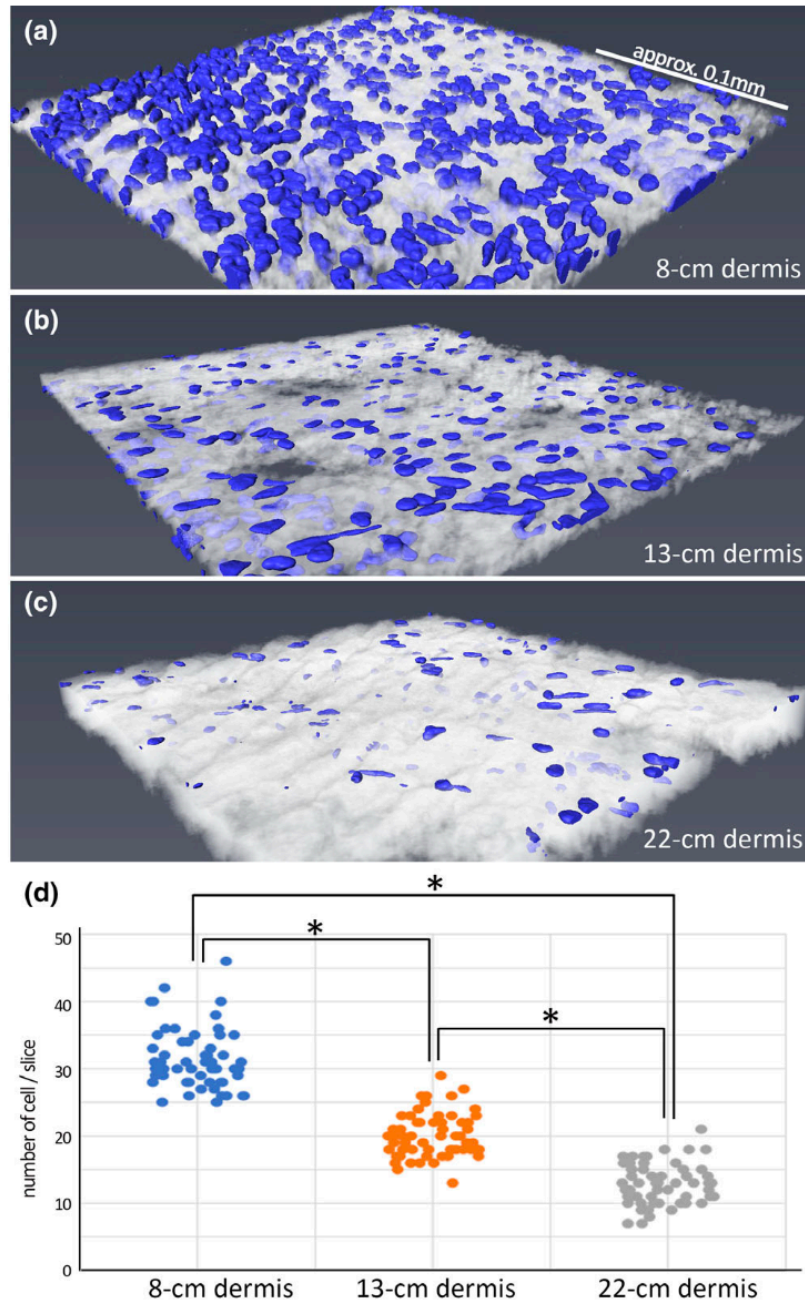


Figure 3: The decrease in the number of cells around the *st.Comp.* in the aged skin

The collagens were visualized using secondary harmonic generation (SHG). The captured images were reconstituted using Amira. The blue color indicates nucleus and white indicates collagen fibers (A–C). (D) The quantification of the cell number around the *st.Comp.* The counting was achieved on the reconstituted XZ cross sections. One-way ANOVA with Turkey's post hoc test was performed for calculating the statistical differences. *** $p < 0.05$. The representative images were chosen from three independent samples.

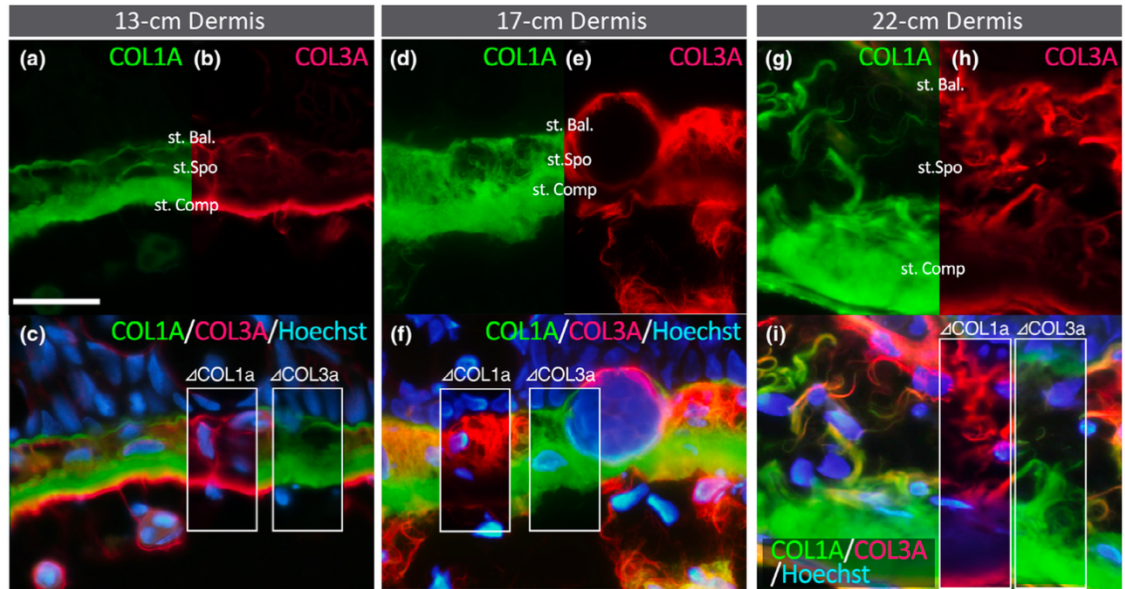


Figure 4: The COL1a and COL3a expression pattern in the mature/aged axolotl dermis

The COL1a (green) and COL3a (red) were visualized by immunofluorescence. Nuclei were visualized by Hoechst (blue). A–C, D–F, G–I are the identical section, respectively. The boxes in C, F, and I indicate the one-color elimination from triple-colored image. Scale bar in A is 50 μ m. All images are taken with the same magnification. The representative images were chosen from at least five independent samples. *st. Bal.* = *stratum baladachinum*, *st. Spo.* = *stratum spongiosum*, *st. Comp.* = *stratum compactum*.

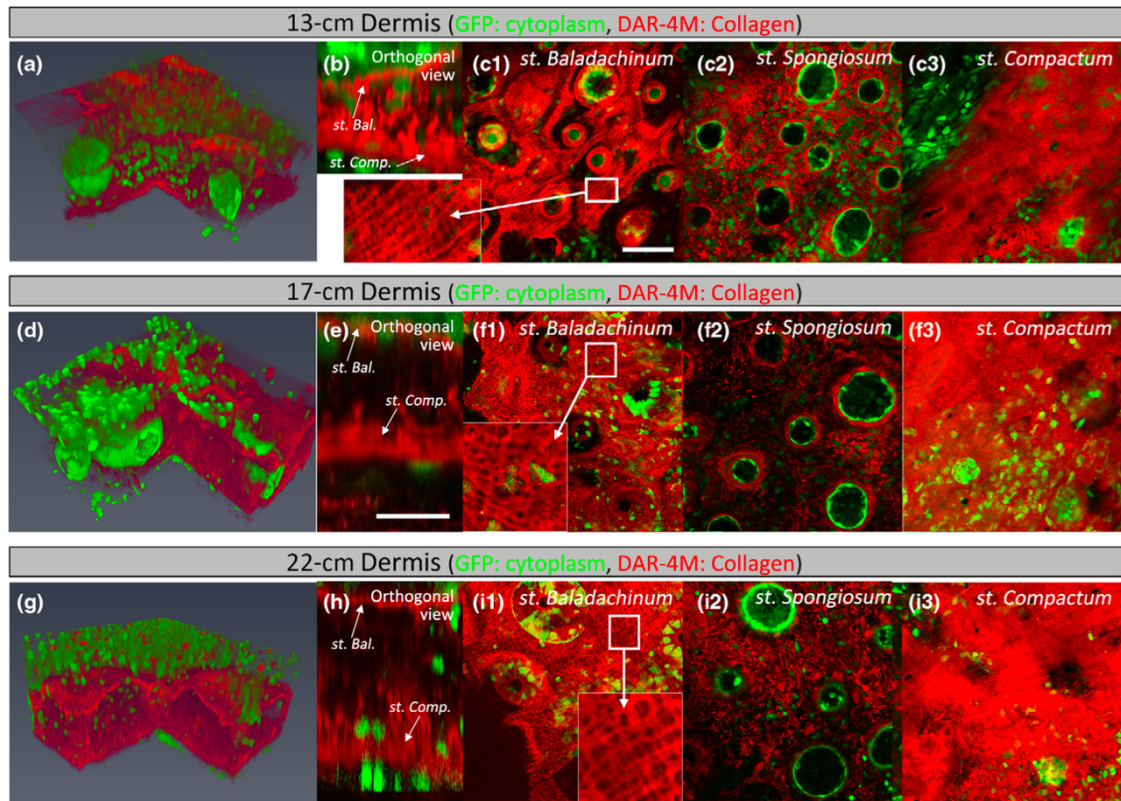


Figure 5: Whole structure imaging of cells in the dermis and the dermal collagens

Collagens were visualized by DAR-4M (red) and cells were labeled by GFP (green). (A, D, G). 3D images were projected using Amira Software. (B, E, H) The reconstituted side view for the confocal images. (C, F, I) The representative images were shown at each dermal layer; *st. Baladachinum*, *st. Spongiosum*, and *st. Compactum*. The inserts are the higher magnification views of the boxed area. Scale bars in B and C1 are 100 and 150 μm , respectively. C1–3, F1–3, and I–3 are the same magnification. A, D, and G are the same magnification. The representative images were chosen from at least four independent samples. The reconstituted 3D images (A, D, and G) and other confocal images were derived from different specimens.

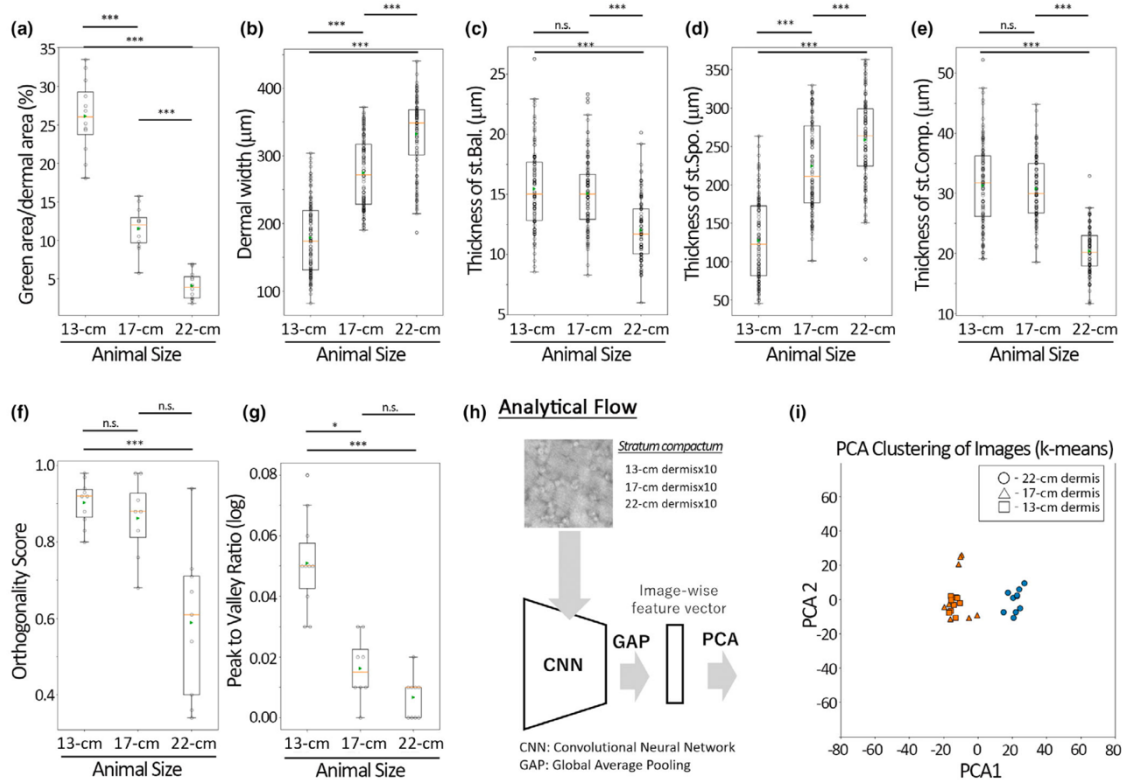


Figure 6: Statistical analysis of Changes in Dermal Collagen layers with Aging

The Figure 5 data with some experimental duplications were statistically analyzed. (A) Cell occupancy in the dermis, suggesting the presence of the cells in the whole dermis. (B) The thickness of the entire dermal collagen layer. (C) The thickness of the *st.Bal.* (D) The thickness of the *st.Spo.* (E) The thickness of the *st.Comp.* (F) The orthogonality scores. The score = 1 means that the fibers cross perpendicularly. (G) The peak to valley ratio (log). Statistical differences were analyzed using ANOVA followed by Tukey's HSD post-hoc test. * indicates $p < 0.05$, and *** indicates $p < 0.01$. (H–I) Principal Component Analysis (PCA) was performed and the dimension was reduced to two dimensions. The color indicates the two-classified group. The circle, the square, and the triangle indicate data from 22, 17, 13^{cm} dermis, respectively. *st.Bal.* = *stratum baladachinum*, *st.Spo.* = *stratum spongiosum*, *st.Comp.* = *stratum compactum*.

Reference

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General discussion

In those studies, I used the axolotl, a model that allows fluorescence-based observation of the skin, to examine dermal type I collagen from its formation through aging in detail. In Chapter 1, I demonstrated that epidermal cells produce type I collagen specifically during the early stages of dermal formation. Furthermore, I obtained the results suggesting that type I collagen production by epidermal cells is a conserved mechanism in embryos of animal species. In Chapter 2, I investigated skin aging and found that axolotl skin exhibits age-dependent reductions in cell number and *Colla1* expression, accompanied by alterations in the lattice-like collagen structure. These changes suggest that aging in the axolotl skin shares similarities with age-associated decline observed in mammals. Collectively, our findings demonstrate conserved mechanisms underlying dermal structure formation and aging across animal species and highlight new possibilities in skin biology.

In Chapter 1, I demonstrated that during the early stages of dermal formation, keratinocytes produce type I collagen and contribute to the establishment of dermal structure. Dermal type I collagen has long been thought to be produced exclusively by fibroblasts, and accordingly, most studies on skin collagen have focused on fibroblasts. However, our findings reveal that, at early stages of dermal development, not only fibroblasts but also epidermal cells actively synthesize type I collagen. Furthermore, I showed that collagen production by epidermal cells is likely a conserved mechanism across a wide range of animal species. This finding challenges a long-standing paradigm in skin biology. Nevertheless, the mechanisms by which collagen produced by keratinocytes traverses the basement membrane and reaches the dermis remain unclear. In addition, the regulatory mechanisms responsible for the disappearance of collagen production by epidermal cells during later stages of dermal formation have yet to be elucidated. Future studies addressing

these issues will be essential for understanding how collagen production by epidermal cells is controlled during skin development.

In Chapter 2, I investigated skin aging in the axolotl and demonstrated that dermal structure undergoes marked changes with age. However, how these age-related structural alterations affect the physical properties of the skin remains unknown. In addition, changes in the turnover of type I collagen during aging were not addressed in this study. Future investigations using axolotls, combined with comparative analyses in mammalian models, will enable a more detailed understanding of age-associated changes in skin structure and may help identify skin aging mechanisms that are conserved across animal species.

Through those studies, I elucidated a series of events underlying the formation and aging of dermal type I collagen in axolotl skin. Investigating extracellular matrix components such as type I collagen in mammalian skin is technically challenging due to tissue opacity and limited accessibility. In this respect, axolotls represent a highly advantageous model system. The approaches established in my study are expected to facilitate further advances in skin biology and contribute to a deeper understanding of dermal extracellular matrix dynamics.

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