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Ultrastructural evidence of receptor-mediated endocytosis during early mouse limb morphogenesis: a three dimensional analysis using deep etching and scanning electron microscopy

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INTRODUCTION

Epithelial - mesenchymal interactions are essential to normal embryonic development and morphogenesis. One classic example, which is the subject of considerable experimental interest among developmental, cell and molecular biologists, is the vertebrate limb bud. These structures develop at their distal tips a thickened epithelium called the apical ectodermal ridge. The ridge functions as an inducer of limb development, since upon removal, further development ceases and only those limb parts established prior to ridge removal will form [10, 12]. In addition, a necessary relationship exists between limb mesoderm and the overlying ectoderm. Mesodermal cells destined to form limb induce the adjacent ectoderm. Mesodermal cells destined to form the limb induce the adjacent ectoderm to become active, i.e. to acquire properties of the apical ridge [6]. Once this is accomplished, mesodermal cells maintain the ridge in its active thickened form until all limb parts have been specified [13].

Although the phenomenon of morphogenetic tissue interaction is well known and thoroughly studied, little information is available about mechanisms which either effect or mediate embryonic induction. Wolpert [11], in his hypothesis outlining the concept of positional information in development, included the possibility of morphogenetic signaling molecules, or "morphogens". Since it is known that interacting cells in limb mesoderm and ectoderm do not directly contact one another (they are separated by the complex molecular nature of a basal lamina; Kelley, [4], Kelley and Fallon, [5]), it would seem that developmental signals would be required to traverse intercellular distance containing extracellular matrix, and furthermore, intersect the responding cell's plasma membrane before information (either instructive or permissive) is transmited into cell's interior.

In this regard, cell biologists have long recognized ligand-receptor interaction as a mechanism for intercellular signalling (see Goldstein, et al, [2], for review) and receptor-mediated endocytosis is thought to be among the mechanisms used by cells to remove membrane bound signals once cell has responded [8]. The structures which participate in membrane receptor localization and internalization area clathrin-coated pits and vesicles (reviewed recently by Pearse and Crowther [9]).

To investigate the possibility morphogenetic signals involve cell surfaces during early ridge induction and thickening, and, further, that structural evidence of signal-receptor interaction might provide both temporal and spatial information on the nature of ridge induction, techniques of electron microscopy were applied to mouse embryos at stages prior to and during thickening of ridge ectoderm. This paper reports structural evidence of receptor-mediated endocytosis in pre-ridge limb ectoderm. The presence of coated pits and vesicles suggests receptor activity at cell surfaces, and, further, suggests a potentially important role for receptor-mediated events in limb morphogenesis.

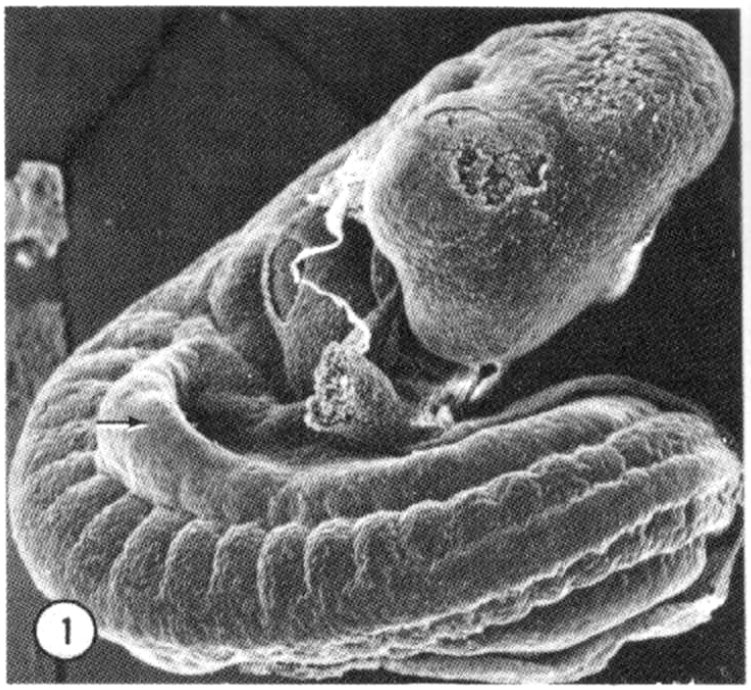


Fig. 1. Scanning electron micrograph of 9d mouse embryo. Note development of forelimb bud (arrow) and rudimentary presence of hindlimb. X 200

MATERIALS AND METHODS

Swiss Webster mouse embryos at days 9, 9.5, 10, 10.5 and 11 (day 1 calculated as the day copulation plugs were observed) were fixed in 2.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 4° C), postfixed in 2.0% osmium tetroxide in 0.1 M cacodylate buffer (4° C), and either embedded in epon for thin sectioning, or critical point dried for scanning electron microscopy.
Other limb buds were dissected, quick-frozen in either liquid freon or nitrogen and deep-etched in a Balzers BAF 301 apparatus [3]. Specimens and replicas were examined with an Hitachi H-600 transmission and S-800 scanning electron microscopes.

OBSERVATIONS

The mouse embryo exhibits a prominent forelimb bud, but only a rudimentary hindlimb bud, at day 9 (Fig.1). At 9.5d (Fig.2), the hind limb bud enlarges, although both buds lack and apical ectodermal ridge at this stage. The ridge (Fig. 3) is clearly developed in the forelimb but at 10.5d and appears by day 11 in the hindlimb. The ridge regresses in both limbs by 12.5d. Figure 3 reveals that the murine ridge is a stratified epithelium in contrast to the simple cuboidal organization of adjacent, non-ridge ectoderm.

As ridge development begins in the forelimb (day 10-10.5), the relationship to underlying mesoderm is illustrated in Fig. 4. A thin, continuous basal lamina separates mesoderm from ectoderm at all stages. Numerous projections from mesenchymal cells explore the matrix at the mesodermal surface of the basal lamina (Figs. 4 and 5), but do not penetrate the lamina to contact the basal membrane of adjacent ectoderm cells. Cell division and production of matrix are the principal activities of subridge mesodermal cells (Fig. 4).

By 9.5d, numerous coated pits are observed at basal (Fig. 6) and basolateral (Fig. 7) surfaces of membranes of cells which abut the basal lamina. It is noted that few coated pits are observed in flank ectoderm at 8.5d and in cells of limb ectoderm which will not participate in the ridge by day 9. However, these pits are infrequently located at basal membrane surface and do not appear to be as numerous as in the distal limb tip prior to ectodermal thickening. Internalization of coated pit membrane is evidence at 9.5d by the presence of numerous coated vesicles in the

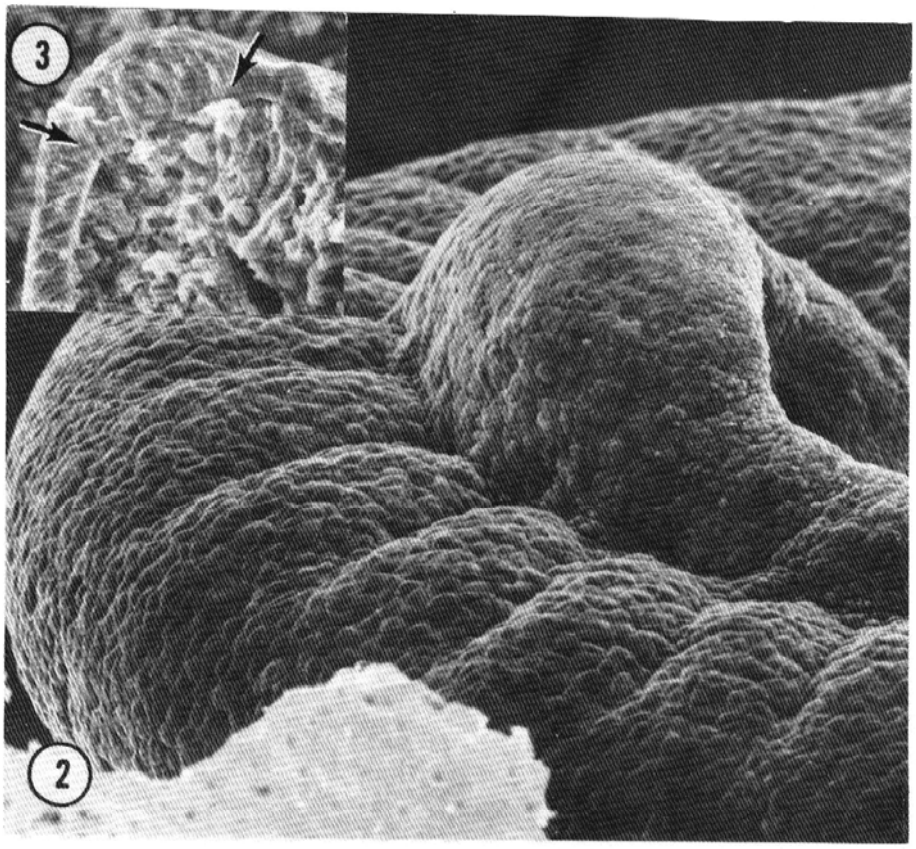
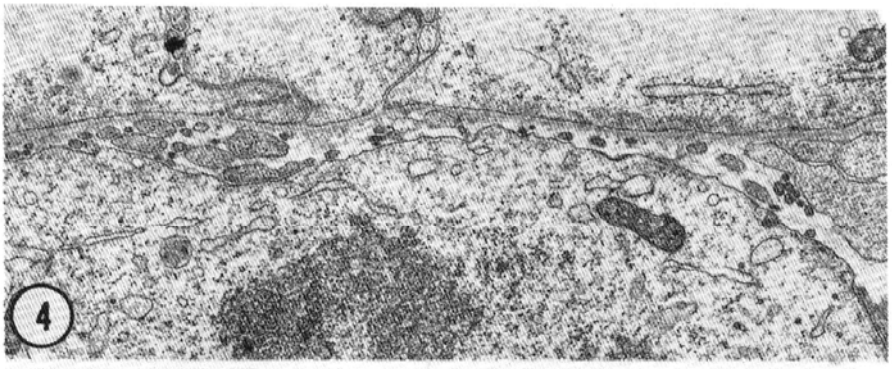


Fig. 2. Forelimb bud of 9.5d mouse embryo. The bud exhibits polarity in relation to other body parts and axes. Note the prominent development of the bud, but absence of apical thickening of ectoderm. X 500.

Fig. 3 . By breaking the specimen open, the thickened apical ectoderm is clearly contrasted to the thinner dorsal and ventral ectoderm. In addition, subjacent mesoderm and investing extracellular matrix is revealed. X 450.



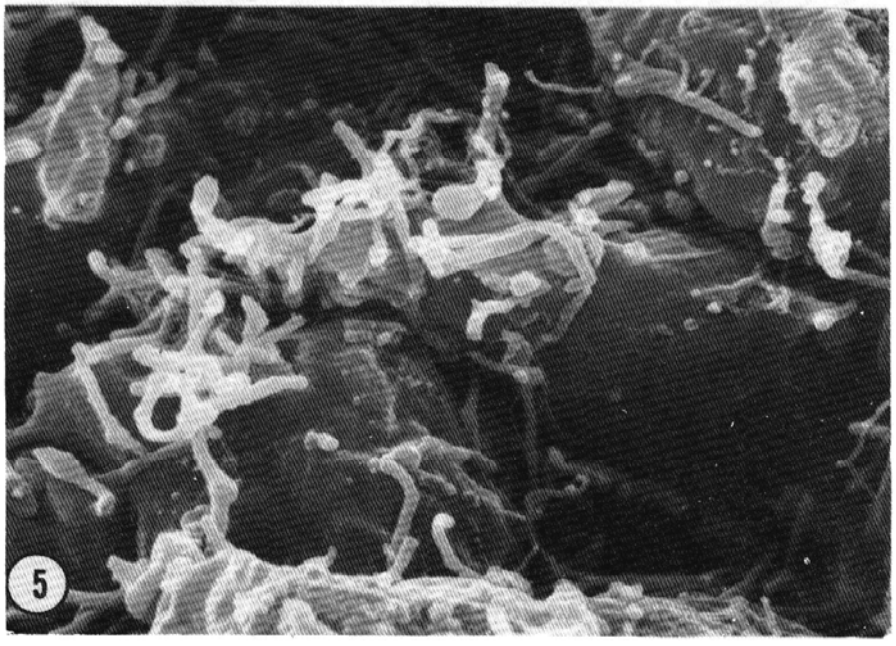
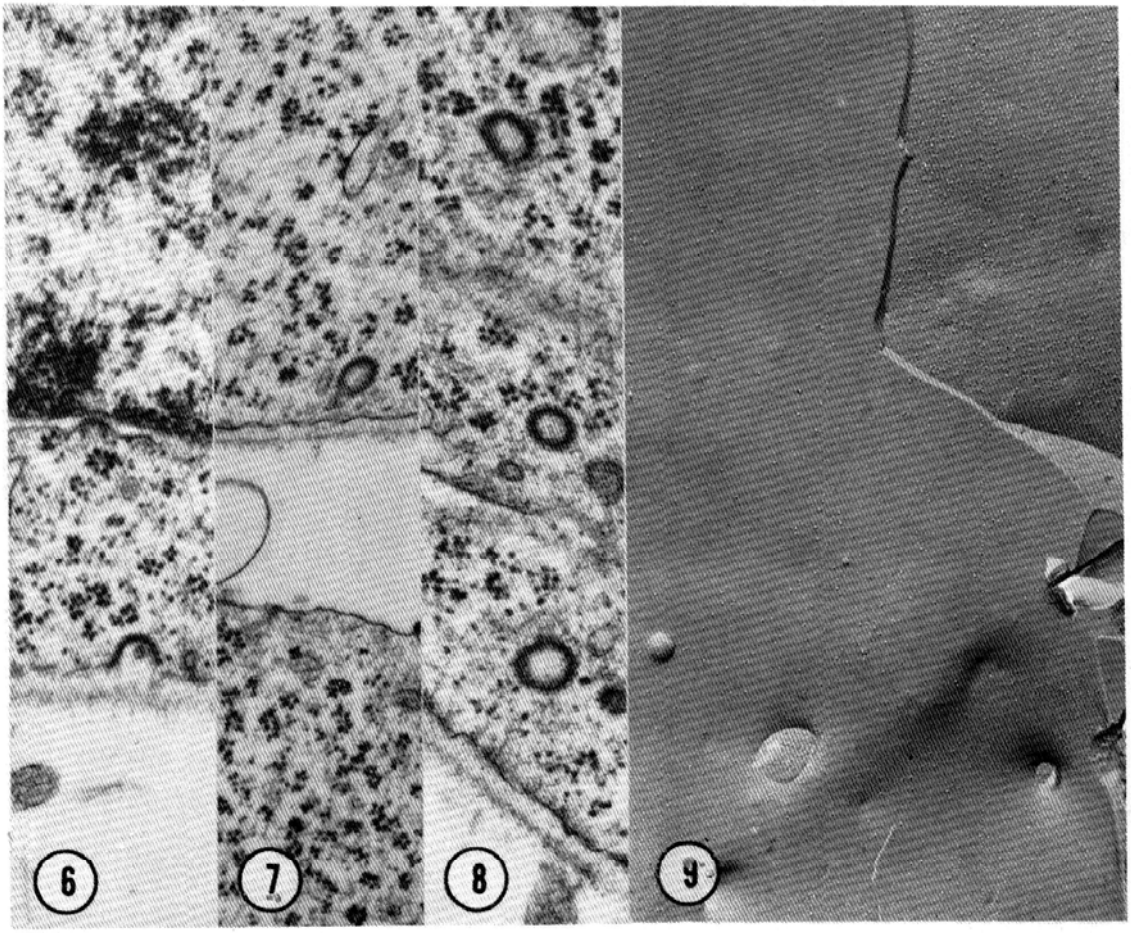


Fig. 4 . Transmission electron micrograph of ectodermal-mesodermal interface. A dividing mesodermal cell extends projections which intersect a basal lamina subtending the ectoderm. X 10,000.

Fig. 5. Scanning electron micrograph of mesodermal cell surfaces immediately beneath the apical ridge. Processes extend both towards the epithelial surface and towards adjacent cells within the limb mesoderm. X 20,000.

cytoplasm of cells destined to form ridge tissue (Fig. 8). A replica of fractured membranes of pre-ridge ectodermal cells (Fig.9) illustrates the distribution of pits forming in adjacent cells. Similar membrane organization persists through day 12 in both ridge and non-ridge ectoderm, with the former exhibiting the more prominent distribution of coated pits and vesicles.

Once internalized, coated vesicles appear to become intimately associated with the cytoskeleton in epithelial cells forming the ridge. Fig. 10 illustrates two vesicles surrounded by filamentous elements of the cytoplasm, whereas Fig. 11 shows an extensive array of vesicles (not all coated vasicles) associated with the cytoskeleton. Current thought suggests that the cytoskeleton may participate in membrane traffic from the surface to selected sites within the cell (e.g. the Golgi apparatus, lysosomes, fusion with other vesicles, etc.) where spent receptors are processed and new membrane is returned to the cell surface.



Figs. 6-9. Formation of coated pit in the basal membrane of apical epithelial cell at 9.5d. X50,000 (Fig. 6). Internalization of the coated segment of membrane yields a coated vesicle. X 50,000 (Fig. 7). Multiple coated vesicles are observed at 9.5d prior to morphological appearence of apical thickening. X 50,000 (Fig. 8). Freeze fracture exhibits membrane faces which show distribution of depressions and bumps which may forming pits ad vesicles X 30,000. (Fig. 9)

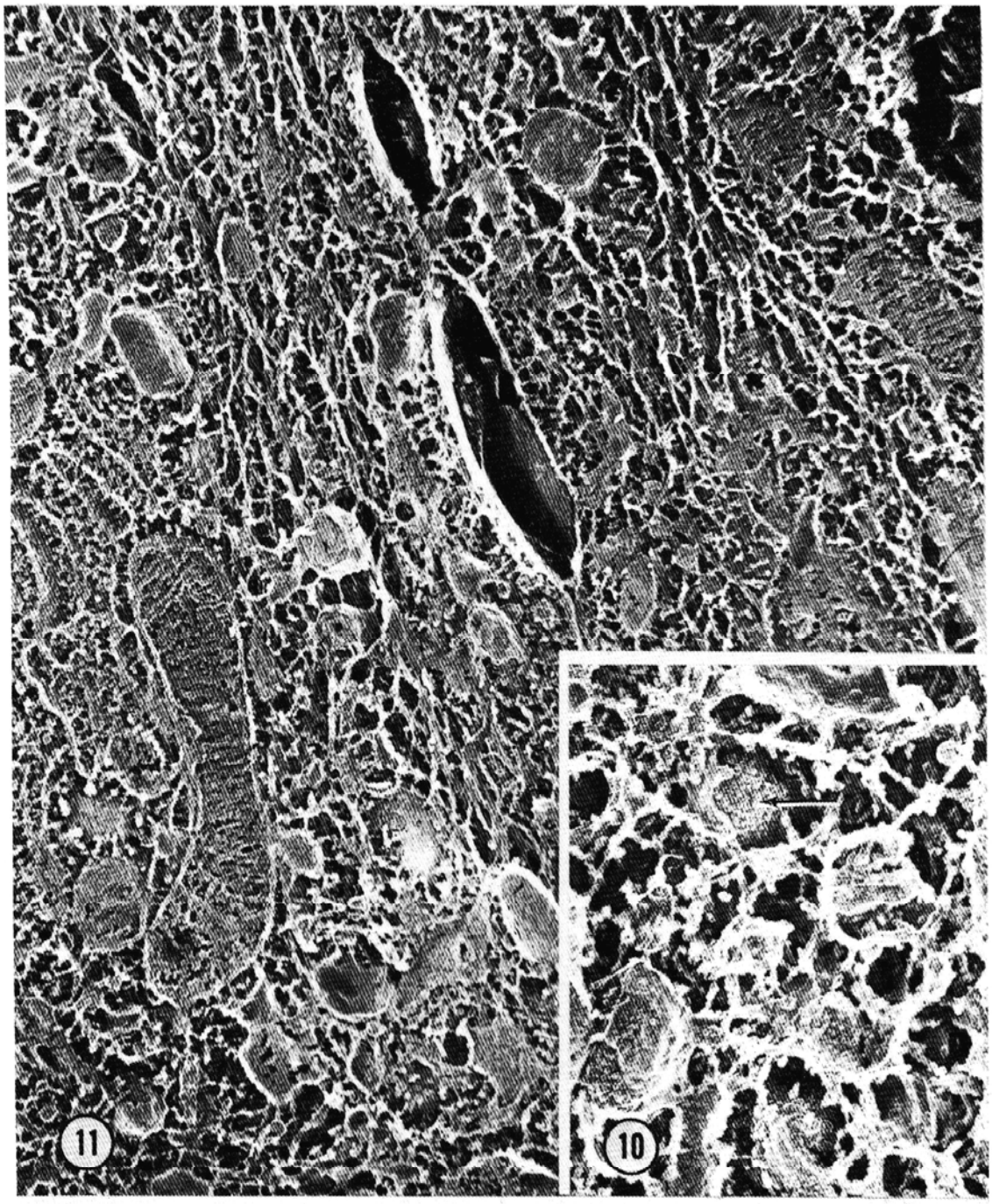


Fig. 10. Replica of quick-freeze, deep etch specimen revealing internalized vesicles (arrows) in close association with components of the cytoskeleton. X 80,000.
Fig. 11. Lower magnification image shows distribution of vesicles (arrows) along basolateral borders of epithelial cells. X 60,000.

DISCUSSION

It is reasonable to interpret experimental results obtained with the chick limb [13] to mean that the apical ectoderm of the limb bud is instructed and maintained in its active form only by limb mesoderm. The investigations of Milaire [7] and Cairns [1] suggest that the same is true in mammals. What remains unclear, however, is (1) the nature of gene products from mesoderm which may serve as "morphogens" to instruct ectoderm to thicken and, in turn, permit growth of underlying mesoderm, and (2) what structural mechanisms cells in intact embryonic tissues employ to effect the transduction of those signals across cell surfaces once they are intercepted by the responding cell.

The observations presented in this brief note sugget that cells engaged in the epithelial-mesenchymal interactions which result in the appearance of an apical ridge in limb buds emloy membrane-mediated mechanisms for internalization of ligand-receptor complexes. Althought it is not known what the nature of such a complex might be, it is clear from the structural features of interacting cell surfaces that the coated pit-coated vesicle mechanism is active in these tissues. Coated pits, which are present in a wide variety of cells and first described by electron microscopy in the early 1960's, are associated with the plasma membrane and are involved in endocytosis.

Numerous studies have shown that many different ligands (such as epidermal growth factor, fibroblast growth factor, platelet-derived growth factor , and others) enter cells by way of the large coated pits of the plasma membrane [9]. The observation of receptor-mediated endocytosis in pre-ridge limb ectoderm provides structural evidence which permits the suggestion that (1) a growth-like factor may be transmitted from mesodermal cells; (2) this factor locates its receptor on component pre-ridge ectoderm cells and (3) in turn, provides the initial signal for these cells to form the stratified layer of epithelium which has the active qualities of the apical ridge. This hypotesis is attractive in that regulation of receptor availability through gene action of cells in the limb ectoderm may serve as the basis for morphogenesis of ridge pattern (i.e. the apical position of the ridge and the presence of cells in limb ectoderm which do not respond to mesodermal influence by forming ridge tissue).

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