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ORIGINAL ARTICLE

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Apoptosis in the early involuting stellate reticulum of rat molar tooth germs

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Abstract When the enamel organ of the rat tooth germ is fully developed at the tip of the prospective cusp, amelogenesis begins, and at this site the overlaying stellate reticulum begins its involution. During the involution process, there is a gradual decrease in intercellular spaces, invasion by blood vessels, appearance of macrophage-like cells and reduction in the number of stellate reticulum cells. Since reduction or disappearance of cells during embryonic development in organs and tissues has been shown to occur by apoptosis, we decided to examine early involuting regions of the stellate reticulum in the hope of detecting apoptosis. For this purpose, upper first molars of Wistar newborn rats aged 1 and 3 days were fixed in formaldehyde for the TUNEL method and in glutaraldehyde-formaldehyde for light and electron microscopy. Paraffin sections revealed TUNEL-positive structures, i.e. brown-yellow-stained bodies, in the central portion of the stellate reticulum, and next to the outer enamel epithelium and stratum intermedium. Examination of ultrathin sections confirmed the TUNEL findings: some stellate reticulum cells showed nuclei containing crescent-like electron-opaque condensed masses of peripheral chromatin, typical of apoptosis. Also, apoptotic bodies of various sizes and appearances were frequently observed within stellate reticulum cells. We should like to suggest that apoptosis is associated with the reduction in the number of cells during regression of the reticulum.

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Introduction

Tooth development is characterized by a series of complex morphogenetic and differentiation events that involve oral epithelial cells and ectomesenchymal cells of neurocrestal origin. Oral epithelial cells form the enamel organ that will ultimately be responsible for production of enamel, while ectomesenchymal cells form the dental papilla and the dental follicle that will produce dentine, pulp and the periodontal apparatus (Ten Cate l995). The enamel organ develops to form the inner enamel epithelium, outer enamel epithelium, stratum intermedium and the stellate reticulum. The stellate reticulum consists of epithelial cells originated from the central cells of the early enamel organ. These central epithelial cells become gradually separated, forming large intercellular spaces and the cells become, consequently, star-shaped (Ten Cate 1994). The stellate reticulum cells are connected by desmosomes, gap junctions and occasional focal tight junctions (Sasaki et al. 1984). When the enamel organ of a rat tooth germ is fully developed at the tip of the prospective cusp, amelogenesis begins and at these sites the overlaying stellate reticulum starts its involution. However, in the rat molar tooth germ, amelogenesis does not occur at the uppermost region of a prospective cusp.

Involution or regression of the stellate reticulum involves a series of events but does not occur simultaneously in all its regions. Thus, soon after beginning of amelogenesis there is invasion of blood vessels, removal of extracellular matrix, reduction of the intercellular spaces, appearance of macrophage-like cells and gradual decrease in the number of cells (Pannese 1960, 1961; Kallenbach 1975).

Although the morphological events that accompany the development of the stellate reticulum are well documented (Pannese 1961; Kallenbach 1975), there is little information on the processes that occur during its involution. It has been established that disappearance of cells

during embryonic development of various organs and tissues occurs through apoptosis (Majno and Joris 1995; Haanen and Vermes 1996). Since there is reduction in the number of cells during stellate reticulum involution, we felt that apoptosis might be involved in this process. We have therefore examined the stellate reticulum of rat tooth germs at early stages of involution using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) method for detection of DNA fragmentation, and light and electron microscopy.

Materials and methods

Animals

The tooth germs of upper first molars of 1- and 3-day-old Wistar albino rats, from both sexes, obtained from the Federal University of São Paulo Animal House (OUTB EPM-1, Br Epm-2c), were used in the experiments. The animals were anaesthetized with ether, decapitated, the mandible removed, and the upper jaw exposed. With the help of a dissecting microscope the first molar tooth germs were extracted in toto and placed in the fixative solution (see below). Principles of laboratory animal care (NIH publication 85-23, 1985) and national laws on animal use were observed.

Light microscopy

The specimens were fixed in a mixture of 4% glutaraldehyde and 4% formaldehyde (freshly derived from paraformaldehyde), buffered at pH 7.4 (0.1 M sodium cacodylate) at room temperature (RT) for 4 h and left at 4°C for 12–16 h (Katchburian and Holt 1972). After fixation, they were dehydrated in graded ethanols and embedded in glycol metacrylate (Technovit 7100, Kulzer, GmbH). Sections (3-μm-thick) were stained with hematoxylin and eosin (HE) and examined in an Olympus BX-50 light microscope.

TUNEL method for apoptosis

The Oncor Apoptosis Detection Kit-Peroxidase (ApopTag-Plus, Oncor, Gaithersburg, USA) was used (Gavrieli et al. 1992). The specimens were fixed in 4% neutral formaldehyde for 24 h at 4°C. After dehydration, they were embedded in paraffin and 5-μm sections were obtained. Deparaffinized sections were washed in PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl) for 5 min and pre-digested with proteinase K solution (20 μg/ml in PBS) for 15 min at RT to expose the DNA strands. The sections were subsequently treated with 2% hydrogen peroxide for 5 min to block endogenous peroxidase and immersed in an equilibration buffer for 15 s. This was followed by incubation in a solution containing terminal deoxynucleotidyl transferase (TdT) at 37°C for 1 h in a humid chamber. The reaction was stopped by incubation in the stop/wash buffer solution at 37°C for 30 min. The sections were then incubated in the anti-digoxigenin-peroxidase solution in a humid chamber for 30 min at RT. After washing in PBS, they were treated with 0.02% 3,3'-diaminobenzidin TRIS-HCl for 5 min at RT. The sections were counterstained with methyl green, washed in distilled water, treated with butanol and processed routinely.

The same procedure as above was used on sections of mammary gland (positive control) provided by the manufacturer of the kit, and also on sections of involuting rat prostate glands. Negative controls were prepared by replacing TdT enzyme by distilled water.

Transmission electron microscopy

The specimens were fixed in a mixture of 4% glutaraldehyde and 4% formaldehyde (freshly derived from paraformaldehyde) buffered at pH 7.4 (0.1 M sodium cacodylate) for 4 h at RT with constant agitation and were subsequently left at 4°C for 12–16 h. After washing in cacodylate buffer, the specimens were transferred to a solution of cacodylate buffered 1% osmium tetroxide for 2 h at RT. After dehydration in graded ethanols, the specimens were treated with propilene oxide prior to embedding in Araldite. Toluidine-stained semithin sections were examined with a light microscope and selected regions were trimmed. Ultrathin sections were collected onto grids and stained with lead citrate and uranyl acetate before examination in a JEOL-100 CX II electron microscope.

Results

Our results showed brown-stained structures – typical of TUNEL positivity – within the early involuting stellate reticulum (Figs. 1a–c), i.e. in regions of the stellate re-

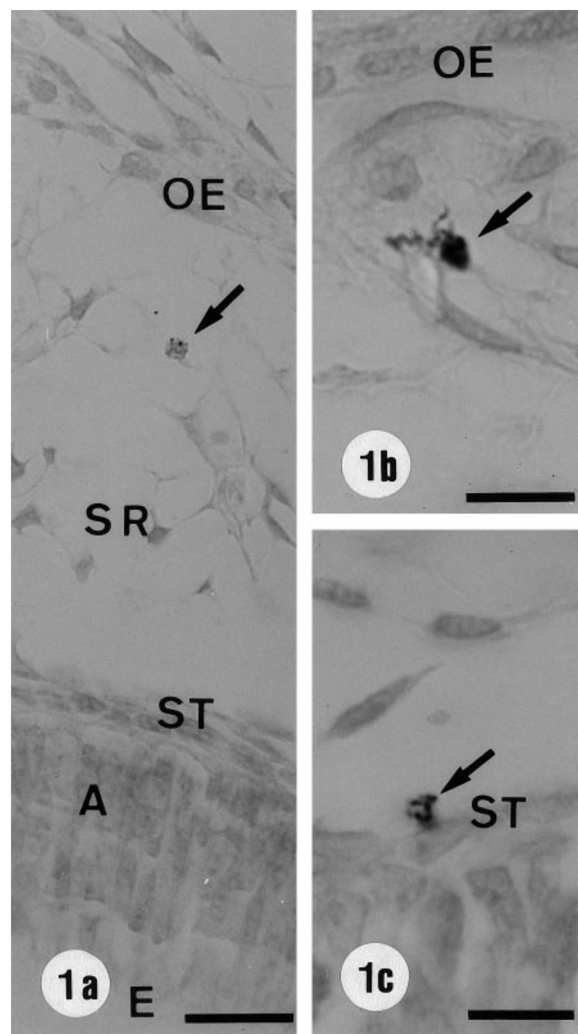

Fig. 1a–c Light micrographs of tooth germs stained by the TUNEL method and counterstained with methyl green. **a** general view of the stellate reticulum (*SR*) at the onset of its involution. A TUNEL positive structure (*dark colour*, *arrow*) is present in the central region. (*A* ameloblasts, *ST* stratum intermedium, *OE* outer enamel epithelium, *E* early enamel). *Bar* 100 μm. **b** TUNEL-positive structure (*arrow*) near the outer enamel epithelium (*OE*). *Bar* 25 μm. **c** Another positive structure (*arrow*) near the stratum intermedium (*ST*). *Bar* 25 μm

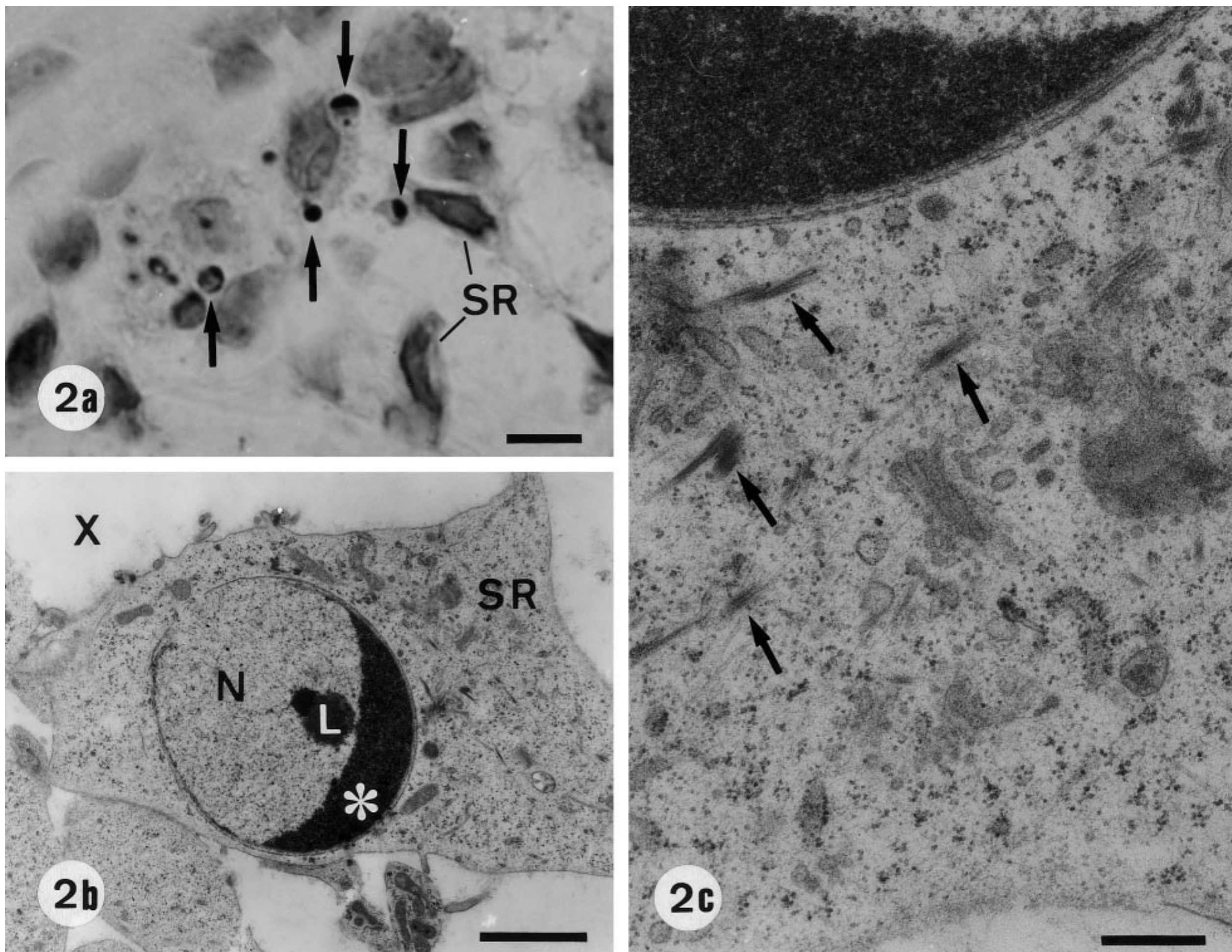

Fig. 2a–c Light micrograph of a portion of the involuting stellate reticulum showing round/oval structures containing densely stained bodies (*arrows*; *SR* stellate reticulum cells). HE staining. *Bar* 6 μm. **b** Electron micrograph of a portion of a stellate reticulum cell (*SR*) showing the nucleus (*N*) containing crescent-like electron opaque masses of condensed peripheral chromatin (*). A nucleolar structure (*L*) is also present (*X* intercellular space). Lead/uranyl staining. *Bar* 2 μm. **c** High magnification view of a portion of the cell in **b** showing the typical bundles of tonofilaments (*arrows*) within the cytoplasm of the stellate reticulum cell. Lead/uranyl staining. *Bar* 0.5 μm

ticulum that overlay early developing enamel. The positive and irregularly shaped structures were observed in the central regions of the stellate reticulum (Fig. 1a), close to outer enamel epithelium (Fig. 1b), and near the stratum intermedium (Fig. 1c). As expected, the TUNEL-positive structures were not abundant and were not present in all sections examined. Controls for the TUNEL method using involuting mammary gland or prostate sections revealed the presence of positive structures. Incubation of sections in medium lacking TdT enzyme were negative.

HE-stained sections showed images of dense round/ovoid structures, near or possibly within stellate reticulum cells; however, such images were not present in all sections examined (Fig. 2a).

Ultrastructural results revealed that some tonofilament-rich stellate reticulum cells contained crescent-like electron-opaque masses of condensed peripheral chromatin in their nuclei (Fig. 2b, c). Other stellate reticulum cells exhibited large, electron-opaque and compact cytoplasmic inclusions of variable appearance (Figs. 3, 4). Desmosome connected stellate reticulum cells occasionally showed one single compact and electron-opaque inclusion (Fig. 3). Sometimes, a cell containing large electron-opaque inclusions was surrounded by a cytoplasmic process of a stellate reticulum cell, also rich in electron-opaque inclusions (Fig. 4). Some of the electron-opaque inclusions showed surface blebs, while others appeared to be disintegrating. Other inclusions were more complex and heterogeneous, containing granular material of variable electron-opacity, irregular electron-opaque bodies and often an intact mitochondrion (Fig. 4).

Discussion

Our results have shown that TUNEL-positive structures were present in the early involuting stellate reticulum, i.e. in regions overlaying sites of incipient amelogenesis, indicating thereby that apoptosis is taking place. The TUNEL test reveals DNA breaking points (Gavrieli et al.

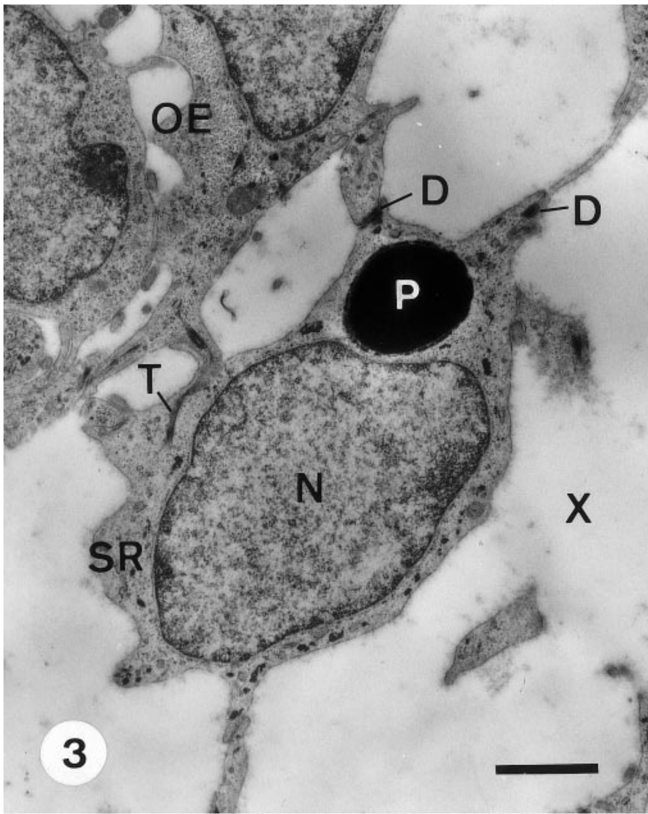

Fig. 3 Electron micrograph of a portion of the involuting stellate reticulum. A stellate reticulum cell (*SR*), close to the outer enamel epithelium (*OE*) shows a large and extremely electron-opaque body (*P*) within its cytoplasm (*D* desmosomes, *N* nucleus, *T* tonofilaments). Lead/uranyl staining. *Bar* 1 μm

1992; Wijsman et al. 1993; Gold et al. 1994), being therefore characteristic of part of the cascade of events that leads finally to cell death. Our TUNEL results are supported by the images of corresponding regions observed in HE preparations and in ultrathin sections. HE preparations showed round/ovoid structures containing densely stained semicircular/round bodies, strongly suggestive of apoptosis. Our ultrastructural findings revealed the presence of stellate reticulum cells with nuclei containing typical crescent-like electron-opaque masses of condensed peripheral chromatin. This kind of image has been described as characteristic of cells undergoing apoptosis (Kerr et al. l972; Majno and Joris 1995). Moreover, several desmosome-connected stellate reticulum cells exhibited typical images of apoptotic bodies within vacuoles.

As in most other tissues, images of apoptosis as observed by the TUNEL method, by HE, or by electron microscopy were few in our specimens. In the thymus, for example, 97% of the developing thymocytes die but only 0.2% of these cells are TUNEL-positive (Coles et al.

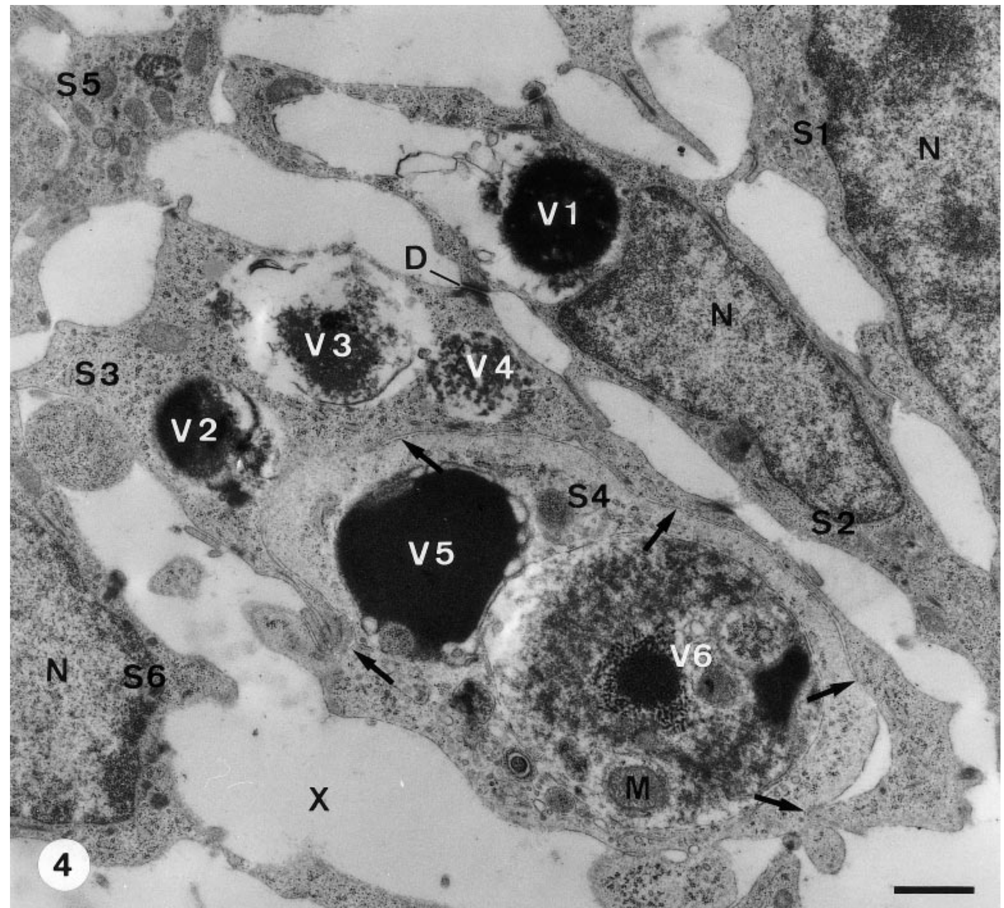

Fig. 4 Electron micrograph of a portion of the involuting stellate reticulum. This complex image shows portions of six stellate reticulum cells (*S1, S2, S3, S4, S5, S6*). Cells S2, S3 and S4 show large and electron-opaque inclusions of varying appearances. Cell S2 shows a single electron-opaque body (*V1*) inside an irregular clear space within the cytoplasm. Cell S3 shows three electron-opaque inclusions (*V2, V3, V4*). Two of the inclusions (*V3, V4*) appear to be fragmented. The same cell (*S3*) partially encircles another cell (*S4*; *arrows*) that contains two large vacuoles (*V5, V6*); V5 is compact but shows peripheral blebs; V6 is heterogeneous in appearance and contains electron-opaque structures with variable electron-opacity, granular material and a single mitochondrion (*M*). Lead/uranyl staining. *Bar* 1 μm

1993). The apoptotic cells quickly become engulfed and digested by other cells. Therefore, the likelihood of finding an apoptotic cell is quite small (Gavrieli et al. 1992), and we also had to examine numerous sections to detect apoptosis.

Our images were similar to those already described in other tissues (Kerr et al. 1972; Wyllie et al. 1980; Abrams et al. 1993; Majno and Joris 1995) and were not observed in regions of the stellate reticulum considered to be non-involuting. However, some of the compact and electron-opaque inclusions were indistinguishable from granules described in the dental follicle and in the stellate reticulum that were found to be inhibitory to tooth eruption (Wise et al. 1990; Lin et al. 1992a,b; Wise and Lin 1995). Thus, there is another type of granule in stellate reticulum cells that is unrelated to apoptosis.

Our combined results demonstrate that some stellate reticulum cells undergo apoptosis in regions of the enamel organ where the stellate reticulum is beginning to involute. Apoptosis or programmed cell death occurs in most tissues during their development, e.g. in regression of the interdigital webs during limb development (Kerr et al. 1994) and tadpole tail regression during metamorphosis (Kerr et al. 1974). In adult mammals, apoptosis occurs in normal slow- and fast-proliferating tissues (Kerr 1971; Benedetti et al. 1988; Kerr and Searle 1973; Potten 1977) and in pathological conditions such as malignant tumors (Wyllie 1985).

In the oral cavity, several epithelial structures undergo apoptosis during development (Abiko et al. 1994; Lesot et al. 1996; Peterková et al. 1996), e.g. the dental and vestibular laminae, stratum intermedium (Vaahtokari et al. 1996), ameloblasts (Nishikawa and Sasaki 1995; Shibata et al. 1995) and the inner enamel epithelium of rat enamel-free areas (Yamamoto et al 1998). It has also been shown that apoptosis occurs in cells located in the region of the enamel knots (Vaahtokari et al. 1996) during initial stages of tooth development, suggesting an association with events leading to the shaping of the cusps (Jernvall et al. 1994). Cell death, during tooth eruption in the rat, was also observed in the surrounding tissues of the crown (Kaneko et al. 1997).

Our results showed apoptosis in early involuting regions of the stellate reticulum, suggesting that apoptosis is also associated with the reduction in the number of stellate reticulum cells. To confirm this, further studies including examination of all stages of the stellate reticulum development, using serial sections and quantitative analysis, will be necessary.

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