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Origin of Rodlet Cells and Mapping Their Distribution in Ruby-Red-Fin Shark (Rainbow Shark) *Epalzeorhynchos frenatum* (Teleostei: Cyprinidae): Light, Immunohistochemistry and Ultrastructure Study

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Abstract

The present study describes for the first time histology, histochemistry, and ultrastructure of all rodlet stages. By ultrastructure, we identified rodlet progenitors in the stroma of the olfactory organ in Red-Fin shark. Rodlet progenitors were mesenchymal-like cells synthesis the primitive rodlet granules. Rodlet progenitors differentiated to vesicular rodlet cells, which was rich in vesicles and vacuoles, and accumulate intracellular fibrillar-like components. Granular rodlet cells were observed in the basal epithelia, contained premature rodlet granules and began to organize the fibrillar components of the rodlet capsule. Transitional rodlet cells continued to deposit the fibrils of the capsule and synthesis of rodlet granules. Mature rodlet cells were polarized bear-shape and had typical rodlet granules. Histochemistry showed rodlet cells had a wide range of staining affinities including carbohydrate, lipid, and protein staining. All stages of rodlet cells revealed the presence of PAS-positive granules in the cytoplasm of rodlet cells where, While the ruptured ones stained strongly with alcian blue, sliver stain and Sudan black B. Rodlet granule was positive for bromophenol blue Rodlet cells were positively immunostained against Matrix Metalloproteinase-9 (MPP-9) in all stages indicate invasion properties of rodlet cells in tissue. Conclusion rodlet cells originated from the stroma of the olfactory organ.

Keywords: Rodlet cell; Olfactory organ; MPP-9; TEM; SEM; Histochemistry

Introduction

Rodlet cells are a specific type of cells found in teleosts either fresh or marine fish [1]. Unique morphological features enable rodlet cells to be easily distinguished in different tissues and organs. Rodlet cells are pear-shaped cells with a basal nucleus, fibrous layer in the inner aspect of the plasma membrane, club or rod-shaped granules, mitochondria, endoplasmic reticulum, Golgi [2]. Rodlet cells are described in different organs. They were detected in the digestive tract [3-6], pancreases [7], vascular system [8], the bulbus arteriosus [5], kidney tubules [4,6,9] the gonads [10], the skin [7], the epithelium of the operculum and gill

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raker [11], gill [3,4,6,9], and abdominal cavity among the mesothelial cells [6].

Rodlert cell is identified by Thèlohan in 1892. Many scientists believed rodlet cells as an exogenous parasite; Rhabdospora thelohani regarding the morphological similarity and the uneven distribution of fish species and organs and detection of RNA [12,13]. Rodlet cell considered as an endogenous cell respond to environmental changes [14]. Rodlet cells implicate to perform many functions. The secretory function is described in the epithelium and discharges their contents via holocrine mode [15,16]. They also attributed in the mediation of immune response and considered as a type of the granulaocytes [17,18]. Rodlet cells act as ion transporting cells and have a role in osmoregulation [19]. Involvement in sensory function is mentioned for rodlet cells [20].

Concerning to existence in different organs and description of a wide variety of functions of typical rodlet cells, a great confusion has arisen regarding their origin. There is still a debate about the nature of the rodlet progenitors and site of rodlet development. A previous study demonstrated the presence of mother cells of the rodlet cells in the olfactory organ in red tail shark [16]. The current study was carried out using Ruby shark which is belong to the same family; the Cyprinidae. The Ruby Shark has morphological similarities with the Red Tailed Black Shark except for appendages colors. Rubby shark (*Epalzeorhynchos frenatum*) is commonly known as Red Finned Shark, Ruby shark, Rainbow shark. Red finned-shark is freshwater fish and exists in Southeast Asia [21].

The current study elucidated the exact origin of rodlet cells, represented a detailed sequential differentiation of rodlet cells by TEM and SEM, identification of different stages of rodlet cells by histochemical techniques and rodlet cell nature and identify enzymatic activity responsible for the penetration of rodlet cells by immunostaining. Identification of rodlet cells origin may help to recognize the biological role of rodlet cells in teleosts.

Materials and Methods

Ten healthy fishes of Red-Fin shark (*Epalzeorhynchos frenatum*) were commercially purchased from an ornamental shop in Assuit city, Egypt and brought to the laboratory, five fish samples were used for light histological and immunohistochemical analysis and three fish samples for used transmission electron microscopic and two fish samples were processed for scanning electron microscopic analysis. All fish measured and deeply anaesthetized with benzocaine (4 mg/L). Fish were ranging from 10 to 12 cm in standard body length. Fish were deeply anaesthetized with benzocaine (4 mg/L).

Histological investigations

The whole five fish were immersed in a mixture of 20 mL of 2.5% glutaraldehyde and 80 mL 0.1 M Na-phosphate buffer pH 7.2-7.4). Then Specimens were washed by 0.1 M Na-phosphate buffer (pH 7.2-7.4) then immediately fixed in Bouin's fluid for 2 h. Bouin`s fixed samples were extensively washed in 70% ethanol (3 × 24 h) to get rid of the fixative before the subsequent steps of tissue processing for preparation of paraffin blocks. Cleared in methyl benzoate and embedded in paraffin wax. The embedding time was no more than 8 h.

Serial longitudinal sections from three fish and transverse section were obtained from other two fish at 5 µm. Paraffin. Representative sections were stained with Hematoxylin and Eosin satin [22] and used for general histological examination, were dewaxed (2 × 30 min), rehydrated in a descending series of ethanol (100%, 95%, and 70%) and distilled water. After staining, the sections were dehydrated again in an ascending series of ethanol (70%, 95%, and 100%), cleared in xylene (2 × 10 min) and mounted with DPX.

Other samples of the olfactory organ were fixed in Karnovsky fixative (10 mL paraformaldehyde 25%, 10 mL glutaraldhyde 50%, 50 mL, phosphatebuffer and 30 mL DW) was used for semi thin section and EM examination.

Histochemical investigations

The fixed specimens in Bouin's fluid were examined for neutral and acidic mucous by Periodic acid-Schiff reaction after [23] and Combined PAS-Alcian Blue techniques (pH 2.5) was used to differentiate between neutral and acidic polysaccharides [24]. Sudan Black B was used for detection of lipid [25]. Safranin O was used for detection glycosaminoglycans [26]. Other histochemical stains were used including Resorcin-fuchsin-nuclear red after [27] and van Gieson's method, after [28], Grimelius's silver nitrate method [29], Heidenhain's Iron-Hx [30], Mallory triple trichrome stain [31], Mercury bromphenol blue method for total protein [32] and Gimesa stain [33].

All staining were cited in Bancroft's theory and practice of histological [33]. Stained sections were examined by Leitz Dialux 20 Microscope. Photos were taken using a Canon digital camera (Canon Powershot A95).

Preparations of resin embedding samples

Small specimens from olfactory rosettes and gills from the three fish were used for semithin sections. Small pieces 2.0-3.0 mm long were fixed in Karnovsky fixative [34] at 4°C overnight. They were washed 4 times for 15 min in 0.1 M sodium phosphatebuffer (pH 7.2) then were post-fixed in 1% osmic acid in 0.1 M Na-phosphate buffer at 4°C for 2 h. The samples were again washed 3 times for 20 min in 0.1 M phosphate buffer (pH 7.2). Dehydration was performed through graded ethanol to propylene oxide. Samples were dehydrated in ascending graded series of ethanol (50% (for 30 min), 70% (overnight), 90% (for 30 min), and 100% I (for 30 min) and 100% II (60 min). The dehydrated samples were embedded in resin (Epon-araldite) as the following; propylene oxide (Merck, Darmstadt, Germany) for 30 min, Epon: Propylene oxide about (1:1) (for 30 min) then in Epon (for 3 h). Epon was prepared (5 mL, Epon812 (Polysciences, Eppelheim, Germany) +5 mL, Araldite +12 mL DDSA) Epon was mixed thoroughly by shaker Incubation at 60°C. Polymerization of samples was performed by using Epon mix and accelerator (DMP30) (1.5%). The blocks were incubated for 3 days as the following; 60°C at first day and at 70°C in second day and 75°C at third day. Semithin sections (1 µm) were cut using an ultram icrotome Ultracut E (Reichert-Leica, Germany) and stained with toluidine blue (Sodium tetraborate (borax) 1 g, toluidine blue 1 g, and Distilled water 100 mL) [33].

Resin embedded specimens were also used in histochemical studies. Resin sections were treated with a saturated alcoholic solution of sodium hydroxide for 15 min to dissolve the resin [35]. The semithin sections were stained by Periodic acid-Schiff reaction (PAS) after [23].

Ultrathin sections were obtained by a Reichert ultra-microtome. The sections (70 nm) were stained with uranyl acetate and lead citrate [24] and examined by JEOL100CX II transmission electron microscope at the Electron Microscopy Unit of Assiut University.

Samples preparation for SEM

The head of fish after opening the outer covering of the skin and exposed the two olfactory rosettes and small specimens of gills washed by 0.1 M Na-phosphate buffer. Then they were fixed in Karnovsky fixative [34] for 4 h. at 4°C. Thereafter, they were washed in the same buffer used in fixation 5 min × 4 times and post-fixed in 1% osmic acid in 0.1 M Na-phosphate buffer for further 2 h at room temperature. They were washed by 0.1 M Na-phosphate buffer 15 min × 4 times. The samples were dehydrated by alcohol 50%, 70%, 90% for 30 min in each concentration and 100% for 2 days with changes many times followed by isoamyl acetate for 2 days and then subjected to critical point drying method with a polaron apparatus. Finally, they were coated with gold using JEOL-1100 E ion sputtering Device and observed with JEOL scanning electron microscope (JSM-5400 LV) at KV10.

Immunohistochemical investigations (IHC)

We used immunostaining to identify invasion properties of rodlet cells in tissue, using mouse anti-rabbit antibody against matrix metalloproteinase-9 (MPP-9). Immunohistochemical staining was performed on paraffin sections of the whole fish section after choose the sections contains gills, olfactory rosette and pharyngeal bad area using superfrost plus microscope slides. Antigen localization was achieved using mouse anti-rabbit antibody against matrix metalloproteinase-9 (MPP-9) combined with the avidin-biotin complex (ABC) technique using the Reagent of Ultra Vision Detection System (Anti-Polyvalent, HRP/DAB (ready to use, Table 1) Thermo Fischer Scientific TP-015-HD) according to the manufacturer's instructions (Table 1) [36].

Staining was done according to the following protocol. Sections (5 µm) of paraffin-embedded sections were dewaxed, rehydrated, and rinsed in PBS pH 7.4 (3 times for 5 min). Endogenous peroxidase was inhibited by adding drops of hydrogen Peroxide block at room temperature followed by intense washing under running tap water for additional 10 min. For antigen retrieval, slides were placed in 10 mm sodium citrate buffer (pH 6.0) and heated to 95-98 in a water bath for 20 min followed by cooling for 20 min at room temperature. Sections were then rinsed in PBS (pH 7.4, 3 times for 5 min). Sections were covered with Ultra V block, (Table 1, Thermo Fisher scientific, USA) by adding drops cover the sections for 5 min at room temperature to block non-specific background staining. (Note: Do not exceed 10 min or there may be a reduction in the desired stain.). Sections were then incubated with the primary antibody overnight at 4°C (mouse anti-rabbit antibody against matrix metalloproteinase-9 (MPP-9, RB-9423-PO Thermo Fisher Scientific, UK. Lab Vision corporation; USA) at dilution (1:25) in the PBS. Slides were washed with PBS (pH 7.4, 3 times for 5 min), followed by incubation for 10 min at room temperature with drops of a biotinylated secondary antibody (Table 1, Biotinylated goat Anti-Polyvalent, Anti-mouse igg+Anti -Rabbit igg, Thermo Fisher Scientific, UK. Lab Vision corporation; USA). The slides were thereafter rinsed in PBS (pH 7.4, 3 times for 5 min) followed by incubation with streptavidin- peroxidase complex (Table 1, Thermo Fisher Scientific, UK. LabVision corporation; USA) for10 min at room temperature. Visualization of the bound antibodies was carried out by adding 1 drop of DAB plus chromogen to 2 mL of DAB plus substrate. Mix well and apply a drop on the tissue. Incubate for 5 min at room temperature (Table 1). Consider, all incubations were performed in a humid chamber. The sections were counterstained with Harris haematoxylin for 30 seconds. The sections were dehydrated in a graded series of alcohols (96% ethanol, isopropanol I and II), cleared with xylene, and covered with DPX. Negative controls were performed by omission of the primary antibodies.

TP-015-HD	Component
TA-015-HP	Hydroen Peroxide Block
TP-015-UB	Ultra V Block
TP-015-BN	Biotinylated goat Anti-Polyvalent
TS-015-HR	Streptaidin Peroxidase
TP-015-HSX	DAB Plus Substrate
TA-001-HCX	DAB Plus Chromogen

Table 1: Reagent of Ultra Vision Detection System (Anti-Polyvalent, HRP/DAB (ready to use) Thermo Fischer Scientific TP-015-HD.

Immunohistochemical staining was evaluated by Leitz Dialux 20 Microscope and photos were photographed by cannon digital camera (Cannon Powershot A95) (Table 2).

Results

The current study was carried out to explore the site of rodlet cells emergence, identification of rodlet progenitors, differential stages of rodlet by transmission electron microscope, different histochemical staining, recognition of enzymatic activity responsible for penetration of rodlet cells by immunostaining, and identification of stages of rodlet cells existed in other organs.

Various differential stages were observed in the respiratory organs of ruby shark fish. Rodlet cells underwent sequential differentiation from mesenchymal progenitors to mature functioning rodlet cells in the stroma and the covering epithelium of the olfactory organs. Rodlet stages could be classified to six distinctive forms; each had its morphological characteristics; mesenchymal like cells, vesicular cells, granular cells, transitional rodlet cells, mature rodlet and ruptured cells (Figure 1).

Mesenchymal like cells (or rodlet progenitors) appeared small spindle shaped with numerous cytoplasmic processes. Rodlet progenitors began synthesis vesicles and typical rodlet granules. They had dark cytoplasm and active nucleus with euchromatin and prominent nucleolus (Figure 2). Rodlet vesicles gradually increased and transformed to the vesicular stage (Figure 2E).

The Vesicularstage, vesicular rodlet cells eccentric nucleus with euchromatin, peripheral heterochromatin, and prominent nucleolus. They were characterized by extensive cytoplasmic vacuolation, vesicle formation, and synthesis fibrillar-like components. Early morphological markers of rodlet progenitor's development were small cytoplasmic vesicles. Enlargement of the cells was accompanied

Buffer	Chemicals	Amount
PBS-buffer (pH 7.2-7.4):	NaCl	42.5 g
	$Na_2HPO_4\,2H_2O$	6.35 g
	$NaH_2PO_4\,H_2O$	1.95 g
	Aqua dest.	add 5 l
Citrate-buffer (pH 6.0):	Solution A:	
	Citrate $C_6H_8O_7\,H_2O$	21.01 g
	Aqua dest.	add 1 l
	Solution B:	
	Natriumcitrate $Na_3C_6H_5O_7\,2H_2O$	29.41 g
	Aqua dest.	add 1 l
	Using solution:	
	Solution A:	9 ml
	Solution B:	41 ml
	Aqua dest.	add 500 ml

Table 2: Buffers used in semithin, transmission electron microscope, and immunohistochemistry.

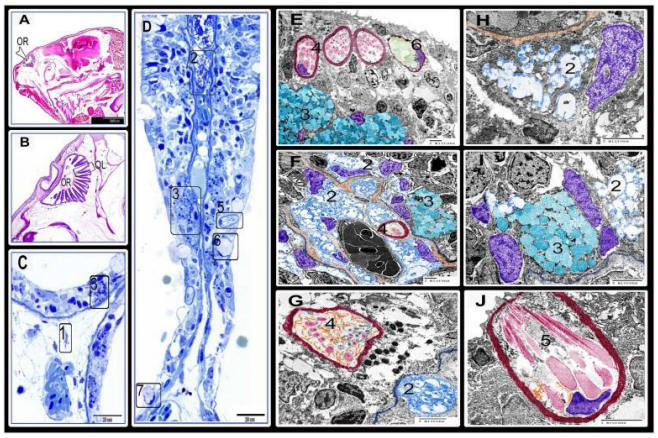

Figure 1: Different stages of rodlet cells. *Paraffin sections stained by H&E "A", Alcian blue PAS "B", semi-thin sections stained by toluidine blue "C, D" and colored ultra-thin sections "E-J". A: Head of red tailed shark. "OR" refers to olfactory organ. B: Olfactory organ "OR" consisted of concentrically arranged olfactory lamellae "OL". C: the basal part of the olfactory lamella; note lamina propria contained mesenchymal-like cells "1", granular rodlet cell "3" located in the basal layer of the olfactory epithelium. D: Olfactory lamella consisted of connective tissue core contained vesicular rodlet cell "2" and epithelial covering contained granular "3", mature "5", pre-rupture "6", rupture "7" rodlet cells. E-J: colored electron micrographs of rodlet cells; vesicles and vacuoles "blue color", nucleus "violet color", RER "orange color", primitive rodlet granules " turquoise color", rodlet granules" pink color", " external lamina" brown color", capsule o rodlet cells "dark red color", basement membrane "dark blue". E, F: showed the localization of rodlet precursors in the stroma of the olfactory organ and various stages of differentiating rodlet cells in the olfactory epithelium. Note; the vesicular rodlet cells "2" was characterized by abundant vesicles and vacuoles "blue" and located in the connective core and the basal layer of the olfactory epithelium. Transitional stages of rodlet cells "4" were common in the middle layers of the olfactory epithelium and few cells were located in lamina propria of the olfactory organ. The pre-ruptured stage had a thin capsule" red colored", electron lucent granules "green colored". G: transitional rodlet cell "4" was rich in RER and rodlet granules. H: vesicular rodlet cells "2" contained occupied by many vesicles and vacuoles "blue". I: granular rodlet cell "3" was marked by presence of immature granules " turquoise color" . Vesicular rodlet cell "2" located in the basal layer of the olfactory epithelium. J: mature rodlet cell "6" had thick capsule "red color" and club-shaped rodlet granules "pink color".

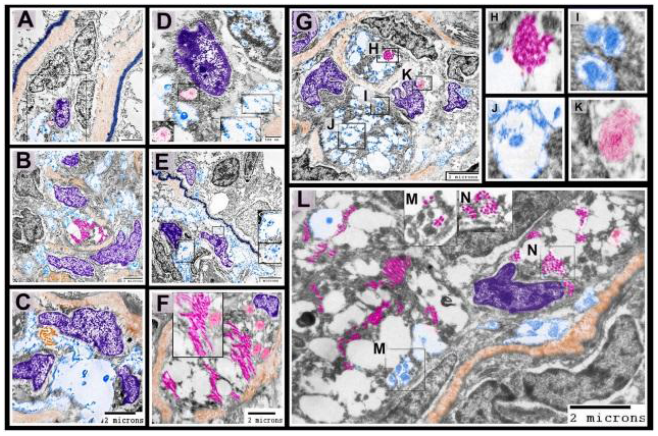

Figure 2: Morphological transformation of rodlet precursors to the vesicular stage in the olfactory stroma.

*Colored electron micrographs of rodlet cells; vesicles and vacuoles "blue color", nucleus "violet color", RER "orange color", rodlet granules" pink color", fibrils-like components " dark pink ", external lamina" brown color", capsule of rodlet cells "dark red color", basement membrane "dark blue". A, D: "D" represented a high magnification of the squared area in "A". Colored cells represented a mesenchymal-like cell which was small cell had cell processes "arrows" and oval nucleus with prominent nucleolus "arrowhead". Note, the cytoplasm contained small vesicles and vacuoles "blue colored" and small rodlet granule "pink color"; the granules was composed of central electron dense core and an electron lucent matrix. The vesicles and rodlet granules were magnified. B-F: showed the vesicular stage located in groups and were surrounded by an external lamina "brown color". Rodlet cells began to accumulate intra-cytoplasmic vesicles and vacuoles "blue colored". Vesicular rodlet cells synthesized intra-cytoplasmic fibril-like components "dark pink" which were organized in a parallel manner. Note; RER "orange colored", rodlet granules "light pink colored". G: enlargement of the vesicular rodlet cells was associated with accumulation of more vesicles. H-K: represent a high magnification of the squared areas. H: showed cross sectioned intra-cytoplasmic fibril-like components "dark pink colored". I: small vesicles. J: large vesicles. K: Rodlet granule "light pink colored". L: large vesicular rodlet cells contained intracellular fibril-like components and large vesicles and vacuoles of varying sizes. M: high magnification of small vesicles "blue colored". N: high magnification of intra-cytoplasmic fibril-like components "dark pink colored".

by increase cytoplasmic vacuolation and formation of more vesicles contained electron dense bodies which had different forms and size. Large vesicular rodlet cells accumulated intra-cytoplasmic small fibrils occurred as individual fibers or well-organized in bundles (Figure 2B-2N). Vesicular cells could penetrate the basal lamina to the basal epithelial layer and differentiated to mature rodlet cells which further on undergo rupture (Figure 3C and 3F). Vesicular cells were found encapsulated in groups by carbohydrate-rich external lamina stained positive for PAS (Figures 3A, 3B and 4E). The lamina was composed of well-organized parallel fibrils (Figure 3).

The Granular stage, granular rodlet cells could be observed in the basal layer of the epithelium (Figure 3D and 3G). Granular rodlet cells

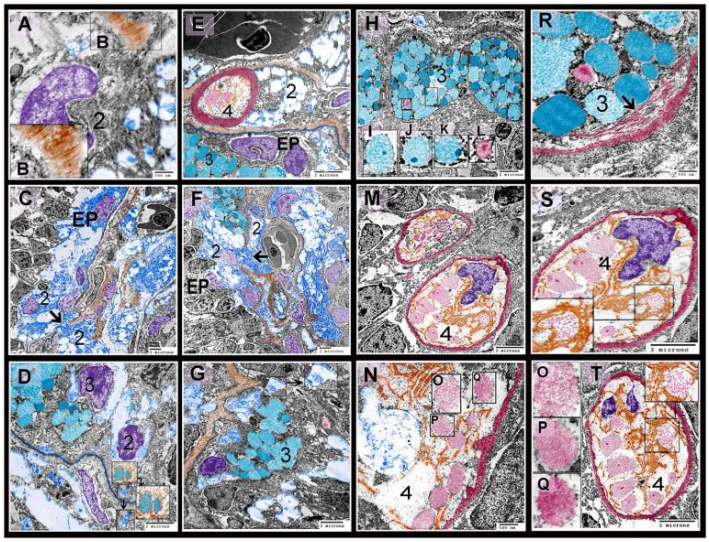

Figure 3: Migration of the rodlet precursor to epithelial tissue and differentiation to granular and transitional rodlet cells. Colored electron micrographs of rodlet cells; vesicles and vacuoles "blue color", nucleus "violet color", RER "orange color", rodlet granules" pink color", external lamina" brown color", capsule of rodlet cells "dark red color", basement membrane "dark blue". A: vesicular rodlet cell "2" in the connective tissue was surrounded by external lamina which was composed of small fibers "B". C, F: the vesicular rodlet cells penetrated through the basement membrane "arrows" to locate in the basal layer of the epithelium "EP". D: the vesicular rodlet cells among the basal epithelial cells began synthesis of primitive rodlet granules "turquoise color", granular rodlet cell was characterized by existence of immature rodlet granules "turquoise color" of varying electron densities. E: Transitional rodlet cells "4" could be observed in the connective tissue surrounded by the vesicular type "2". Note; granular rodlet cells "3" in the basal layer of the olfactory epithelium. G: the granular rodlet cells "3" contained immature rodlet granules "turquoise color" and few vesicles "blue colored". H: showed different stages of rodlet granules maturation in "I-L". I, J: immature rodlet granule was large with granular matrix and small electron dense central core. K: the granule was smaller the granular matrix become condenser. L: granule contained electron lucent matrix and central electron dense core resembling the rodlet granules. R: the granular rodlet cells deposited the capsular fibrils (arrow) on the cytoplasmic aspect of the cell membrane. M, S, T: Transnational rodlet cells "4" note; immature rodlet granules "the squared area" with granular matrix in vicinity of RER "orange colored". N: Stages of rodlet granules maturation in "O-Q". O: the immature rodlet granule had granular matrix with electron dense core. P, Q: the granules became smaller and condensed the matrix components and the central core was more obvious.

were dominated by secretory granules of varying electron densities which represent different stages of granules maturation. Premature rodlet granules had electron lucent matrix and central electron dense core. The matrix of the mature granules became more condensed and appeared electron dense. Transitional stages of granules contained a matrix of grading electron destinies (Figure 3H and 3R). Marked reduction of the fibrillar components was accompanied by the initial formation of rodlet cell capsule (Figure 3R).

In transitional rodlet cells, the secretory vesicles acquired the unique shape of rodlet granules. Most granules contained a central electron dense core and surrounded by a matrix of moderate electron density. Transitional rodlet cells appeared oval with well-developed RER. Various stages of rodlet granules formation could be observed near RER (Figure 3M-3T). The first stage represented by a small central core enclosed by faint granular matrix (Figure 3O). Moderate electron dense central core was surrounded by compact matrix (Figure 3P). The ripened granules had electron dense central core and less electron dense compact matrix (Figure 3Q). Transitional rodlet cells can be seen in lamina propria (Figure 3E).

Mature rodlet cells were polarized bear-shape. The cytoplasm contained few cisternae of RER around the nucleus, abundant SER in the apical part and almost cytoplasm was occupied by rodlet granules. Rodlet granules had the characteristic shape; the rod-like core; and appeared as club-shaped with widened basal part and tapered apical end (Figure 5A and 5B).

The Stage of rupture, Mature rodlet cells underwent rupture, liberated its contents in the epithelia surface. Early stages of ruptured cell contained a large rodelt granules with elelctron lucent matrix and a small part of the central core ((Figure 5C and 5D) and the granular conentes was secreted (Figure 5E-5J). This stage was positive for alcian blue (Figure 4G), sudan black (Figure 6I). In the final stages, ruptured rodelt cells became empty (Figure 7).

Granular, transitional and mature rodlet cells were positive for MMP-9 immunostaining (Figure 8A-8H). BY H&E, vesicular rodlet cell appeared large cells with eosinophilic vacuolated cytoplasm (Figure 6B). The cytoplasm of granular rodlet cells had abundant acidophiilic granules (Figure 6C). Rodlet granule appeared as elongated acidophilic granules filled the cytoplasm of mature rodlet cell (Figure 6D). Rodlet granules could be observed by giemsa and appeared pink in mature rodlet cells (Figure 6E). The section of rodlet cells in ruptured stage appeared violet (Figure 6F). Vesicular rodlet cells were negatively stained with silver stain (Figure 6G), while rodlet granules in mature cells stained strong positive (Figure 6H). The cytoplasm of mature rodlet cells was positive for sudan-black and rodlet granules were strongly positive for sudan-black. The extruded cytoplasm of ruptured rodlet cell stained positive for sudan-black (Figure 6I).

Rodlet cells had different affinity for Crossman's trichrome during differentiation. The cytoplasm of vesicular rodlet cell appeared vacuolated red colored (Figure 4A). The granular stage had two types of granules; red and green stained granules (Figure 9F). Mature rodlet cells had dichromatic rodlet granules the expanded basal appeared blue while the apical part of rodlet granules appeared red.

Mallory trichrome stained rodlet granules at different stages of maturation of rodlet cells. In the granular stage, rodlet granules appeared blue. Rodlet granules in the mature stage appeared either blue

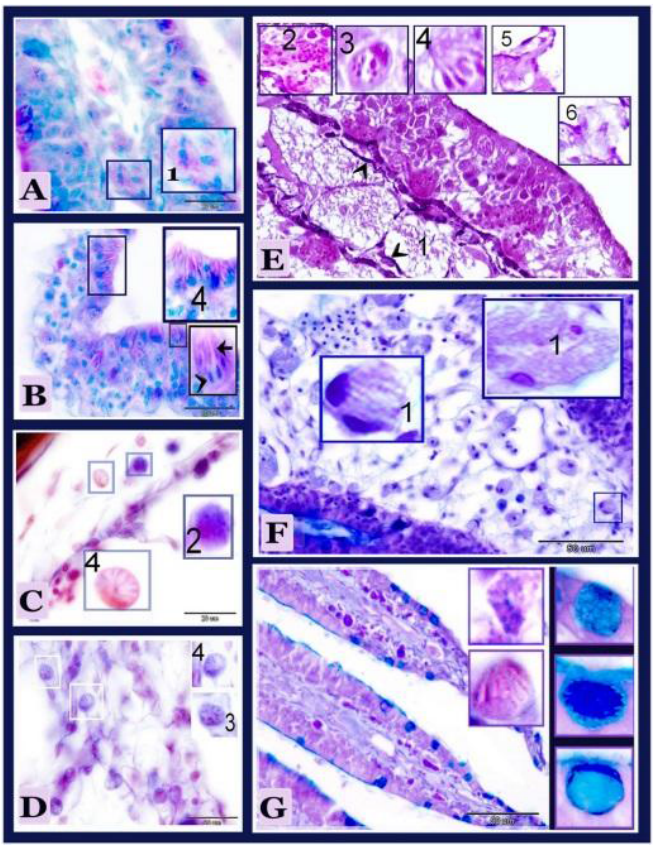

Figure 4: Affinity of different stages of rodlet cells for Crossman's, Mallory trichrome, PAS, combination of alcian blue and PAS. *Paraffin (A-D, F-H) and semi-thin (E) sections stained by Crossman's "A, B" and Mallory "C, D" trichrome, PAS "E combination of Alcian blue/PAS "F-H". A: The cytoplasm of vesicular rodlet cell (1) appeared vacuolated red colored. B: Mature rodlet granules appeared dichromatic by Crossman's trichrome; the expanded basal appeared blue (arrowhead) while the apical part of rodlet granules appeared red (arrow). C, D: Mallory trichrome stained the granular rodlet cells (2) by blue color; rodlet granules appeared blue in transitional stage (3) while rodlet granules in mature cells (4) may appear either blue or red. E: the vesicular rodlet cells appeared vacuolated and surrounded by PAS positive- external lamina (arrowheads). Rodlet granules in granular (2), transitional (3) and mature (4) stages had a strong affinity for PAS in all stages. Ruptured rodlet cells (5) lost PAS staining affinity. F: the vesicular rodlet cells appeared had no staining affinity for alcian blue/PAS staining. G: olfactory lamellae stained by alcian blue/PAS staining. Stages of rodlet showed different staining reaction. Granular rodlet cells (2) contained dichromatic granules. Mature rodlet cell (4) contained red rodlet granules. Pre-ruptured rodlet cell (5) had blue granules. Early ruptured rodlet cells (6) with homogenous cytoplasm stained dark blue. Late ruptured rodlet cell (7) stained light blue.

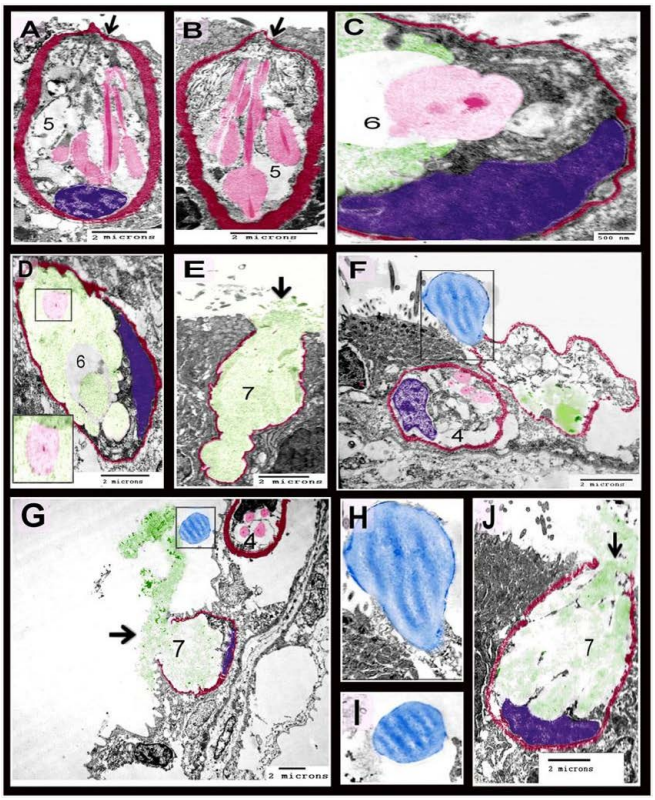

Figure 5: Maturation and rupture of rodlet cells. *Colored electron micrographs of rodlet cells; nucleus "violet color", rodlet granules" pink color", capsule of rodlet cells "dark red color", granules during rupture stage "green granules", striated granules "blue colored granules". A, B: mature rodlet cells were pear-shaped, had a thin capsule, the apical capsule became thin. Rodlet granules were club-shaped; had an expanded basal part and tapered end. C, D: ruptured stage C-E: the apical capsule of rodlet cells was opened "arrow", the entire capsule became thin, the central core disappeared remained only the electron lucent matrix components "green". Some granules had a central core "pink". F, G, J: ruptured rodlet cells with wide opening evacuated their content to the surface of the olfactory epithelium. Large granules "blue" were observed in association the secretion in the terminal stages. Note; transitional rodlet cells "4".

or red (Figure 4C and 4D). Rodlet granules had a strong affinity for PAS staining in all stages of rodlet cells differentiation. PAS-positive external lamina was observed around the vesicular rodlet cells in lamina propria (Figure 4E). Stages of rodlet showed different staining reaction by combined staining of alcian blue/PAS while the vesicular rodlet cells had no affinity for alcian blue/PAS staining. Granular rodlet cells contained dichromatic granules. Mature rodlet cell contained red rodlet granules by alcian blue/PAS. Pre-ruptured rodlet cell had blue granules. The cytoplasm of early ruptured rodlet cells was homogenous and stained dark blue by alcian blue/PAS. Late ruptured rodlet cell stained light blue (Figure 4G).

Rodlet granules were stained by safranin O staining in granular, transitional and mature stages (Figures 9C and 10M), by Weigert Van Gieson in granular and transitional stages (Figure 10F), Iron hematoxylin (Figure 10E and 10I), and bromopHenol blue (Figure 10C). Pre-ruptured rodlet cells had a strong affinity for sudan black, while only the capsule of ruptured rodlet cells stained positive reaction for sudan black staining (Figure 10D and 11).

Rodlet cells were distributed in different organs of ruby shark. Mesenchymal like cells, the vesicular, granular, transitional, mature and ruptured rodlet cells were recognized in the olfactory organ while other respiratory organs including gills and pharyngeal pad contained granular, transitional, mature and ruptured stages (Figure 4). Granular, transitional, mature and ruptured rodlet cells were observed in different organs in the body of redtail shark including brain (Figure 9A-9E), body cavity (Figure 9E and 9F), eye (H,I), epidermal (Figure 10A, 10C and 10D) and dermal (Figure 10B, 10E and 10F) layers of skin, skeletal muscles (Figure 10G-10I), bone (Figure 10J-10L), heart (Figure 10M and 10N) blood vessels (Figure 10O and 10P), intestine (Figure 12A and 12B), liver (Figure 12C-12E), pancreas (Figure 12F-12H), thymus (Figure 12J), melano-macrophage center of kidney (Figure 12K-12N), swim bladder (Figure 12O and 12P).

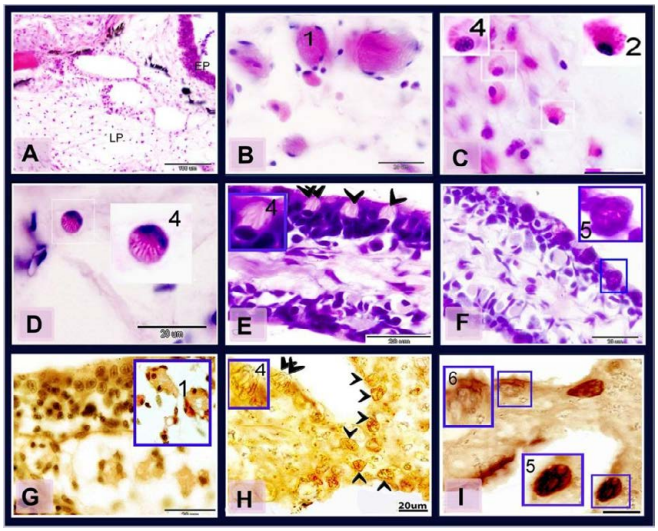

Figure 6: Affinity of different stages of rodlet cells for H&E, giemsa, silver and sudan black. *Paraffin sections stained by H&E "A-D", giemsa stain "E, F", silver "G, H", sudan black "I". A: the wall of the olfactory organ. Note, epithelium (EP) and lamina propria (LP) were rich in rodlet cells (arrows). B, C, D: higher magnifications in lamina propria of the olfactory organ showed different stages of rodlet cells, vesicular stage (1) had eosinophilic vacuolated cytoplasm. Granular stage (2) contained prominent eosinophilic granules. Mature rodlet cells (4) characterized by presences of rodlet granules. E: mature rodlet cells arrowheads contained rodlet granules which stained red by giemsa stain. Double arrowhead refers to the magnified rodlet cell (4). F: show the ruptured rodlet cell (5) appeared deep blue cytoplasm. G: vesicular rodlet cells (1) stained negative for silver. H: rodlet granules stained potive for a silver stain in mature rodlet cells (arrowheads). Double arrowhead refers to the magnified rodlet cell (4). I: the cytoplasmic content of mature rodlet cell (4) was sudan black-positive and rodlet granules were strongly positive for sudan black. The extruded cytoplasm in ruptured rodlet cell (6) stained positive for sudan-black.

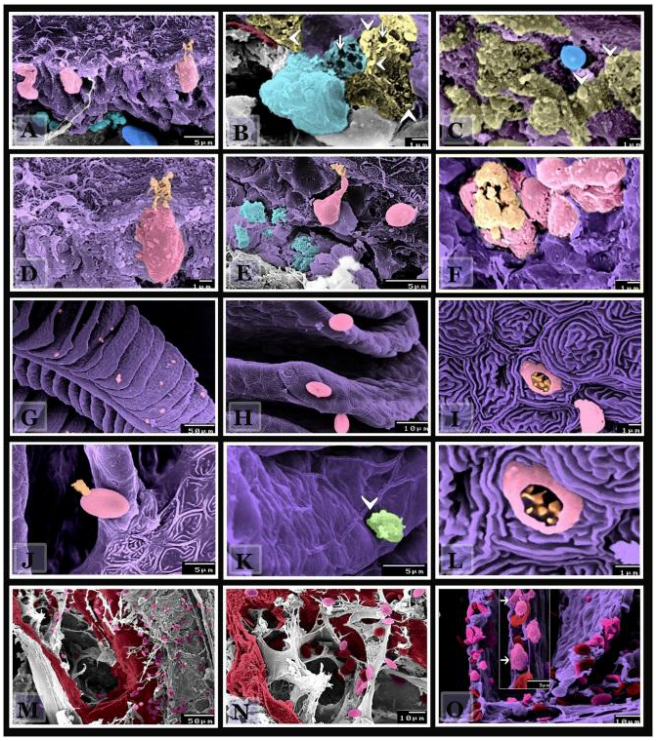

Figure 7: different stage of rodlet cells in olfactory and gill lamellae. *Colored scanned electron micrographs of rodlet cells in olfactory lamellae "A-F" and gill lamllae :G-O"; epithelium "violet color", mature "pink color", transitional "blue color", granular "yellow color", vesicular rodlet " turquoise color" , rodlet secretion "orange color", red blood cells "red color", secretion of the ruptured rodlet "green color", basement membrane "red color". A: vesicular " turquoise color" and transitional" blue color" rodlet located under the epithelium, mature rodlet "pink color" existed at the superficial layer of the epithelium. B: vesicular rodlet "turquoise color" penetrate the basement membrane "red color" and extended to the basal layer of the epithelium, note; arrows refer to vesicles. Granular rodlet cells "yellow colored" contained numerous granules "arrowheads" and few vesicles "arrow". C: a large population of the granular rodlet cells "yellow colored", note the arrowheads refer to rodlet granules. Transitional rodlet cell "blue colored". D: mature rodlet cell at the superficial layer of the olfactory epithelium, note needle-like rodlet secretion "orange colored" projected at the surface of the epithelium. E: olfactory epithelium "violet colored" contained vesicular rodlet cells "turquoise color" in the basal layers and mature rodlet cells in the superficial layers; note rodlet secretion. F: ruptured rodlet cells. Note wide opening of the rodlet cells which librated their secretion in terminal stages "orange color". G, F: showed rodlet cells on the surface of the gill lamellae. I, J, L: early stages of ruptured rodlet cells. Note: needle-like granules. K: arrowhead refers to the area of ruptured rodlet cell, large granule "green colored" discharged from the ruptured cell. M, N: rodlet cells "pink colored" located in and around the blood vessel "red colored". O: rodlet cells located within the epithelium of gill lamellae "arrows".

Discussion

Rodlet cells are documented in digestive, cardiovascular, integument, urinary, respiratory, genital tracts which render a great doubt concerning to nature of rodlet progenitors and organ of origin. The current study for the first time described the site of rodlet cells emergence, identification of rodlet progenitors, differential stages of rodlet by transmission electron microscope, and different histochemical staining, and identify enzymatic activity responsible for penetration of rodlet cells by immunostaining. Identification of stages of rodlet existed in other organs.

In the current study, we identified rodlet progenitors in the stroma of the olfactory organ. Rodlet progenitors were mesenchymal-like cells synthesis the primitive rodlet granules. Rodlet progenitors differentiated to vesicular rodlet cells which was rich in vesicles and vacuoles and accumulate intracellular fibrillar-like components. Vesicular rodlet cells were surrounded by an external lamina which was composed of fine fibers and was stained Positive for PAS. Granular rodlet cells were observed in the basal epithelia, contained premature rodlet granules. The disappearance of fibrillar-like components in the granular stage was associated with initiation of capsular organization; hence the fibrillar-like components may involved in formation of the rodlet capsule. Transitional rodlet cells continued to deposit the fibrils of the capsule and synthesis of rodlet granules. Mature rodlet cells were polarized bear-shape and had typical rodlet granules. Several authors have documented the origin of rodlet cells based on the location and stages of differentiation. The wide distribution of rodlet cells in various organs in the fish body; render the origin of rodlet cells a subject of controversy. Leino suggested rodlet cells arise form epithelial origin and rodlet cells precursors located near the basement membrane and migrate toward the surface [15], however, mature rodlet cell are also described in the subepithlial connective tissue. Rodlet cells are described as blood-cell derived secretory cell [4]. In carp (Cyprinus carpio), typical rodlet cells are detected at 5 days post-fertilization between kidney and intestine, at 6 days post-fertilization in the intestine, 8 days post-fertilization in kidney, abdominal cavity among the mesothelial cells and 14 days post-fertilization in gills [6]. However, the authors describe the sites of typical rodlet cells and not mentioned rodlet progenitors, differential stages, and their localization. Immature stages of rodlet cells are recognized in the intermediate and basal

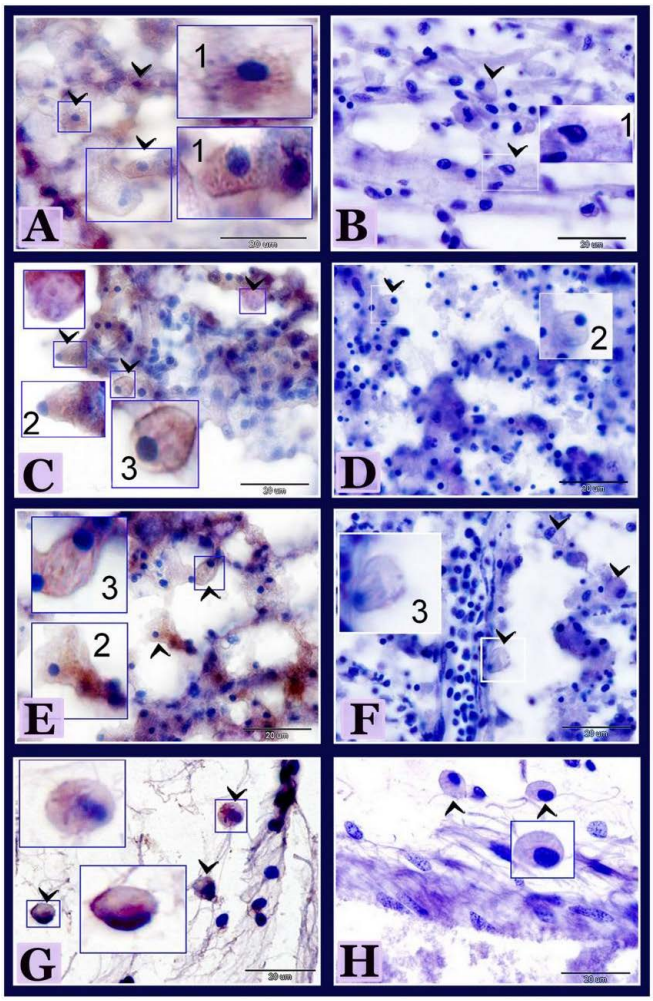

Figure 8: Immunohistochemical staining of the respiratory organs of redtail shark Using MMP-9. *Immunostained paraffin sections for MMP-9 (A, C, E G) and control negative (B, D, F, H). A: Granular rodlet cells (arrowheads) in gills were positive for MMP-9 immunostaining. Note the squared area shows granular rodlet cell (1). B: rodlet cells (arrowheads) in gills. Note the squared area shows granular rodlet cell (1). C: transitional (2) and mature (3) rodlet cells in gills were positive for MMP-9 immunostaining. Arrowheads refer to immunostained positive rodlet cells for MMP-9. D: showed rodlet cells (arrowheads) in gills. Note the squared area shows transitional rodlet cell. E: Arrowheads refer to immunostained positive rodlet cells in gills for MMP-9. Note the squared area shows transitional (2) mature (3) rodlet cells. F: rodlet cells (arrowheads) in gills. Note the squared area shows the mature rodlet cell. G: rodlet cells (arrowheads) in respiratory stroma were positive for MMP-9 immunostaining. H: rodlet cells in the respiratory stroma (arrowheads).

zones of the operculum and gill raker epithelium while mature rodlet cells present along the epithelial surface of operculum and gill raker [11]. Mature rodlet cells are also mentioned in the olfactory organ in zebrafish [37] and in both in the non-sensory and in the sensory epithelium of Caecobarbus. Geertsi and Barbus barbus plebejus) [38]. Cells, morphologically identical to the granular stage of rodlet cells as represented in the current study, has been described as the mother of rodlet cells in the olfactory organ of the red tial shark [16].

In the current study, TEM and SEM revealed ruptured rodlet cells secret their contents through the apical pores and extrude the whole contents to the epithelial surface (holocrine mode of secretion). Similar Ultra-structural observation is mentioned by [15]. Merocrine secretions have been documented in rodlet cells in the kidney of the

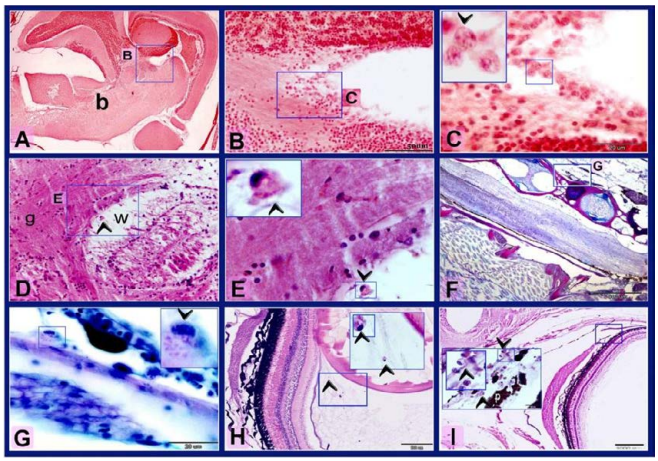

Figure 9: localization of rodlet cells in CNS and eye. Paraffin sections stained by safranin O (A-C), H&E (D, E, H, I) and alcian blue PAS (F, G). A: sagittal section in the head of the red-finned shark showing brain (b). B: heigher magnification of squared area in (A). C: higher magnification of the squared area in (B) note rodlet cells (arrowhead) in brain ventricle. D, E: rodlet cell (arrowhead) between white (w) and grey (g) matter of the brain. Note (E) represent a high magnification of the squared are in (D). F, G: rodlet cell (arrowhead) in body cavity closed to the vertebrae. Note (G) represent high magnification of the squared area in (F). H: Rodlet cell (arrowhead) inside the vitreous chambers. I: rodlet cells (arrowhead) around the eye. Note retinal pigmented layer (P).

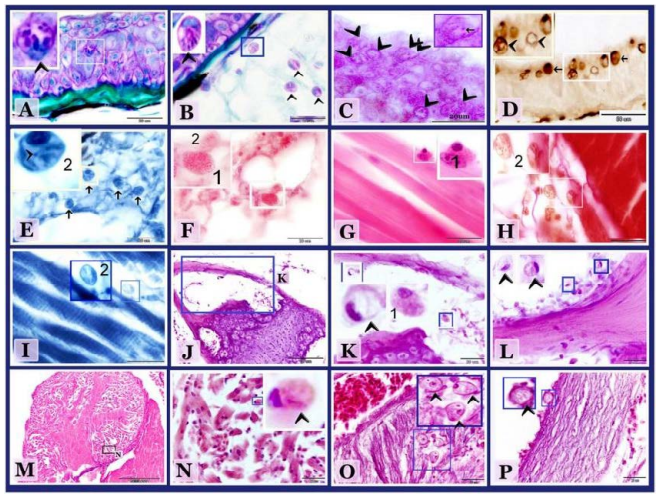

Figure 10: Mature and immature rodlet cells in skin, musculoskeletal, cardiovascular system. Paraffin sections stained by Crossman's trichrome (A, B), bromophenol blue (C),sudan black (D), Iron hematoxylin (E, I), Weigert Van Gieson (F, O, P), safranin O (H), H&E (G, J-N). A: rodlet cells in the epidermis. B: rodlet cells in dermal tissue note blue rodlet granules. C: rodlet cells (arrowheads) contained bromophenol blue-positive stained rodlet granules (arrow). D: pre-ruptured rodlet cells (arrows) stained positive with sudan black. The capsule of ruptured rodlet cells (arrowheads) showed a positive reaction for sudan black staining. E: rodlet cells (arrows) in the dermal connective tissue. Note rodlet granules (arrowhead) stained positive for Iron hematoxylin. F: Granular rodlet cells (1) contained primitive rodlet granules which stained positive for Weigert Van Gieson. Transitional rodlet cell (2). Note rodlet granules were positive for Weigert Van Gieson. G: granular rodlet cell (1) in contact with skeletal muscle. H: transitional rodlet cells in between skeletal muscle fibers. Note safranin O-positive rodlet granules. I: transitional rodlet cells stained by iron hematoxylin located between the skeletal muscles. J, K: rodlet cells (arrow) inside the medullar cavity of the bone. Note: granular rodlet cell (1). L: rodlet cells in closed contact to the periostium. M, N: rodlet cell (arrow) inside the heat between the cardiac muscles. O: rodlet cells (arrowheads) penetrate through the wall of the branchial vessels.

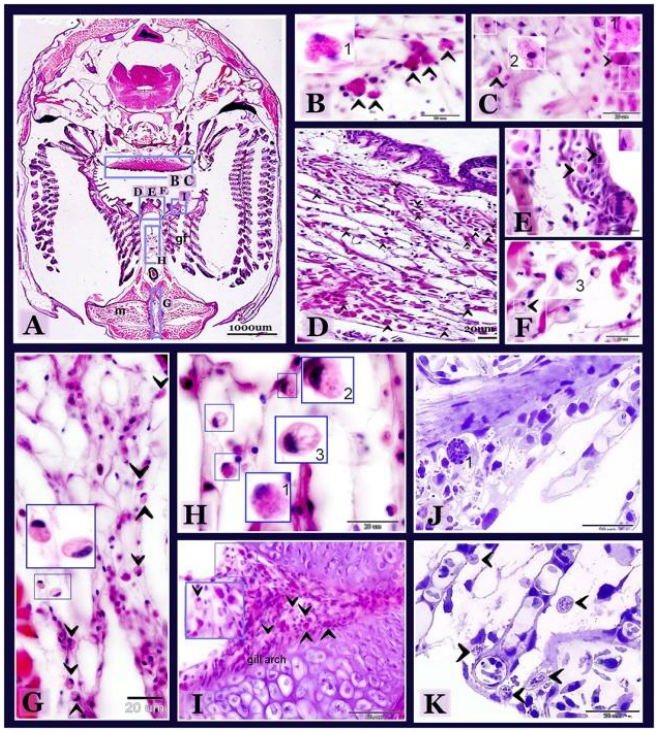

Figure 11: Localization of granular, transitional and mature rodlet cells in gills and pharyngeal wall. Paraffin sections stained with H&E (A-I) and semi-thin sections stained with toluidine blue (J, K). A: cross section in the head of the red-finned shark. The squared areas are highly magnified in B, C (pharyngeal pad), D, E, F, I, H (gill arch), G (connective tissue between muscles), J (Gill filament), K (gill lamella). B, C: lamina propria of the pharyngeal pad had abundant rodlet cells (arrowheads). Note the granular rodlet cell (1), transitional rodlet cell (2). D: the wall of the gill arch was rich in rodlet cells (arrowheads). E: rodlet cells were located in the basal and the superficial layer of the epithelium of the gill arch. F: rodlet cells (arrowheads) in the connective tissue of gill arch Note mature rodlet cell (3). G: Rodlet cells (arrowheads) located in the connective tissue which connected between muscles (m). H: Different stages of rodlet located in the connective tissue between gill filaments (gf). Note the granular rodlet cell (1), transitional rodlet cell (2), mature rodlet cell (3). I: Rodlet cells (arrowheads) around the branchial cartilage. J: granular rodlet cell (1) situated in the epithelial covering the gill filament. K: rodlet cells (arrowheads) within the respiratory lamellar epithelium.

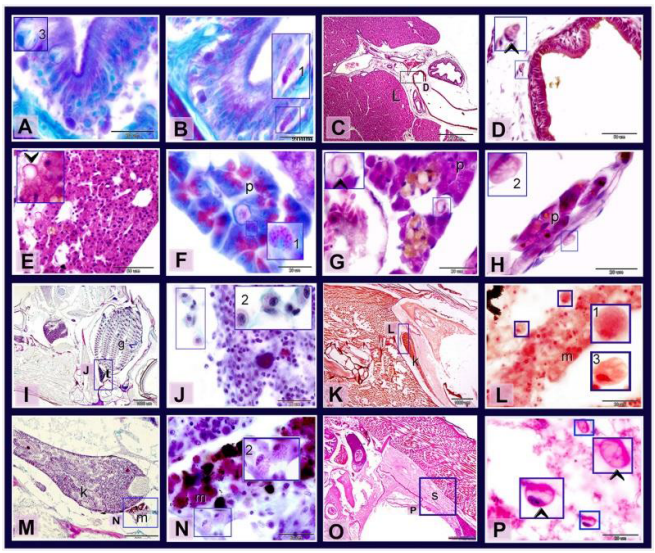

Figure 12: Mature and immature rodlet cells in tubular and parenchymatous organs. Paraffin sections stained by Crossman's trichrome (A, B, F), H&E (C, D, E, G, H, O, P), alcian Blue/PAS (I, J, M, N), safranin O (K, L). A: Mature rodlet cell (3) in the intestinal epithelium. B: Granular rodlet cell (1) in the intestinal epithelium. C: liver (L) note high magnification of the squared area in D showing rodlet cell near bile duct. E: rodlet cell (arrowhead) in liver. F: granular rodlet cell in between pancreatic acini. Note two types of granules; red and green stained granules (P). G: rodlet cell (arrowhead) between pancreatic acini (P). H: transitional rodlet cell between pancreatic acini (P). I: sagittal section id redtail shark. Note gill (g) and thymus (t). J: higher magnification of the squared area in (I) showed transitional rodlet cells (2) in the thymus. K: sagittal section of redtail shark showing kidney (k). L: higher magnification of the squared are in (k) showing granular (1) and mature (3) rodlet cells in the melano-macrophage center (m). M: note kidney (k) and melano-macrophage center (m). N: transitional rodlet cells (2) located in the melano-macrophage center. O: Sagittal section of redtail shark showing swim bladder (s). P: higher magnification of the squared area in (O) showing rodlet cells (arrows) located in swim bladder.

estuarine teleost, particularly the spot *Leiostomus xanthurus* [14]. Rodlet cells excrete their products via apocrine mode of section in kidney of different teleost species including pronephros of *Cobitis taenia* (L.), mesonephros of *Tinca tinca* (L.), *Rutilus rutilus* (L.), *Stizostedion volgense* (Gmelin), *Stizostedion lucioperca* (L.), *Perca fluviatilis* (L.), *Trachurus mediterraneus* (Staindachner) and *Diplodus annularis* (L.) [39].

In the current study, penetration of rodlet cells in the wall of the bronchial artery is properly regarding the invasive activities of the rodlet cells. Various stages of rodlet cells including granular, transitional and mature stages expressed MMP-9 which permitted their free penetration through layers of organs. MMP-9 or gelatinase B is a member of metal-dependent endopeptidases. MMP- 9 destruct extracellular matrix components such as Collagen IV, V, xik´, xivl´, elastin, aggrecan, link protein, decorinr, lamininn, entactin, sparcq, myelin basic proteinm,∞ 2Mn, ∞1Pli, IL-1βj, protnf-∞k [40]. MMP-9 promotes angiogenesis, migration of immune cells, activation of cytokines and chemokines, cancer progression [41].

Conventional histological staining indicates that rodlet cells had a wide range of staining including general stains, specific carbohydrate, lipid, and protein staining. Different stages of rodlet cells could be recognized by general stains including H&E and giemsa. The vesicular stage was marked by vacuolated cytoplasm. Rodlet granules appeared eosinophilic by H&E and pink by giemsa. Rodlet cells acquired characters of protein secreting cells. Rodlet granules in mature cells stained positive for bromophenol blue. Bromophenol Blue interact with proteins in acidic solution [42]. Lein [3] explored the presence of some proteases in the core of the rodlet granules.

Granules of rodlet cells had a strong affinity for carbohydrate staining. The Strong reaction of Rodlet granules for PAS staining indicate secretion of neutral polysaccharides. A similar result is recorded by ref. [3]. A combined alcin blue/PAS staining revealed granular rodlet cells had acidic and neutral polysaccharides. Mature rodlet cells changed the polysaccharides to neutral type. Predominance of acidic polysaccharide ocurred in ruptured rodlet cells. Lein [3] mentioned that rodlet cells have no reaction for Alcian blue (pH 1.0). Rodlet granules were postive for Safranin O which is a cationic dye bind to sulfated glycosaminoglycans [43].

Rodlet cells exhibited different reactivity for connective tissue staining during their differentiation. By Crossman's trichrome, vesicular rodlet cell had vacuolated red cytoplasm. The granular stage had two types of granules; red and green stained granules which may be regarded to the predominance of premature, transitional form of rodlet granules. Mature rodlet cells had dichromatic rodlet granules which

may indicate that the granule contained different components in the basal and the apical parts. Mallory trichrome stained rodlet granules in the granular and the mature stage. Granules of mature rodlet cells exhibited different reaction either blue or red which remained unclear. Moreover, rodlet granules had staining reactivity for Van Giesion stain in granular and transitional rodlet cells.

Pre-ruptured rodlet cells had a strong affinity for sudan black stain which indicated lipid inclusions. Moreover, the capsule of the ruptured rodlet cells was positive for sudan black which reveal the lipid comprised a major constituent of rodlet capsule. Lein [3] documented that rodlet cells have no reaction for sudan black. The affinity of rodlet granules for silver staining was unclear.

Two possible pathways could be suggested for rodlet migration from the respiratory tract to other organs in the body. Immature rodlet could penetrate the wall of one of the branches of the efferent branchial vessels which circulate to the blood and distribute to the other organs. The second pathway; rodlet cells could penetrate through the walls of the respiratory organs in the body cavity to migrate to other organs. Immature stage (particularly granular stage) presented in the connective tissue of these organs and rodlet maturation occurred either in the connective tissue of the organs or in the epithelium of the tubular organs and skin.

Conclusion

Different stages of rodlet cells were identified. Rodlet progenitor appeared as a Mesenchymal-like cell which transformed into vesicular, granular, transitional, mature, and ruptured stages. Rodlet progenitors were detected in the olfactory organs the red-finned shark while granular, transitional, mature, and ruptured were observed in other organs. They expressed MMP-9 which degrade the matrix components and permit their penetration and localization in other organs and tissues. Rodlet cells have histochemical staining affinity for protein, lipid and carbohydrates.

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