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Fish Gill Morphology: Inside Out

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ABSTRACT In this short review of fish gill morphology we cover some basic gross anatomy as well as in some more detail the microscopic anatomy of the branchial epithelia from representatives of the major extant groups of fishes (Agnathans, Elasmobranchs, and Teleosts). The agnathan hagfishes have primitive gill pouches, while the lampreys have arch-like gills similar to the higher fishes. In the lampreys and elasmobranchs, the gill filaments are supported by a complete interbranchial septum and water exits via external branchial slits or pores. In contrast, the teleost interbranchial septum is much reduced, leaving the ends of the filaments unattached, and the multiple gill openings are replaced by the single caudal opening of the operculum. The basic functional unit of the gill is the filament, which supports rows of plate-like lamellae. The lamellae are designed for gas exchange with a large surface area and a thin epithelium surrounding a well-vascularized core of pillar cell capillaries. The lamellae are positioned for the blood flow to be counter-current to the water flow over the gills. Despite marked differences in the gross anatomy of the gill among the various groups, the cellular constituents of the epithelium are remarkably similar. The lamellar gas-exchange surface is covered by squamous pavement cells, while large, mitochondria-rich, ionocytes and mucocytes are found in greatest frequency in the filament epithelium. Demands for ionoregulation can often upset this balance. There has been much study of the structure and function of the branchial mitochondria-rich cells. These cells are generally characterized by a high mitochondrial density and an amplification of the basolateral membrane through folding or the presence of an intracellular tubular system. Morphological subtypes of MRCs as well as some methods of MRC detection are discussed. *J. Exp. Zool. 293:192–213, 2002.*

The last comprehensive reviews of fish gill anatomy date from the 1980s (Hughes, '84; Laurent, '84, '89). Rather than marking the end of morphological studies of the gills, they have served as a reference point from which work has continued. The fish's gills anatomical complexity and functional importance and diversity have contributed to its continuing study.

This anatomical review will include description of the basic gross anatomy of the gills followed by the finer levels of organization, the filament and lamellae, and a detailed description of the cell types in the branchial epithelia. We will limit ourselves to a summary of the gross and microscopic morphology of representatives from the classes Agnatha (jawless fishes), Elasmobranchiomorphi (cartilaginous fishes), and Osteichthyes (bony fishes). From the latter class we will only cover the teleost fishes and refer the reader to the excellent reviews of the gross and microscopic anatomy of the gill that have been made by Hughes ('84) and Laurent ('84) for information on the Chondrostei (e.g., *Acipenser*), Holostei (e.g., *Amia, Lepisosteus*), and Dipnoi (e.g., *Neoceratodus*, *Lepidosiren*, *Protopterus*). We will, however, make some cursory reference to representatives from these groups when interesting points arise. We refer to recent morphological studies when ever possible while also including citations of important earlier works.

In addition to the reviews by Hughes ('84) and Laurent ('84, '89), additional information can be sought in a number of reviews made on particular aspects of fish gill morphology related to technique (TEM by Pisam and Rambourg, '91; SEM by

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Olson, '96) and function (Laurent and Perry, '95; Jurss and Bastrop, '96; Perry, '97, '98).

GROSS ANATOMY

The location and basic structures of the gills of the agnathan hagfishes and lampreys, elasmobranchs, and teleosts are illustrated in Fig. 1. In all fishes, the gills are bilaterally situated on either side of the pharynx and are composed of a series of pouch-like or arch-like structures that provide the physical support for the delicate gill filaments also termed primary lamellae. The gills of the lampreys and higher fishes share a similar arch-like arrangement with intervening branchial slits for the water to pass laterally from the buccopharyngeal cavity through the gills and out. The hagfish gill does not have recognizable arch-like structures, and so its pouch gills will be dealt with separately.

Lining the sides of the gill arches are rows of regularly spaced filaments projecting posteriolaterally. Each row or stack of filaments constitutes a hemibranch, while a set of hemibranchs, one on each side of the arch, constitutes a holobranch. The gills of teleost fishes are composed of four such holobranchs spaced between five branchial slits (chambers) (Fig. 1d) while the elasmobranchs have an extra hemibranch on the anterior side of the first branchial slit (Fig. 1c). The posterior side of the final branchial chamber does not have a hemibranch. Not included in the above count of

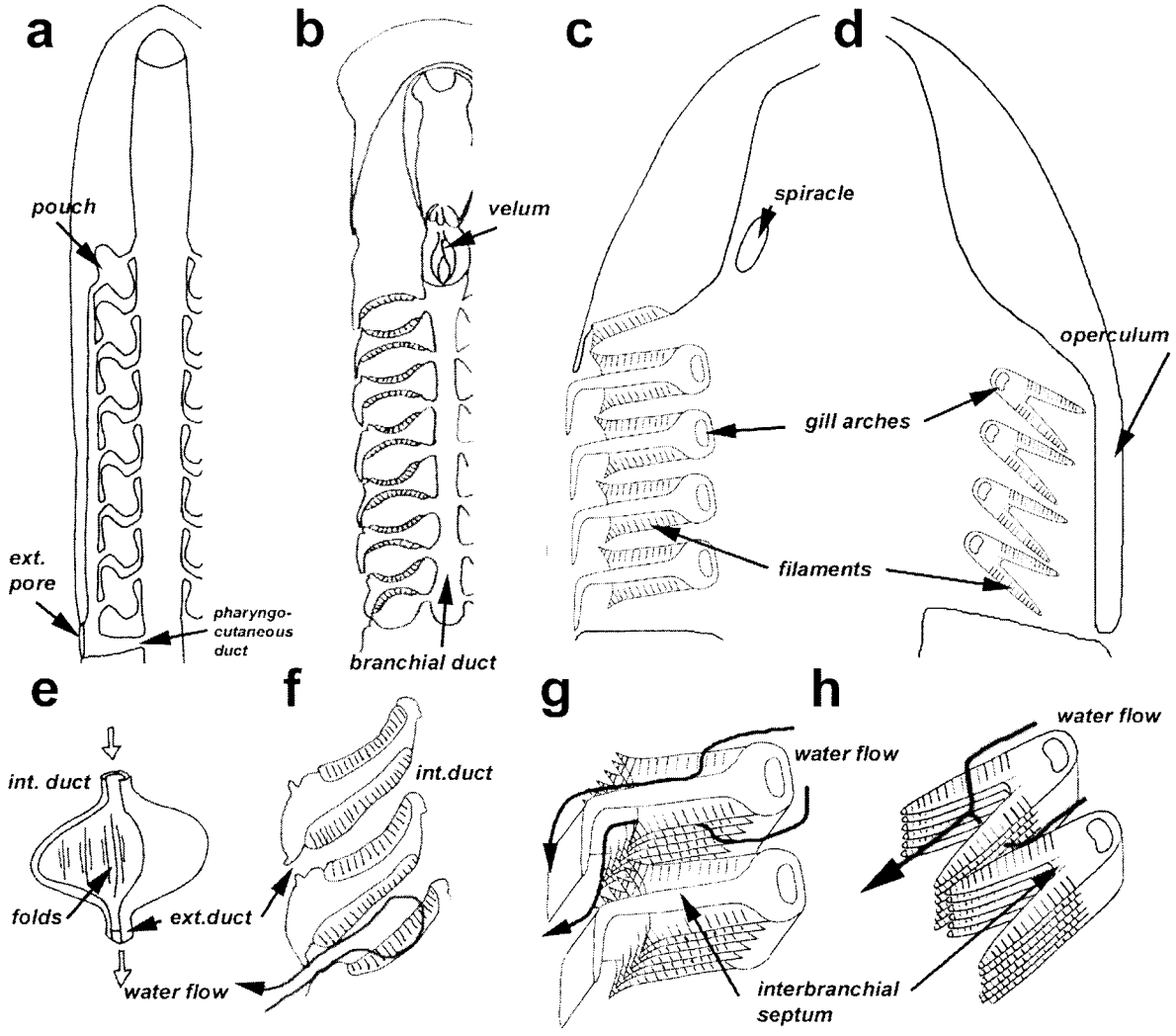

Fig. 1. General schematics of (**a**) hagfish, (**b**) lamprey, (**c**) elasmobranch, and (**d**) teleost gills. More detailed illustrations of the (**e**) hagfish pouches and (**f,g,h**) branchial arches of the respective groups are in the lower panels. Arrows indicate the direction of water flow.

hemibranchs is the pseudobranch, which is a reduced hemibranch present in most teleosts and elasmobranchs in association with the spiracles (Fig. 1c; Laurent and Dunel, '84). The spiracles function as one-way valves for the entry of respiratory water. Within the elasmobranchs, the spiracle is reduced in fast-swimming pelagic species, while in bottom dwelling species the spiracle is enlarged. In the teleosts, the spiracles are lost but the pseudobranch remains. The pseudobranch does not function in gas exchange like the other hemibranchs and in fact receives oxygenated blood. Its function remains somewhat of a mystery (Laurent and Dunel, '84; Bridges et al., '98). The lampreys have six holobranchs and seven branchial slits (pouches). The hagfish gill pouches (5–14 pairs) are either disk shaped or biloped, and each pouch is composed of the hemibranchs of adjacent gill arches. The hagfish gill lacks distinct holobranchs.

In the lampreys and elasmobranchs, the filaments are supported for almost their entire length by an interbranchial septum containing a vertical sheet of connective tissue that runs from the arch to the outer body wall (Fig. 1f,g). The afferent or trailing edge[1] of the filament is continuous with the interbranchial septum, while the efferent or leading edge of the filament is unattached. In the teleosts, the interbranchial septum is reduced and the majority of the distal length of the filaments are unattached or free, giving them a filamentous appearance.

In the teleosts and elasmobranchs, the individual gill arches are supported by vertical elements of the cartilaginous branchial skeleton, which run through the medial portion of each arch and are partially calcified in the teleosts. In the elasmobranchs, the interbranchial septum is supported by skeletal branchial rays, which extend from the main skeletal support in each arch. In the teleosts, these branchial rays are present in the filaments and in conjunction with abductor muscles are important in the positioning of the filament in the water flow (see Hughes, '84). The tips of filaments from opposite hemibranchs in the branchial slit chambers come into close proximity to each other to form a sieve like arrangement (Fig. 1g,h). The agnathan visceral skeleton is totally unlike that of the jawed fishes as it forms a fenestrated framework of continuous cartilage immediately under the skin surrounding the pharynx. This branchial basket is less developed in the hagfish than the lamprey.

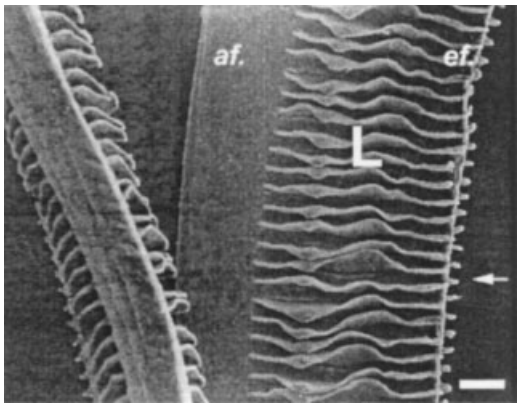

Fig. 2. Scanning electron micrograph of two gill filaments of the teleost tilapia (*O. mossambicus*). One filament is lying flat while the other (left) has its leading edge facing up. From these two views the orientation of the lamellae (L), which are present on both faces of the filament, is made obvious. The arrow indicates the direction of water flow across the filaments. The filament edge facing the water flow is the leading or efferent edge, referring to water and blood flows, respectively. The trailing or afferent edge of the filament is very flat and typically contains MRCs. SEM morphometric studies of the MRC typically make use of this area because it is flat (see Fig. 10c). Scale bar=100 μm (from J.M. Wilson, unpublished). Abbreviations: af., afferent; ef., efferent; L, Lamellae.

The filaments are dorsoventrally flattened, and their surface area is greatly increased by secondary folding to form lamellae (Fig. 2). The lamellae have a thin plate-like appearance with a vascular core covered by a thin epithelium on either side (Fig. 3c). The lamellar blood space is lined with pillar cells. The gill filament is considered the basic functional unit or subdivision of the gill. Its epithelium contains ionoregulatory cells while it also supports the lamellae, which are the basic respiratory unit.

The lamellae of lampreys and higher fishes are produced by cross-folding (perpendicular to long axis of filament) of the upper and lower sides of the filament epithelium (Fig. 2). The lamellae are in evenly spaced rows along the length of the filament (Fig. 2, 3f,g), although in the elasmobranchs they are at an angle slightly oblique to the long axis of the filament (efferent edge closer to the pharynx than the afferent edge). The outer margins of the lamellae from adjacent filaments come into close proximity to each other completing

[1]In all fishes, the flow of water across the gill is counter-current to the flow of blood. When referring to the flow of blood, the terms "afferent" and "efferent" ("to" and "from" the lamellae) are used. The terms "leading" and "trailing" edge are used in relation to the direction of water flow across the filament and lamellae.

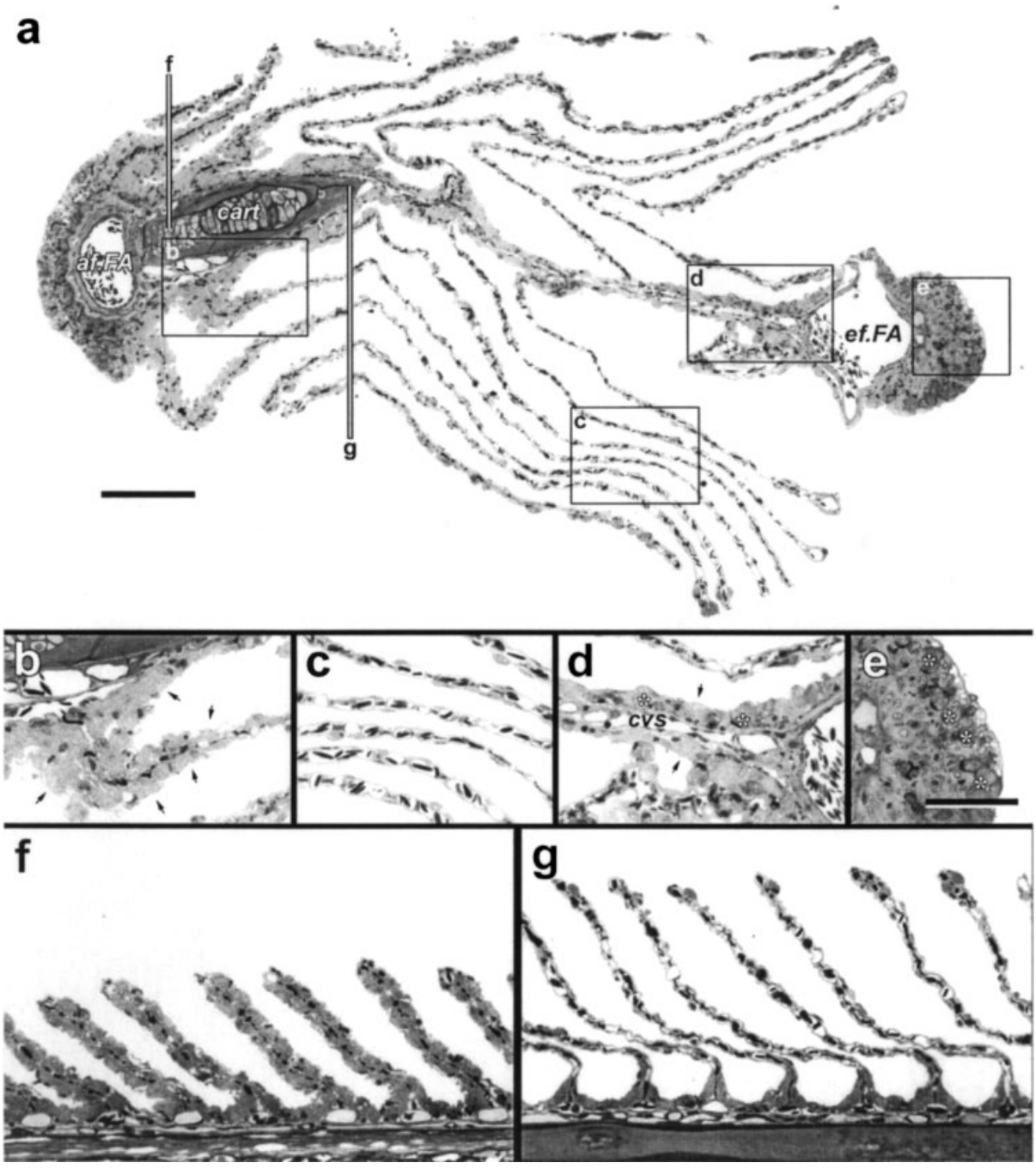

Fig. 3. (**a**) Cross-section through a filament of the teleost parrotfish (*Scarus*) to illustrate the different regions of the filament epithelium and associated vasculature. (**a**) Lower magnification view to give orientation of the higher magnification insets of (**b**) interlamellar space of the filament epithelium near the afferent edge, (**c**) the lamellar epithelium, (**d**) the interlamellar region of the efferent edge of the filament epithelium, (**e**) the efferent or leading edge of the filament and (**f,g**) cross-sections through the lamellae. MRCs are visible as large granular cells (indicated by arrows) (**b,d,f,g**) while mucous cells are indicated by asterisks and are most common on the extreme edges of the filament (**d,e**). In the extreme afferent edge of the lamellae, MRCs are found all the way to the marginal channel. This is not a typical condition. The typical thin epithelium of the lamellae is seen in (**c,g**). The afferent (afFA) and efferent (efFA) filament arteries are seen at the extreme ends of the filament (**a**). The central venous sinus (cvs) is evident within the filament beneath the bases of the lamellae (**d**). Scale bars: (**a,f,g**) 100 μm, (**b–e**) 50 μm (J.M. Wilson, unpublished). Abbreviations: af.FA, afferent filament artery; cart, cartilage; cvs, central venous sinus; ef.FA, efferent filament artery.

a sieve-like arrangement for the respiratory water to pass through. There is a space left between the trailing edge of the lamellae and the lateral wall of the interbranchial septum so the water that has passed between the lamellae can flow on out behind the rows of lamellae. This water channel or canal facilitates the flow of water counter to the lamellar blood flow.

The analogous structure to the gill filament in the hagfish is the primary gill fold. The primary gill folds are arranged radially around the central axis of the pouch like the lines of longitude on a globe (Fig. 1e). Some filaments extend to the centre of the pouch while others have a low profile. The filament can be subdivided into afferent, respiratory, and efferent regions (Fig. 4). In the respiratory region, lamellae are present as subdivisions and branchings of the filament through third to seventh order folding. The lamellae in hagfish are orientated in the same plane as the filament, which is unlike the arrangement in lamprey and other fishes in which the lamellae are perpendicular to the long axis of the filament. Water and blood flows are counter-current.

Hughes ('84) has reviewed the relationship between morphometric parameters of the gill (surface area, diffusion distances) in relation to habit. A comparison of fast-swimming pelagic (e.g., tuna *Thunnus albacares*), sluggish benthic (e.g., toadfish *Opsanus tua*), and air-breathing (e.g., tree-climbing perch *Anabas testudineus*) fishes illustrates the striking differences in filament and lamellae size, shape, number, and spacing that reflect the respiratory needs of the animals (total gill area relative to body mass$=a \cdot W^b$; W=mass in g). Fast-swimming tunas have a larger gas-exchange surface ($3{,}151 \cdot W^{0.875}$), while the sluggish toadfish ($560.7 \cdot W^{0.790}$) and air-breathing climbing perch ($556 \cdot W^{0.615}$) fishes have reduced gas-exchange surfaces. The thickness of the blood to water distance across the lamella is also much smaller in the tuna (0.533 μm) compared to the perch (10 μm) (cf. Hughes '84). Air-breathing fishes generally make use of alternate gas-exchange organs (modified opercular chamber and swim bladders) for gas exchange. The morphometrics of the gills of air-breathing fishes has been reviewed recently by Graham ('97) and Roy and Munshi ('96).

In the elasmobranchs and agnathans the branchial slits open individually to the outside, although there are some exceptions. Outer folds of skin create the vertical slit like opening to the outside in the elasmobranchs and the branchiopores of the agnathans (Fig. 1a,b,c). These openings are generally lateral, although in the skates and rays they are ventral. The teleosts are characterized by the universal presence of a bony operculum. The operculum develops from a fold in the hyoid arch and is a hard structure composed of dermal bone, with a crescent-shaped caudal opening (Fig. 1d). The ventral edges are connected by an expandable membrane beneath the pharynx, and the space enclosed is the opercular cavity or space. The presence of the operculum likely led to the loss of the first hemibranch in the teleosts

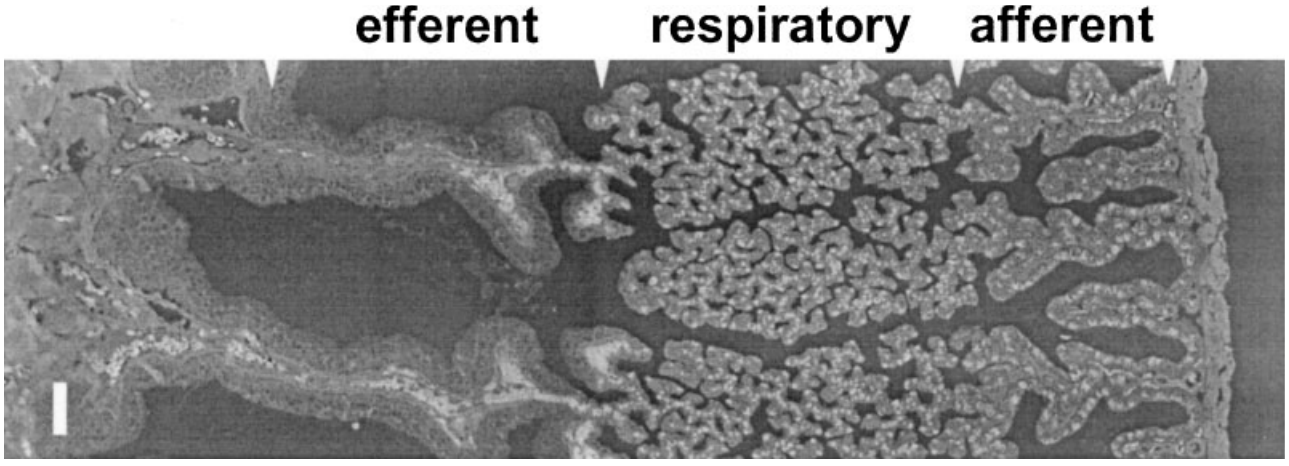

Fig. 4. Section through the gill pouch of the Pacific hagfish (*Eptatretus stouti*) to illustrate the different subdivision of the primary fold: afferent, respiratory, and efferent regions. This section is actually immunolabeled for Na^+,K^+-ATPase similar to Choe et al. ('99). However, in this case due to section pretreatment the erythrocytes as well as the MRCs produce a detectable signal. Scale bars=100 μm (JM Wilson, unpublished).

since water delivery to this area is poor (Romer and Parsons, '86). The chimeras, which are also members of the chondrichthyes, differ markedly from the elasmobranchs in the gross morphology of their gills and associated structures. The numerous gill slits are replaced by a single opening covered by a large fleshy operculum that extends caudally. The interbranchial septa are also shorter, the last branchial chamber is closed, and the spiracles are lost. In these respects, they more closely resemble the teleosts.

In the hagfish, water enters the individual pouches from the pharynx medially via internal branchial ducts and passes out laterally via an external branchial duct (Fig. 1a,e). In the *Bdellostoma* hagslime, the external branchial ducts lead directly to the outside via individual branchiopores, while in the *Myxine* hagfish the ducts extend caudally to form a common external opening. At the end of the series of gill pouches on the left side only is the pharyngocutaneous duct that connects the pharynx to the outside. This duct serves as an entry point for respiratory water when the hagfish has its head buried into its prey (dead fish). Hagfish also have a nasal duct dorsal to the mouth that opens into the pharynx and serves as an important entry point for respiratory water when the body of the hagfish is buried in the mud.

The water flow through the gills in lampreys is modified in order to be compatible with their specialized suctorial feeding mechanism (Randall, '72). Lampreys are parasitic, and when their disc-shaped mouths are attached to a host fish or to the substrate, the water flow via this route to the gills is blocked. The lampreys make use of their external branchiopores for both water inhalation and exhalation (tidal breathing) (Fig. 1f). They have also been observed to draw water in through their first few anterior pouches, which is then exhaled through their posterior pouches (Nekvasil and Olson, unpublished observations). The lattice-like cartilaginous skeleton supporting the pouches and a well-developed musculature produce the pumping action to circulate the water in the gill pouches. The respiratory and feeding mechanisms are separated internally by a modification of the pharynx. The pharynx in the adult lamprey is split into two ducts: one (dorsal) leading to the esophagus and a ventral blind-ended respiratory tube (branchial duct) that adjoins the internal gill ducts. The flow of water through the entrance of the respiratory tube is controlled by a value, the velum (Fig. 1b). During feeding, the velum is closed and the body juices sucked from the host pass directly from the mouth to the digestive tract without affecting respiration and vice versa. This tidal mechanism also appears to be the preferred route of water circulation in free-swimming lampreys and differs from the unidirectional flow through arrangement seen in all other fishes (with the exception of the sturgeon; Burggren and Randall, '78).

EPITHELIA

The epithelia covering the filament and lamellae can be differentiated, obviously by location, but also by thickness, blood circulation (arterioarterial vs. arteriovenous, see Olson, 2002, this issue), and cell type composition (Fig. 3). In the hagfish, the thickness of the epithelium decreases gradually as the divisions of the surface are more complicated (up to sixth-order folding) compared to the lamellae of all other fishes, which represent only first-order folding of the filament (Fig. 4).

In most other fishes, the epithelium covering the filament includes both the afferent and efferent edges as well as the spaces between the bases of the lamellae, which is commonly referred to as the filament interlamellar space (ILS) (Fig. 3f,g). Within the filament, and bordering much of the filament epithelium, is the large central venous sinus (CVS) which is part of the arteriovenous circulation (Olson, 2002, this issue). The filamental epithelium is thicker than the lamellar epithelium, typically being composed of three or more cell layers. The majority of the surface is covered by cuboidal and squamous pavement cells, while basal undifferentiated cells contact the basal lamina and intermediate undifferentiated cells fill the intervening space. Notably, also within the filament epithelium are numerous, large mitochondria-rich cells and mucous cells (see below).

In contrast, the lamellar epithelium overlays the arterioarterial circulation (Olson, 2002, this issue). The epithelium is typically one to three cell layers and composed of squamous pavement cells and basal and intermediate non-differentiated cells. Modified endothelial cells, called pillar cells, support and define the lamellar blood spaces. The epithelium sits on a typical basal lamina composed of an electron-dense and a thinner electron lucent layer. The thickness of the epithelium and basal lamina is often less in fishes trying to minimize their blood to water diffusion distances in order to optimize gas exchange (e.g., active pelagic teleost

T. albacares, 0.55 μm, versus sedentary benthic teleost *Solea variegata*, 2.80 μm, mean total blood–water distance; see Hughes, '84). Mucous cells and MRCs are not as commonly encountered in the lamellar epithelium, although there are exceptional cases.

CELL TYPES

Pillar cells (PC)

The pillar cell is a type of modified endothelial cell, which defines the blood spaces within the lamellae and is a cell type unique to the fish gill. They give the lamellae the appearance of a string of beads when viewed in cross section (Fig. 3c,f,g). The pillar cell body spans the lamellar blood space and has a centrally located polymorphic nucleus (Fig. 5). The ends of the pillar cell flare out at the point of contact with the interstitial connective tissue adjacent the basal lamina of the lamellar epithelia to form thin flanges that extend to neighboring pillar cells. The flanges of neighboring pillar cells may form simple abutments or complex interdigitations. In the hagfish and lamprey, communicating gap junctions have been found between neighboring pillar cells [Bartels and Decker ('85) and Bartels and Potter ('93), respectively]. The flanges are kept thin (0.02–1 μm; Hughes '84) to minimize the blood to water diffusion distances and are generally devoid of organelles except for microfilaments. Associated with the pillar cell body are columns of collagen bundles that entwine with collagen fibrils in the interstitial connective tissue on the vascular side of the basal lamina. These collagen bundles remain extracellular but are enveloped by the pillar cell and sealed in by tight junctions (Newstead, '67). The hagfish is an exception, since the collagen bundles are not deeply enveloped by the pillar cell body and tight junctions are absent. Instead, the collagen bundles are surrounded by groups of pillar cells (Mallatt and Paulsen, '86). It is important to isolate the collagen from the blood space because it will cause blood clotting. These collagen bundles presumably function to prevent distension and collapse of the blood space. The pillar cells also have bundles of microfilaments in the peripheral cytoplasm that run parallel to the collagen bundles and have been found to be contractile although nervous control is not present (Laurent, '84). Also crowded within the pillar cell body are mitochondria, free ribosomes and various membrane bound organelles, although a Golgi apparatus, rough and smooth endoplasmic reticulum (RER and SER, respectively) are not common. Unlike true endothelial cells, pillar cells lack dense core Weibel-Palade bodies (Newstead, '67). True endothelial cells do, however, line the outer marginal channels of the lamellae (Newstead, '67; Hughes and Weibel, '72).

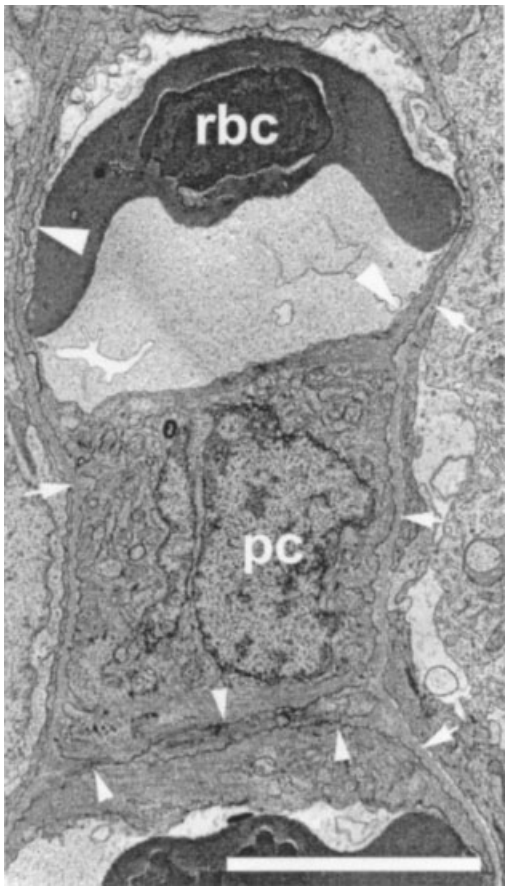

Fig. 5. Transmission electron micrograph (TEM) of a cross-section through the lamellae pillar cell of the rainbow trout (*O. mykiss*). Note the thin flanges (large arrowhead) and centrally located polymorphic nucleus of the pillar cell (pc). Smaller arrows indicate the basal lamina and the smaller arrowheads the extracellular collagen bundles spanning the blood space while embedded in the pillar cell. Electron-dense red blood cells (rbc) are seen within the blood spaces. Scale bar=5 μm (J.M. Wilson, unpublished).

Pavement cells (PVC)

The most abundant cell type covering the epithelium (>90% of the surface area) is the squamous to cuboid-shaped cell commonly referred to as the pavement or respiratory cell. The apical surface of PVCs is usually large and polygonal and may have microridges (referred to

microplicae when viewed in cross-section) or microvilli (finger-like projections) (Laurent, '84; Crespo, '82; Olson, '96; Wilson et al., 2002) (Figs. 6, 7, 8a, 10b, c, and 11). When microridges are present, they typically form concentric rings. A double ridge marks the boarder of neighboring PVCs (Fig. 10c). The types of apical features present show variation between species and also within and between the different gill epithelia. Pavement cells typically have low mitochondrial densities and unelaborated basolateral membranes. The nucleus in squamous pavement cells is compressed while in cuboidal pavement cells it is rounded. Pavement cells have the typical intracellular organelles, rough and smooth endoplasmic reticulum, Golgi apparatus, lysosomes, and vesicles with contents of various electron densities. In the non-teleost fishes, pavement cells display abundant mucous secretory granules in the apical cytoplasm (Laurent, '84; Bartels, '85; Mallatt and Paulsen, '86) (Fig. 6,11). These vesicles fuse with the plasma membrane and expel their contents. Pavement cells are joined by desmosomes, and the peripheral cytoplasm contains microfilaments. The tight junctions associated with pavement cells have multiple strands and are not characterized as leaky (Sardet et al., '79; Kawahara et al., '82; Bartels, '88). In the hagfish and lamprey, communicating gap junctions have been found between neighboring pavement cells, and pavement cells and undifferentiated cells, however, not with MRCs (Bartels '88; Bartels and Potter '93, respectively). The presence of communicating gap junctions is significant because they allow the electrochemical coupling of the cells of the epithelium which is important in coordinating cell growth, differentiation, and function. No studies have been undertaken in elasmobranchs to specifically determine the presence of gap junctions.

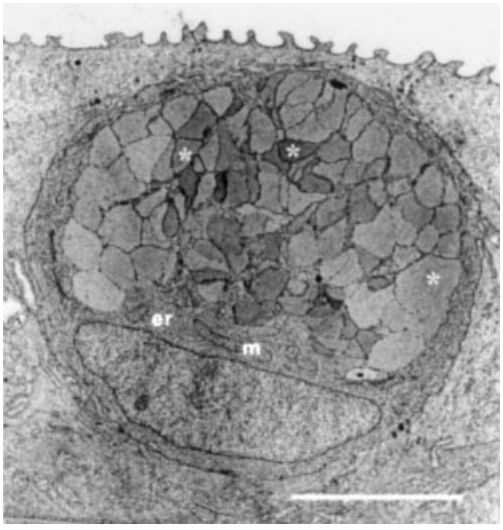

Fig. 7. TEM of a mucocyte of the freshwater chum salmon larvae (*Oncorhynchus keta*). Note the flattened basally located nucleus, peripheral cytoplasm with abundant endoplasmic reticulum (er), and some mitochondria (m), and the large mucin granules dominating the space of the cell (asterisks). Scale bar=5 μm (J.M. Wilson and E.P. Groot, unpublished).

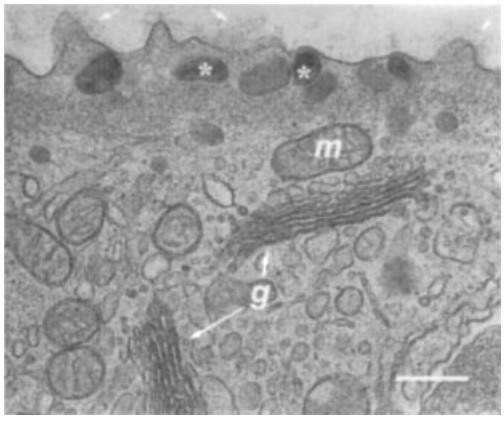

Fig. 6. TEM of the apical region of a pavement cell from the dogfish *Squalus acanthias*). Evident are the apical secretory granules (asterisks) and microfilament network, as well as mitochondria (m), Golgi apparatus (g), and vesicles of various shapes and sizes. Glycocalyx is also recognizable attached to the exterior surface. Scale bar=0.5 μm (J.M. Wilson, unpublished).

In teleost fishes there is conflicting evidence for the presence of PVC gap junctions in the branchial epithelium. In freeze-fracture electron microscopy studies on five euryhaline species (*Anguilla anguilla*, *Oncorhynchus mykiss*, *Fundulus heteroclitus*, *Lebistes reticulatus*, and *Mugil capito*), Sardet and co-workers (Sardet, '77; Sardet et al., '79) were unable find evidence for the presence of gap junctions; however, Kawahara and co-workers ('82) using the same approach detected gap junctions between gill PVCs of *Oplegnethus fasciatus*. Kawahara et al. ('82) were unable to find gap junctions between PVCs and MRCs. In a recent study by Sandbacka et al. ('98), communicating junctions between trout (*O. mykiss*) gill pavement cells in primary culture were demonstrated using a dye-coupling technique. They also suggested that the clustered intramembranous particles of the P-face shown in Fig. 3 of Sardet et al. ('79) are gap junctions although they are not recognized as such. Unfortunately, this figure

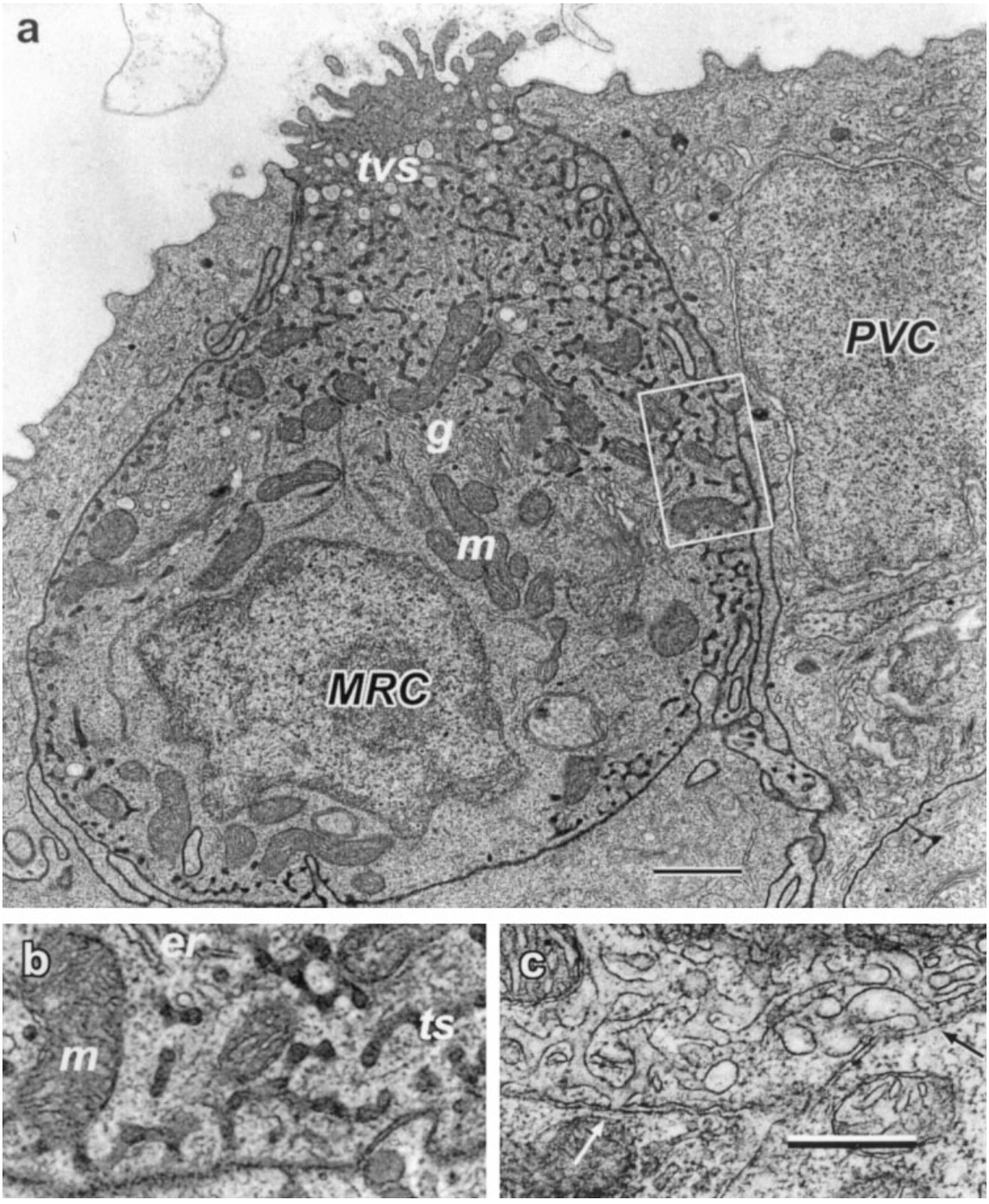

Fig. 8. (**a,b**) TEMs of the MRC of the freshwater chum salmon larvae (*O. keta*) using the potassium ferrocyanide-reduced osmium stain described by Pisam et al. ('87). Note the strong staining of the tubular system (ts) and the plasma membrane while other membrane systems remain weakly stained. (**c**) TEM showing the connections of the tubular system with the basolateral membrane (indicated by arrows) in the neon tetra (*Paracheirodon innesi*) MRC using conventional heavy metal staining. Scale bars: (**a**) 1 μm; (**b,c**) 0.5 μm [(**a,b**) J.M. Wilson and E.P. Groot, unpublished; (**c**) J.M. Wilson, unpublished]. Abbreviations: MRC, mitochondria-rich cell; PVC, pavement cell; tvs, tubulovesicular system.

shows the fracture face of the apical membranes of two neighboring pavement cells and not the lateral membranes as would be necessary to make such a claim. It is possible that gap junctions in primary cultures are an artefact of being under culture conditions as in situ attempts to demonstrate dye-coupling were unsuccessful due to technique reasons (mucus layer). Thus, further work seems justified to clarify this important question.

PVCs have been shown to be involved in the covering and uncovering of MRCs under certain conditions (Goss et al., '94; Bartels et al., '96; Daborn et al., 2001), but the role of the PVC in the process is somewhat secondary to that of the MRC. Generally PVCs themselves have been shown to be morphologically unresponsive to changes in environmental conditions (Laurent, '84). Part of the problem stems from the fact that the MRCs are generally the focus of attention in morphological studies and the changes in PVCs may be overlooked. It also might be difficult to recognize these changes if they only occur in a subpopulation of PVCs since there are proportionally many more PVCs. With that said there have been two studies that have shown that PVCs increase apical surface area (densities of microvilli and microplicae) in response to hypercapnia and alkaline exposure [Goss et al. ('94) and Laurent et al. (2000), respectively]. Laurent et al. ('94) have also found studded subapical vesicles that resemble the proton pump containing vesicles from other animals (Brown et al., '87). These morphological characteristics of PVCs help to support the hypothesis that they are the sites of proton pump-driven sodium uptake (Perry, '97; Wilson et al., 2000a). Perhaps more attention focused on these cells will reveal more adaptational changes of this somewhat underrated cell type.

Nondifferentiated cells (basal and intermediate)

The basal and intermediate layers of the epithelium contain cells that are characterized by a high nucleus to cytoplasm ratio. These cells appear undifferentiated and serve as the progenitors for the other terminally differentiated epithelial cell types (PVCs and MRCs). In teleost fishes, at least, mitosis of undifferentiated cells is more common in the filament than lamellar epithelium (Chretien and Pisam, '86; Laurent et al., '94). The non-differentiated cells share some common features with the PVCs (RER, lysosomes, vesicles, and vacuoles). However, relative to PVCs, non-differentiated cells have more abundant free ribosomes. In the lamellar epithelium, the nucleus of the basal undifferentiated cells often sits on the basal lamina above the pillar cell body. In epithelia that are thicker than two cell layers (filament epithelium), intermediate non-differentiated cells are found. Desmosomes are found between non-differentiated cells and pavement cells. Hemidesmosomes connect the basal undifferentiated cells to the basal lamina anchoring the epithelium. Caveolae, non-clathrin-coated plasma membrane invaginations, can also be found along this cell boarder as well.

Mucous cells (goblet cells)

Goblet type mucous cells are large ovoid cells that are composed mostly of large apical mucous secretory granules (Figs. 3 and 7). The nucleus and cytoplasm are usually flattened and in a basal position. Within the cytoplasm is the cellular machinery for producing the mucin (ER, Golgi, mitochondria). The mucin granules are generally electron lucent or only moderately electron dense with some variability between granules within the same cell. The mucous cells are commonly found in the filament epithelium in the following frequency: efferent edges > afferent edge > interlamellar space > base of lamellae outer margin of lamellae. However, their distribution and numbers do show variability from this general plan. There tends to be a reduction in the number of mucous cells in seawater versus freshwater fish (Laurent and Hebibi, '89). Goblet cells have been found in all fishes with the exception of the hagfishes (Mallatt and Paulsen, '86).

Mucous cells have also been characterized by the types of mucin in their granules. The periodic acid-Schiff (PAS) method is used to detect neutral mucins, and the variations of the alcian blue method to detect acidic mucins (pH 2.5, carboxylated and sulfated mucins; pH 0.5, sulfated mucins, and Quintarelli's acid hydrolysis sialomucins) (e.g., Sabóic-Moraes et al., '96; Watrin and Mayer-Gostan, '96). Rojo et al. ('96) have also used lectin histochemistry to describe different types of mucous cells in the brown trout (*Salmo trutta*). The different lectins bind specifically to the defined carbohydrate moieties present in the mucin granules.

Neuroepithelial cells (NEC)

Neuroepithelial cells are found deep within the filament epithelium along the full length of the efferent edge although concentrated near filament tips (Dunel-Erb et al., '94). Neuroepithelial cells have been described in elasmobranch and teleost fishes. The fish neuroepithelial cells share common characteristics with the neuroepithelial cells described within the wall of the respiratory tract of mammals (Laurent, '84). These cells can be readily identified by detecting the biogenic amine fluorescence resulting from formaldehyde treatment (Falck et al., '62), which has been confirmed to include 5-HT by immunohistochemistry (Baily et al., '92). A defining feature of these cells is the presence of dense-cored vesicles (80–100 nm) and their location directly adjacent the basal lamina. The most significant characteristic of this cell is its innervation. Within the filament is a dense subepithelial nervous network composed of mostly unmyelinated nerve fibers. Processes of unmyelinated nerve fibers cross the basal lamina, and multiple contacts are made with the same NEC. These cells are believed to function as oxygen sensors and to be involved in the regulation of blood flow (see Sudin and Nilsson, 2002, this issue).

Mitochondria-rich cells (MRCs) or chloride cells (CCs)

The descriptive morphological study of the gill is dominated by the mitochondria-rich chloride cell and its subtypes (Pisam and Rambourg, '91; Jürss and Bastrop, '95; Perry, '97; Evans et al., '99). MRCs tend to be concentrated in the afferent region of the filament epithelium and have an intimate association with the arteriovenous circulation, notably the central venous sinus, although in the interlamellar region MRCs are also associated with the basal channels of the lamellar arterioarterial circulation (Laurent, '84; Olson, 2002, this issue). A nice example of the distribution within the tilapia (*Oreochromis mossambicus*) gill filament can be found in the paper by Uchida et al. (2000) in which laser scanning confocal microscopy was used to localize MRCs. The defining feature of this cell type is a high mitochondrial density. MRCs also generally have an amplification of their basolateral membrane either through folding or the presence of a tubular system (Fig. 8), although the lamprey freshwater-type MRCs are an exception. Associated with the basolateral membrane amplification is the important ionoregulatory enzyme Na^+,K^+-ATPase (see Marshall, 2002, this issue). In the apical region of MRCs is a collection of tubules and vesicles (tubulovesicular system) that is distinct from the tubular system (Fig. 9). The apical membrane can be quite variable in appearance ranging from concave to convex, sometimes forming deep crypts. In addition, the surface topography has been shown to be smooth, or having microvilli of varying densities and lengths as well as highly branched, to give a sponge-like appearance. The use of morphological features has led to the classification of different types of MRCs.

The term ''chloride cell'' relates to the function of the MRC in Cl^- elimination. In seawater teleosts, the MRCs have quite convincingly been shown to be sites of active Cl^- elimination and hence the name is fitting (Marshall, 2002, this issue). In the agnathans, elasmobranchs, and freshwater teleosts, the evidence that MRCs are involved in Cl^- fluxes is indirect or lacking all together. So in order to avoid any confusion on the matter, we will only use the term ''chloride cell'' when referring to the seawater teleost MRC unless otherwise noted. However, in general, numerous mitochondria in these cells are thought to supply the ATP for ion-transport proteins to drive the vectorial transport of ions as part of ion and acid–base regulation (see Claiborne, 2002; Marshall, 2002).

Teleost MRCs

The teleost MRC is characterized by its elaborate intracellular system of branching tubules that are continuous with the basolateral membrane (tubular system; Fig. 8). This network of anastomosing tubules is closely associated with the mitochondria and is only excluded from the area of the Golgi apparatus and the band immediately beneath the apical membrane. The elements of the tubular system can be found along side the endoplasmic reticulum making identification somewhat problematic. Extracellular space markers such as horseradish peroxidase and ruthenium red have been used to clearly establish the basolateral continuity of the tubular system and its separation from the ER (Philpott, '80). Pisam and co-workers ('87) have made effective use of the potassium ferrocyanide reduced osmium stain developed by Karnovsky ('71) to unambiguously differentiate the membrane elements of the tubular system from the ER without the need of extracellular markers. The selective heavy

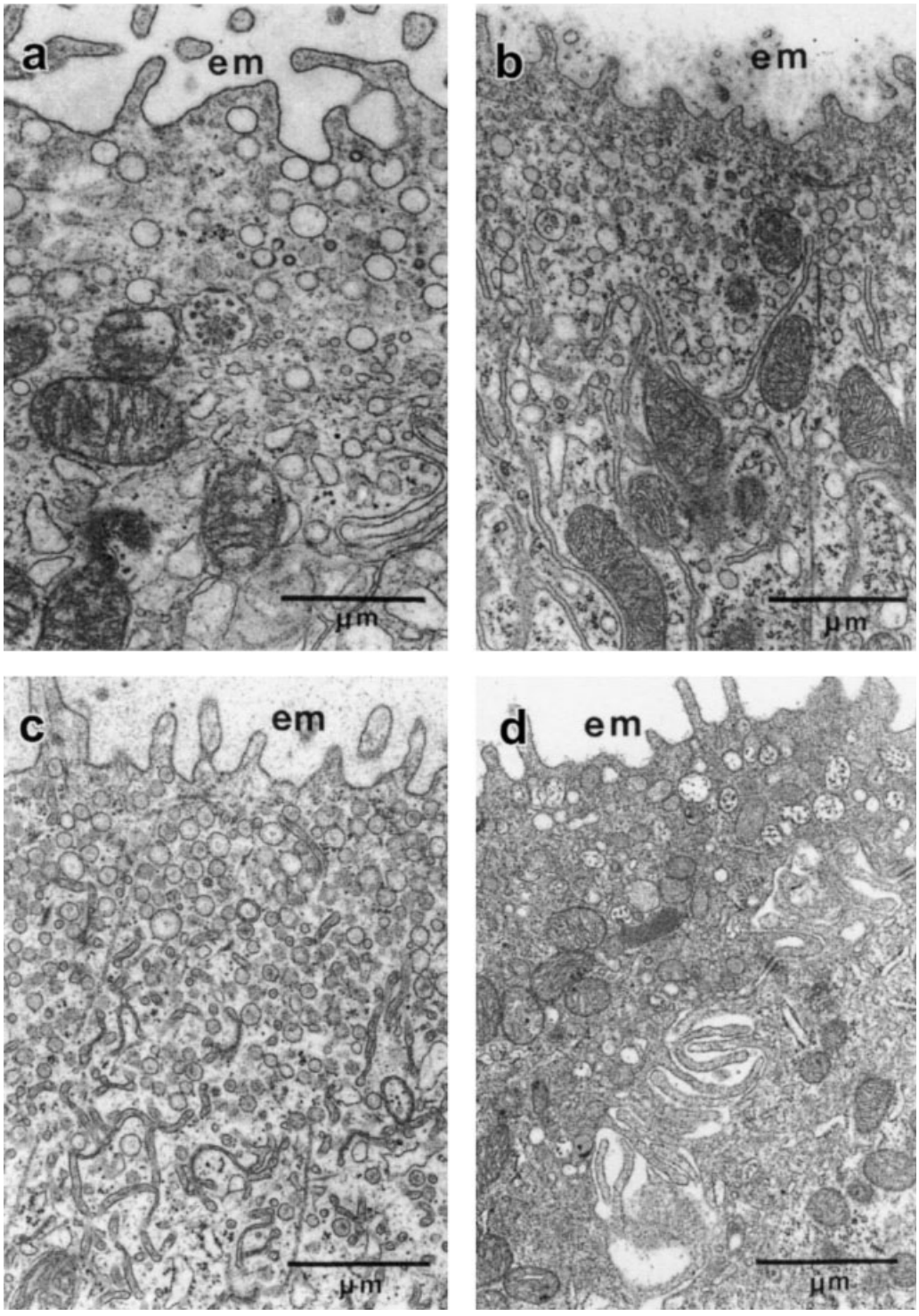

Fig. 9. TEMs of the apical regions of the MRCs of (**a**) *Raja erinacei*, (**b**) *Acipenser beari*, (**c**) *Perca fluviatilis*, and (**d**) *Protopterus annectens* showing the tubulovesicular system. Scale bars=1 µm (P. Laurent, unpublished). Abbreviation: em, external milieu.

staining of the tubular system contrasted markedly with the poorly stained endoplasmic reticulum (Pisam, '81) (Fig. 8a,b). Environment ionic conditions have a marked effect on the morphology of the teleost MRCs, which of course relates to the role of these cells in active ion transport

(Perry, '97; Evans et al., '99). In sea water, the gill is involved in active ion elimination while in freshwater active ion-uptake takes place.

The mitochondria-rich chloride cell (CC) and accessory cell (AC) are two types of MRC that are universally expressed in seawater teleosts (Fig. 10). These cells form multicellular complexes in the filament epithelium. The larger chloride cell may be elongate, ovoid, or cuboidal in shape, depending upon the species. The tubular and the tubulovesicular systems are highly developed, and the cell is packed with mitochondria. High packing of Na^+,K^+-ATPase is associated with the CC tubular system (Sardet et al., '79; Marshall, 2002, this issue). The apical membrane of a CC is recessed from the surface of neighboring PVCs and may be further deepened through invagination (Fig. 10c). The smaller accessory cell is superficially located, semi-lunar or pear shaped, and generally has a less extensive tubular system and a poorly developed tubulovesicular system. Notably, they have low levels of Na^+,K^+-ATPase unlike the CCs (Hootman and Philpott, '80). The AC sends cytoplasmic processes into the larger CC, which emerge at the apical surface to form a complex mosaic. Notably, the tight junctions found between CC and AC contain fewer strands than do those formed with PVCs (Sardet et al., '79; Kawakara et al., '82). Therefore the AC interdigitations within the CC apical membrane would greatly increase the linear distance of leaky tight junction, which is functionally important for the paracellular sodium efflux (see Marshall, 2002, this issue). There is also ample evidence that Cl^- efflux occurs transcellularly through the CC (Marshall, 2002, this issue).

In freshwater teleost fishes there is less consensus about clear MRC subtypes that might relate to the instability and variability of the freshwater environment itself. Pisam and coworkers, however, have been able to describe α and β sub-types[2] of MRCs with electron-lucent (light or pale) and electron-dense (dark) cytoplasms, respectively, in a number of species, including the guppy (*L.reticulatus*), loach (*Cobitis taenia*), grudgeon (*Gobio gobio*), Atlantic salmon (*Salmo salar*), and Nile tilapia (*Oreochromis niloticus*) (Pisam et al., '87, '90, '95). In addition to a pale cytoplasm, α cells were found at the edges of the interlamellar space (ILS) in contact with the basal lamina opposite the arterioarterial circulation. The β cells, on the other hand, were generally observed in the ILS in contact with the basal lamina associated with the arteriovenous circulation. Also a manganese-lead (Mn-Pb) stain was found to selectively stain the contents of a population of variably sized membrane bound bodies in the apical cytoplasm of only the β-cell type. The material in the apical vesicles of the β-cell has been shown to include carbohydrate material (Pisam et al., '95) and recently Tsai and Hwang ('98) have found binding of the lectin WGA (wheat germ agglutinin: sialic acid and *N*-acetylglucosaminyl residues) to the apical vesicles of a subpopulation of MRCs in tilapia (presumably β-cells). In a complementary study by Shikano and Fujio ('98) on the guppy (*L. reticulatus*), it was shown that the α-cell has high levels of Na^+,K^+-ATPase immunoreactivity while the β-cell has only weak labeling. In a study on rainbow trout (*O. mykiss*), and an earlier study on Atlantic salmon, differentiation of these two subtypes was not made (Pisam et al., '88, '89).

Transfer to sea water of euryhaline freshwater fishes, results in the degeneration of the β-cell through apoptosis and hypertrophy of the α-cell (Pisam et al., '87; Wendellar Bonga and van der Meij, '89). The α-cell is transformed into what is recognized as the seawater chloride cell (CC). The transformation consisted of an increase in the size of the α-cell, density of the cytoplasm, number of mitochondria, the tubular system network with a tighter polygonal mesh size, and the apical tubulovesicular system. A mitochondria-poor cell type has been described by van der Heijden et al. ('99) that possesses a well-developed tubular system but has relatively few mitochondria. This cell increases in frequency following seawater acclimation and infrequently contacts the water. No function was suggested for this cell type.

Pisam et al. ('88, '89, '90) aided by their specific heavy metal stains have also characterized the accessory cell type in some freshwater euryhaline species. These cells are associated with the α-cell type in tilapia and Atlantic salmon but β-cell type in brown trout (*S. trutta*) and are similar to the AC described in the seawater fish gill, but they are less common and do not send cytoplasmic processes into the apical cytoplasm of the CC. Upon transfer to sea water or in anticipation of transfer (smolting), their numbers increase and interdigitations are present. The AC are presumably present in fishes living in fresh water in order to facilitate the rapid switch to NaCl elimination. In the tilapia

[2]It should be noted that the α and β cells described by Pisam and coworkers are not meant to be analogous to the α and β cells of the turtle bladder (e.g., Steinmetz and Andersen, '82).

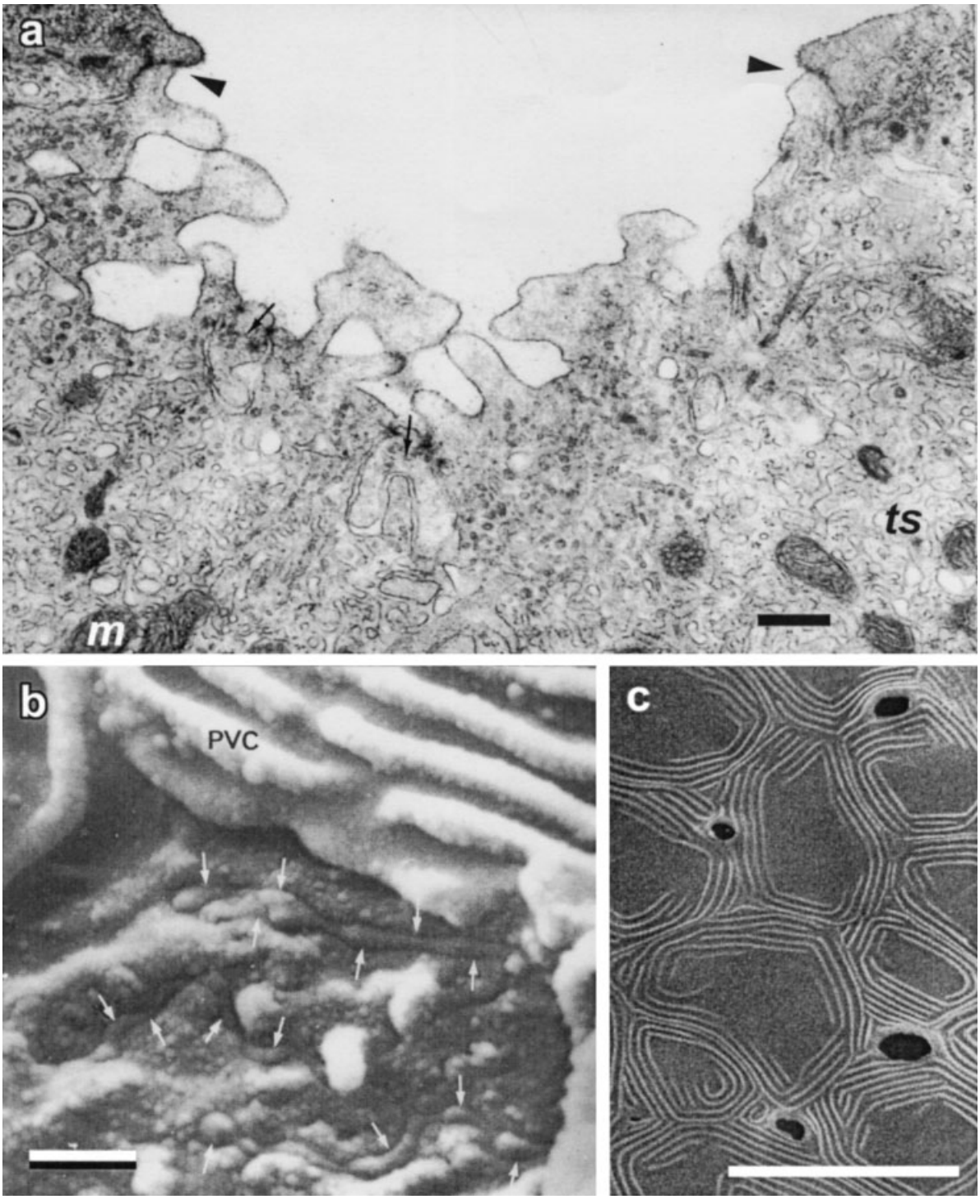

Fig. 10. Transmission (TEM) and scanning electron (SEM) micrographs of the apical crypt of the seawater mitochondria-rich chloride cell (CC) and accessory cell (AC). (**a**) TEM showing CC and AC sharing an apical crypt. Arrows indicate AC inclusions into the CC. Compare the shallow tight junctions found between the AC and CC with those of the pavement cells PVCs (arrowheads). (**b**) High-magnification SEM of the apical crypt showing AC inclusions into the CC (arrows). (**c**) Lower-magnification SEM showing deep-hole appearance of MRCs and the large neighboring PVCs with their microridged surface arranged concentrically [(**a**) *Morone labrax*, P. Laurent and S. Dunel-Erb, unpublished; (**b**) *Solea solea*, modified from Laurent ('84) with permission from Academic Press; (**c**) *O. mossambicus*, J.M. Wilson, unpublished]. Scale bars: (**a,b**) 0.5 μm, (**c**) 10 μm.

(*Alcolapia grahami*) of extremely alkaline (pH 10) Lake Magadi in Kenya, Laurent et al. ('95) have found active seawater ACs associated with larger CC type MRCs. The lake water has very high HCO_3^- and CO_3^{2-} concentrations of 40 and 265 $mEq \cdot L^{-1}$, respectively, with Na^+ and Cl^- at 342 and 108 $mEq \cdot L^{-1}$, respectively. In the proposed model, the CC–AC complex facilitates paracellular Na^+ efflux via the leaky tight junction while HCO_3^- is pumped out by the CC.

In freshwater tilapia (*O. mossambicus*), Lee and co-workers ('96) have defined three MRC types according to the apical membrane appearance by SEM and categorized them as either wavy-convex, shallow-basin, or deep-hole. The ''wavy-convex'' type has a convex apically exposed area with variable ornamentation with microvilli and a relatively large two-dimensional area. The surface of the ''shallow-basin'' type MRCs is flat but recessed below neighboring PVCs. The density of the ''wavy-convex'' type MRCs increases in low NaCl and the ''shallow-basin'' type MRCs increase in low Ca^{2+} water, suggesting roles in Na^+Cl^- and Ca^{2+} uptake, respectively (Chang et al., 2001). The ''deep-hole'' MRCs are also recessed between PVCs like the ''shallow-basin'' type, but they form a deeper apical crypt characteristic of seawater CCs (Lee et al., 2000). In the fathead minnow (*Pimephales promelas*), the ''deep-hole'' MRCs appear in response to exposure to acid conditions, suggesting a role in acid excretion (Leino and McCormick, '84). However, in other species, like the rainbow trout, this system of classification is not applicable, and it remains to be determined if the subtypes of MRCs can be differentiated on apical appearance alone (Galvez et al., 2002).

Although MRCs tend not to be as frequently found in the lamellae as in the filament epithelium in freshwater and especially seawater fishes, acclimation of trout to ion-poor (< 0.01 mM $[Na^+]$) conditions, elicits a massive proliferation of MRCs in the lamellar epithelium (Laurent et al., '85; Perry and Laurent, '89; Greco et al., '96). The number and area of exposed surface of these cells both increase dramatically. The effects of ion-poor water on MRC proliferation can also be reproduced using hormone treatments with cortisol and growth hormone (Laurent and Perry, '90; Bindon et al., '94). Since MRC are generally quite large, proliferation on the lamellae results in the thickening of the epithelium, and, consequently, the blood to water diffusion distance is increased (reviewed by Perry, '98). This has negative consequences for gas exchange as blood PO_2 and hypoxia tolerance are reduced in fish with lamellar MRC proliferation. It should be noted, however, that ion-poor conditions are not always associated with the such massive numbers of lamellar MRC as is evident in the neon tetra (*Paracheirodon innesi*), which is native to the ion poor waters of the Amazon (Wilson et al., '96).

The estuarine, air-breathing mudskipper *Periophthalmodon schlosseri* has quite exceptional gill lamellae because the basal layer is composed almost exclusively of MRCs with a superficial layer of very electron-dense pavement cells (Wilson et al., '99). The MRCs communicate to the outside via very deep apical crypts. Very few ACs could be found and MRC were isolated from each other by intervening filament-rich cells. The lamellae also fuse together at various points, and the interlamellar space is reduced to a narrow irregular channel that can no longer accommodate a ventilatory flow of water. It has been hypothesized that the numerous lamellar MRCs are involved in the active NH_4^+ elimination observed in these animals (Randall et al., '99; Wilson et al., 2000b). The apical marginal channels are still quite thick, and the functional gas exchange surface of these animals is found on the inside of the operculum where intraepithelial capillaries are found. Curiously, these intraepithelial capillaries can also be found on the edges of the filament making the gills of this fish quite atypical.

Elasmobranch MRCs

The elasmobranch MRCs are generally found singly in the filament epithelium and are cuboidal or ovoid in shape with a basally located nucleus (Laurent '84; Fig. 11). In addition to their high mitochondrial densities, they have a basolateral membrane amplified through heavy folding into a basal labyrinth and a densely packed sub-apical tubulovesicular system. The tight junctions between MRCs and neighboring PVCs consist of many strands and are not considered leaky. The apical surfaces of elasmobranch MRCs range from being deeply invaginated to convexed and are covered by microvilli of varying densities (Crespo, '82; Wilson et al., 2002).

Although the salt-secreting rectal gland replaces the gill as the dominant salt secretory organ, branchial MRCs are still present in the gills in significant numbers. Also the important ionoregulatory enzyme Na^+,K^+-ATPase is associated with these cells (Piermarini and Evans, 2001; Wilson et al., 2002); however, leaky tight junctions

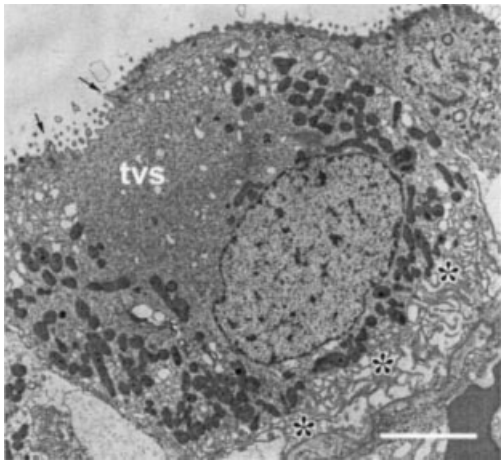

Fig. 11. TEM of the MRC from the dogfish (*S. acanthias*). Note the peripheral location of the mitochondria and the extensive basolateral folding (asterisks), and the dense collection of vesicles and tubules of the subapical tubulovesicular system (tvs). Arrows indicate the deep tight junctions joining the MRC to neighboring pavement cells. Scale bar=5 μm (J.M. Wilson, unpublished data).

are absent, suggesting an alternative function to NaCl elimination. Piermarini and Evans (2001) suggest roles in acid-base regulation (see Claiborne, 2002, this issue).

Agnatha MRCs

The agnathans or jawless fishes represent the most primitive group of fishes and are represented by the osmoconforming marine hagfish (*Myxine*, *Eptatretus*) and hagslime (*Bdellostoma*) and the osmoregulating, anadromous lamprey (*Lampretra*, *Petromyzon*, *Geotria*). These differences in osmoregulatory abilities are reflected in the differences in MRC morphology in the agnathans (Morris and Pickering, '76; Youson and Freeman, '76; Bartels et al., '98). MRCs are found in both freshwater and seawater lampreys as well as in the marine hagfish. The morphological properties of the MRCs differ within these groups. Freshwater lamprey have three different types of MRC (Bartels et al., '98), one of which is associated with the preadaptation to sea water and is the only type found in the seawater-adapted lamprey. The seawater type is commonly referred to as the chloride cell due to its morphological similarity with the teleost chloride cell. In seaward-migrating freshwater lamprey, the seawater-type (SW) MRCs are found in a continuous row along the length of the interlamellar space of the filament epithelium (Bartels et al., '96). The swMRCs are disc shaped and covered over by neighboring PVCs with the apically exposed surface containing microvilli limited to a diameter of 3–4 μm. Following acclimation to sea water, the overlying PVCs retract and the apical surface increases to extend the full width of the interlamellar space. The surface bulges slightly and is relatively smooth with few microvilli. The number of strands in the tight junction between neighboring seawater MRCs or CC also decreases following seawater transfer (Bartels and Potter, '93). The shallow tight junction is an important morphological characteristic that gives a good indication of the cell's function, namely, NaCl elimination.

In the lamprey, the freshwater MRC types are sometimes referred to as intercalated MRCs and one of the noticeable differences to the swMRC is the lack of a tubular system (Bartels et al., '98). One type is only present in the ammocoete and is characterized by electron-dense mitochondria, small secretory mucus granules, short microvilli, and/or microplicae and the presence of globular particles in its apical membrane (as shown by freeze-fracture). It is also found in groups in both the lamellar and filament epithelia. The function of this MRC type is not known, and it disappears following metamorphosis. The second type is generally found singly or in pairs in the interlamellar region and at the base of the lamellae, where it intercalates with neighboring pavement cells and/or other MRC types. It is characterized by an apical membrane with elaborate microplicae, the absence of secretory mucous granules, the presence of numerous small vesicles and membranous tubules, and the presence of rod-shaped particles in either the apical or lateral membranes (as determined by freeze-fracture electron microscopy; Bartels et al., '98). These rod-shaped particles are similar to those described in the mitochondria-rich intercalated cells of the mammalian kidney collecting duct and toad and turtle bladders (Stetson and Steinmetz, '85; Brown et al., '87). The plasma membrane domains having dense rod-shaped particle aggregates also show strong immunoreactivity for the proton pump (vacuolar type H^+-ATPase) (Brown et al., '87). When the proton pumps are in the apical locations, these cells function in acid excretion (A cells), while in cells in which the particles have a basolateral location (B cells), the function is for base excretion. It would thus seem, on the basis of homology with the amphibian and mammalian epithelia, that the hagfish and lamprey also have a proton pump in their MRCs. A direct demonstration

using immunohistochemical techniques has yet to be made in the lamprey.

In the hagfish, MRCs are restricted to the lateral wall of gill pouches and the lateral half of gill folds. All MRCs occur singly, being separated by PVCs, and lack leaky tight junctions (Bartels, '88). The hagfish MRCs have abundant, large mitochondria and a tubular system continuous with the basolateral membrane along with a subapical vesiculotubular system (Bartels, '88). These cells also have perinuclear glycogen and high levels of carbonic anhydrase and Na^+,K^+-ATPase (Conley and Mallatt, '87; Choe et al., '99). Communicating gap junctions are not found between hagfish MRCs and its neighbors (PVCs, non-differentiated cells) (Bartels and Potter, '90). Their apical membrane contains microvilli and is usually convex, and deep apical crypts are not common. Freeze-fracture electron microscopy has shown the presence of rod-shaped particles in the apical membrane (Bartels, '88), which may represent proton pumps as in the lamprey. Since hagfish are osmoconformers and therefore lack active NaCl regulation, the presence of an apical proton pump would suggest the involvement of their MRCs in acid–base regulation (see Claiborne, 2002, this issue).

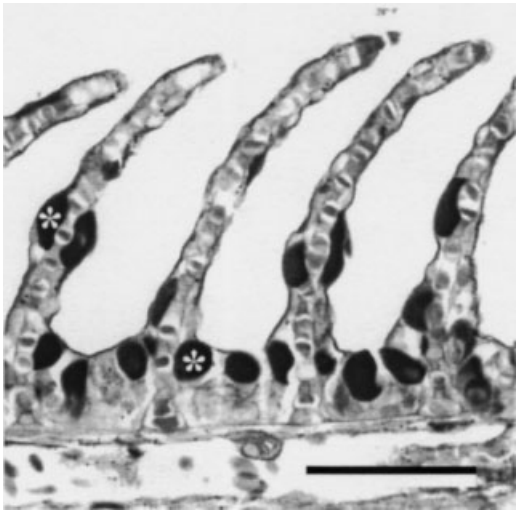

Fig. 12. Localization of MRCs in the gills of a coho salmon (*Oncorhynchus kisutch*) using the Champy-Malliet solution originally used in fish by Garcia-Romeu and Masoni ('70). The technique preferentially stains the cells because of their ample intracellular membrane systems (tubulovesicular and tubular systems; see Figs. 8 and 9). Asterisks indicate some positively labeled cells in the filament and lamellae. Note the similarity in labelling with the Na^+,K^+-ATPase immunohistochemical technique (Fig. 13). Scale bar=50 μm (J.M. Wilson, unpublished data).

DETECTION OF MRCs

In addition to identifying MRC on the basis of their fine structure using electron microscopy, a number of other techniques have been used for localization and quantification of this cell type.

Keys and Willmer's ('32) chloride-secreting cells were identified on the basis of their greater affinity for eosin, ovoid shape, and granular cytoplasm. Since then, some more specific histological stains have been used with success to identify and enumerate MRCs. The acid Fuschin stain was used by Shirai and Utida ('70) to identify MRCs in eel. The use of Champy-Maillet's fixative ($ZnI\text{-}OsO_4$) originally applied to fish MRCs by Garcia-Romeu and Masoni ('70) has been used by a number of other investigators to quantify and localize MRCs (Avella et al., '87; Madsen, '90; Watrin and Mayer-Gostan, '96; Wilson et al., 2002). This stain reacts specifically with membranes, and the reaction with the abundant membrane systems of the MRC results in its selective blackening (Fig. 12).

Some techniques are directed at the detection of the mitochondria. With higher numbers of mitochondria associated with these cells, they give a stronger signal than background. The most common detection methods used are the mitochondrial vital stains DASPMI (dimethylaminostyrylmethylpyridinium iodide, emission wavelength 609 nm) and DASPEI (dimethylaminostyrylethylpyridinium iodide, emission wavelength 530 nm) (e.g., Karnaky et al. '84; Li et al., '95; Witters et al., '96; van der Heijden et al., '97; Rombough, '99), which accumulate in active mitochondria. This limits their use to fresh, unfixed tissues. There are, however, newer products on the market that are retained in the mitochondria after fixation and are being used on fish MRCs (Mitotracker, Molecular Probes; Galvez et al., 2002; Marshall et al., 2002). There have also been a few studies using immunohistochemical techniques to detect mitochondria using antibodies directed against mitochondria specific proteins (HSP 60, Mickle et al., 2000; Ab-AMA, Sturla et al., 2001).

Another approach has been to target the enzyme Na^+,K^+-ATPase, which has been shown to be expressed in high concentrations in MRCs. Ouabain specifically binds and inhibits the Na^+,K^+-ATPase and both [^{3}H]ouabain autoradiography and anthroylouabain fluorescence have been found to localize to MRCs (Karnaky et al., '76; McCormick, '90). Histochemical methods have

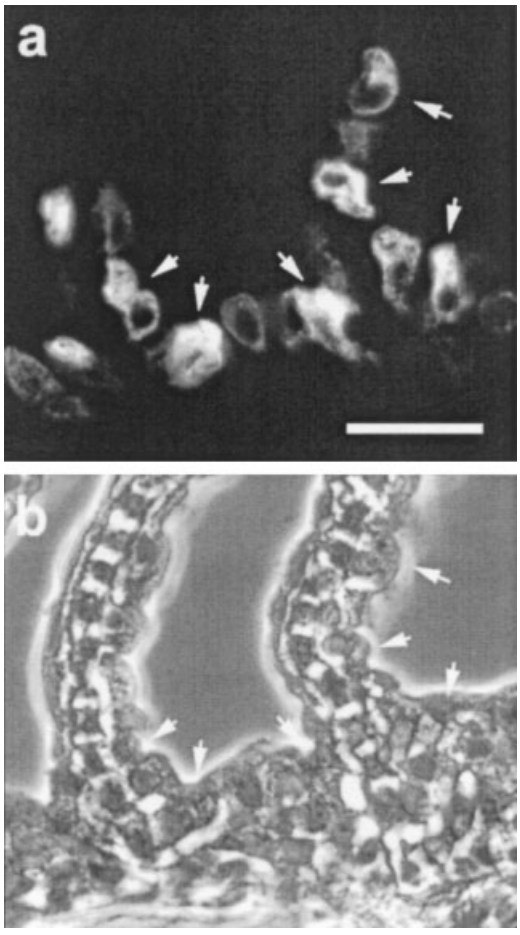

Fig. 13. (**a**) Immunohistochemical localization of Na^+,K^+-ATPase α-subunit in rainbow trout (*O. mykiss*) gill using the mouse monoclonal α_5 antibody in conjunction with a goat anti-mouse conjugated Alexa 488 (Molecular Probes) secondary antibody. Arrows indicate some immunopositive cells in both the filament and along the lamellae. Note the similarities in labeling with the coho salmon (Fig. 12). (**b**) Corresponding phase-contrast image showing the tissue. Scale bar=25 μm (J.M. Wilson, unpublished data).

been used in the past to localize Na^+,K^+-ATPase to MRCs (de Renzis and Bornancin, '84; Conley and Mallatt, '87). The use of antibodies directed against the Na^+,K^+-ATPase has proven very useful, as is evident in the large number of studies published recently using this approach (Fig. 13). Practically all of these studies make use of either the mouse monoclonal or rabbit polyclonal antibodies directed against conserved epitopes or regions of the Na^+,K^+-ATPase α subunit originally employed by Witters et al. ('96) and Ura et al. ('96), respectively. In addition to the teleosts, the mouse monoclonal has been used on a wide range of fishes with positive results (Atlantic hagfish *Myxine*, Choe et al., '99; lamprey, J.M. Wilson, unpublished; Atlantic sting ray, Piermarini and Evans, 2000, 2001; dogfish, Wilson et al., 2002). Lee et al. ('98) have also used other antibodies against specific α-subunit isoforms that have been found to localize to MRCs in the tilapia (*O. mossambicus*) while Hwang et al. ('98) have also developed a specific tilapia Na^+,K^+-ATPase α-subunit antibody.

Localization studies employing double-labeling techniques have also been used that demonstrate whether the apical surface of the MRC is exposed as covered MRCs are presumably not active in ion transport. To this end Li et al. ('95), van der Heijden et al. ('97), and Lee et al. (2000) have used fluorochrome-conjugated concanavalin A (Con-A), a lectin that binds specifically to α-mannopyranosyl and α-glucopyranosyl glycoprotein residues, to localize the apical region of MRCs identified with either a mitochondrial stain or Na^+,K^+-ATPase marker. PNA (peanut agglutinin binds specifically to terminal β-galactose residues) and WGA (wheat germ agglutinin binds specifically to *N*-acetylglucosamine and *N*-acetylneuraminic acid (sialic acid) residues) have been used by Galvez et al. (2002) and Tsai and Hwang ('98), respectively, to identify specific subpopulation of MRCs.

In hagfish, the PAS technique has also been used to identify MRC through the reaction with the abundant glycogen associated with the perinuclear region. EM studies have shown that the hagfish gill does not have goblet type mucous cells precluding the possibility that the PAS reaction is associated with the neutral mucins found in some mucous cells (Mallatt and Paulsen, '86).

OTHER EPITHELIAL CELL TYPES

In addition to the main cell types described above are lesser numbers of eosinophilic granule cells (EGC) and rodlet cells. The eosinophilic granule cells may be homologous to mammalian mast cells (Holland and Rowley, '98). The granules are PAS negative and some show immunoreactivity with monoclonal antibody 21G6, which has been shown to be reactive with trout leukocytes and intestinal EGC. The rodlet cells, which have a well-defined capsule, and intracellular rodlets are likely parasitic in origin (Barber et al., '79). The mudskipper (*P.schlosseri*) possesses a unique filament rich cell (Wilson et al., '99), which has also been found in the inner opercular epithelium of the killifish (*F. heteroclitus*; Lacy, '83). In the

mudskipper, these cells are laterally compressed between neighboring MRCs in the lamellar epithelium and appear to confer structural support to the epithelium. The septum epithelium of the elasmobranch *Scyliorhinus canicula* contains the usual complement of cells (PVCs, MRCs, undifferentiated cells, mucocytes) as well as large cells (10–30 μm in diameter) that contain a large apical vacuole (8–27 μm in diameter) (Wright, '73). The vacuoles are usually electron lucent while some show moderate electron density.

PROSPECTIVE STUDIES

The morphological approach will continue to be an integral part of the understanding and interpretation of the physiological functions of the gill (gas exchange, ionoregulation, acid–base regulation, detoxification). There will continue to be both quantitative and qualitative morphological data collected in studies of undescribed species as well as changes during development and in response to endogenous and exogenous factors (i.e., hormones and changes in environment, respectively). Importantly, more direct indicators of branchial cell function will also be studied by looking directly at the expression of the proteins that facilitate these various physiological processes. Detection of ion-transport proteins, using immunohistochemical techniques, has yielded some insight into the roles of both pavement cells and mitochondria rich cells in ion regulation in freshwater fishes (see Marshall, 2002, this issue). Our understanding of the roles of these cells in freshwater ion regulation has been handicapped by the lack of a suitable surrogate model to apply electrophysiological techniques which have provided insightful in the study of the seawater fish gill (see Marshall, 2002). Most immunohistochemical studies to date have relied on nonhomologous antibodies and thus suffer from complications of species cross reactive, but in the future fish specific homologous antibodies will likely feature more prominently. The cloning of fish ion transport proteins will facilitate the design and production of these antibodies. It is also now possible to detect low levels of mRNA expression in tissue sections using in situ PCR. Uncertainty surrounding the functional role of MRCs in the agnathans, elasmobranchs, and freshwater fishes, the presence of gap junctions in teleosts fishes, and the role of accessory MRCs will likely be addressed through the use of these techniques.

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