

## The Source of Lamellar Mitochondria-Rich Cells in the Air-Breathing Fish, *Trichogaster leeri*

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**ABSTRACT** Pavement cells and the mitochondria-rich cells (MRCs) are two of the main types of cells in fish gill epithelia. The pavement cells are generally responsible for gas exchange and MRCs for ion regulation. MRCs are found especially in the trailing edge and the interlamellar region of gill filament. In some species, MRCs are also observed in the gill lamellae. A previous study reported the likelihood of having lamellar MRCs in air-breathing fishes. Nevertheless, the source of lamellar MRCs is unclear. We used the air-breathing fish, *Trichogaster leeri*, to investigate the source of proliferated cells on the lamellae when 5-bromo-2-deoxyuridine (BrdU) was injected at different times before fish were sampled from deionized water. There were two major findings in this study. First, undifferentiated cells were found in the lamellae, as well as in the filaments. And, within 12–24 hr, a proliferated cell, identified as BrdU cell, could differentiate to an MRC in the gill lamellae. Second, the filaments and the lamellae in *T. leeri* responded to ionic stress differently but the proportion of the proliferated MRCs to the BrdU cells remained constant. Our results suggested that the lamellar MRCs were mainly differentiated from the cells that proliferated earlier from the lamellae. *J. Exp. Zool.* 309A:198–205, 2008. © 2008 Wiley-Liss, Inc.

**How to cite this article:** Lee W, Huang C-Y, Lin H-C. 2008. The source of lamellar mitochondria-rich cells in the air-breathing fish, *Trichogaster leeri*. *J. Exp. Zool.* 309A:198–205.

Fish gills are an organ with multiple functions, including gas exchange, ion regulation, nitrogenous waste excretion and acid–base balance (Perry and Laurent, '93; Jurss and Bastrop, '95; Perry, '97, '98; Marshall and Bryson, '98; Randall and Brauner, '98; Perry et al., 2003; Evans et al., 2005). The four pairs of branchial arches in teleosts consist of filaments and lamellae, both covered with epithelial cells that can be further characterized into pavement cells, mitochondria-rich cells (MRCs), mucous cells and undifferentiated cells (Laurent and Dunel, '80; Perry, '98; Evans et al., '99; Wilson and Laurent, 2002). Pavement cells account for more than 90% of the gill surface (mostly in the lamellae) and are considered the site of gas exchange (Laurent and Dunel, '80; Perry, '98). MRCs are mainly found in the trailing edge of gill filament epithelia of teleosts. In general, MRCs are the sites of ion extrusion in seawater (SW) and ion uptake in freshwater (FW; Laurent and Perry, '90; Perry, '97; Evans et al., '99; Piermarini and Evans, 2000).

In addition to the induction of lamellar MRCs in certain species by acclimation in FW or deionized water (DW; Greco et al., '95; Perry, '98; Piermarini and Evans, 2000) or by hormonal treatment (Perry et al., '92a,b; Bindon et al., '94; Perry, '98; Dang et al., 2000), several studies have reported the presence of lamellar MRCs in various teleost species. Among them, Lin and Sung (2003) surveyed the distribution of MRCs in the gills of 66 fish species (including 29 species from previous literature) from 12 orders, 28 families and 56 genera, and found that lamellar MRCs are more likely to be found in air-breathing fishes.

Grant sponsor: National Science Council; Grant numbers: NSC 90-2611-B-029-001; and NSC 92-2321-B-029-006; Grant sponsor: Academia Sinica; Grant number: AS-92-TP-B01.

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Received 13 September 2007; Revised 1 December 2007; Accepted 16 January 2008

Published online 15 February 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.446

A significant association was found between the lamellar MRCs and the mode of breathing at three levels of systematic classification (species, genus and family). That is, when a fish can exchange gases from other parts of the body, the gill lamellae will play a role in alternative physiological functions such as osmoregulation.

There is disagreement as to whether there are undifferentiated cells in gill lamellae. Chrétien and Pisam ('86) and Wilson and Laurent (2002) stated that the undifferentiated cells were found in both filaments and lamellae. On the other hand, Laurent et al. ('94) and Uchida and Kaneko ('96) reported that there were no undifferentiated cells in the lamellae. The discrepancy is, in part, owing to the different techniques in these studies. However, most studies that reported the presence of lamellar MRCs did not indicate their sources.

It was suggested that MRCs proliferated in the filaments before migrating to the lamellae (Laurent et al., '94). However, most studies on the source of MRCs used fish species that do not usually have lamellar MRCs or that belong to fish that have a complex life cycle, such as the anadromous *Oncorhynchus keta* (Uchida and Kaneko, '96) and catadromous Japanese eel (*Anguilla japonica*) (Sasai et al., '98). It is, therefore, necessary to find a fish species that normally has MRCs in the lamellae through life. The air-breathing fish that can exchange gas directly with the aerial environment (Graham, '97) are ideal for studying the trade-off and morphological plasticity in fish gills (Huang et al., 2008).

In this study, we chose the aquatic air-breathing *Trichogaster leeri*. It belongs to Anabantoidei, which is a suborder with five families, and all species have a labyrinth apparatus specialized for assisting gas exchange (Graham, '97). We used 5-bromo-2-deoxyuridine (BrdU), an analog of thymidine, which can be incorporated into DNA during the S phase and has been used to study cell proliferation (Laurent et al., '94; Uchida and Kaneko, '96; Tsai and Hwang, '98; Lin et al., '99). The specific objectives of this study were to clarify the existence of proliferated cells in the lamellae (BrdU cells), and to examine the source of lamellar MRCs.

## MATERIAL AND METHODS

### *Animals*

The experiments and handling of the animals comply with the current laws of Taiwan. Depend-

ing on their behavioral and habitat conditions, air-breathing fishes are categorized into two modes, amphibious and aquatic (Graham, '97). The pearl gourami (*T. leeri*) is a stenohaline FW and aquatic air-breathing fish (order Perciformes, suborder Anabantoidei, family Osphronemidae). It is mainly distributed in Southeast Asia, including the Malay Peninsula, Thailand and Indonesia (Sumatra and Borneo). The streamlets or marshes they inhabit accumulate humic substance and often create a hypoxic environment. In the previous study, Lin and Sung (2003) indicated that pearl gourami was one of the 18 air-breathing fish species investigated that had lamellar MRCs in the gills. It is available all year round and relatively easy to maintain in the laboratory.

Mature adult pearl gourami (either sex, 7–12 cm in standard length and 5–11 g in wet weight) purchased from local fish shop was maintained in plastic tanks (45 × 25 × 30 cm<sup>3</sup>). Aerated, circulated local tap water was filled up to 20 cm. One-fifth of the water was replaced every 2 days. Three fish were raised in one tank. They were acclimated at 28 ± 1°C under a 13:11 hr light:dark cycle and fed with commercial fish food (JBL, Neuhofen, Germany) once daily for at least a week before the experiment. The fish were not fed 1 day before the experiment. Water chemistry was provided in one of our recent reports (Huang et al., 2008).

### *The sources of lamellar MRCs*

There were three experimental designs on the timing of BrdU injection and acclimation duration. The first experiment was to determine the presence of BrdU-positive cells in the lamellae. Fish were transferred from local tap water to DW. They were sacrificed and the gill dissected at 1, 2, 3 and 7 days after transferal. BrdU was injected 1 hr before sampling.

The second experiment was to examine whether the lamellar MRCs were proliferated, in situ, from lamellae. Fish were transferred from local tap water to DW. BrdU was injected 36 hr after transferal and gills were dissected after another 12 hr of acclimation.

To distinguish the possible source of the lamellar MRCs from the filament epithelia, the last part of the experiment included fish injected with BrdU before transferal from local tap water to DW. The gill was dissected 1, 2, 3, 4 and 7 days after transferal. The sample was stained for the proliferated mitochondria-rich cells (PMRCs).

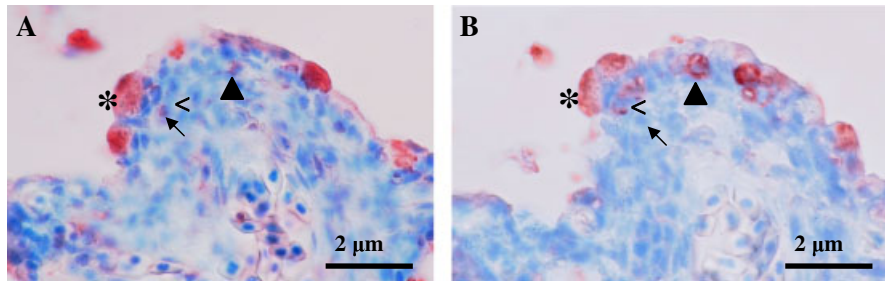


Fig. 1. Micrograph of proliferated mitochondria-rich cells (PMRCs) labeling with (A) anti-BrdU monoclonal antibody and (B) anti- $\text{Na}^+$ ,  $\text{K}^+$ -ATPase monoclonal antibody. Symbols are ▲: a PMRC; <: an MRC but not a BrdU cell; ↑: a BrdU cell but not an MRC; \*: mucous cell. BrdU, 5-bromo-2-deoxyuridine; MRC, mitochondria-rich cell.

### ***BrdU application***

Because of the low solubility of BrdU, we prepared a stock containing 20 mg BrdU/mL Ringer's solution using ultrasonic processor (Sonnics and Materials, Danbury, CT). After a further dilution of 1:100 with Ringer's solution, fish were anesthetized with 0.4 mg/mL MS-222 (3-amino-benzoic acid ethyl ester, Sigma, Missouri) and injected intraperitoneally with BrdU at 0.2 mg/g body weight in Ringer's solution.

### ***The paraffin method***

All four gills were excised in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M phosphate buffer at 4°C and fixed in the dark for 12 hr. They were rinsed with phosphate-buffered saline (PBS) containing (in mM) NaCl 136.9, KCl 2.68,  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  6.39 and  $\text{KH}_2\text{PO}_4$  1.76, with pH adjusted to 7.4 for 6 hr followed by ethanol-xylene series dehydration. After embedding in paraffin, tissue sections were prepared with a thickness of 3–5  $\mu\text{m}$  (RM2025RT, Leica, Nussloch) and placed on slides pre-coated with poly-L-lysine solution (Sigma).

### ***The labeling and quantification of total MRCs, BrdU cells and PMRCs***

From the paraffin ribbon, three continuous sections were carefully obtained and separately placed on the same slide. After de-waxing and rehydration, the specimens received different treatments. The first section was incubated in 2 N HCl for 30 min for DNA denaturation. Next, the slide with the three sections was immersed in 3%  $\text{H}_2\text{O}_2$  (in 100% methanol) for 7 min to remove any endogenous reaction followed by a 2-min PBS wash three times. Anti-BrdU monoclonal antibody (1:500) (Developmental Studies Hybridoma Bank, IA) was applied to the first section and  $\text{Na}^+$ ,  $\text{K}^+$ -

ATPase primary antibody (1:1,000) ( $\alpha$ -5 monoclonal antibody against the a-subunit of chicken  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, Developmental Studies Hybridoma Bank) to the second section for 1 hr in the dark at room temperature. After a 2-min PBS wash three times, the secondary antibody (horseradish peroxidase/Fab polymer conjugate, Zymed, South San Francisco, CA) and the color reagent (aminoethyl carbazole signal solution chromogen kit, Zymed) were applied for 30 and 15 min, respectively. Lastly, the samples were stained by hematoxylin (Zymed) for 10 min. The samples were mounted (GVA (Glycerol vinyl alcohol aqueous) mounting solution, Zymed) and examined by a light microscope (E600, Nikon, Tokyo, Japan). The pictures were taken by a digital camera (D1, Nikon, Japan) and saved in a computer. The third section that served as a negative control without the application of the primary antibody was prepared simultaneously. The specimens labeled with anti-BrdU monoclonal antibody and anti- $\text{Na}^+$ ,  $\text{K}^+$ -ATPase monoclonal antibody were identified as proliferated cells (BrdU cells; Fig. 1A) and MRCs (Fig. 1B), respectively. If both antibodies were on the same location, the cells were identified as PMRCs (Fig. 1).

There were ten fish for each treatment. Two filaments from each individual were randomly chosen to determine the number of total MRCs (TMRCs), BrdU cells and PMRCs in both filament and lamellar epithelia. We measured the lengths of the trailing edge of the filament and the full length of the lamella obtained, respectively, from the cross-sections and sagittal sections on image processing software (Image-Pro Plus 4.5, Media Cybernetics, Silver Spring, MD). The numbers of MRCs and BrdU cells per mm were calculated. For the analysis on the filaments, we included only the trailing side of the filament epithelia in the analysis. The widest axis across the afferent-vascular edge of the filament was to represent

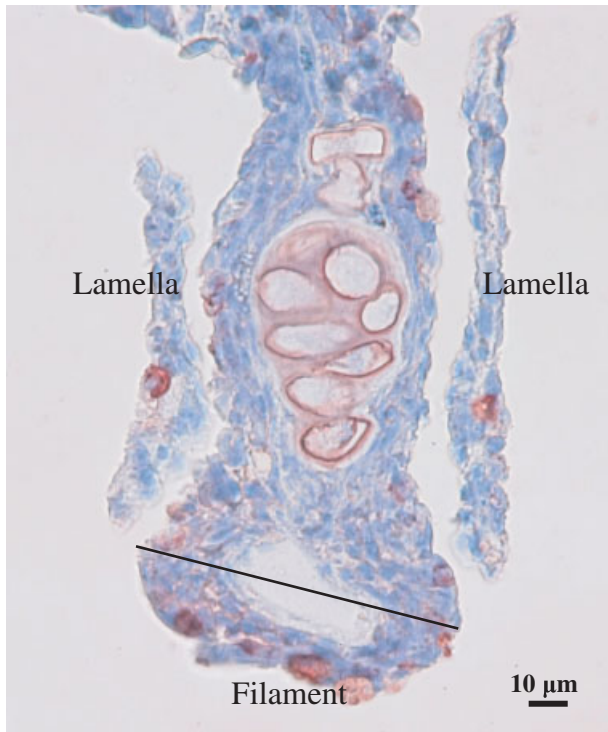


Fig. 2. The determination of the numbers of mitochondria-rich cells (MRCs) and the proliferated cells (BrdU cells) per mm length of the trailing edge in the filament. The longest axis of trailing edge side was taken and the MRCs distal to the axis counted. BrdU, 5-bromo-2-deoxyuridine.

the relative area of the trailing edge, and the MRCs distal to this axis were counted (Fig. 2).

For quantifying the numbers of the MRCs in the lamellae, two filaments were randomly chosen. Only those that were 5  $\mu\text{m}$  away from the base of the lamella were included. The length of 50 lamellae was determined and averaged by image analysis software (Image-Pro Plus 4.5). The number of lamellar MRCs per mm lamella was obtained.

### Statistics

In the control and DW group, the numbers of MRCs, BrdU cells and PMRCs were analyzed by two-way analysis of variance. If there were significant differences, the least-squares mean (LSMEANS) was used to compare those data. All the analysis took  $P < 0.05$  as significantly different. The statistical software was SAS 8e(Cary).

### RESULTS

As BrdU was injected 1 hr before each sampling time, only the cells that were proliferating were

labeled. We found BrdU cells both in the lamellae and in the interlamellar regions 1, 2, 3 and 7 days after transferal to DW. Therefore, there was a constant supply of proliferating cells in the lamellae, as well as in the filaments.

In the second experimental design, we extended our time of sampling from 1 to 12 hr after BrdU injection and determined the minimum time necessary to detect a PMRC. For example, to examine the gill at the end of a 2-day incubation, *T. leeri* were subjected to BrdU injection upon 36 hr of transferal to DW. Again, only BrdU cells on lamellae were found and no new MRC was observed on the lamellae. This implies that the differentiation from a proliferated cell to an MRC takes longer than 12 hr.

In the next experiment, *T. leeri* were injected with BrdU before transferal to DW. Fish gills obtained on days 1–4 and 7 after transferal were examined to determine the numbers of MRCs, BrdU cells and PMRCs in both filament and lamellar epithelia, respectively. In the gill filaments, TMRCs were not significantly different between water treatments ( $P = 0.053$ , Table 1). The numbers of BrdU cells within the 7 days of observation and between two water treatments were not significantly different (Table 1). This implied a constant supply and turnover of new cells in the gill filaments. Significantly more PMRCs in the gill filaments were found in tap water than in DW (Table 1A). However, the number of PMRCs was not significantly different within the 7-day observation (Table 1B). Within 12–24 hr in DW condition, no PMRC was found in the gill filaments. The ratio of PMRCs to TMRCs (PMRCs/TMRCs) increased and remained at about 30% from day 4 (Table 1A). This was different from that found in DW where it peaked on day 3 (15.6%) and decreased to 7% at day 7. Among the BrdU cells, up to 33.14% became MRCs (PMRCs/BrdU cells) at day 3 in tap water (Table 1A).

In the lamellae, significantly more TMRCs were found in the DW group than in the tap-water group ( $P = 0.006$ , Table 1B). The average number of the BrdU cells in the lamellae was significantly different among sampling days. It increased from 6.7 to 10.4 per mm in tap water and from 4.2 to 9.4 per mm in DW (LSMEANS,  $P < 0.05$ ). These are 155 and 223% increases, respectively. The number of PMRCs in the DW group was not significantly different from the tap-water group. However, a gradual and significant increase in PMRCs was found among the 7-day observation (Table 1B);

TABLE 1. (A) The density and ratio of total mitochondria-rich cells (TMRCs), proliferated cells (BrdU cells) and proliferated mitochondria-rich cells (PMRCs) on the afferent edge of filament epithelia when transferring to tap water and DW and (B) ANOVA table for the effects of water and time after transfer on total mitochondria-rich cells (TMRCs), proliferated cells (BrdU cells) and proliferated mitochondria-rich cells (PMRCs)

			Sampling time (day)					
(A)	Parameter	Site	Treatment	1	2	3	4	7
	TMRCs (mm <sup>-1</sup> )	F	Tap water	28.9±3.9	33.9±4.6	24.6±4.6	32.4±6.3	22.4±1.2
			DW	16.2±5.1	21.2±2.7	27.5±6.1	23.7±4.6	25.6±2.7
		L	Tap water	5.7±1.7	5.9±1.8	4.2±0.3	6.0±1.0	5.6±1.2
			DW	7.1±1.1	5.6±1.2	10.3±3.2	12.7±1.7	19.4±5.5
	BrdU cells (mm <sup>-1</sup> )	F	Tap water	24.5±2.8	25.4±5.4	16.9±2.7	31.9±2.6	29.1±4.7
			DW	20.0±7.6	20.9±4.9	23.9±5.4	21.4±7.5	18.7±4.3
		L	Tap water	6.7±3.5	5.1±0.9	5.1±1.0	8.9±2.1	10.4±1.6
			DW	4.2±0.7	4.7±1.0	7.5±1.3	8.0±2.7	9.4±1.5
	PMRCs (mm <sup>-1</sup> )	F	Tap water	4.0±2.8	5.8±2.1	5.6±2.9	9.7±5.9	6.8±1.2
			DW	0.0±0.0	3.0±2.2	4.3±2.3	2.7±1.8	1.8±1.2
		L	Tap water	0.2±0.2	0.3±0.2	0.4±0.4	1.0±0.5	1.2±0.2
			DW	0.3±0.2	0.5±0.2	0.8±0.3	0.7±0.4	1.2±0.4
	PMRCs/TMRCs (%)	F	Tap water	13.8	17.1	22.8	29.9	30.4
			DW	0	14.2	15.6	11.4	7.0
		L	Tap water	3.5	5.1	9.5	16.7	21.4
			DW	4.3	8.9	7.8	5.5	6.2
	PMRCs/BrdU cells (%)	F	Tap water	16.3	22.8	33.1	30.4	23.4
			DW	0	14.4	18.0	12.6	9.6
		L	Tap water	3.0	5.9	7.8	11.2	11.5
			DW	7.1	10.6	10.7	8.8	12.8
	Sample size (n)		Tap water	5	6	4	4	4
			DW	8	8	7	7	8

		Filaments				Lamellae			
(B)	Source	df	MS	F	P	df	MS	F	P
TMRC	Water	1	609.23	3.91	0.053	1	423.53	7.99	0.007**
	Day	4	71.92	0.46	0.763	4	130.75	2.47	0.06
	W*D	4	167.44	1.07	0.379	4	77.17	1.46	0.23
	Error	51	155.86			51	52.99		
BrdU cells	Water	1	301.76	1.21	0.278	1	5.07	0.28	0.60
	Day	4	26.77	0.11	0.98	4	49.75	2.70	0.04*
	W*D	4	136.34	0.54	0.70	4	8.23	0.45	0.77
	Error	50	250.33			50	18.42		
PMRCs	Water	1	225.48	7.56	0.008**	1	0.06	0.10	0.60
	Day	4	26.25	0.88	0.48	4	1.93	3.25	0.02*
	W*D	4	11.89	0.40	0.81	4	0.20	0.34	0.85
	Error	51	29.83			51	0.59		

BrdU, 5-bromo-2-deoxyuridine; DW, deionized water; ANOVA, analysis of variance.

day 7 was significantly higher than other sampling days (LSMEANS,  $P < 0.05$ ). The ratio of PMRCs to TMRCs (PMRCs/TMRCs) in the control group increased, but the PMRCs/TMRCs ratio in the DW group remained constant and ranged from 4.3 to 8.9%. From the ratios of PMRCs/BrdU cells in the lamellae, we found that a higher proportion of BrdU cells became PMRCs within 3 days of DW acclimation.

## DISCUSSION

The major findings of this study are as follows: (1) There were proliferating cells in the lamellae, as well as in the filaments. (2) A different response to ionic stress between filament and lamellae was found in the numbers of TMRCs, BrdU cells and PMRCs. In the past, there were not many studies that focused on the source of lamellar MRCs. Most

studies were on euryhaline or anadromous fishes (Chrétien and Pisam, '86; Laurent et al., '94; Uchida and Kaneko, '96; Tsai and Hwang, '98; Hirai et al., '99; Chang et al., 2001, 2003). These fish normally do not have lamellar MRCs or they have them only at a certain life stage, for example, the chum salmon fry (*O. keta*) (Uchida and Kaneko, '96; Shikano and Fujio, '98a,b). Some reports have indicated that a change in the environmental ion concentration induces the proliferation of the lamellar MRCs (Bindon et al., '94; Perry, '98; Sakuragui et al., 2003). In these studies, the new lamellar MRCs were not specifically labeled and an increase in total lamellar MRCs was interpreted as the proliferation of new MRCs.

There were at least three studies on the source of the PMRCs. First, Laurent et al. ('94) injected cortisol and growth hormone to *O. mykiss* and transferred them to DW. BrdU was injected 1 hr before the gills were collected at different time intervals and the numbers of BrdU cells were determined. Again, their results could only represent the number of BrdU-positive cells at each time interval. In addition, the authors determined the number of lamellar MRCs by a scanning electron microscope and found a significant increase in TMRCs when rainbow trout were transferred to DW. Laurent et al. ('94) concluded that the new MRCs were either differentiated in situ from the stem cells of the lamellae or from the filament epithelia and migrated to the bases of lamellae.

In this study, BrdU was applied at the beginning of the 7-day experiment. One would suspect the cumulative effect of BrdU labeling if there was a constant profile of increase in BrdU cells in both the location of the gills (filaments and lamellae) and the water conditions (tap water and DW). However, we found that the BrdU cells remained constant in the filaments among treatments but they increased significantly in the lamellae. Particularly, significant increases were in the lamellar BrdU cells and PMRCs (the "day" effects in the analysis for lamellae in Table 1B). The discrepancy between filaments and lamellae deserved further investigation.

Uchida and Kaneko ('96) injected BrdU to *O. keta* before salinity transferal. Then, serial sections were prepared and the BrdU-positive cells and the MRCs were labeled simultaneously. The turnover rate of the MRCs can be monitored with this method and can be distinguished from the proliferated cell owing to salinity transferal.

*O. keta* is an anadromous fish and it only possesses lamellar MRCs during a certain life stage. In the experiment, *O. keta* was about to have downstream migration and the number of lamellar MRCs was regressing. Therefore, the BrdU-positive cells were rarely found and the authors combined the numbers of the filament and lamellar MRCs together. In their experiment, Uchida and Kaneko ('96) successfully labeled PMRCs on the first day of observation and the turnover rate was higher upon transferal to SW than to FW. Because most of the BrdU cells were found around the central venous sinus, Uchida and Kaneko ('96) had a similar conclusion to that of Laurent et al. ('94) and suggested that the lamellar MRCs proliferated from the filament epithelia and migrated to the lamellae.

Lastly, Hirai et al. ('99) transferred *Lateolabrax japonicus* from SW to FW and labeled MRCs by immuno-histological staining for Na<sup>+</sup>, K<sup>+</sup>-AT-Pase. They counted the numbers of filament and lamellar MRCs separately and measured the distance of lamellar MRCs from the filament epithelium. When *L. japonicus* was transferred from SW to FW, the number of filament MRCs decreased, whereas that of the lamellar MRCs increased. They concluded that the lamellar MRCs moved from the filament epithelia because of the increasing distance of lamellar MRCs from the filament epithelium.

In this study, we found that the filaments and the lamellae in *T. leeri* responded to ionic stress differently in at least three aspects. First, in the filaments, more TMRCs were found in the tap-water-acclimated fish than in the DW-acclimated ones. However, it was the DW-acclimated fish that had more TMRCs in the lamellae. In addition, deionized condition did not result in a significant change in TMRCs in the filaments within 7 days. On the contrary, a 2.7-fold increase (19.4/7.1) was found in the lamellae at the end of 7-day acclimation.

Second, there was a constant cell renewal in both TMRCs and BrdU cells in the filaments but ionic stress did not induce more cell proliferation. In the lamellae, there was a gradual and significant increase in BrdU cells within 7-day acclimation. Particularly, the number of BrdU cells increased more than two-fold in deionized condition by day 7.

Third, there were more PMRCs per unit length in the filaments when *T. leeri* were in tap-water condition than in DW condition. This trend of difference between the two water treatments did not change within 7 days of experiment. Similar to

those found in BrdU cells, the number of the PMRCs in the lamellae in both tap-water and DW conditions increased four to six times by day 7.

The PMRCs could be observed in the lamellae 1 day after transferal, but not in the filament epithelium. This was an indication that the lamellar PMRCs emerged very quickly. It is of interest to mention that PMRCs accounted for a higher proportion of TMRCs in the filaments compared with the lamellae in both water conditions. Together with what was found in the greater proportion of BrdU cells that differentiated into PMRCs (i.e., PMRCs/BrdU cells) in the filaments, it is suggested that the cell renewal activity was higher in the filaments. This higher cell renewal does not mean a migration of these cells to the lamellae in the DW condition, because the ratios of PMRCs/TMRCs and PMRCs/BrdU cells did not increase accordingly through time of acclimation. In other words, our results suggested that the rise in lamellar TMRCs in DW condition ( $19.4 \pm 5.5$  per mm lamella) was mainly differentiated in situ from the cells that proliferated earlier from the lamellae rather than from either the new PMRCs in the lamellae or the cells migrating from the filaments.

### ACKNOWLEDGMENTS

H. C. L. was supported by grants from National Science Council (NSC 90-2611-B-029-001 and NSC 92-2321-B-029-006) and Academia Sinica (AS-92-TP-B01).

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