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Assessment of Proliferative Activity by Proliferative Cell Nuclear Antigen (PCNA) and Anti-Bromodeoxyuridine (BrdU) Immunolabeling in the Tissues of Japanese Eels (*Anguilla japonica*)

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Abstract

Five Japanese eels (*Anguilla japonica*) were examined by immunolabeling with proliferating cell nuclear antigen (PCNA) and bromodeoxyuridine (BrdU) incorporation for assessment of proliferative activities in various tissues of Japanese eels. PCNA protein was expressed in all tissues of eels, mainly in the haematopoietic tissues, especially in the anterior kidneys, an indication for the role of PCNA in haematopoiesis. Also, positive PCNA immunolabeling was frequently seen in the spleen. PCNA labeling index in kidney and spleen of Japanese eels was correlated well with BrdU incorporation which could be indicating higher proliferative activity of these organs. Absence of correlation between PCNA and BrdU in the testes may might refer to the expression of PCNA in germ and other somatic cells, while BrdU immunostaining was only noticed in S-phase dividing spermatogenic cells. Therefore, our results demonstrated over expression of PCNA in haematopoietic tissues and testes suggest the role of PCNA in haematopoiesis and spermatogenesis in Japanese eels. Moreover, PCNA and BrdU labeling indices could be a valuable approach for analyzing cell proliferation activity in the kidney and spleen of eel tissues.

Keywords: Cell proliferation, PCNA, BrdU, Japanese Eels (*Anguilla japonica*).

Introduction

In an organism, the rate of cell division is a tightly regulated process that is intimately associated with proliferation, differentiation and tissue turnover. Several methods have been developed to study cellular proliferation, one of which is proliferating cell nuclear antigen (PCNA). PCNA is an auxiliary protein of DNA polymerases and enzymes necessary for DNA synthesis (Kelman, 1997), and is expressed during different stages late in the G1-, S- and G2-phases of cell cycles (Morris and Mathews, 1989). PCNA can be expressed in the spermatogenic cells of Japanese eel, and also over-expressed during embryonic and adult haematopoiesis in zebrafish (Miura *et al.*, 2002; Leung *et al.*, 2005). To date, little data has been available on the role of PCNA in eel tissues, and only a small number of studies have been carried out on spermatogenic cells (Ito and Abé, 1999; Miura *et al.*, 2002).

BrdU is an analog of thymidine, which incorporates into replicating nuclear DNA in S-stage dividing cells (Gratzner, 1982; Alison, 1995). BrdU can be detected by immunolabeling with a specific anti-BrdU monoclonal antibody as described by Gratzner (1982). The BrdU immunolabeling method has been used in place of 3H-thymidine autoradiography to detect DNA-synthesizing cells, both in cell cultures and in whole mount preparations (Hamada, 1985; Plickert and Kroiher, 1988). Previously, it has been used as proliferating marker in the retinas of zebrafish (Hitchcock and Raymond, 2004), the testes of mosquito fish (Koya and Iwase, 2004), the skin of channel catfish (Zhao *et al.*, 2008) and the gills of killifish (Pierre *et al.*, 2006).

Although several lines of evidence have been reported in teleosts, to our knowledge, there have been almost no data available concerning applications to different eel tissues. The present study was undertaken to assess proliferative activities in the various tissues of Japanese eels using PCNA immunolabeling, as well as BrdU incorporation.

Materials and methods

Animals

Five male Japanese eels (*Anguilla japonica*) with a body weight of approximately 200 g were kept in a glass tank and fed a commercial fish diet. All were administered BrdU (5-bromo-2'-deoxy-uridine) (Sigma-Aldrich Co. Saint Louis, MO, USA)

intraperitoneally 1 hour before euthanization. Forty mg of BrdU was dissolved in 80 µl dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) and 320 µl of physiological saline solution was administered to each eel in a dose of 200 mg/kg body weight.

Samples from the skin, gastrointestinal tract, kidney, spleen, pancreas, gill, eye and testes were collected, sliced at a thickness of 5 mm, and fixed in 10% neutral buffered formalin and subjected to heat without boiling for 15 minutes for rapid fixation, then left at room temperature for 24 hours. After dehydration and clearance, the tissues were embedded in paraffin and sectioned in 5 mm thickness. The serial sections were subjected to staining with hematoxylin and eosin or immunostaining.

Immunohistochemistry

Immunolabeling of PCNA antigen antibody was performed as described (Ishikawa *et al.*, 2005). Briefly, the serial sections were dewaxed, hydrated, and immersed in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) for 15 minutes and autoclaved at 121°C for 15 minutes. They were then treated with hydrogen peroxide 0.3% and protein block, followed by incubation with anti-PCNA (clone PC10; 1: 200 Dako, Glostrup, Denmark) at 4°C overnight. The slides were rinsed three times with PBS, incubated with anti-mouse IgG secondary antibodies for 30 minutes at room temperature, visualized with di-aminobenzidine commercial kits, and finally counterstained with Mayer's haematoxylin. As a negative control procedure, the primary antibody was replaced by normal mouse serum.

Immunolabeling of BrdU-incorporating cells was performed as described (Leung *et al.*, 2005). After dewaxing and hydrating, serial sections were hydrolysed with 5N HCl at 37°C for 30 minutes before being neutralized in Palitisch's boric acid-NaCl-borate buffer (pH 7.6) at 4°C for 15 minutes. The sections were subjected to a digestive process with 0.05 and 0.02% trypsin (Wako Pure Chemical Industries, Osaka, Japan) in Tris buffer at 37°C for 30 minutes. The remaining procedures were performed in the same manner as immunolabeling with PCNA, after inactivation of endogenous peroxidase and blocking of non-specific binding of the antibody, the primary anti-BrdU mouse monoclonal antibodies (1:50 dilution; Dako, Glostrup, Denmark). Most of the slides treated with trypsin 0.05% showed good immunolabeling but lower haematoxyline stainability, thus, some were counterstained with eosin stain.

Assessment of Immunolabelling and Statistical Analysis

Immunolabeling reactions were scored semiquantitatively for PCNA, and BrdU, only a distinct brown-coloured nucleus was accepted as a positively labeled cell. PCNA-positive cells and BrdU-positive cells were counted in total cells from 5 fields of 20 high-power views to give the PCNA labeling index and BrdU labeling index. Immunolabeling reactions were indicated as $^{-}$ meaning an absence of positive cells, (<5%) as $^{+}$ meaning a small number of positive cells, (25–50%) as $^{++}$ meaning a moderate number of positive cells, and as $^{+++}$ (>50%) meaning numerous positive cells. The correlation between PCNA LI and BrdU LI was analysed by linear regression and Pearson's coefficient of correlation.

Results

The cell proliferative activity in the integumentary system (skin and fins), gastrointestinal tract, haematopoietic tissues (kidney and spleen), pancreas, testes and gills of Japanese eels (*Anguilla japonica)* was examined using PCNA immunohistochemistry and 5-bromodeoxyuridine (BrdU) incorporation. In the skin, frequent positive immunoreaction for PCNA was observed in the undifferentiated germinal cells consisting of the basal (stratum basale) and supra basal layers and the epithelial cells of the taste buds, while no reactions were seen in the mucous or alarm cells (Figure 1a). In the esophagus, positive PCNA expression was frequently observed in the basal cell layer of both the stratified anterior part and the columnar part of the esophagus. The gastric mucosa showed frequent positive reactions for PCNA in the surface mucosa and columnar lining epithelial cells of the distal gastric glands (Figure 1b). Also, the small and large intestine showed that PCNA expression occasionally immunolocalised to the epithelial cells at the bottom mucosal folds, which may be the proliferative zone (Figure 1c).

In the urinary system, the kidneys were located on a midline longitudinally and were attached to the dorsal wall of the body cavity, and the cellular morphology was characterized as comprising both the excretory and haematopoietic tissues. The anterior part of the kidneys (the head) had larger numbers of haematopoietic cells, where the posterior part (the trunk) contained more dominant excretory tubules. These haematopoietic cells were interspersed among the excretory tissues. Among the haematopoietic elements, mature erythrocytes, unlike their mammalian counterparts, were characterized by an ellipsoid shape and elongated nuclei. There were frequent cells with a relatively open chromatin texture and a high nucleus-to-cytoplasm (N/C) ratio, reminiscent of human blast and promyelocytic cells (herein referred to as "progenitor cells") (Figure 1d). There was prominent PCNA positive immunolabeling in these haematopoietic compartments in the kidneys, the expression of which was confined to the nuclei of the haematopoietic cells, which had larger nuclei and a higher N/C ratio. This corresponded morphologically to the progenitor cells under H&E

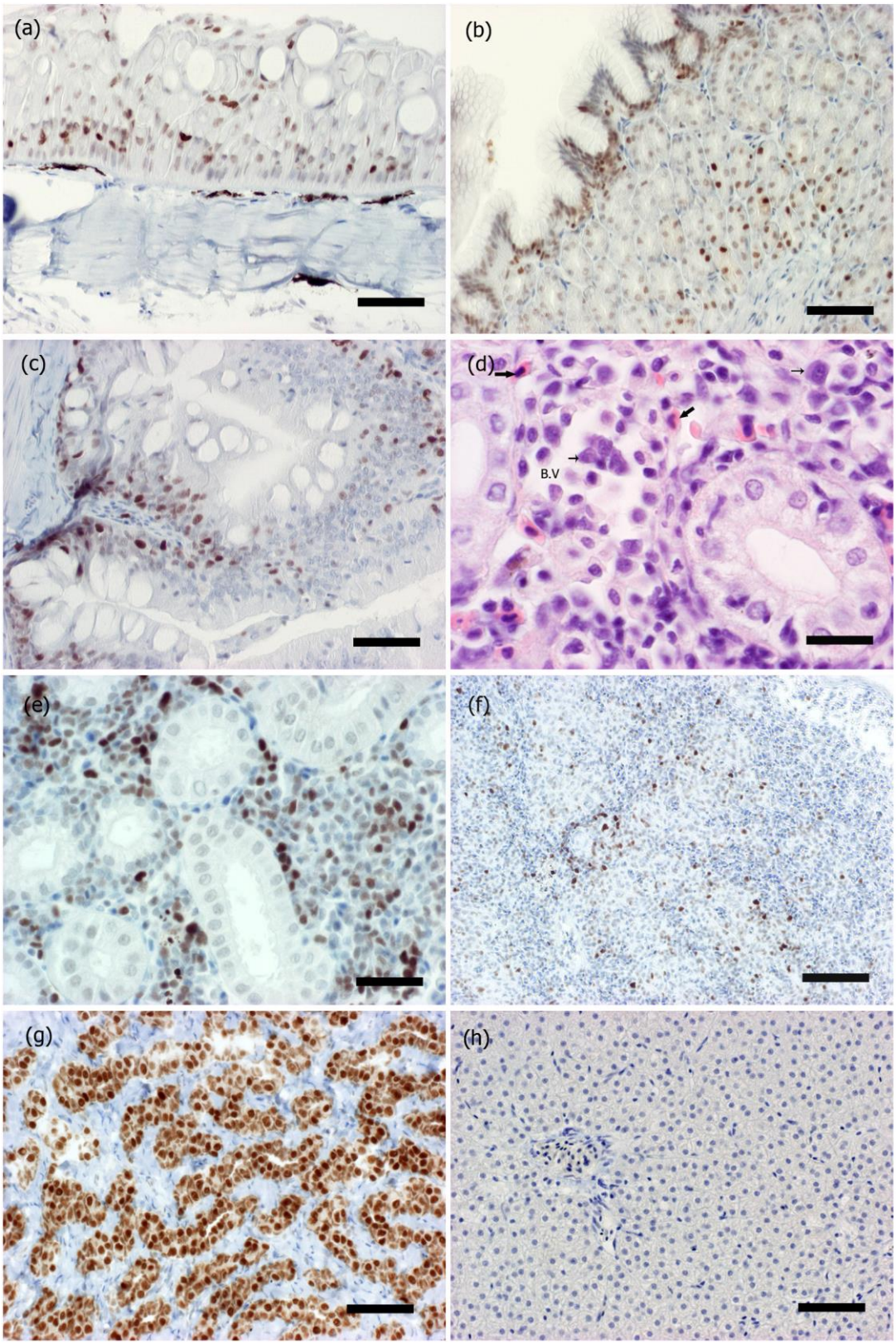

Figure 1. PCNA immunolabeling in Japanese eel tissues. (a) PCNA expression in the skin trunk. (X 200, bar = 50 µm). (b) Fundic region of the stomach showing PCNA positive cells located at the columnar epithelium and glandular epithelium of the basal layer. (bar = 50 µm). (c) PCNA positive cells located at the basal layer of the intestinal crypt. (X 200, bar = 50 µm). (d) Photomicrograph of the anterior kidney showing progenitor haematopoietic cells. HE. (X 400, bar =25 µm). B.V Blood vessels. The heavy arrow indicates haematopoietic progenitor cells and the thin arrow RBCs. (e) Anterior kidney showing positive PCNA progenitor haematopoietic cells. (X 200, bar = 50 µm). (f) Positive PCNA cells on the margin of the white pulp of the spleen. H&E. (X 100, bar = 100 µm). (g) Immature testes showing intense elaboration of PCNA labeling. (X 200, bar = 50 µm). (h) Liver showing PCNA expression. No positive reaction is seen. (X 200, bar = 50 µm).

staining. In some areas in the kidneys, these PCNA positive cells were in close proximity to the renal tubular cells, where in other areas they were within blood capillaries (Figure 1e). In spleen, there were frequent PCNA positive reactions in cells closely related to the red pulp or at the margin of the white pulp (Figure 1f). In the testes of immature Japanese eels, spermatogenic cells in addition to the supporting somatic cells, mostly likely Sertoli cells are PCNA labeled (Figure 1g). In the gills, there were frequent PCNA positive reactions in the epithelial cells lining the primary lamellae. There were almost no PCNA positive cells in the liver or pancreas in any of the sections examined (Figure 1h).

BrdU immunostaining: BrdU incorporated into the nuclei of proliferating S-phase cells in the skin, gastrointestinal tract, testes, kidneys, spleen and retina. In the skin, slight to moderate numbers of BrdU incorporated cells were mainly seen the basal cell layer rather than in the upper cell layers. In the gastrointestinal tract, moderate numbers of BrdU incorporated cells were seen in the base of the intestinal folds (Figure 2a). Haematopoietic organs such as the kidneys revealed frequent BrdU incorporated (S-phase) cells in the interstitial haematopoietic tissue and occasional S-phase cells in the renal tubular epithelium. Most of the positive BrdU incorporated cells were consistent with haematopoietic cells stained with H&E. Also, in some of these cells, the nucleus exhibited a marked dotted pattern or granules at the periphery (Figure 2b). The spleen also exhibited frequent positive BrdU cells in both the red pulp and white pulp (Figure 2c). Testes of Japanese eel, showed several nuclei of germ cells which were strongly BrdU-stained, and others not so strongly stained. The supporting somatic cells appeared to be BrdU-negative (Figure 2d). The retinal epithelium of the eye of the Japanese eel was seen to be generally organized as in other vertebrates, with internal nervous tissue layers, overlying rod and cone receptor cells, and a black pigmented layer found peripherally. These make up a total of eight specific layers in the retina, in which the inner nuclear layer contains the cells with the most strongly BrdU-immunostained (Figure 2e).

PCNA and BrdU immunolabeling indexes are shown in (Table 1): There was a strong correlation and prominent immunolabeling (+++) among the PCNA index and BrdU labeling index, especially in the haematopoietic organs. For PCNA and BrdU, there was some discrepancy between the PCNA index (moderate ++) and BrdU incorporating index (occasional +) in the esophagus, stomach, intestinal tracts, and skin. In the testes, there were frequent cells that were positive for PCNA expression (+++) in the seminiferous tubules, while only occasional cells demonstrated (+) a positive reaction to BrdU in the nuclei.

Pearson's coefficient of correlation and linear regression analysis revealed that the averages of PCNA LI and Brdu LI in haematopoietic organs (kidney and spleen), stomach and intestine of each eel were significantly correlated (r_s=0.8446, 0.8215, 0.8934 and 0.9027 respectively, $P<0.05$ with

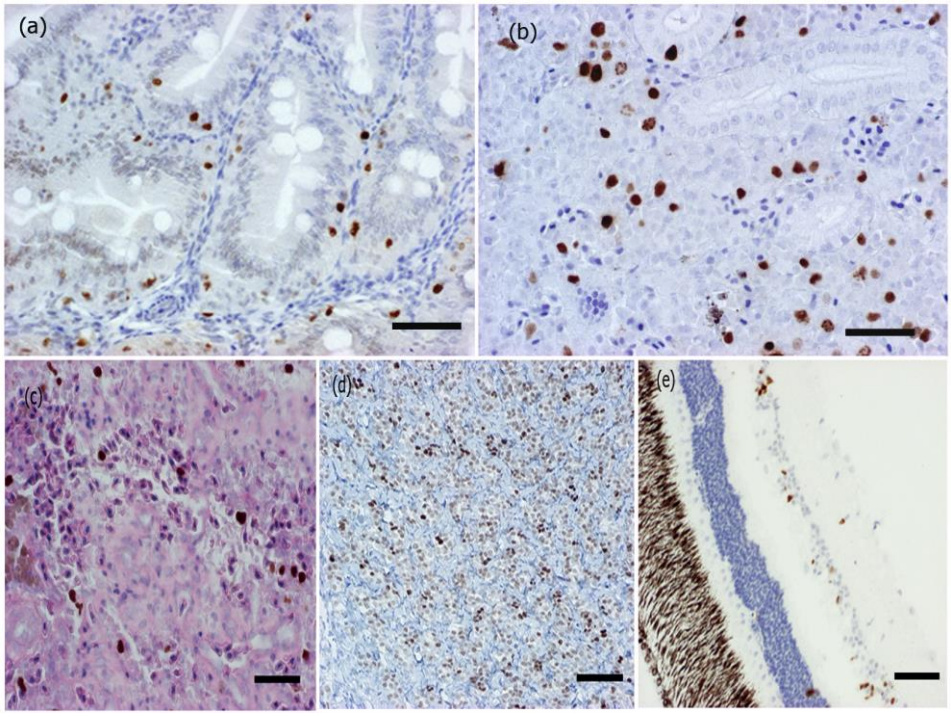

Figure 2. BrdU immunostaining. (a) BrdU labeled cells at the basal layer of the intestinal crypt. (X 200, bar = 50 µm). (b) Anterior kidney showing BrdU labeled cells. (X 200, bar = 50 µm). (c) Spleen showing BrdU labeled cells in the red pulp with HE counterstain. (X 200, bar = 50 µm) (d) Testes showing BrdU labeled cells. (X 100, bar = 100 µm). (e) Eye showing BrdU labeled cells in the inner nuclear layer of the retina. (X 200, bar = 50 µm).

Pearson's coefficient of correlation; r=0.919, 0.906, 0.945, 0.950, P<0.05 with linear regression analysis). There was no significant correlation between anti-Brdu and anti-PCNA LIs in testes, esophagus and gills (r_s=0.2971, 0.486, 0.047 respectively, P<0.05 with Pearson's coefficient of correlation; r=0.545, 0.221, 0.216 respectively P<0.05 with linear regression analysis) (Figures 3, 4).

Discussion

In the present study, a higher PCNA LI, rather than BrdU LI, was observed in the integumentary system, gastrointestinal tracts and gills. It's noteworthy that PCNA not only functions as a DNA sliding clamp for replicative DNA polymerases, but also has a marked ability to interact with multiple partners involved in multiple pathways, DNA repair, and cell cycle regulation (Maga and Hubscher, 2003). While, immunohistochemical localization of BrdU incorporated in the nuclei was limited to S-phase divided cells (van de Kant *et al.*, 1990; Rosiepen *et al.*, 1994).

The absence of a correlation between PCNA expression and BrdU incorporation in certain organs was in agreement with a number of previous reports in which no correlation was observed between anti-PCNA LI and other indices of proliferation (Jain *et al.*, 1991; Yu *et al.*, 1991; Leonardi *et al.*, 1992; Rosa *et al.*, 1992; Visakorpi, 1992).

Of particular note is the overexpression of PCNA in the anterior kidney and spleen of Japanese eels, which suggests the role of PCNA in the proliferation of haematopoietic cells. This finding coincides with results reported on the kidneys of wild-type male adult zebrafish (Leung *et al.*, 2005); the authors of that study also explained the role of PCNA in embryonic haematopoiesis through up-regulation of

Table 1 Anti-PCNA and anti-BrdU LIs average in different eels tissues

Organ	PCNA LI	BrdU LI
Esophagus	26.3±10.26	8.84±3.93
Stomach	32.22±14.74	9.42±6.59
Intestine	37.1±7.75	12.26±6.25
Skin	38.56±7.24	22±5.38
Gills	29.76±6.93	18.92±5.24
Testes	70.18±9.89	21.2±9.94
Kidney	65.9±11.35	57.1± 9.8
Spleen	43.96±9.76	36.86±13.59

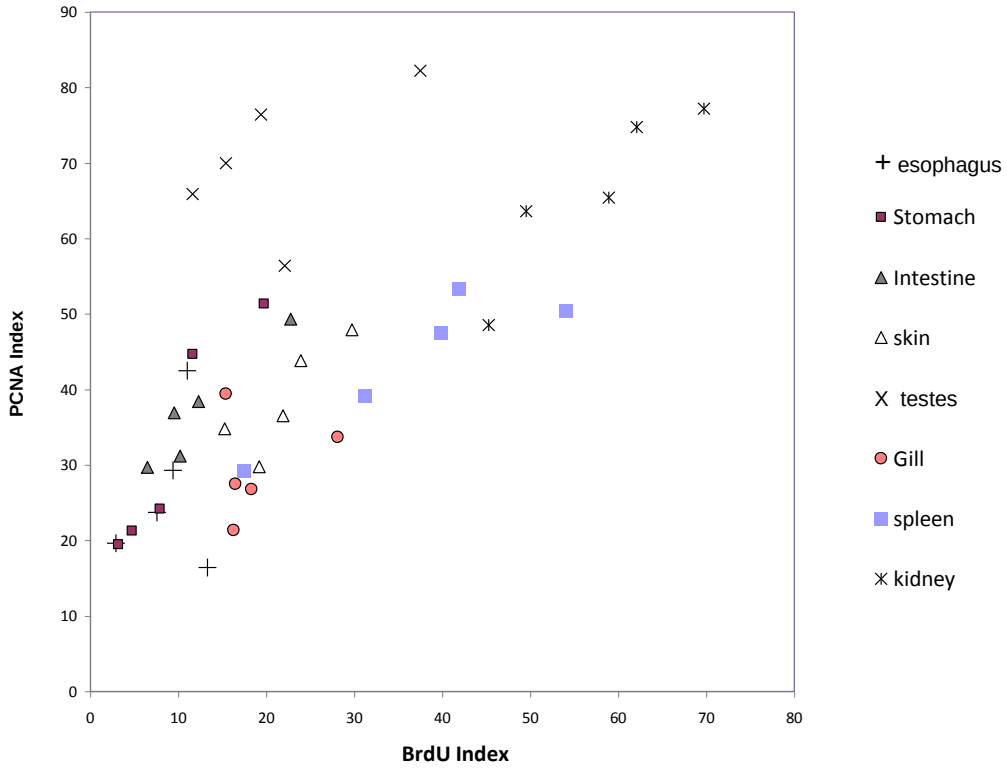

Figure 3. Correlation between anti-PCNA and anti-BrdU LIs in eel tissues.

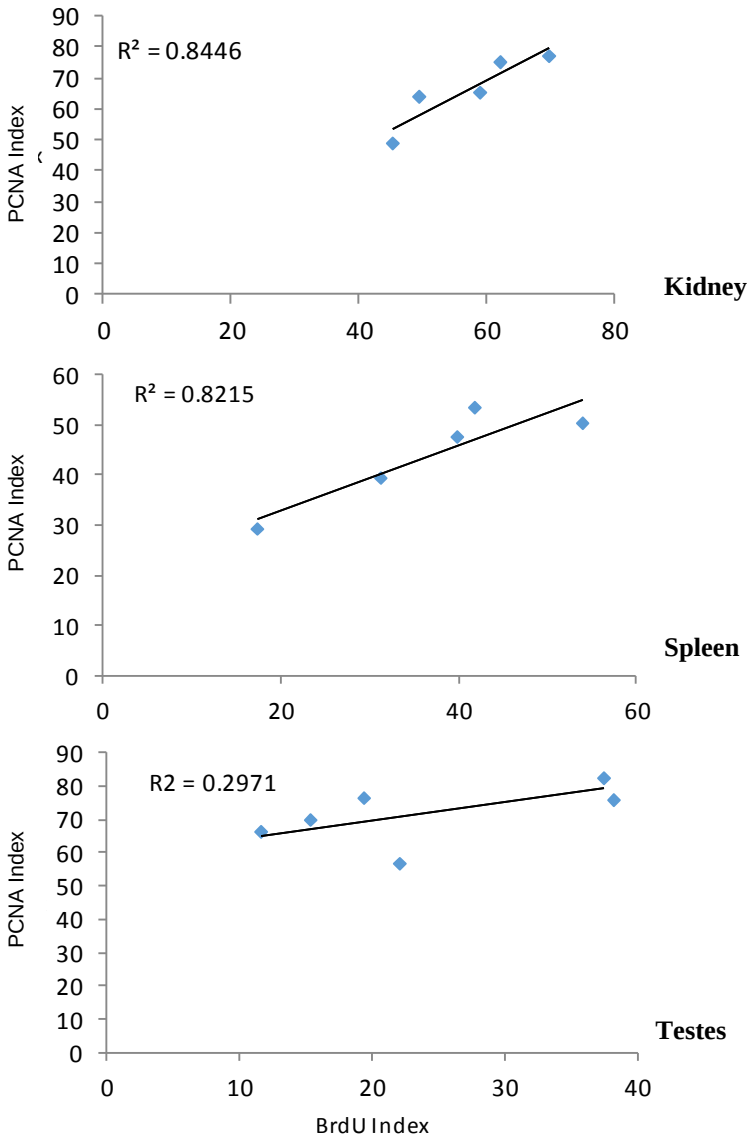

Figure 4. Linear regression between anti-PCNA and anti-BrdU LIs in kidney, spleen and testes of Japanese eel.

PCNA in the primitive Intermediate Cell Mass (ICM), which is considered to be the site of primitive haematopoiesis in the embryos of zebrafish. Meanwhile, they also mentioned that PCNA immunostaining was absent in the spleens of zebrafish (Leung *et al.*, 2005). Interestingly, our result revealed PCNA was expressed in both the white pulp and red pulp of the spleens of Japanese eels, thus further study may be needed to investigate the role of the spleen in haematopoiesis in eels. Frequent BrdU incorporation in the nuclei in the spleen and kidneys also indicated the highly proliferative activity of haematopoietic cells. The nuclei exhibited a dotted or granular appearance when immunostained against BrdU. The dotted appearance in nuclei could be indicating the early S-phase divided cells while, larger labeled granules in BrdU immunostained might formed in the late S-phase (Valero *et al.*, 2004).

In the immature testes of Japanese eels, PCNA was predominantly expressed in the nuclei of the spermatogonial stem and other somatic cells as Sertoli cells, which indicates that the PCNA protein plays an important role in spermatogenesis. The germ cells of a Japanese eel underwent active cell division demonstrating a 32 k Da PCNA protein expression (Miura *et al.*, 2002). On the other hand, occasional positive nuclei for BrdU incorporation in Japanese eel testes may be indicate S-phase dividing germ cells only.

In summary, PCNA expression is a potentially useful method for analyzing cell proliferation in kidney and spleen of Japanese eel, and also correlates well with BrdU incorporation, which can be used for identification of S-stage dividing cells.

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