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p53 Mutation suppresses adult neurogenesis in medaka fish (*Oryzias latipes*)

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ABSTRACT

Tumor suppressor *p53* negatively regulates self-renewal of neural stem cells in the adult murine brain. Here, we report that the *p53* null mutation in medaka fish (*Oryzias latipes*) suppressed neurogenesis in the telencephalon, independent of cell death. By using 5-bromo-29-deoxyuridine (BrdU) immunohistochemistry, we identified 18 proliferation zones in the brains of young medaka fish; *in situ* hybridization showed that *p53* was expressed selectively in at least 12 proliferation zones. We also compared the number of BrdU-positive cells present in the whole telencephalon of wild-type (WT) and *p53* mutant fish. Immediately after BrdU exposure, the number of BrdU-positive cells did not differ significantly between them. One week after BrdU-exposure, the BrdU-positive cells migrated from the proliferation zone, which was accompanied by an increased number in the WT brain. In contrast, no significant increase was observed in the *p53* mutant brain. Terminal deoxynucleotidyl transferase (dUTP) nick end-labeling revealed that there was no significant difference in the number of apoptotic cells in the telencephalon of *p53* mutant and WT medaka, suggesting that the decreased number of BrdU-positive cells in the mutant may be due to the suppression of proliferation rather than the enhancement of neural cell death. These results suggest that *p53* positively regulates neurogenesis via cell proliferation.

1. Introduction

In the adult brain of teleosts, most proliferating cells are observed in well-defined zones of the brain (called proliferation zones) [1]. The whole brain of teleosts, such as medaka (*Oryzias latipes*) [2], zebrafish (*Danio rerio*) [3], gymnotiform fish (*Apteronotus leptorhynchus*) [4], and three-spined stickleback (*Gasterosteus aculeatus*) [5], contains a large number of proliferation zones. Previously, we identified 17 proliferation zones (Zones A–Q) in the adult medaka brain using sexually mature fish (age, more than 3 months) and demonstrated that there is persistent cell proliferation in these brain regions in the adult brain, irrespective of sex, body color, or growth environment [2]. Further, the distribution of proliferation zones is largely conserved among some fish species [2], suggesting that this distribution in the adult teleost brain is important for the maintenance and development of the fundamental structure of fish brains [2].

To clarify the molecular basis underlying adult neurogenesis in teleost fish, we focused on medaka *p53* mutants [6]. p53 is a sequence-specific DNA-binding transcription factor that induces apoptosis or cell cycle arrest in response to genotoxic stress, thus preventing DNA mutations from transmitting to progeny cells [7]. In murine brains, the *p53* null mutation enhanced cell proliferation in the adult subventricular zone (SVZ) and, in association with their rapid differentiation, resulted in an increased number of newborn neurons and oligodendrocytes [8–11]. Here, we show the distribution of proliferating zones largely overlapped that of *p53*-expressing cells in the medaka brain. Furthermore, the medaka *p53* null mutant phenotype suggested that *p53* positively regulates neurogenesis.

2. Materials and methods

2.1. Fish

Medaka fish (*O. latipes*), Cab strain and *p53* mutants [6], were maintained in groups in plastic aquariums (12 × 13 × 19 cm). Sexually immature medaka fish (approximately 1 month after hatching; body length, 15 mm) without secondary sexual characteristics were used for immunohistochemistry and *in situ* hybridization studies.

2.2. Detection of mitotic cells in the young medaka brain

The detection of mitotic cells was performed as described previously [2]. Dividing cells were labeled with 5-bromo-29-deoxyuridine (BrdU), by exposure to water containing 1 g/L BrdU (Sigma Aldrich, Tokyo) for 4 h. BrdU-positive cells were detected by anti-BrdU immunohistochemistry. Paraffin sections (10-μm thick) were cut with a microtome (LR-85, Yamato Kohki, Tokyo). Immunostain-

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ing was performed following standard procedures. Cell nuclei were detected with DAPI staining (Invitrogen, Tokyo). BrdU-positive cells were counted as described previously [2].

2.3. In situ hybridization

In situ hybridization of tissue sections was performed as described previously [12,13]. The *p53* cDNA fragment was amplified with forward primer 5′-**TGTTACATTTTATAGCTGTGGAGCA**-3′ and reverse primer 5′-**TTGGGCTGAAAACAGCACAACCATAGTT**-3′ using cDNA clone number orbr44c15 (Medaka National BioResource Project [14]) as a template. The digoxigenin (DIG)-labeled riboprobes were synthesized by T7 or SP6 polymerase with a DIG labeling mix (Roche, Tokyo) from a template containing the *p53* cDNA fragment. Micrographs were obtained with a BX50 optical microscope (Olympus, Tokyo). The micrographs were processed with Photoshop software (Adobe, San Jose, CA).

2.4. TUNEL (TdT-mediated dUTP-biotin nick-end labeling) staining

Medaka brains were fixed in 4% paraformaldehyde (prepared in phosphate buffer saline) overnight and embedded in paraffin. Each brain was sliced into 10-μm sections. Apoptotic cells were detected using a DeadEnd™ Fluorometric TUNEL System (Promega, Tokyo), according to the manufacturer's protocol.

3. Results

3.1. Distribution of proliferation zones and p53-expressing cells in brains of young medaka fish

To elucidate the molecular basis underlying cell proliferation in the medaka brain, we focused on medaka *p53* [6]. *p53* is expressed in proliferating and newly formed neurons of the adult murine brain [15]. To examine whether medaka *p53*-expressing cells were

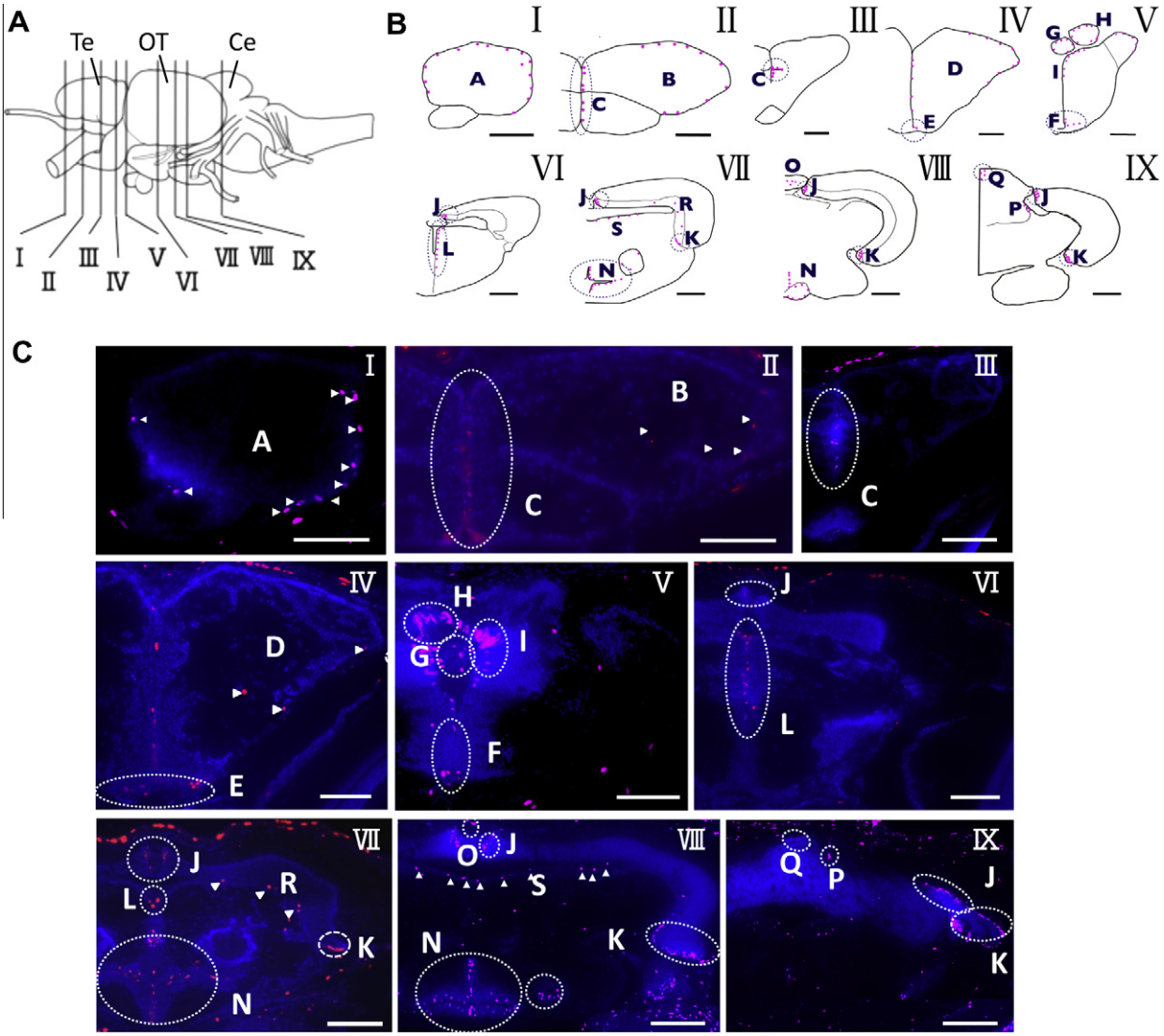

Fig. 1. Mapping proliferation zones in the brain of young medaka. (a) Schematic drawing of the lateral view of the medaka brain. The positions of sections I–IX are indicated by the lines. Te: telencephalon, OT: optic tectum, Cb: cerebellum. (b) Schematic representation of the distribution of the 18 proliferation zones. Red dots indicate proliferating cells. Zone A: marginal zones of the anterior part of the telencephalon, Zone B: marginal zones of the dorsolateral part of the telencephalon, Zone C: medial zones of the telencephalon, Zone D: dorsolateral part of the posterior part of the telencephalon, Zones E and F: preoptic area, Zone G: pineal body, Zone H: habenular nucleus, Zone I: ventromedial nucleus, Zones J and K: optic tectum, Zone L: anterior part of marginal zones of third ventricular zone, Zone N: hypothalamus, Zones O–Q: cerebellum, Zone R: periventricular grey zone (layer 3), and Zone S: Ependyme. Roman numerals in the panels correspond to section numbers shown in (a). Proliferation zones were determined according to the medaka fish brain atlas (Supplemental Fig. 1). (c) Distribution of BrdU-positive cells in the different proliferation zones. A magnified photo for zones P and Q (cerebellum) in panel XI is shown in Supplemental Fig. 2. Scale bars indicate 100 μm.