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Diethylnitrosamine-induced expression of germline-specific genes and pluripotency factors, including *vasa* and *oct4*, in medaka somatic cells

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ABSTRACT

Various methods have been developed to reprogram mammalian somatic cells into pluripotent cells as well as to directly reprogram somatic cells into other cell lineages. We are interested in applying these methods to fish, and here, we examined whether mRNA expression of germline-specific genes (*vasa*, *nanos2*, *-3*) and pluripotency factors (*oct4*, *sox2*, *c-myc*, *nanog*) is inducible in somatic cells of Japanese medaka (*Oryzias latipes*). We found that the expression of *vasa* is induced in the gut and regenerating fin by exposure to a carcinogen, diethylnitrosamine (DEN). Induction of *vasa* in the gut started on the 5th day of treatment with >50 ppm DEN. In addition, *nanos2*, *-3*, *oct4*, *sox2*, *klf4*, *c-myc*, and *nanog* were also expressed simultaneously in some *vasa*-positive gut and regenerating fin samples. Vasa-positive cells were detected by immunohistochemistry (IHC) in the muscle surrounding the gut and in the wound epidermis, blastema, and fibroblast-like cells in regenerating fin. In *vasa:GFP* transgenic medaka, green fluorescent protein (GFP) fluorescence appeared in the wound epidermis and fibroblast-like cells in the regenerating fin following DEN exposure, in agreement with the IHC data. Our data show that mRNA expression of genes relevant to germ cell specification and pluripotency can be induced in fish somatic cells by exposure to DEN, suggesting the possibility of efficient and rapid cell reprogramming of fish somatic cells.

1. Introduction

The development of induced pluripotent stem (iPS) cells has made it possible to reprogram somatic cells into an embryonic stem (ES) cell-like state by exogenous expression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* [1]. The field of direct cell lineage reprogramming has undergone rapid development in recent years. It is now possible to directly convert somatic cells into a variety of other cell types using specific factors [2]. iPS cells as well as direct cell reprogramming technologies have been used to successfully reprogram somatic cells in animals and humans [3]. Cell reprogramming technologies are of great importance in medicine and drug screening. Such technologies would also be very useful in fish, since they could be applied toward the cryopreservation of valuable strains. Although considerable effort has been expended, efficient methods for programming fish somatic cells into a long-lasting pluripotent state have not been developed [4].

The precise mechanism underlying the process of somatic cell reprogramming remains unclear, although it is now known that pluripotent stem cells and cancer cells share many characteristics [5]. During pluripotent cell derivation, the capacity for self-renewal is necessary for somatic differentiated cells, which is also critical for carcinogenesis [6]. Moreover, epigenetic regulation during cancer development induces pluripotency and confers the capacity for self-renewal on somatic cells [7]. These findings suggest that there is a functional relationship between cancer development and somatic cell reprogramming [8].

In zebrafish, germ plasm containing *vasa* and *nanos* mRNAs is stocked maternally in the oocytes, and embryonic cells containing the germ plasm differentiate into primordial germ cells (PGCs) [9,10]. Therefore, it is thought that even if ES-like or iPS-like cells are established in fish, they do not contribute to the germ cell lineage when transplanted into host embryos. Hence, the ability to induce germline-specific genes such as *vasa* and *nanos* [11] along

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with pluripotency factors would contribute to the development of a new system for manipulating germ cells in fish. *Vasa* is both over-expressed in cancer cells and associated with malignant tumor growth [12,13]. *Nanos* is among a group of cancer-germline gene that are activated in a significant fraction of tumors of various histologic types [14,15]. Thus, we examined whether the expression of germline-specific genes and pluripotency factors could be induced in somatic cells of fish in response to transient exposure to a carcinogen.

Diethylnitrosamine (DEN) can induce tumors in a variety of organs, including the liver, skin, gastrointestinal tract, and respiratory system, and is widely used to induce cancer experimentally in fish as well as mice [16]. In this study, we used DEN to examine whether mRNA expression of various germline-specific genes (*vasa*, *nanos2*, *-3*) and pluripotency factors (*oct4*, *sox2*, *klf4*, *c-myc*, *nanog*) is induced in somatic cells of Japanese medaka (*Oryzias latipes*) following exposure to the carcinogen DEN. We found that all of these genes were induced in the gut and regenerating fins by the fifth day of DEN exposure.

2. Materials and methods

2.1. Experimental fish

Japanese medaka were purchased from a local pet shop. The medaka *vasa:GFP* Tg line, Tg(olvas-GFP) (ID: TG870) [17], in which expression of GFP is driven by the medaka *vasa* promoter, was obtained from the NBRP-Medaka. Fish were maintained at 26°C in standard tanks equipped with a circulation system optimized for medaka and zebrafish culture and fed commercial fish food and *Artemia nauplii*. Fish maintenance and experimental protocols were approved by the Tohoku University Committee of Animal Experiments.

2.2. DEN treatment

DEN (Sigma-Aldrich) was added to 1 L of water to a final concentration of 20, 50, or 100 ppm. Fish were exposed to DEN for 3–7 days. Females were used for DEN-exposure experiments, and their ovaries were used as positive controls for *vasa* expression in reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) analyses. Prior to DEN exposure, the caudal fin was amputated at approximately 1 mm from the fringe.

2.3. RT-PCR

Total RNA was extracted using an ISOGEN with Spin Column kit (Nippon Gene) and treated with DNase I (Takara) to remove contaminating genomic DNA. Regenerating fin explants of approximately 2 mm in width were cut from the regenerating area of the fin after amputation. The extracted total RNA (0.5 μg) was reverse transcribed to first-strand cDNA using ReverTra Ace (Toyobo) with a random hexamer primer. The primer sequences used for PCR (*vasa*, *nanos2*, *nanos3*, *oct4*, *sox2*, *klf4*, *c-myc*, *nanog*, *β-actin*) are shown in Table 1. PCR was performed at an annealing temperature of 58°C with 40 cycles for *vasa* and *nanos2*, *-3* and with 30 cycles for *β-actin*. Thirty-five cycles were used for the other genes.

2.4. IHC

The gut and regenerating fin were fixed with Bouin's fixative and cut into 7-μm paraffin sections. To guarantee the specificity of IHC analyses, DEN-untreated and DEN-treated tissue and ovary (positive control) sections were mounted on a single slide to ensure that the exact same IHC conditions were used for each group. In addition, both fluorescent- and enzyme-labeled secondary antibodies were used. Sections were incubated for 1 h with anti-zebrafish Vasa antibody (GTX128306, GeneTex) diluted 1:500 with phosphate-buffered saline containing 0.1% Tween 20 (PBST); we ascertained that the anti-zebrafish Vasa antibody used reacts specifically with the oocytes in female medaka (data not shown). After washing with PBST, the sections were incubated with Histofine Simple Stain AP (MULTI) (Nichirei Biosciences) or Alexa Fluor 488 goat anti-rabbit SFX (A31627, Invitrogen). The sections incubated with Histofine were developed using NBT/NCIP stock solution (Roche). After washing, sections incubated with Alexa Fluor 488 were observed under a fluorescent microscope. Hematoxylin and eosin (HE) staining was carried out according to the standard protocol.

2.5. Photography

Photomicrographs were taken using a Leica DFC500 or 450C digital camera (Leica) attached to a Leica DM2500 or Leica DM IRB microscope. The captured images were processed using Photoshop CS5 (Adobe).

3. Results

3.1. Induction of germline-specific genes following DEN exposure

To test the ability of DEN to induce germline-specific genes in somatic cells, we first incubated medaka in 100 ppm DEN for 6 days and compared *vasa* expression in the regenerating fin, ovary, brain, liver, gill, muscle, and gut using RT-PCR. All three fish examined began to express *vasa* in the gut after DEN exposure (Fig. 1A). In tests involving a larger number of fish ($n = 22$), we observed that DEN induced *vasa* expression in both the gut and regenerating fin in some of the examined fish (Fig. 1B). The ratio of fish that expressed *vasa* in the gut after DEN exposure was 72% (16 of 22), and the ratio in the regenerating fin was 36% (8 of 22) (Fig. 1C). Induction of *vasa* expression in the regenerating fin was observed in the fish that were positive for gut expression.

To examine the time- and dose-dependence of *vasa* induction by DEN, medaka were incubated in 20, 50, or 100 ppm DEN, and gut samples were examined using RT-PCR on the first, third, fifth, and seventh days of incubation. The data showed that *vasa* expression was initiated between days 3 and 5 at a DEN concentration >50 ppm (Fig. 1F).

Induction of the medaka germline-specific genes *nanos2* and *nanos3* [18] was also observed in the gut and regenerating fin of some of the DEN-exposed fish but at a ratio lower than that of *vasa* expression (7 of 22 and 4 of 22, respectively, versus 16 of 22 and 8 of 22, respectively) (Fig. 1C). In the regenerating fins, *nanos2* and *nanos3* induction by DEN was restricted to samples positive for *vasa* expression (4 of 8 *vasa*-positive samples) (Fig. 1C).

Fin amputation induces blastema formation and can be a stimulation for reprogramming. However, in the absence of DEN, the regenerating fins did not express *vasa*, *nanos2*, or *nanos3* ($n = 8$) (Fig. 1D). DEN stimulation alone (without amputation) induced the expression of *vasa* (1 of 9), and *nanos3* (2 of 9) in the fin, although the ratios were markedly lower compared with combined DEN stimulation and amputation (Fig. 1E, G).

3.2. Induction of pluripotency factors by DEN

We next examined whether the pluripotency factors *oct4* and *nanog* are induced together with *vasa* and *nanos2*, *-3* by DEN exposure. In the gut, *oct4* and *nanog* were simultaneously

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