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ANALYSIS OF OSTEOPOROSIS-LIKE PHENOTYPE OF THE RANKL: HSE:CFP HOMOZYGOUS TRANSGENIC MEDAKA FISH LINE C1C6 MODEL FOR OSTEOPOROSIS

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Abstract. The transgenic medaka *rankl:* HSE:CFP was generated to model human osteoporosis, a bone disease characterized by reduced bone mass, deteriorated bone structure, and high risk of bone fragility. The fish expresses the transgene *rankl* encoding for the osteoclast-stimulating factor Rankl under the control of a heat-inducible promotor so it could exhibit damaged mineralized bone structures or an osteoporosis-like phenotype upon a heat-shock. In the previous study, the original transgenic fish carrying multiple insertions of the transgene were segregated into genetically homogeneous lines, each had one insertion of the transgene, including the line named c1c6 that was heterozygous for the transgene. However, for the studies to screen for substances of anti-osteoporosis potential, homozygous fish is very important to provide large numbers of heterozygous embryos. In this study, we testcrossed 12 fish of the F5 generation, which were offspring obtained from inbreeding of heterozygous F4 siblings, with wild type fish and found 3 female and 3 male F5 fish homozygous for the transgene. These F5 individuals were inbred to generate homozygous fish of F6 and F7 generations. Osteoporosis-like phenotype and levels of bone damage of these homozygous fish larvae were analyzed and compared with those of heterozygous larvae. Results showed that homozygous c1c6 Rankl fish had stable osteoporosis-like phenotype but a lower level of bone mineralized damage (70,6% for F6 and 65,8% for F7) than heterozygous fish (88,4% for F6 and 81,1% for F7). These homozygous fish are essential for further studies to screen for anti-osteoporosis substances.

Keywords: medaka, *rankl*: HSE: CFP, c1c6, homozygous, heterozygous, osteoporosis.

1. Introduction

The medaka fish *(Oryzias latipes)*, a small teleost fish that is commonly found in rice fields and small water bodies in East Asia and Southeast Asia, has been studied and used

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in laboratories worldwide for physiological and pathological studies of many human diseases due to its high similarity with humans in biological processes and its good experimental characteristics such as high fecundity, short generation time, external fertilization, easy maintenance, and low maintenance cost [1]. In particular, a small-sized and transparent embryo that develops outside the body facilitates genetic manipulation so that transgenic and mutant fish can be easily generated and life processes can be simply observed at cellular levels using fluorescence imaging tools [2-5].

Medaka fish is also an important animal model for bone research. It shares similar molecular and cellular mechanisms underlying bone metabolism with humans [6, 7] as indicated by the similarity in formation and differentiation of bone-forming cells expressing osteoblast-specific genes at different stages, such as twist [8], collagen 10a1 [2], osterix [3] and osteocalcin [9] as well as in the differentiation of osteoclasts [4, 6]. Direct interactions between bone-resorbing cells osteoblasts and bone-resorbing cells osteoclasts have been observed and recorded *in vivo* in medaka [6, 10].

Tight interaction and balance in activities of osteoclasts and osteoclasts ensure normal bone renewal and thus maintain bone health and development. Bone diseases resulted from imbalance in activities of these two cell types, especially increased bone resorption may lead to osteoporosis, a common bone disease manifested by impaired bone density and structure, and high risk of bone fragility [11]. Research on osteoporosis needs good animals to model the disease to develop better and more effective drugs and therapies for an increasing number of patients worldwide [12, 13]. Medaka fish has been proved in many studies as a promising and suitable model [14].

RANKL (Receptor Activator of Nuclear Factor kappa-β ligand) is a protein encoded by the *RANKL* gene expressed by osteoblasts. Binding of RANKL to its receptor RANK on the surface of osteoclast precursors triggers RANKL/RANK pathway promoting formation, differentiation, and activation of osteoclasts [15-17]; thus, increased RANKL can lead to increased bone destruction and osteoporosis. In 2012, To et al. at the National University of Singapore generated the transgenic medaka *rankl:* HSE: CFP as a model for osteoporosis by introducing into the genome of wild-type fish a heat-inducible bidirectional promotor that simultaneously controls the expression of two genes, one encoding for Rankl and the other for the cyan fluorescent reporter protein CFP [6] (*hereinafter this* fish can be called Rankl, or c1c6, or Rankl c1c6 fish). Therefore, after a heat-shock induction at 9 days of age, the fish (identified by CFP fluorescent signal) expressed ectopic Rankl that caused formation and activation of osteoclasts which destructed early mineralized bone structures, especially vertebral bodies and neural arches of the vertebrae [6, 18, 19]. This fish has been maintained and optimized to be used as a model to screen for substances with the potential to be developed into anti-osteoporosis drugs [19].

In a previous study we segregated the original *rankl*: HSE: CFP fish having multiple insertions or copies of the transgene *rankl* into three fish lines determined by Mendelian segregation ratio; each line was genetically homogeneous with a single copy of the transgene and named c1c6, c1c7, or c1c8. These three fish lines had three different levels of bone damage under the same heat-shock condition; namely, the c1c6 had mild, the c1c8 had moderate, and the c1c7 had severe phenotype [18]. Depending on the

experimental design, a fish line with an appropriate degree of bone damage should be used. However, these lines were firstly created in a heterozygous genotype for the transgene [18], so each of these fish individuals, when crossed with a wild-type fish, could provide only 50% Rankl transgenic offspring. This limits experimental progress that requires large numbers of transgenic fish. Therefore, in this study we continued to testcross the fish to generate homozygous c1c6 fish and analyzed their bone damage phenotype, aiming to ensure an adequate supply of osteoporosis fish model for further studies.

2. Content

2.1. Methods and materials

** Fish lines and fish maintenance*

The study used medaka *rankl*:HSE:CFP c1c6 heterozygous with the transgene [18] and wild-type fish. Fish were raised and maintained according to established procedures described previously [18, 20, 21] that set the temperature at 28-30 ^{0}C, light cycles of 14 h light and 10 h dark. Embryos were screened for expression of fluorescent reporter CFP at 11 days post fertilization (dpf), that was, 2 days after heat-shock.

** Procedures for the screening of homozygous Rankl fish*

The Rankl transgenic sibling fish of F4 generation heterozygous for the transgene were inbred to obtain F5 offspring that included both homozygous and heterozygous fish. Each F5 fish individual was testcrossed with wild-type fish then F6 embryos were collected, heat-shocked at 1 day after fertilization (dpf) and checked for CFP transgenic signal at 2dpf. If 100% of F6 fish embryos were CFP positive, then their F5 parent was homozygous for the transgene. These homozygous individuals were further maintained and inbred to produce homozygous fish of F6 and F7 generations. The stability of osteoporosis-like phenotype and level of bone damage of homozygous fish were evaluated in fish larvae of F6 and F7 generations, in comparison with those of heterozygous fish [18, 20, 21].

** Heat-shock procedure to induce osteoporosis-like phenotype*

To induce osteoporosis-like phenotype, transgenic fish larvae at 9dpf were heat-shocked at 39^{o}C for 90 minutes then maintained until 11 days of age when they were fixed and their mineralized bone structures were stained by alizarin red (2012) [6, 19].

** Staining of mineralized bone structures*

Fish larvae at 11 dpf were fixed and stained with alizarin red (Sigma A5533) to visualize mineralized matrix, as previously described [6, 19, 22].

** Quantification of the level of bone mineralization and bone mineralization damage*

The level of bone mineralization and bone mineralization damage of the fish larvae were determined via the Index of bone mineralization (I_M) and the Index of mineralization damage (I_D), respectively using the I_M method published previously [11]. Mineralized neural arches of the 11 dpf fish larvae were chosen as representative bone structures to be analyzed as bone damage occurred mostly in these structures of the Rankl fish larvae. I_M was defined as the sum of the lengths of the first 15 mineralized neural arches that is calculated by the formular $I_M = \sum_{k=1}^{15} L$, where k is the ordinal number of neural arch and

L is the length of each arch. Based on the I_M of Rankl fish and of WT fish, the Index of mineralization damage I_D of Rank fish was calculated by the formular $I_D = [I_M (WT) – I_M (Rankl)]/I_M (WT) \times 100$ %, where I_D is the percentage of mineralization damage of neural arches of an embryo, I_M (WT) is the Index of bone mineralization of wild-type fish, and I_M (Rankl) is Index of bone mineralization of the corresponding Rankl fish. I_M is inversely correlated to I_D.

** Statistical analysis*

Student t-tests (two-tailed, unequal variance) or one-way ANOVA followed by Tukey's multiple comparison test were used for comparing groups and determining significance, using Prism 5 (GraphPad Software Inc., San Diego, CA). Differences with a p-value less than 0.05 were considered statistically significant and marked with one asterisk (*); three asterisks (***) indicate $p < 0.001$, and four (****) indicate $p < 0.0001$. Results are presented as mean ± S.E.M. Data were selected by Thomson method which allowed I_M values in the range of Mean - ST <IM < Mean + ST (S: standard deviation; T: the value of Tau corresponds to the number of samples) (Outliers, John M. Cimbala, Penn State University).

2.2. Results and discussion

2.2.1. Screening for *rankl*:HSE:CFP c1c6 fish homozygous for the transgene

From inbreeding of sibling *rankl*:HSE:CFP heterozygous fish of c1c6 line of the F4 generation (from here on, it is called as Rankl fish or fish of generation denoted by the letter F and the number representing the ordinal number its generation), we obtained 12 F5 fish, including 6 females and 7 males to be screened for homozygous fish.

Homozygous F5 fish were determined by testcrossing each fish individual with wild-type (WT) using a ratio of 1 F5 female:1 WT male or 1 F5 male:2 WT females (to increase the number of offspring embryos). Resultant transgenic F6 embryos were identified and screened via CFP fluorescence signal after heat-shock; if 100% of F6 fish embryos expressing CFP then their F5 parent was homozygous, or if 50% of F6 embryos expressing CFP, then their F5 parent was heterozygous with the transgene. Results of the screening for F5 homozygous fish individuals are shown in Table 1.

Table 1. Results of testcrosses and screens for homozygous Rankl fish

F5 individual	Number of CFP^+ embryos	Number of CFP- embryos	*rankl* genotype of F5
C1c6F5c1 × WT	199	0	**homozygous**
C1c6F5c2 × WT	104	0	**homozygous**
C1c6F5c3 × WT	144	0	**homozygous**
C1c6F5c4 × WT	31	26	heterozygous
C1c6F5c5 × WT	22	26	heterozygous
C1c6F5d1 ×WT	16	18	heterozygous
C1c6F5d2 × WT	27	24	heterozygous
C1c6F5d3 × WT	29	10	heterozygous
C1c6F5d5 **× WT**	69	0	**homozygous**
C1c6F5d6 × WT	5	18	heterozygous
C1c6F5d7 **× WT**	137	0	**homozygous**

Thus, by testcrossing, we found 6 F5 fish individuals homozygous for the transgene including 3 females (c1c6F5c1, c1c6F5c2, c1c6F5c3) and 3 males (c1c6F5d4, c1c6F5d5, c1c6F5d7).

2.2.2. Osteoporosis-like phenotype of homozygous fish of F6 and F7 generation

After finding homozygous parent fish of the F5 generation, we continued to generate homozygous fish of the F6 and F7 generations by inbreeding within siblings of corresponding generations. Fish larvae at 9dpf were often used for the assessment of bone protective activity of tested substances, so we examined the pattern of mineralized bone damage and the stability of this phenotype in the F6 and F7 homozygous fish, compared with heterozygous fish larvae of the same generation.

Four groups of 9dpf homozygous and heterozygous larvae of F6 and F7 generations, and one group of WT fish were heat-shocked for 90 minutes at 39 ° C, then fixed and stained with alizarin red for mineralized structures at 11 dpf (Figure 1A). Images of the first 15 vertebrae of the spine of the representative fish of each group were taken and shown in Figure 1B.

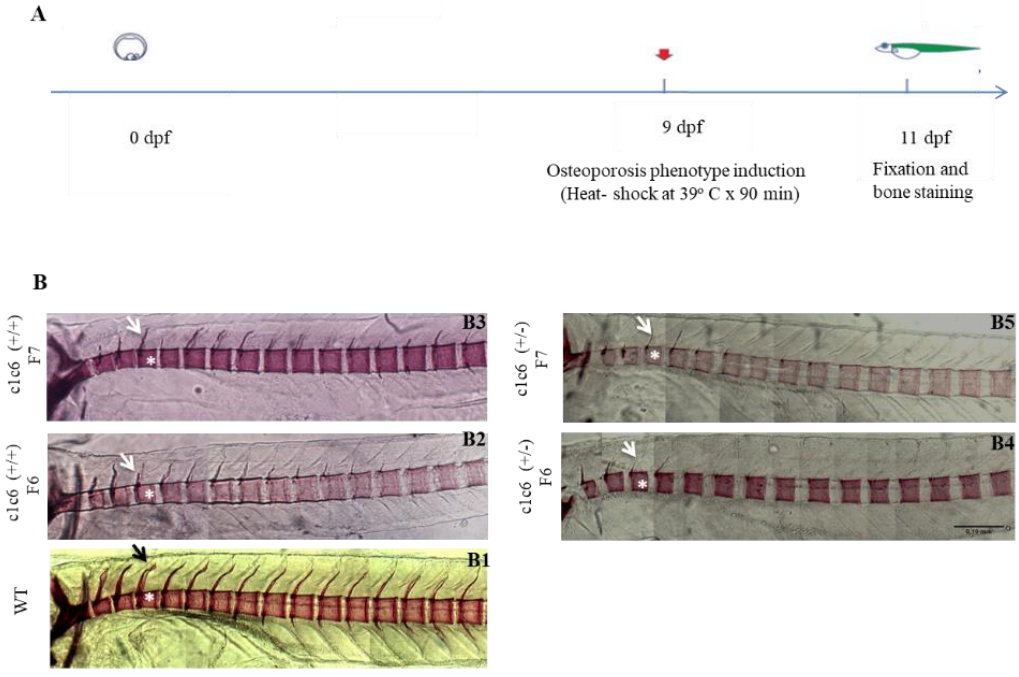

Figure 1. Bone lesion pattern of 11dpf homozygous fish larvae of the F6 and F7 generations, compared with that of heterozygous and wild-type control (WT)

*A. Timeline depicting a procedure for induction of osteoporosis-like phenotype in Rankl fish with the red arrow indicating heat-shock induction and fish in green symbolizing one with heat-shocked induced Rankl. B. Images of the first 15 mineralized vertebrae of fish representative for a group of B1. wild-type fish (WT); B2. F6 homozygous Rankl fish (c1c6 (+/+) F6); B3. F7 homozygous Rankl fish (c1c6 (+/+) F7; B4. F6 heterozygous Rankl fish (c1c6 (+/-) F6); B5. F7 homozygous Rankl fish (c1c6 (+ / +) F7). * indicates intact mineralized vertebral body, black or white arrow represents intact or damaged neural arch, respectively.*

Wild-type fish at 11dpf have intact vertebrae (Figure 1B1), each vertebra consisting of a vertebral body (asterisk in Figure 1B1) and a neural arch (black arrow in Figure 1B1). Homozygous fish of the F6 and F7 generations did have intact vertebrae (asterisks in Figure 1B2, B3) but their neural arches were partially damaged to different degrees (white arrows in Figures 1B2, B3). The bone lesion pattern of the F6 and F7 homozygous fish are comparable indicating the stability of osteoporosis-like phenotype of homozygous fish, but interestingly, homozygous fish showed less damage in neural aches than heterozygous fish of the same generation as the heterozygous F6 and F7 fish had neural arches that almost completely damaged (white arrows in Figures 1B4, B5).

2.2.3. Level of mineralized bone damage of homozygous fish

To quantify the level of bone damage of homozygous fish and compare it with heterozygous fish, we measured the lengths of neural arches and calculated the Index of bone mineralization (I_M) and Index of mineralization damage I_D of fish groups (each group had a number of fish as indicated by n in the Figure 2A) following I_M method. The mean value of the I_M of each fish group was calculated as the mean of the total length of the 15 mineralized neural arches of all fish in the group (Figure 2A). From the the I_M values of Rankl and WT fish, the Index of bone damage of each Rankl fish group I_D was also determined (Figure 2B).

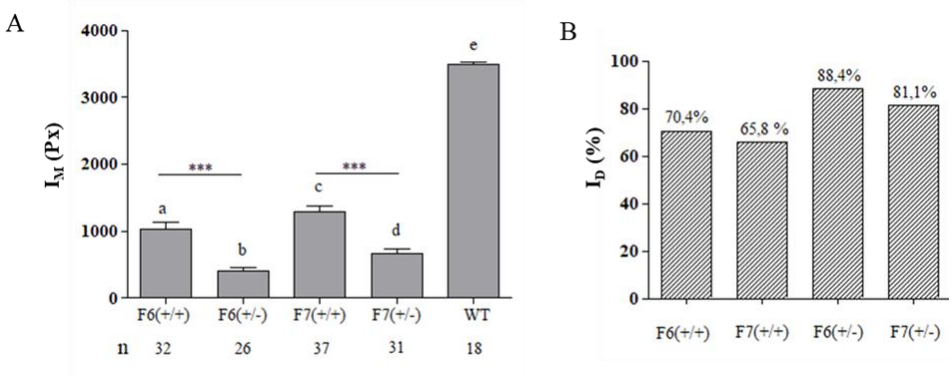

Figure 2. Index of bone mineralization (I_M) and Index of mineralization damage (I_D) of homozygous fish compared with heterozygous fish

*A. Mean values of Index of bone mineralization I_M of F6, F7 homozygous (+/+), heterozygous (+/-) and wild-type fish groups (WT). a - e: IM value of the corresponding fish group, which is 1029.84; 403,184; 1190,156; 658,49; 3486,53, respectively. (Px). Px: pixels. ***p < 0.001, n: number of fish in the respective group. Bars indicate S.E.M. B. Index of mineralization damage of Rankl fish group.*

The Index of bone mineralization of the F6 and F7 homozygous fish larvae were 1029,84 and 1190,156, respectively (Figure 2A), corresponding to the Index of mineralization damage I_D of 70,6% for F6 and 65,8% for F7 fish (Figure 2.B). The difference in the level of bone mineralization of homozygous fish in the F6 and F7 generations was not statistically significant (p> 0,05), indicating the stability in osteoporosis-like phenotype and the degree of bone damage of homozygous fish through generations. However, as seen in Figure 2, the Index of bone mineralization I_M of homozygous fish is significantly higher than that of heterozygous fish of the same

generation (I_M F6 (+/-): 403,18; I_M F7 (+/-): 658,49), so the Index of bone damage of homozygous fish is lower than that of heterozygous fish (I_D F6 (+/): 88,4%; I_D F7 (+/-): 81,1% (F7) (Figure 2B).

Thus, in this study, we have generated c1c6 *rankl*: HSE: CFP fish homozygous with the transgene *rankl* from the first six F5 homozygous individuals identified. Homozygous fish were confirmed because all (100%) of their offspring obtained from testcrosses with wild-type fish showed CFP signals. Currently, we have maintained the fish to F6 and F7 generations with a total of about 30 individuals, which are very important fish resources for our further studies.

Osteoporosis-like phenotype and level of bone damage of homozygous fish were determined to be stable over generations as seen incomparable level of bone damage of fish of the F6 and F7 generations. However, what we have yet to explain is why homozygous fish with two versions of the transgene *rankl* in the genome exhibited weaker osteoporosis-like phenotype and lower level of bone damage than heterozygotes which had only one version of the transgene. A newly published study on the *rankl*:HSE:CFP fish showed that Rankl is a factor that stimulates the transformation and differentiation of a group of macrophages into osteoclasts, causing these cells to migrate to bone tissue and thus leading to the damaged mineralized bone matrix or osteoporosis-like phenotype [4]. Rankl has been also known to be involved in regulating and controlling many life processes, especially ones involving the regulation of immune functions and organ development [23]. High level of Rankl expression in homozygous fish may activate these processes simultaneously, causing interaction and inhibitory effects on Rankl/rank pathway for osteoclastogenesis so that homozygous fish exhibited a lower level of bone damage that heterozygous ones. However, we have successfully generated the *rankl*: HSE: CFP medaka of c1c6 line homozygous with the transgene that exhibited a stable osteoporosis-like phenotype. These fish will serve as important models for our study in screening for natural and synthetic substances with anti-osteoporosis potential. Specifically, homozygous c1c6 fish will be crossed with wild-type fish to provide 100% of heterozygous c1c6 offspring larvae for experimenting at a large scale.

3. Conclusions

The *rankl*:HSE:CFP homozygous medaka fish line c1c6 generated in this study had stable bone damage patterns in the mineralized neural arches and a level of bone damage of about 70%. This ensures the use of these fish as important osteoporosis models for further studies to screen for the substances with anti-osteoporosis potential.

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