Collagen10a1 GEN EXPRESSION AND BONE MINERALIZATION IN THE *col10a1*:nlGFP TRANSGENTIC MEDAKA FISH LARVAE

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ABSTRACT

Objective*: To assess the expression level of the osteoblast-specific collagen10a1 gene and the degree of bone mineralization during the early larval stages of col10a1:nlGFP transgenic medaka fish to identify a specific developmental time window suitable for conducting bone anabolic effect evaluations of bioactive substances.* ***Methods:*** *Homozygous and hemizygous col10a1:nlGFP fish larval groups at 7, 9, 10, 11, and 14 days post fertilization (dpf) were used in the study (n=…/group). The fish were subjected to live staining with alizarin red S (ARS) dye to visualize their mineralized bone structures. Fluorescent images were captured using a fluorescent stereoscope to document both the GFP and ARS signals in the fish. The density of the GFP signal was utilized to assess the expression level of the collagen10a1 gene in osteoblasts, while the density of the ARS signal was used to evaluate the extent of bone mineralization. ImageJ software was employed for this analysis.* ***Results****: The intensity of GFP signal exhibited an upward trend as the fish grew from 7 to 9 dpf, followed by a gradual decline from 9 to 14 dpf. Along with that, the intensity of ARS signal increased as the fish grew from 7 to 10 dpf, followed by a gradual decrease from 10 to 14 dpf. These findings indicate that the optimal developmental window for conducting tests on the fish with bioactive compounds, utilizing a stereo fluorescence microscope, is between 7 and 9 dpf.*

Key words: *medaka, transgenics, col10a1, osteoblasts, mineralization, GFP.*

1. INTRODUCTION

In vivo studies conducted on bone and bone disease typically utilize mammalian models such as mice, rabbits, dogs, and primates [1]. However, more recently, the use of medaka fish *(Oryzias latipes)* has gained momentum and has emerged as a promising alternative to mammalian models [4, 5]. Medaka fish exhibit bone metabolism mechanisms at both the molecular and cellular levels with striking similarities to those found in humans [4, 5]. Moreover, the fish offers various advantages as laboratory animals, including ease of care, prolific egg production, rapid growth period, external fertilization, transparent embryos, and the ability to perform techniques and observe bone cell activity in live fish in real time [4, 5].

In 2013, researchers at the National University of Singapore generated the transgenic medaka fish using the *col10a1*:nlGFP construct. This construct enabled the expression of the *colagen10a1 (col10a1)* gene through the production of green fluorescent protein (GFP) in osteoblasts, the bone forming cells [3]. The transgene construct consisted of a 5.865 kb promoter region from the medaka *col10a1* gene, followed by the GFP coding sequence and a nuclear localization signal (nlGFP). The construct was inserted into the medaka genome using the meganuclease transgenic technique. It was observed that the *col10a1* gene is expressed early during the differentiation of osteoblastic cells in the mineralized bone structures of the fish. It appears both before and during the mineralization of the vertebral bodies, thus suggesting that it could serve as an indicator for bone formation in fish [6]. Osteogenic cells appear as individually spaced cells located on the outer surface of the notochord and

mineralized structures. Therefore, the *col10a1*:nlGFP transgenic medaka fish model is used in studies on bone formation and osteoblasts in relation to bone diseases and to evaluate the bone protective and bone anabolic effects of bioactive compounds for drug development [2].

The *col10a1*:nlGFP fish has been raised and maintained in our laboratory since 2013. Recently, our group has examined and identified the stability of the *col10a1* transgene of the fish and test crossed them to obtain homozygous and hemizygous fish with the transgene [6]. Given that investigations examining the impacts of substances on bone formation are commonly carried out on fish larvae, which refers to the period from post-hatching (around 7 days) to approximately two weeks of age, our study aimed to determine the optimal time frame during larval development for evaluating the effects of substances. To this end, we measured the level of expression of the *col10a1* gene via the density of GFP signal and assessed the level of bone mineralization through ARS signal of the fish at various time points during the early larval stages, seeking to identify the developmental time window suitable for conducting bone anabolic effect evaluations of bioactive substances.

2. MATERIAL AND METHODS

2.1. Fish lines and fish maintenance

In this study, homozygous and heterozygous *col10a1*:nlGFP were used [6]. Fish were maintained following established procedures [2] with temperature set at 28ºC using an air conditioner and LED lighting cycles of 14-h light-10-h dark. Fish embryos were raised in E3 medium as previously reported [2].

2.2. Fish crossing and embryo collection

Male and female fish are kept together in the same tank with a ratio of 1 male: 3 females. After the fish spawns, embryos were collected into a petri dish. Wash the embryos with 1X E3 medium with a little methylene blue added to avoid mold growth, unfertilized and dead embryos were removed.

2.3. Live bone staining with Alizarin red S and fluorescent imaging

Fish were stained with ARS (0.03%, 3 hours) in a 24-well plate at a density of 5 fish per well. After staining, the fish was washed with E3 water, left to rest for 1 hour and then taken for imaging. Before taking photos, fish were anesthetized with tricaine (0.03%) and mounted in a 35mm glass-bottomed petri dish with low melting agarose (0.15%) [4, 6].

col10a1:nlGFP expression and ARS stained mineralized bone in fish larvae were imaged by a fluorescent stereoscope (STEREO Discovery V8) using GFP and RFP filter settings, respectively. Images were captured at 8X magnification using BUC5F-2000C Microscope Digital Camera (Bestcope) and analyzed using ImageJ software [6].

2.5. Statistical analysis

One-way Anova was used to evaluate differences in GFP density as well as ARS density between fish groups. The level of significance was set as follows: *0.01 < p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. Graphs were created using Graphpad Prism 9 software.

3. RESULTS

3.1. Osteoblastic GFP intensity in *col10a1*:nlGFP homozygous and hemizygous fish at 7, 9, 10, 11, 14 days of age

Figure 1 shows representative fluorescent images taken for *col10a1*:nlGFP homozygous (+/+) and hemizygous (+/-) fish of 7, 9, 10, 11, 14 dpf. GFP signal was observed in bone

structures of the head, the vertebral column and the tails of both (+/+) and (+/-) tested fish. The vertebral column consists of vertebrae, each when fully developed has a vertebral body and a neural arch and a hemal arch. In 7dpf fish, GFP was seen only in vertebral bodies and neural arches of some anterior vertebrae, while the signal was present in these bone structures of all vertebrae in 14dpf fish.

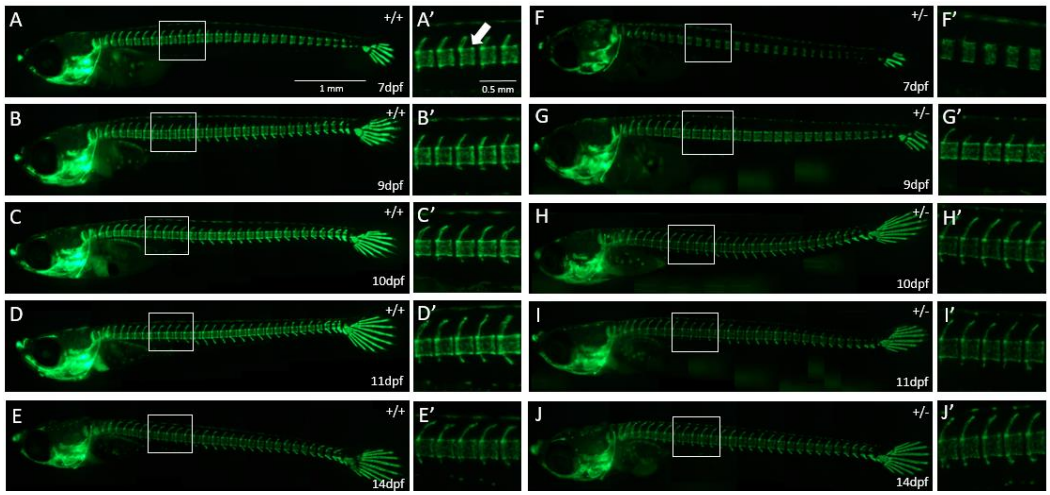

Figure 1. GFP signals of homozygous and hemizygous col10a1:nl GFP fish larvae at 7, 9, 10, 11, 14 dpf.

Fluorescent images of homozygous (+/+, A-E) and heterozygous (+/-, F-J) fish at days of age indicated by dpf; A'-E'; F'-J': Magnified images of five vertebrae (8-12) boxed in A-E; F-J, respectively.

Intensity of GFP signal of five vertebrae (vertebrae 8-12; boxed in Figure 1 A-E, A'-E' for homozygous fish; F-J, F'-J' for hemizygous fish) (n=7/group) was measured and chosen as representative for the expression of *col10a1* gene of each fish. Statistical analysis of GFP intensity of all tested fish groups (n=7/group) is shown in Figure 2. It reveals that GFP signal intensity of homozygous fish is significantly higher than that of hemizygous fish of the same age and in both homozygous and hemizygous fish, GFP signal intensity increased when the fish grew from 7 to 9dpf ($p < 0.0001$ for homozygous and $p < 0.001$ for hemizygous fish) and then gradually decreased when the fish grew older to 10, 11, and 14dpf.

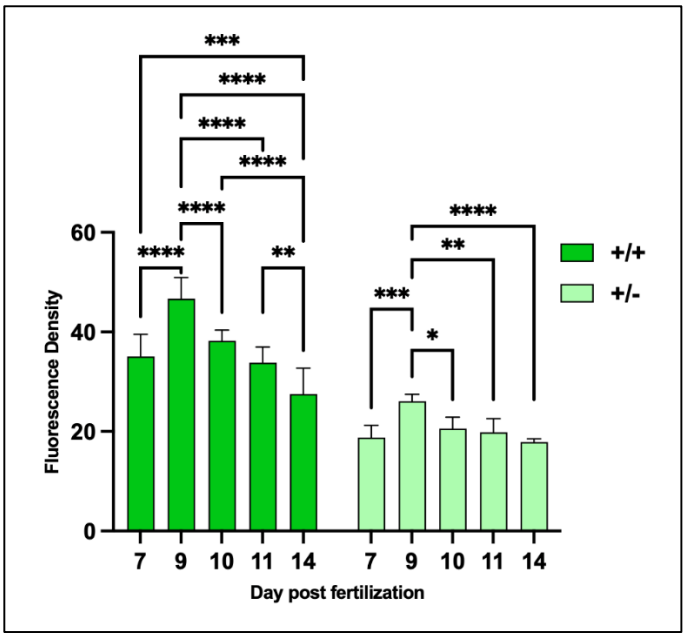

Figure 2. *Average GFP intensity of homozygous (+/+) and hemizygous (+/-) fish groups at 7, 9, 10, 11, 14 dpf (n=7/group).*

**0.01 < p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.*

3.2. Bone mineralization level of homozygous and hemizygous fish at 7, 9, 10, 11, 14 days of age

To evaluate the level of bone mineralization, we stained live fish larvae with the fluorescent dye alirazin red S (ARS). Representative fluorescent images taken for ARS signal of *col10a1*:nlGFP homozygous (+/+) and hemizygous (+/-) fish groups of 7, 9, 10, 11, 14 dpf are presented in **Figure 3.**

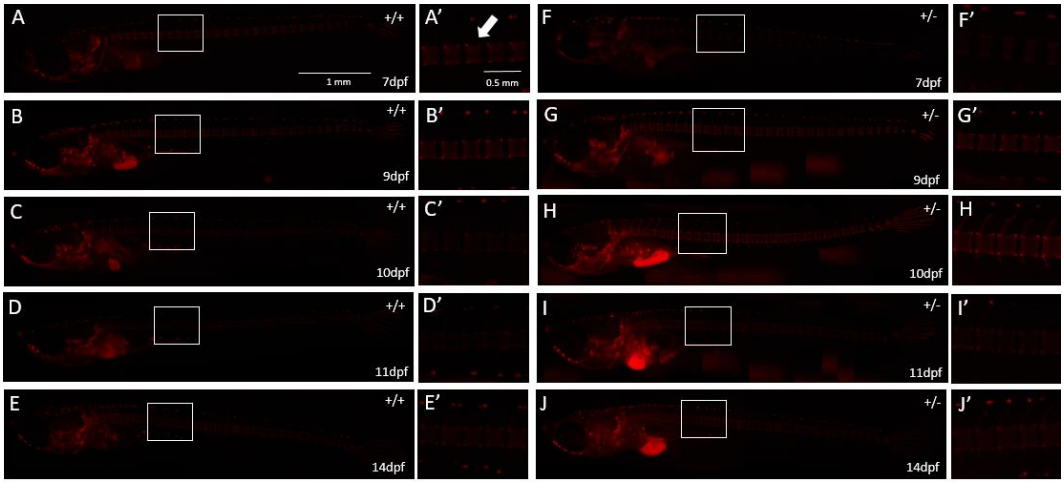

Figure 3: ARS fluorescence signals of homozygous and hemizygous col10a1:nl GFP fish larvae at 7, 9, 10, 11, 14 dpf.

Fluorescent images for ARS signal of homozygous (+/+, A-E) and heterozygous (+/-, F-J) fish at days of age indicated by dpf; A'-E'; F'-J': Magnified images of five vertebrae (8-12) boxed in A-E; F-J, respectively.

As expected, ARS signal was seen in mineralized bone structures in the head, the vertebral column and the tail of

fish of all tested groups. The ARS intensity seemed to correspond to the level of bone development in fish larvae (**Figure 3**).

We also chose to measure ARS signal intensity of 5 vertebrae (vertebrae 8-12) as representative for the level of mineralization of each fish. Average ARS density of fish groups (n=7/group) and statistical analysis are presented in **Figure 4**.

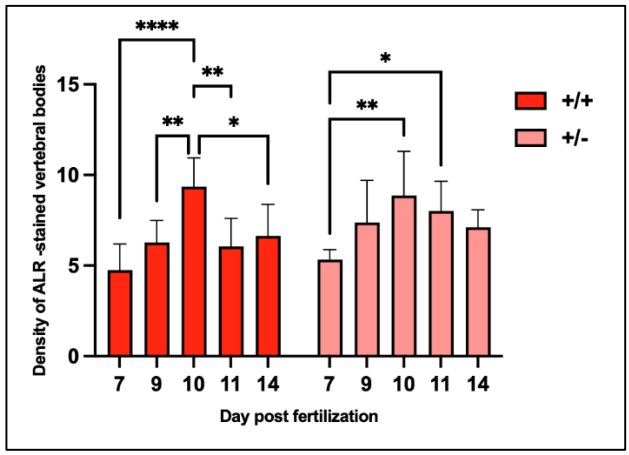

Figure 4. Average ARS signal level of homozygous (+/+) and hemizygous (+/-) fish groups at 7, 9, 10, 11, 14 days of age (n=7/group)

**0.01 < p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.*

The results show that the level of ARS signal increased as fish grew from 7 to 10 dpf ($p < 0.0001$ for homozygous fish and $p < 0.01$ in hemizygous fish), followed by a decreasing trend when the fish grew older to 11 and 14 dpf. When comparing the ARS density between homozygous and hemizygous fish of the same age, there is no significant difference.

4. DISCUSSION

In this study, we evaluated the level of *col10a1* expression in osteoblasts and level of bone mineralization via the intensity of GFP and ARS signals, respectively, of both homozygous and hemizygous *col10a1*:nlGFP transgenic medaka at larval stages of 7, 9, 10, 11, 14 dpf.

GFP signal was observed in the fish as previously described [3, 6], indicating the stability of the *col10a1*:nlGFP transgene in the fish.

Quantification of GFP and ARS intensity of the representative vertebrae of the fish groups indicates that the GFP intensity increased when the fish developed from 7 to 9 days of age and then decreased in the following days as the fish gets older. Along with that, the ARS intensity also increased in fish during the period of 7 - 10 days of age and decreased as the fish gets older.

The decrease in GFP intensity in fish 10 days old or older can be explained by the fact that GFP signal is the reporter for the expression of *collagen10a1,* which is expressed in osteoblast precursors, the early stage of osteoblast differentiation, so its expression decreases as osteoblasts become more differentiated and the fish gets older [3]. However, it is possible that this result is due to technical limitations when using a fluorescence stereoscope to image the fish. The fish grew and became thicker, which made it difficult for the fluorescence stereoscope to capture the GFP signal from deep within the vertebrae. The limitation of the imaging technique is a likely explanation for this decrease in GFP intensity, as the

measured ARS density also showed a similar decrease in fish older than 10 days old. This seems unreasonable, as ARS density represents the level of mineralization of the fish and bone mineralization should increase during larval growth. A confocal microscope using a laser light to scan deep tissue and provide high-resolution images may help to clarify this issue [4]. However, fluorescence stereoscope was the only way to image live fish larvae in our laboratory. Therefore, our results suggest to assess intensity of GFP and ARS signal of the *col10a1*:nlGFP larvae from 7-9 dpf to evaluate bone anabolic effect of bioactive substances when using a fluorescence stereoscope.

5. CONCLUSION

Osteoblast-specific *collagen10a1* expression increases in the *col10a1*:nlGFP fish larvae as the fish grow from 7 to 9 days post-fertilization (dpf), and the mineralization level of the fish increases as they grow from 7 to 10 dpf. The bone anabolic effect of bioactive substances can be tested on the fish during the period of 7 to 9 dpf using a fluorescence stereoscope.

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