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学 位 論 文 題 目

Induced androgenetic diploids without egg irradiation in loach and zebrafish

（卵の照射によらないドジョウとゼブラフィッシュの雄性発生二倍体誘起）

The eggs of fish have the possibility for chromosome manipulation such as induced polyploidy, gynogenesis, and androgenesis. The androgenesis is a type of reproduction that the progeny inherits chromosomes only from the male parent. Traditional way to induce androgenesis needs irradiation of eggs using gamma rays, X-rays, or UV rays to inactivate the egg nucleus; and during the irradiation, the special protection solution is needed to protect eggs from dryness and maintain the fertilization ability. So it is not suitable for the routine use. Morishima *et al.* (2011) reported a new method to induce haploid androgenesis by cold-shocking just fertilized eggs in loach. This method provides new possibilities for artificial androgenesis without the need for irradiation in fish, thus simplifying and facilitating the induction procedure. In this dissertation, viable diploid androgenics were induced without irradiation of eggs in loach and zebrafish, and an androgenetic clonal line was also established.

In chapter 1, the diploid androgenics were produced without egg irradiation in the loach. Eggs of wild-type diploid females were fertilized with the diploid sperm of a neo-tetraploid male, and then cold-shock treated at 3 ± 0.5 °C for 30 min just after fertilization to eliminate the female nucleus. After hatching, the ploidy status of hatched larvae was analyzed by flow cytometry, which revealed putative diploid androgenics as well as larvae possessing other

ploidies. Five independent microsatellite DNA markers were genotyped to confirm all-male inheritance of the resultant diploid larvae. The yield rate of diploid androgenetic larvae to total eggs used was $12.29 \pm 3.25\%$ in the cold-shock group, and $22.23 \pm 13.42\%$ in the UV-irradiated group, and the difference was not statistically significant ($P > 0.05$). No diploid androgenetic larvae were detected in the intact control group.

In chapter 2, the androgenetic doubled haploids (DHs) were induced in the loach without irradiation of the eggs. The eggs of wild-type females were fertilized with the intact sperm of an orange-phenotype male, and treated (within 10 s of fertilization) at 3 ± 0.5 °C for 30 min, to eliminate the female nucleus. The eggs were then incubated in a water bath at 20 ± 0.5 °C for 35 min. Finally, diploidy was restored (65 min after fertilization) by heat-shock treatment at 42 ± 0.5 °C for 2 min. Under these conditions, the yield rate of putative DHs relative to the total number of eggs used was $10.43 \pm 1.69\%$; it was significantly higher ($P < 0.05$) than the yield rates obtained under the remaining heat-shock initiation conditions (55, 60 and 70 min after fertilization). The ploidy status of the putative DH was analyzed by using flow cytometry. All-male inheritance was confirmed by the expression of the recessive orange body color trait and microsatellite genotypes. No maternally derived alleles or heterozygous genotypes were detected at any of the 28 loci (covering 27 linkage groups) of loach, indicating the exclusively paternal inheritance and homozygosity of the obtained androgenetic DHs.

In chapter 3, the androgenetic zebrafish DHs were induced by cold-shocking just fertilized eggs, and then the eggs were heat-shocked to double the chromosome set. The yield rate of haploid progeny in 7 ± 0.5 °C, 30 min cold-shock treatment group was the highest ($23.23 \pm 8.81\%$) than other groups (1 ± 0.5 , 4 ± 0.5 , and 10 ± 0.5 °C)($P < 0.05$). However, the difference

was not significant ($P > 0.05$) in the haploid rates among the cold-shocked groups (7 ± 0.5 °C, for 20, 30, 40, 50 and 60 min). All-male inheritance of haploid progeny was confirmed by microsatellite genotyping at four loci. To restore the diploidy, the cold-shocked eggs were incubated in a water bath at 28.5 ± 0.5 °C for 13 min, and then heat-shocked at 41.4 ± 0.5 °C for 2 min. The yield rate of putative DHs relative to the total number of eggs used was $1.10 \pm 0.19\%$. Microsatellite genotyping of the putative DHs using 30 loci that cover all 25 linkage groups detected no heterozygous loci, thus confirming the homozygosity of the DHs.

In chapter 4, the cytological mechanism of cold-shock androgenesis in zebrafish was elucidated by observing the early development process of cold- and heat-shocked eggs with histological sections. The developmental process and morphological feature of zebrafish normally fertilized eggs are similar to other fishes. In the cold-shocked egg, based on the histological section, the nuclear and blastodisc behaviors could be categorized into three types: (1) all chromosomes in second meiotic spindle were released together with second polar body. There was only male nucleus existed in the egg. The eggs of this type developed into androgenesis, resulting in haploid progeny. (2) The second meiotic chromosomes were enclosed, but the second polar body released without chromosomes. The unreleased diploid female nucleus and haploid male nucleus fused together, resulting in triploid progeny. (3) The second polar body released with a certain number of chromosomes. The number of chromosomes released could be 25 (half of diploid zebrafish chromosomes), less than 25 or more than 25. If the released chromosome number was 25, the unreleased haploid female nucleus would fuse with male nucleus, resulting in the diploid progeny. If the released chromosome number was not 25, the egg will have aneuploid chromosomes, resulting in abnormal progeny. The results

obtained here are the same with loach, indicating that both zebrafish and loach share the same mechanism for the cold-shock induced androgenesis.

In chapter 5, a clonal line was established by inducing the second cycle of androgenesis using cold- and heat-shock with the sperm of a golden-type DH male in zebrafish. Totally, seven normal diploid progeny with golden phenotype were obtained. These progeny together with the male parent were analyzed by DNA fingerprint with AFLP to check the clonality. For all 64-primer sets used, they had identical DNA fingerprints to reveal the band sharing index (BSI) with 1; while in the intact control, the BSI of 31 primer sets was 0.76 ± 0.13 . This result indicated that the seven progeny hatched from androgenesis were isogenic to the male parent, so they were clones.

In conclusion, for the first time, the viable androgenetic DHs were induced without irradiation of eggs, just by a combination of cold- and heat-shock in loach and zebrafish, and also an androgenetic clonal line was established by using this method. By eliminating the requirement for specialized protection media and irradiation, this method simplifies the process of androgenesis induction in fish, and has the potential to be widely used in other fish species.