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Review

Growth hormone gene transfer in common carp

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Abstract

The first successful case of transgenic fish was achieved in 1984. It is in a model system that the integration and expression of recombinant human growth hormone (*hGH*) in host red common carp (*Cyprinus carpio*, red var.) have been thoroughly studied. Recently, the integration sites have been recovered and characterized. Compared with non-transgenic peers, *hGH*-transgenic fish are prior in dietary utilization and growth performance. In view of bio-safety and bio-ethics, an "all-fish" construct *CAgcGH*, grass carp growth hormone fused with common carp β-actin promoter, has been generated and transferred into Yellow River carp (*C. carpio*, local strain in Yellow River) fertilized eggs. Under middle-scale trial, *CAgcGH*-transgenics show higher growth rate and food conversion efficiency than the controls, which is consistent to laboratory findings. To avoid the potential impact of transgenic fish on the environment, a sterile strain of transgenic triploid fish has been successfully produced. The "all-fish" transgenic common carp is also approved safe enough as daily food, according to a test based on the pathological principles of new medicines issued by the Ministry of Health of China. The "all-fish" transgenic common carp with growth enhancement is now ready for market, but looking for governmental authorization.

Résumé

Transfert du gène de l'hormone de croissance chez la carpe commune. Le premier cas de poisson transgénique a été réalisé avec succès en 1984. L'intégration et l'expression de l'hormone de croissance humaine recombinante (*hGH*) ont été étudiées chez la carpe commune var. rouge (*Cyprinus carpio*, red var.) en tant que système modèle. Récemment, les sites d'intégration ont été trouvés et caractérisés. Comparées aux carpes non-transgéniques, les carpes transgéniques-*hGH* ont des performances supérieures d'assimilation nutritionnelle et de croissance. En vue de sécurité biologique et de bio-éthique, un promoteur, de *β-actine* de la carpe commune, fusionné avec l'hormone de croissance de la carpe *Ctenopharyngodon idellus*, a été généré en « all-fish » *CAgcGH* et transféré dans des œufs fécondés d'une souche locale de la carpe du fleuve Jaune. Un essai à moyenne échelle de transgéniques-*CAgcGH* montre des taux de croissance et de conversion alimentaire plus élevés que les témoins, ce qui correspond aux résultats obtenus en laboratoire. Afin d'éviter un impact possible des poissons transgéniques sur l'environnement, une souche stérile de poisson triploïde transgénique a été obtenue avec succès. Les carpes « all-fish » transgéniques de la carpe commune sont aussi reconnues comme suffisamment propres à la consommation, d'après un test fondé sur des principes pathologiques des nouvelles médecines du ministère de la santé de la Chine. La carpe transgénique à croissance accélérée est désormais prête pour le marché mais en attente d'autorisation gouvernementale.

Keywords: Growth hormone; Gene transfer; Growth; Safety; Common carp

1. Introduction

Fishes, widely cultured all over the world, have served people as one of the most important food supplies for a million or so years. Fish culture, moreover, is one of the earliest activities of human civilization. The first record of fish culture can be dated back to 2500 years ago. In the *Handbook of Fish Culture*, the author Fan Li described the domestication and cultivation of common carp (*Cyprinus carpio*) in ponds. Ever since, common carp has become one of the most important farmed species in aquaculture.

The techniques of fish culture have been greatly developed along with civilization over the past thousands of years. However, the aquatic productivity is subject to the natural

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performance of the breeding species. Population explosion and overfishing in the past decades has made it impossible to meet the increasing demands on fish protein through traditional aquaculture (FAO, 2000). In order to improve the qualities and quantities of important farmed fishes, it is necessary to develop new breeding techniques, other than those methods that rely on classical genetic selection and hybridization.

Around the late 1970s, Chinese investigators who had a long career on fish nuclear transplantation initiated a study of "total DNA" transferred mud carp (*Cirrhina molitorella*). In this study, the total DNA of common carp, a species of fish that could survive cold winter, was transferred into the fertilized eggs of mud carp, a tropical species. About 8% of the total DNA transferred mud carp showed improvement on cold-resistance (Zhu and Huang, unpublished data). In the early 1980s, with the advancement of molecular cloning and micromanipulation, it was possible to isolate a single gene coding a unique protein and introduce the gene into the fertilized eggs of vertebrate (Bending, 1981). The newly developed biotechnology gave birth to the well-known transgenic "super mouse" (Palmiter et al., 1982). Two or 3 years later, a recombinant human growth hormone gene (*hGH*) was successfully transferred into the fertilized eggs of goldfish (Zhu et al., 1985) and loach (Zhu et al., 1986), which led to the birth of "fast-growing" transgenic fish. For application purpose, an "all-fish" recombinant growth hormone (*GH*) was subsequently constructed and transferred into common carp (Zhu, 1992a; Wang et al., 2001). After rigid safety evaluation, "all-fish" *GH*-transgenic common carp has been proved a successful example for modern aquaculture (Wang et al., 2001; Zhu, 2000).

In addition to produce *GH*-transgenic fish for growth enhancement, many investigators have attempted to produce transgenic fish with other valuable traits. For example, antifreeze proteins gene (*AFP*) was used to achieve freeze-resistant salmon (Hew et al., 1992) and cold-tolerance goldfish (Wang et al., 1995); lysozyme coding sequence was introduced into Atlantic salmon to gain disease resistance (Hew et al., 1995), infectious hematopoietic necrosis virus proteins coding sequences into rainbow trout eggs as DNA vaccination (Anderson et al., 1996), and human lactoferrin gene (*hLF*) into grass carp to promote the resistance to grass carp hemorrhage virus (GCHV) (Zhong et al., 2002). Nevertheless, *GH*-transgenic fish with growth enhancement is so far the one that has drawn the most attention, that has been the most thoroughly investigated, and that is also the most likely to be on the market in the near future. In the following paragraphs, we will briefly review the study of *GH*-transgenic common carp, especially the work conducted in our laboratory.

2. A model study of transgenic fish

In the early 1980s, a few recombinant genes relevant to morphology were available. One of them was *MThGH* construct from Dr. D.H. Hamer (Pavlakis and Hamer, 1983), *hGH* under the control of mouse metallothionein-1 (*MT-1*) promoter. Subsequently, *MThGH* was microinjected into the fertilized eggs of goldfish and a batch of fast growing transgenic fish was produced (Zhu et al., 1985). Other than the technique of microinjection that needs skillful manipulators, a variety of more convenient approaches, including electroporated and sperm-mediated methods, have been successfully employed for generation of transgenic fish (Inoue et al., 1990; Khoo et al., 1992; Xie et al., 1993; Synonds et al., 1994; Li et al., 1996; Zhong et al., 2002).

A study focused on the behavior of the foreign pMThGH gene during the embryogenesis of host common carp had been carried out (Zhu et al., 1989). It was found that the foreign gene endured a dynamic process during this course, including replication, degradation, concatenation and integration. The replication of the foreign gene started immediately once introduced into the fertilized eggs, and the strongest signal of replication occurred from late blastula to early neurula. Concatamers were the dominant form of foreign gene when the embryo developed from multi-cell stage to late gastrula stage. The integration of foreign gene was supposed to take place from the early stage and last for a long time course, which resulted in the transgenic mosaicism, i.e. the integrated transgenes were distributed in different tissues and organs of the transgenic fish along with the embryogenesis. It was obvious that only those transgenes integrated into the genome of germline could be transmitted to the offspring via sexual reproduction. The transcription of the foreign gene could be observed at the late-gastrula stage and radio immunity analysis revealed that different individuals had different levels of transgene expression. As a result of the expression of *MThGH*, some transgenics gained significant improvement on growth performance; whereas, some were smaller than the controls, and even showed morphological deformities. According to the positional effects related to the expression and function of transgene, the manners of transgene integration were divided into three categories: functional integration, silent integration and toxic integration. Compared with silent integration that does not show any effect and toxic integration that blocks the normal development, only functional integration results in the normal expression of hGH and growth enhanced transgenic fish (Zhu et al., 1989; Wei et al., 1992). Therefore, it is of great necessity to set up transgenic lines carrying functional integration for aquaculture purpose. Hybridization between transgenics and non-transgenics has shown to be an efficient way to attain this goal (Hew et al., 1992; Lin et al., 1994).

When transgenic male was hybridized against non-transgenic female, 72–88% offspring were transgene carriers. The results suggested that there were two to three integration sites in the genome of each germ cell according to Mendel's law (Wang et al., 2001). It was also found that *hGH* transgene could be transmitted to F4 generation and initiate its transcription normally (Sun et al., 2000). Besides, the flanking sequences of the integration sites in the F4 genome

were characterized and the relationship between integration sites and transgenic performance was analyzed (Zeng and Zhu, 2001). Recently, the experiment of gene targeting based on the strategy of dual-fluorescence positive–negative selection was successfully performed in cultured cells and living embryos (Wang, 2000). The techniques of cell culture and nuclear transplantation, fortunately, have been developed and become familiar in fish (Zhu and Sun, 2000). It will be another promising way to generate stable line of transgenic fish by nuclear transplantation with in vitro genetically modified cells. This will considerably overcome the integration mosaicism in fish transgenesis.

Why did *GH*-transgenic fish gain higher special growth rate (SGR) than the controls? According to the study on growth and energy budget, *GH*-transgenic common carp channeled less energy to metabolism and more to protein synthesis than the controls. Compared with the controls, F2 *GH*-transgenic common carp showed higher wet body weight, dry body weight and feed conversion efficiency. In total, F2 *GH*-transgenic fish utilize 6.62% more energy for synthesization than the controls. This phenomenon is called "fast-growing and less-eating" effect (Cui et al., 1996). On the other hand, growth and feed utilization by F4 *GH*-transgenic common carp fed diets with different protein levels (20%, 30%, and 40%) were studied. No matter what protein level was fed with, the transgenics showed higher diet conversion efficiency and higher growth rate than the controls. When fed with low protein level diet, the higher growth rate of transgenic common carp mainly depended on taking more food. When dietary protein was adequate, however, higher energy conversion efficiency drove transgenic common carp superior in growth performance. That is to say, *GH*-transgenic fish could not only gain growth improvement but also have a "feed-saving" effect (Fu et al., 1998). Under the same breeding condition, the transgenics had higher contents of dry matter, sarcous protein and amino acids while fewer lipids than the controls. Further analysis revealed there was no significant difference in amino acids composition between the transgenics and the controls (Fu et al., 2000).

It should be concluded that, introduced *GH* gene not only could be integrated into the host genome and inherited to the offspring, but also could express normally, which resulted in the transgenic fish with many valuable traits, such as "fast-growing and less-eating" and "high-protein and low-lipid contents". The model study of *GH*-transgenic common carp has laid a foundation for fish transgenic breeding and shown great potential for aquaculture.

3. "All-fish" *GH*-transgenic common carp

Under the consideration for bio-safety and bio-ethics, both *MT-1* promoter that needs heavy metal ion inducing and *hGH* that codes a kind of human protein are not encouraged to be used for the purpose of fish breeding. For this reason, the tentative plan of constructing "all-fish" gene that contains only piscine sequences has been proposed (Zhu, 1992a). In the early 1990s, common carp β-actin (*CA*) and grass carp (*Ctenopharyngodon idellus*) growth hormone (*gcGH*) were successively cloned (Liu et al., 1990; Zhu et al., 1992). Subsequently, *CA* promoter and the coding sequence of *gcGH* were linked and subcloned to pUC118 to make the "all-fish" constructs, pCAgcGH and pCAgcGH*c* (Zhu, 1992b).

When the "all-fish" constructs were microinjected into the fertilized eggs of common carp, they showed to be efficient by dramatically improving the growth rate of the transgenics. At 4-month, the transgenic common carp reached a weight of 2.75 kg, while the largest control was only 1.4 kg. At 17-month, one transgenics weighted 7.65 kg, more than double of the largest control (Wang, 2000). Furthermore, the middle-scale trial of F1 "all-fish" transgenic common carp has been carried out with parallel experiments. In average, the F1 transgenics gained growth rate by 42–80% over the controls, according to the data collected every 20 days for 5 months. The feeding conversion ratio (total food weight for per unit of gained body weight) of transgenics was 1.10, whereas that of control was 1.35 (Zhu, 2000). It could be concluded that "all-fish" *GH*-transgenic common carp could gain both higher growth rate and feed conversion efficiency than the controls in field farming, just consistent to the findings in laboratory. It is obvious that those farmers who raise "all-fish" transgenic fish will gain the yield of fish production, while the feeding costs will be greatly reduced.

4. Environmental safety of transgenic common carp

Nevertheless, before the application of "all-fish" transgenic common carp in aquaculture, the safety issues concerning their potential impact on environment need urgently to be evaluated. In a broad view of genetics, the crossbreeding is in fact a kind of transgenesis—a whole genome transgenesis—which occurs ever since fishes appeared in natural water body. Compared with crossbreeding, the transgenic fish is merely an "artificial variety" of the normal non-transgenics. In this regard, transgenic fish will likely pose less impact on the environment than those hybrid fishes that have been applied to aquaculture for a long history.

In a general opinion, transgenic fish is actually a newborn to this world, so that we need some carefully conducted studies to reach a conclusion. According to Cui's investigation, the transgene could flow among the same species by natural reproduction while not among different species (Cui, 1998). To avoid the potential risk, sterile technology could be utilized in the breeding of transgenic fish. Fortunately, a fertile strain of tetraploids had been successfully produced by crucian carp × common carp, and the strictly sterile triploid could be obtained by crossing diploid common carp against the tetraploid (Liu et al., 2001). The "all-fish" *GH* transferred tetraploids weighed over three times more than the controls after 240 days rearing (Zeng et al., 2000). By crossing the transgenic common carp diploids against the tetraploids, a

sterile strain of "all-fish" transgenic triploids was successfully produced (Zhu, 2000). Just like *GH*-transgenic diploids, *GH*-transgenic triploids showed significantly improved growth rate and feed conversion efficiency. Under economic estimation, to raise the fast-growing transgenic triploids can benefit the fish farmers by 52% over to raise the normal common carp (Zhu, 2000).

5. Food safety of transgenic common carp

Since the transgenic common carp are expected to be one kind of food supply for human being, the issue of food safety should be considered seriously. Due to the wide application of the transgenic techniques in agriculture, the food safety of transgenics has become the focus of the public concerns. A thorough and careful analysis of the food safety of transgenic fish needs to be conducted and it will be helpful to make the public understand and accept the laboratory-produced organisms.

At present, the widely accepted principle on food safety evaluation of foods produced by modern biotechnology is the "substantial equivalence principle", which was first proposed by OECD (European Organization for Economic Cooperation and Development) (OECD, 1993). When compared with the widely cultured non-transgenic common carp, the "all-fish" *GH*-transferred common carp do not produce any new proteins and other new biological products; and strictly speaking, they only produce a kind of novel piscine growth hormone. While grass carp and common carp belong to the same family and share 97% homology in amino acids sequence of the growth hormones (Zhu et al., 1992). Moreover, the polypeptide of growth hormone is very sensitive to acid, alkali and heat. It is undoubted that the physiological function of novel grass carp GH can be easily destroyed during routine cooking. It is reasonable that the "all-fish" *GH*-transgenic common carp is safe enough as daily food and no further nutrition and toxicology analysis is needed according to the "substantial equivalence principle".

Yet the experiment of food safety evaluation of "all-fish" *GH*-transgenic common carp was conducted to meet public concern. Physiological and pathological analysis of the mice fed with "all-fish" *GH*-transgenic common carp had been carried out according to the pathological principles of new medicines issued by the Ministry of Health of China (Zhang et al., 2000). Test groups of mice were fed with homogenate of transgenics at the dosages of 5 and 10 g/kg body weight for 6 weeks, while the control groups were fed with control fish at the same dosages. In comparison with the control mice, the test mice did not show any significant difference in growth performance, general appearance and biochemical analysis of blood, and histochemical assay of 12 organs, etc. ($P > 0.01$). Feeding with transgenic fish did not cause any impact on the reproduction capacity of the test mice and the development of the sub-generation of test mice. These results revealed that "all-fish" *GH*-transgenic common carp were substantially equivalent to control common carp in the aspect of physiology and pathology, and "all-fish" *GH*-transgenic common carp were safe enough as a kind of food resources.

6. Conclusion

In view of the transgenic breeding, applications of transgenic animals are far behind those in plants. Since the first transgenic plant, the antiviral tobacco was produced in 1983 (Shaw et al., 1983) following the "super mouse", the transgenic "antiviral tomato" has been authorized by the Food and Drug Administration (FDA) of the United States for entering the market 10 years later. By now, there are thousands of transgenic plants all over the world in filed trial, among which more than 40 plants have been commercialized (FDA, 2002). However, there is no report on the commercialization of transgenic animals, since the public and the governments are more cautious to the application of transgenic animals than plants.

The laboratory studies and field trials have shown that "all-fish" *GH*-transgenic common carp are safe enough to the environment and human health. More importantly, they could bring great benefits to both the fish farmers and the consumers. Therefore, "all-fish" transgenic common carp could be considered one of the successful examples of the application of transgenic animals. From scientific point of view, the mature season of applying "all-fish" transgenic common carp to aquaculture is coming; while in practice, the "all-fish" transgenic common carp is waiting for public acceptance and governmental authorization.

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