**TRANSGENIC FISH DEVELOPING TECHNOLOGY**

D.R. Saxena1, G. S. Tiwary2, N.J.Tupkar3 and R. A. Lonkar4.

HOD KNM1 Sakkardara, Nagpur, Research student2, Research student3 Research student4

**Introduction:**

Desirable foreign genes of one or many donor species of fishes are isolated, and spliced into artificially constructed infectious agents that act as vectors to carry genetic traits like enhanced growth rate, flesh quality, colour, flavour, texture, resistance to diseases, etc. When inserted into cells / genomes of recipient fish, produce a transgenic fish to enhance fish production or any aquaculture organism that is suitable as food for human. This has been done by various researchers in loach (Zhu et al., 1988; he was the first to transfer gene into fertilized egg of gold fish, Carassius auratus (1985); in Atlantic salmon, Salmo salar that attain marketable size in a short span of 18 months (Du et. al, 1992), in Tilapia Oreochromis niloticus; Oryzias latipes; Ictalurus species; aquarium ornamental Brachydanio rerio in zebra fish by Pandian et.al., 1991.

**Transgenic fish development Globally and in India:**

Earlier in 1991, the first transgenic fish was developed using imported constructs from foreign genetic engineering laboratory. In India, such work has been done at Madurai Kamraj University; Center for Cellular and Molecular Biology, Hyderabad; and National Matha College Kollam. Here rohu, zebra, cat fish and singhi are transgenic fishes that been already developed. Cirrhinus mrigala and H. fossilis transgenic fishes can now be developed using our indigenous vectors, promoters, genes, etc. That grow rapidly to marketable size to fulfill the ever rapidly expanding consumer demand. The transgenic rohu acquire 50 gm weight in 36 week duration and exhibit eight times larger body size.

Isolation, characterization and cloning of more than 8500 piscine genes and their CDNA sequences by advanced molecular biology has come to rescue to make available transgenes to develop the transgenic fishes currently at the global level. Currently Indian scientists are focusing on auto transgenesis method to obtain multiple copies of growth hormone genes in transgenic fishes to increase flesh content. In several piscine’s the breeding cycle is shorter and breeding frequency is relatively higher, such females yield multitude number of externally fertilized genetically identical eggs via application of autotransgenesis.

China and USA have more labs than Canada, Australia, New Zealand, Israel, Brazil, Cuba, Japan, Singapore, Malaysia, etc., that are involved in producing transgenic fishes that have accelerated growth and comparison to wild or traditionally bred relatives. Salmon and trout are cash crops, but carps and tilapia are mainly nutritional protein requirements in deficient humans. Commercial tie up of labs with few companies aim to enter in trade of transgenic fish (Dunham, 1999). Atlantic Salmon grown in cold water has a good flavour, the antifreeze gene promoter enhance flesh flavour in the Atlantic Salmon that have the pacific salmon growth hormones. The transgenic Coho salmon possessing integrated genetic elements of Sockeye salmon after one year become of marketable size due to faster growth rates (11 to 37 times than the control fish), ( Devlin et al., 1994 & 2001).

The poor-mans food, Tilapia is a transgenic fish cultivated in isolated or confinement production ( Hallerman, 2002) exhibit most experimental genetic modifications; the one with pig growth hormone has size thrice than their non modified siblings (Rahman et. al, 1980). Human insulin gene. Inserted in tilapia is a source of islet- cells for transplantation in diabetes patients. Muir & Howard (1999) produced transgenic medaka, the Oryzias latipes by inserting a gene construct of human growth hormone driven by salmon growth hormone promoter. Large male fish exhibit four times more mating advantage not observable in wild-type medaka. Resistant bacterial pathogens from silk inserted genes of silk moth was developed in transgene medaka to enhance disease free survival.

It was Amsterdam et. al., (1995) who introduced the Green fluorescence protein synthesized by a gene in Jelly fish Aequorea Victoria into zebrafish embryos, it absorbs blue light (395nm) but emits green light (509nm) to produce green fluorescence in muscles as well as other tissues as they developed and also in subsequent generations (Gong. et, al., 2003); similarly in B. rerio a transgenic fish that produce red fluorescence it is denoted as ‘glofish’ first hit the market in 2004 without approval from FDA, USDA and EPA. GFP gene of Aequorea Victoria, introduced into Zebra fish; yellow fluorescent and red fluorescent genes, all were expressed in presence of a potent muscle specific my1z2 promoter in stable lines of transgenic fish (Gong et.al., 2003).

T. Chen has developed transgenic common carp by injecting growth hormone of rainbow trout fused to a sequence from avian sarcoma virus, first generation offspring grew 20-40% faster than their unmanipulated siblings. Nichols and Dunham (1999) introduced an extra copy of a fish growth hormone by microinjection and electroporation strategies into fertilized eggs of carp and catfish. The transgenic piscines grew 20-80 % faster than the control ones.

**Development of Transgenic Pisces applying gene transfer Technology:**

Preferred techniques applied in research laboratories to make transgenetic fishes are microinjection (1985), electroporation of sperms, electroporation of eggs, sperm incubation, etc. At the same time triploid, tetraploid, haploid, gynogenetic and androgenetic strategies involving chromosome manipulation and hormone treatments were employed.

**Outline for gene transfer to produce commercial transgenics are:**

**Preparation of DNA construct :**

A suitable plasmid possessing promoter ‐- enhancer genetic signals and a structural DNA sequence to which is integrated the desired gene / growth hormone genes from fish and mammal (GH transgene )of interest resulting in the formation a recombinant DNA or DNA construct that is finally introduced to join it into the genome of each cell of fertilized egg or embryo, so that it produces increased growth hormones that enhance specifically growth rate of the fish (Type 1 transgene denoted as Gain-of- function. When transgene gauze the strength of promoter ‐- enhancer sequence ( it is designated Type 2 Reporter function transgene) and when the host genes functions is blocked without modification in genome of transgenic individual (it’s called as Transgene Type 3, Loss of function). Transgene are generally inserted outside the normal metabolic control of the transformed cell and are expressed at a escalated levels constitutively. This property of transgene is the basis of above described types of transgenes.

**Antifreeze Protein Gene Transfer for Survival in Cold water:**

AFPs of 3 types and AFGPS of 1 type are glycoproteins possessing similar antifreeze properties but variable protein structures naturally found in sera of marine Polar inhabiting fishes. Due to differences in freezing and melting temperatures of these proteins denoted as ‘Thermal Hysteresis ‘ they constitute above antifreeze glycoproteins that forbid growth of ice crystals in fish body ( Davies and Hew, 1990). According to Davis and Hew (1990) the wflAFP- 6 earlier name HPLC ‐ 8 is a polypeptide with more alanine content and helical organization that is major liver Type 1 AFPs encoded by genomic clone 2A-7 having an insert of AFGP genes isolated from winter flounder, Pleuronectes americanus and transferred into Salmo salar .This antifreeze resistant salmon transgenic fish now can tolerate and survive below ‐- 0.6 to ‐- 0.8 \*c cold water environment ( Hew et.al., 1999).Flounder wflAFP‐ 6 or wflAFP- 8 is one isomer synthesized as prepro AFPs in liver only, the other isomer wfsAFP-2 and wfsAFP- 3 are translated as intracellular mature antifreeze skin- type proteins in peripheral tissues, both type isomers belong to multigene family of 80 to 100 copies (Gong et . al., 2003).

Metallothionein gene promoter, heat shock promoter, and other tissue-specific promoter such as the myosin light polypeptide chain mylz and keratin ; cold or hypoxia stress bearing genetically manipulated piscine were obtained ( Asaduzzaman et.al., 2013; Iwai et.al., 2009; Guan et.al., 2011; Zhong and Fan, 2002).The Danio rerio mylz 2 promoter was integrated in Gymnocorymbus ternetzi for emitting white fluorescence similar to, medaka , farm raised carp Labeo rohita where GFP fluorescence can be seen with aid of fluorescence microscopy (Mohanta et. al., 2014; Pan et.al., 2008; Zong et.al., 2005, a).

**Transfer of Growth Hormone Gene :**

The very efficient pCAZ expression vector containing the grass carp growth hormone cDNA integrated to grass carp carbonic anhydrase beta- actin promoter gene has been developed by researchers Hew et. Al., (1992). Now CAT receptor gene harbouring pCA grass carp growth hormone gene was microinjected through egg-micropyle of a nonmodified common carp to obtain transgenic “ all fish” depicting 137 % enhanced growth property confirmed by Northern blotting and reverse transcriptase techniques ( Zhu, 1992 ; Zhu 1996 and Wang 2001).

**Strategies of Gene Transfer :**

Micropipette to hold fish embryo and microneedle of glass to insert gene of choice require a dissecting microscope. In hard chorion of water wetted fertilized Atlantic Salmon eggs opening can be made by microsurgery. Digest chorion using trypsin enzyme. To facilitate immediate fertilization and soften the rigid chorion use 1mM glutathione solution. Direct intracellular or cellular gene transfer in eggs. Glass slides and coverslips to support the partly reformed cell wall of protoplast aid rapid delivery of genes by microinjection.

Add milt and water to eggs, ten minutes post fertilization trypsinize to remove chorion membrane, now in the germinal disc microinject the choice DNA either in 25 mins before initiation of first cleavage, so that water ‐ incubated eggs to give 30 to 80 % survival rate of hatched eggs in different species of fish.

A) Microinjection method delivers more i.e > 10⁷ DNA copies through 1 to 2 ml solution in cytoplasm in contrast to nucleus( Zhu et.al., 1985 ; Walker ,1993).

B) Gene Transfer by Electroporation : A series of electrical pulses or shocks initiate temporary permeability or form hole that can be stabilized by a suitable dipole interaction with the electric field. DNA uptake occur take place in electroplated fertilized eggs and 25 % more rate of integration and survival occur in embryos. This is a rapid, efficient and easy method and produce slightly better results. The integration of DNA incorporation ranging from 30 to 100% 15 and hatching rates of channel catfish embryos is more than microinjection strategy( D. A. Power et . al., 1992 ; Walker,1993).

C) Retroviral integration method: The vesicular stomatitis virus envelope protein has been incorporated in retroviral vectors ( J.C. Burns et.al., 1994 ) to generate transgenic Cray fish and top minnow ( A. Sarwasik et.al., 2001).

D) Sperm electroporation mediated desired gene transfer to improve transgenic efficiency was used by Liu et.al., ( 2011). Involves foreign DNA incubation with sperm followed by oocyte or egg fertilization as was done in Labeo rohita to obtain transgenic fish ( Barman et. al., 2015).

Transgenic Fish Threats To Environment Evolution And Conservation.: Past Present And Future.

**The following suggestions are proposed by D. R. Saxena (2019-2023 personal critical observations):**

1) They compete with non- transgenic naturally existing native conspecifics for food, space, shelter, mate, ecological niche, initiating interspecific struggle for survival.

2) The release or escape of various already developed transgene fish cause decline and extermination of conspecifics due to loss of “Total Fitness". Moreover their offspring’s survival rate to reach first breeding stage in not 100% it being 20 to 50 % has been reported by researchers. A natural flow of “Conserved Consensus Gene Sequence" by natural predetermined and controlled pathways that can be designated as "Natural Universal Species-Specific Gene Flow Line" is operating since geological ages through the "Molecular Genetic Clock" (MGC) facilitating evolution, survival and extinction of biodiversity on this planet and elsewhere. Chronobiology is an essence of MGC that shape evolution . Transgenic biotechnology and genetic manipulations is costly with its limited temporary efficiency and are against the natures ethics and may bring synchronous biological and environmental hazards in the long run. Nature itself is a great scientist and laboratory, where multiple evolutionary processes sequentially or spontaneously are facilitated according to requirement of time and space.

3) The sterile transgenic fish may spread everywhere and during a certain period of life cycle exhibit an altered abnormal pseudo breeding behavior disrupting the potential normal breeding of their native conspecifics leading to loss of biodiversity locally and globally (Invasive effect).

4) According to author their seems to be a great difference between “Constructive critical applied concepts" in comparison to "Destructive erratic unidirectional non ‐ rational thinking " just for the sake of benefits prove to be disastrous. Use of GMO Plant and animal food may favour horizontal deleterious gene transfer in humans and animals So, it is better to use naturally produced plant and animal food and their pharmaceutical, industrial and other products rather than developing GMO for any purpose whatsoever.

**REFERENCES:**

A. Amsterdam et al., Developmental Biology, 171: 123-129 (1995).

A. Sarwasik et al., Marine Biotechnology, 3: 470-477 (2001).

Anderson E.D. et al., Molecular Marine Biology and Biotechnology, (1996): 5: 114-122.

Anderson, M. (1986): Evolution of condition dependent sex ornaments and mating preferences: sexual selection based on viability differences Evolution, 40: 804-816.

Barman, et.al., ( 2015 ): The beta actin gene promoter of Labeo rohita drives reporter expressions in transgenic rohu and various cell lines including spermatogonial stem cells , Cellular and oMolecular Biology Letters 10.

Bright, C. (1996): Understanding the threat of biological invasions. In Streke L. (ed.).

Burns, J.C.et.al., (1994): Development Biology. 165. 285-289.

Clifford,H.( 2014): Aqua Advantage Salmon ‐ a pioneering application of biotechnology in aquaculture. BMC Proceedings 8.

D M Langenau et al., Science, 299: 888-890 (2003). 26. A.P. Farrell et al., Can. J. Zool, 75: 335-337 (1997).

D.A Power et al., Molecular Marine Biology and Biotechnology, 1:301-309(1992).

D.J.Penman et al. Molecular reproduction and development, 30: 201-206 (1991).

Devlin, R.H.; Yesaki, T.Y; Baiagi, C.A.; Donaldson, E.M.; Swanson, P.; Chan, W,K; (1994): Extraordinary salmon growth. Nature, 371, 209.

Devlin, RH: Baiagi, CA: Yesaki, T.Y; Smailus, J. C; and Byatt, J.C. (2001): Growth of domesticated transgenic fish. Nature, 409, 781-782.

Du, S. J. Gong, Z.; Fletcher, F.L.; Shears, M.A.; King M.J.; Idler, D.R.; Hew, C.L.;(1992).

Duhham R. A. and R. N. Winn., Elsevier, Chapter II, Production of transgenic fish (2014).

Dunham R .A. et al., Transaction of the American Fisheries Society, 116:87-91(1987).

Dunham R. A. et al., Marine Blotechnology, 4: 338-344 (2002e).

Dunham. R.A. (1999): Utilization of transgenic fish in developing countries: Potential benefits and risks. J. World aquacult. Soc. 30:1-11.

F.E. Indiq and Moav Reproduction in Fish: Basic and applied aspects of Endocrinology and genetics, INRA press, 221-225 (1988).

Fish Fish 1: 146-172.

G.L. Fletcher and P.L.Davis.(1991).Transgenic fish for aquaculture, Genet.Eng.13:331‐370.

G.W. Stuart et al., Development, 103: 403-412 (1988).

Gong Z. et al., (2002). Aquatic genomics: Steps towards a great future, springer Verlag, NY. PP 329-339.

Gong Z. et al., Genetica, (2001). 111: 213-225.

Gong Z.; Wan, H.; Tay, T.L.; Wang, H.; Chen, and Yan, T. (2003); Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescence protein in the skeletal muscle. Biochemical and Biophysics Research Communications 58-63.

Gross M. R. (2005): Will farmed Atlantic salmon invade the ecological niches of wild Atlantic salmon? In Gallaugher P and Orr C. (eds.) Aquaculture and protection of wild salmon. 25-28, Simon Frasier University Cont. Stud. Sci. Burnaby, British Coloumbia.

Growth enhancement in Atlantic salmon by the use of an “all fish” chimeric growth hormone gene construct. Biotechnology 10: 176-180.

Guan,B., Ma.,Wang, Y., Hu, Y., Lin, Z., Zhu, Z., Hu,W.( 2011): Vitreoscilla hemoglobin(VHb) overexpression increases hypoxia tolerance in Zebrafish ( Danio rerio).Marine biotechnology 13:336‐344.

H.M. Stern & L.I Zon, Nat. Rev. Cancer, 3: 1-7(2003)

Hallerman, Eric, M. (2002): “Genetically Modified Fish and Shellfish Food for Thought.” Virginia Issues and Answers 8.1, Winter Virginia Tech Publications & Outreach. Communications.

Hedrick, P.W. (2001): Invation of transgenes from salmon or other genetically modified Organism into natural populations, Can. J. Fish Aquat. Sci. 58: 841-8440.

Hew, C. Poon, R: Xiong, F. Gauthier, S: Shears, M.; King, M.; Davis, P and Fletcher, G. (1999): Liver specific and seasonal expression of transgenic Atlantic salmon harboring the winter flounder antifreeze protein gene. Transgenic Research 8 405-414. Academic Publishers, Printed in Netherlands.

Hew, C.L: Fletcher, G.L. and Davis, P.L. (1992): Transgenic Salmon: tailoring the genome for food production. J. Fish Biology 47:1-19.

Hew, C.L: Fletcher, G.L. and Davis, P.L. (1992): Transgenic Salmon: tailoring the genome for food production. J. Fish Biology 47:1-19.

Iwai, T. et.al., ( 2009): Production of transgenic medaka fish carrying fluorescent nuclei and chromosomes. Zoological Science. 26 , 9‐16.

J.C. Burns et al., Developmental Biology, 165: 285-289 (1994).

J.K. Lu et al., Proceedings of the national Academy of Sciences, USA. 93: 3482-3486 (1996).

Kapuscinski, A.R. and Hallerman, EM. (1991): Implications of introduction of Transgenic fish into natural ecosystems. Can J, Fish Aquat. Sci. 48: 99-107.

Krasanov, A., Agren, J.J., Pitaken, T., Molsa., H.(1999):Transfer of GH transgenes into Arctic charr. (Salvelinus alpinus L.) Il.Nutrient partitioning in rapidly growing fish.Genetic analysis: biomolecular engine.15: 99‐105.

Krishnan, N.T. 1993: Economic Entomology, J.J. Publications.

Liu, T., Liu, L.,Wei, Q.,Hong.Y.(2011): Sperm nuclear transfer and transgenic production in the fish medaka. International Journal of Biological Sciences.7: 469‐475.

Liu, Z.J.,Moav, B.,A.J.,Guise, K.S., Kapuscinski, A.R.(1990): Development of expression vectors for transgenic fish.Biotechnogy 8,1268 ‐1272.

Maclean, N. and Laight, RJ. (2000): Transgenic fish: an evaluation for benefit and risks.

Muir, W. M. and Howard, RD. (1999): Possible ecological risks of transgenic organism release when transgenes affect mating success sexual selection and the Trojan gene Hypothesis. Proc. Natl. Acad. Sc. 96: 13853-13856.

Muir, W.M., and Howard, R.D. (2002): Assessment of possible ecological risks and hazards of transgenic fish with implications for other sexually reproducing organism Transgenic Research 11: 101- 114.

P.J. Zhang et al., Molecular reproduction and development, 25: 3-13 (1990).

Penman, D. J. et.al ., ( 1990): Molecular Reproduction Development. 30. 201-206.

Power, D. A. et.al., ( 1992): Molecular Marine Biology and Biotechnology. 1, 301-309.

Power, D.A. et.al., (1991): Studies on transgenic fish: Gene Transfer, expression, inheritance.Biotechnology. 16, 307‐324.

Rahman, M.A.: MAK, R.; Ayed, H. : Smith, A. and Maclean. (1980) : Expressions of a noval piscine growth hormone gene result in growth enhancement in transgenic Tilapia (Oreochromis mossambicus), Transgenic Res. 7: 357-369.

S. Lin et al., Science, 265: 666-669 (1994).

S.D. Walker, Master of Science Thesis, Auburn University, AL (1993).

S.J. Yoon et al., Aquaculture, 85: 21-33 (1989).

Sarmasik , A .et.al., (2001a).Production of live bearing fish and crustaceans produced by transforming immature growth gonadotropin with replication defective pantropic retroviral vectors. Marine Biotechnology 3, S 177‐ S 184.

Sarmasik , A. et.al., (2001b ): Transgenic live bearing fish and crustaceans produced by transforming immature gonads with replication defective pantropic retroviral vectors. Marine biotechnology 3, 470 ‐ 477.

State of World. A World watch Institute Report on Progress of a Sustainable Society pp. 95-113.

Stockstad, E. (2002): Engineered fish: friend or foe of the environment? Science 1797- 1798.

T. MC Evoy et al., Aquaculture, 68: 27-37(1988).

Walker, S. D. M. Sci. Thesis Auburn University , AL.( 1993):

Wang (2001): Construction of all-fish geACGH carp and genetic analysis of its F1 generation. Chinese Science bulletin. 46 (3), 226-229.

Yee et al., (1994): Proceedings National Academy Science, USA, 91: 9564 9568.

Z. Zhu. et al., (1985): Journal of Applied Ichthyology, 1: 31-38 .

Z. Zhu. et al., (1986): Academia Sinica, 31: 988-990.

Zhu et.al., (1985): Novel gene transfer into the fertilized eggs of gold fish ( Carassius auratus, L.) 1758. Journal of Applied Ichtyology. 1, 31-34.

Zhu, Z. (1992): Growth hormone gene and transgenic fish. Agricultural biotechnology. (Edts. C.B. You and Z.Z. Chen). China Science and Technology Press, Beijing, pp 106-116.

Zhu, Z. (1996): Foreign gene introduction via fish germ mediated technique. Chinese J. of Hydrobiology. 20 (3): 242-247.