

Transgenic fish and gene transfer technology

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Transgenic Fish

Technology and Applications

About three decades ago

- Genetic transformation of mouse embryos by injection of purified DNA (Gordon et al., 1980)
- Novel gene transfer into fertilized eggs of goldfish (*Carassius auratus*), Zhu et al. (1985)
- Germ-line transformation of mice (Palmiter and Brinster, 1986)

Why transgenic fish ?

- Improving economic traits in commercial species – food and ornamental fish
- Biofactories
- Transgenic fish can provide insights into the
 - mechanisms of development
 - gene regulation
 - actions of oncogenes and
 - intricate interactions within the immune system



Some facts.....

Genetically Altered Salmon Set to Move Closer to Dinner Table

By ANDREW POLLACK

The Food and Drug Administration is seriously considering whether to approve the first genetically engineered animal that people would eat — salmon that can grow at twice the normal rate.

The developer of the salmon has been trying to get approval for a decade. But the company now seems to have submitted most or all of the data the F.D.A. needs to analyze whether the salmon are safe to eat, nutritionally equivalent to other salmon and safe for the environment, according to government and biotechnology industry officials. A public meeting to discuss the salmon may be held as early as this fall.

Some consumer and environmental groups are likely to raise



AQUABOUNTY

A gene-engineered fish, top, and a natural one of the same age.

objections to approval. Even within the F.D.A., there has been a debate about whether the salmon should be labeled as genetically engineered (genetically engineered crops are not labeled).

The salmon's approval would

help open a path for companies and academic scientists developing other genetically engineered animals, like cattle resistant to mad cow disease or pigs that could supply healthier bacon. Next in line behind the salmon

for possible approval would probably be the "enviropig," developed at a Canadian university, which has less phosphorus pollution in its manure.

The salmon was developed by a company called AquaBounty Technologies and would be raised in fish farms. It is an Atlantic salmon that contains a growth hormone gene from a Chinook salmon as well as a genetic on-switch from the ocean pout, a distant relative of the salmon.

Normally, salmon do not make growth hormone in cold weather. But the pout's on-switch keeps production of the hormone going year round. The result is salmon that can grow to market size in 16 to 18 months instead of three years, though the company says the modified salmon will not end up any bigger than a conventional

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Transgenic Salmon aqua advantage salmon

- ❖ *Aquabounty, USA*
- ❖ *Growth hormone gene from Chinook salmon*
- ❖ *Antifreeze promoter from ocean pout*
- ❖ *Reduces growth to maturity time by 50%*
- ❖ *16-18 months rather than 3 years*
- ❖ *Thus, can **double** production*
- ❖ *Containment : triploidy*

Rainbow Trout



- ❖ *University of Rhode Island , USA*
- ❖ *Mutation of myostatin gene*
- ❖ *Transgenic fish have 20% more muscle mass*
- ❖ *Thus, 20% more production*
- ❖ *Containment: triploidy*

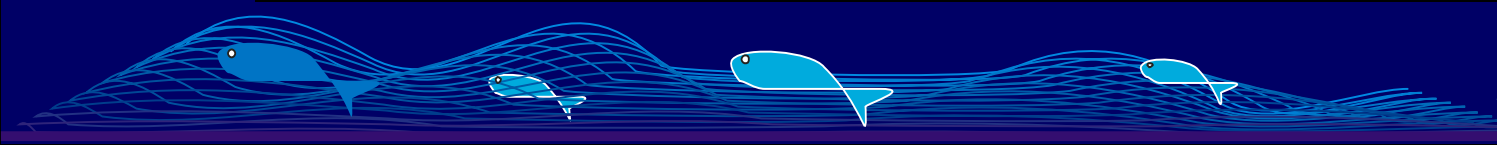
Transgenic Tilapia



- ❖ *Centre for Genetic Engineering and Biotechnology, Havana, Cuba*
- ❖ *School of Biological Sciences, University of Southampton, UK*
- ❖ *Growth hormone gene from Chinook salmon*
- ❖ *Antifreeze promoter from ocean pout*
- ❖ *Increase in growth 2.5 times*
- ❖ *Containment: triploidy*

Common Carp

- ❖ *Chinese Academy of Sciences, Hubei, PRC*
- ❖ *Common carp beta-actin promoter*
- ❖ *Grass carp growth hormone*
- ❖ *Over 50% increase in size (less than half the culture period), doubles production*
- ❖ *Containment: triploidy*



GloFish: transgenic zebrafish

- Glowfish is first transgenic animals made available to the general public in the United States.
- Green fluorescent protein gene from *Aequorea victoria* (a jellyfish) under the control of myosin light chain gene promoter.
- First transgenic animal coming public.

Indian Rohu



- ❖ *CCMB, India*
- ❖ *Autotransgenesis- rohu GH with H1 histone promoter*
- ❖ *Grows and matures in half the time*
- ❖ *Can double production*
- ❖ *Containment - ?*

Cold tolerance

- Antifreeze protein gene of the winter flounder was transferred to produce salmon that could be farmed under Arctic conditions
- Expression levels obtained have been inadequate for increasing the cold tolerance of salmon
- Preliminary results with goldfish show some promise for increasing survival within the normal cold temperature range
- Initial thrusts at improving cold tolerance via gene transfer have been minimally successful

Disease resistance

Antiviral

- Viral coat protein genes
- Antisense expression of viral early genes

Antibacterial

- Lytic peptide genes - Cecropin genes
- Initially translated into precursors of 62-64 a.a. residues
- Processed intra-cellularly into mature peptides of 35-37 a.a.
- Readily incorporate into cellular membranes of bacteria, fungi and parasites, resulting in the formation of pores on the membrane, leading to the inevitable death of pro- and eukaryotic pathogens.

As Biofactories

Short generation time.

High fecundity. Germ line transmitted genes will be expressed in large quantity.

No known viruses or prions infect both humans and fish.

Less chance of proteolytic degradation of recombinant protein. Milk commonly contains lytic enzymes that can hydrolyze heterologous proteins unless they are highly resistant to degradation.

Human biopharmaceuticals in fish

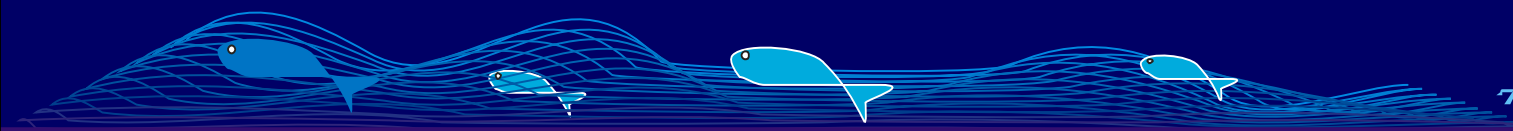
Product	Species	Target organ	company
Factor VII	Tilapia	Mucus	Aqua gene
Insulin	Tilapia	unknown	PCAMD
Human calcitonin	Salmon	Mucus	Diver drugs
Pleurocidine	Salmon	Mucus	Diver drugs
Human defensins	Salmon	Mucus	Diver drugs

Fish as a model system

- Fish and many aquatic organisms produce large quantities of eggs, which are usually fertilized and incubated externally.
- The embryos are relatively easy to obtain, manipulate, incubate and hatch rapidly in warmwater species of fish and shellfish.
- Do not require complex manipulations such as *in vivo* culturing of embryos and transferring of embryos into foster-mothers as required in the mouse model.

Zebrafish and medaka as a model organisms

- Zebrafish and medaka have become an efficient vertebrate model system because of its relatively short reproductive cycle, transparent, non-adhesive eggs which allows for easy microinjection and other manipulation
- Relatively small space is needed to maintain large numbers of offspring at low cost
- They have a completely sequenced genome
- Several gene functions have been found to be conserved in humans
- Are now powerful research systems for functional genomics and metabolic pathways



The technique

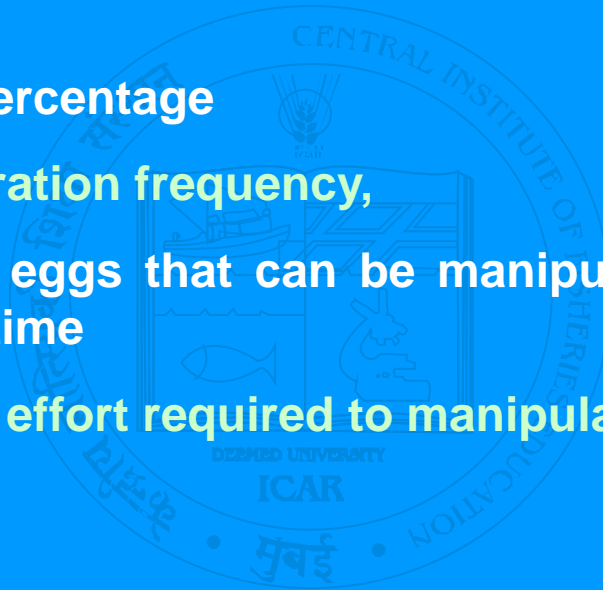
1. The appropriate gene needs to be isolated and cloned.
2. The foreign gene must be transferred to the fish and be integrated in the host's genome.
3. The transgene must be expressed, as transfer of the gene does not guarantee that it will express and function.
4. A positive biological effect must result from expression of the foreign DNA, and no adverse biological or commercial effects occur.
5. Finally, the foreign gene must be inherited by subsequent generations.

Which gene ?

- Prior to the early 1990s, very few fish genes had been isolated.
- Hence, fusion genes with origins from mammals, birds, insects, bacteria and viruses were transferred to fish embryos
- Today the long list of fish genes is growing everyday for the study of gene expression and potential transfer and manipulation in fish and aquatic invertebrates.
- These genes may have application for the enhancement of growth, reproduction, disease resistance, carcass yield, cold tolerance and other economic traits.
- Most of the research until recently has focused on growth hormone (GH) gene transfer (Dunham, 2004).

Efficiency of gene transfer techniques

- Hatching percentage
- Gene integration frequency,
- Number of eggs that can be manipulated in a given amount of time
- Quantity of effort required to manipulate the embryos



Gene transfer techniques

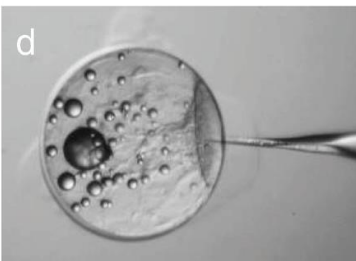
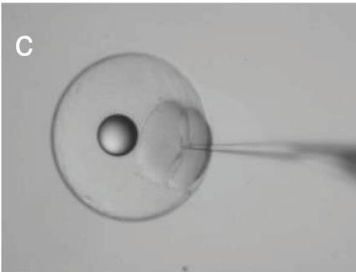
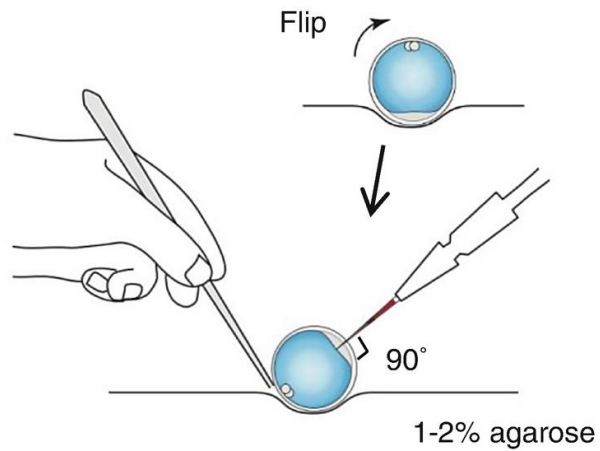
Microinjection

- Fertilized eggs can be held in place by micromanipulation or in specialized grooves made in agarose plates while the DNA is microinjected.
- Ozato *et al.* (1986) injected medaka oocytes removed from ovaries 9h before ovulation. Transgene was found in 4 of 8 medaka embryos.
- Post-microinjection foreign DNA replicates rapidly in the cytoplasm of developing embryo and begins to disappear as development proceeds (Houdebine and Chourrout, 1991).

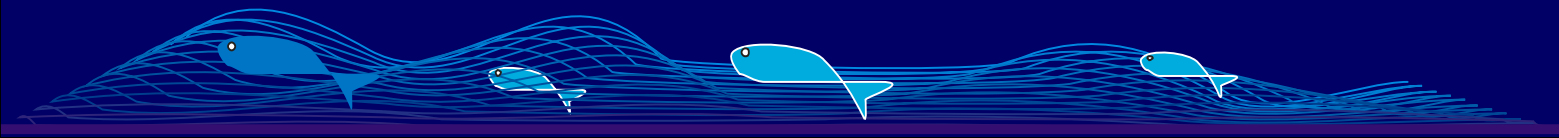
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- **Integration happens in about 5% of the surviving embryos at the two-cell stage or beyond**
- **Integration at the one-cell stage has never been observed, resulting in mosaicism**
- **Tedious and slow procedure**
- **Can result in high egg mortality**
- **More dependent upon the skill of the operator**
- **Maximum speed of about ten-twenty eggs microinjected per minute using the latest techniques.**



Electroporation

- Electroporation involves placing eggs in a buffer solution containing DNA and applying short electrical pulses
- These create transient openings of the cell membrane allowing the transfer of genetic material from the solution into the cell.
- The efficiency is affected by voltage, number and frequency of pulses.
- Higher rates of integration sometimes as much as 30-100% were obtained using electroporation rather than microinjection

- **Electroporation is an excellent method for transfer of DNA into a large number of fish embryos in a short time period (Powers *et al.*, 1992)**
- **15 to 100 catfish eggs can be electroporated every 4 seconds simply by depressing a button.**
- **It is also possible to electroporate the sperms and use them to fertilize non- manipulated eggs.**

Genetically modified sperm

- Primary cultures of zebrafish male germ cells in which the differentiation from spermatogonia to functional sperm can occur *in vitro*, provides an opportunity to produce GM sperm under entirely *in vitro* conditions.
- Retroviral vectors were used to produce transgenic zebrafish
- No mosaics were obtained
- Only 5 out of 89 fish were transgenic (5.6%)
- Eight to nine days are required for sperm to differentiate from actively dividing cells, such as a premeiotic spermatocyte, in this culture system
- Some later stages seem resistant to viral infection
- Efficiencies can be improved by further standardization

The transgene construct

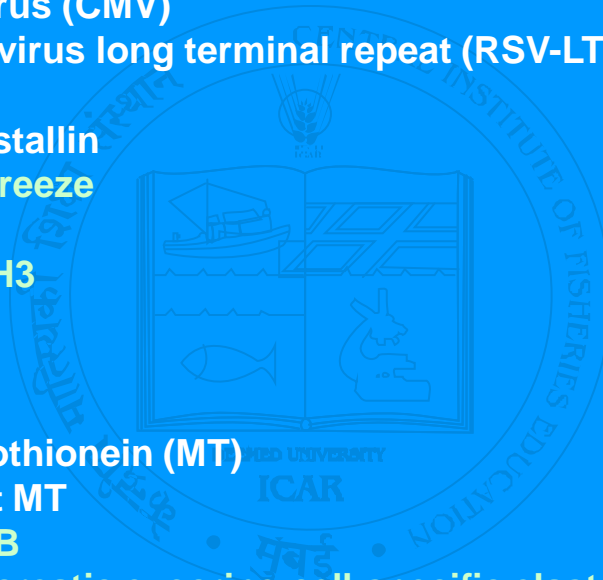
- The transgene needs to include a selected gene along with artificial regulatory sequences
- This allows circumvention of natural regulatory mechanisms that may inhibit/ regulate expression.
- Efficient transgenesis rate and enhanced performance relies on expression vectors with high integration rates and reliable and stable expression
- Earlier promoters were viral and mammalian
- Less research is conducted today with viral promoters because of poor public perception of the word virus, which would make future commercialization extremely difficult.

Constitutive promoters

- Cytomegalovirus (CMV)
- Rous saroma virus long terminal repeat (RSV-LTR)
- Actin
- Chicken δ -crystallin
- Flounder antifreeze
- Carp β actin
- Carp histone H3

Inducible promoters

- Mouse metallothionein (MT)
- Rainbow-trout MT
- Salmonid MT-B
- Zebrafish pancreatic exocrine-cell-specific elastase B
- Zebrafish epidermis specific keratin 8
- Zebrafish fast-muscle-specific myosin light polypeptide 2.



- Growth hormone genes from human, rat, bovine, carp, trout, salmon, catfish, etc. have been used.
- Expression of uninterrupted coding regions from prokaryotic and eukaryotic sources has been successful in fish cells.

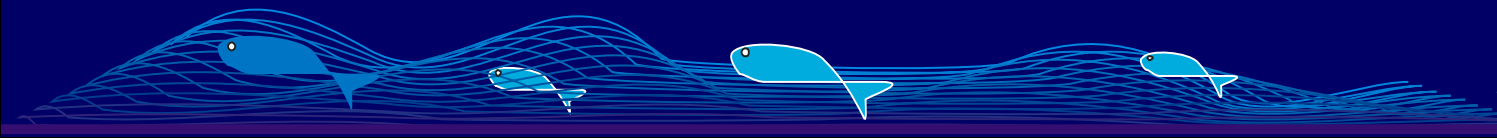
Integration of transgene

- Most of the DNA constructs introduced are lost during the first 10 days after delivery into host embryos.
- Of a million copies of the gene that are introduced into each embryo only about 0.0001% of the constructs take up permanent residence in the fish genome
- Integration between 2 and 30% has been reported.
- Efficient gene transfer would reduce the number of copies to be introduced per embryo
- Will reduce time and cost of screening for transgenic individuals

- Higher integration rates would potentially increase the number of lines generated, with a greater number of genotypes to evaluate and select from
- This would reduce potential problems with inbreeding and founder effects from initiating transgenic populations from a limited number of founders.
- Transposon sequences and retroviral sequences have been studied to overcome integration inefficiencies and position effects on expression

Mosaicism

- This happens when not all tissues contain the transgene and not all cells within the transgenic tissues harbour the transgene in the injected individuals.
- DNA may persist for several cell divisions or few weeks without integration or degradation, so false positives detected
- Delayed integration.
- Cytoplasmic nature of DNA injection causes virtually all founder transgenic fish to be mosaic



Copy number of transgene

- Copy numbers of the integrated transgene can range from one to several thousand at a single locus
- In contrast to the head-to-tail organization observed in the mouse system, in some cases the DNA can also be found organized in all possible concatameric forms
- This suggests random end-to-end ligation of the injected DNA prior to integration.

Transgene expression

- Nature of its promoter (constitutive/ inducible)
- Transgene copy number
- Position of insertion (transcriptionally active or inactive region of the genome)
- Source of the gene and its relatedness to the host organism
- Other factors related to the physiology of the host

Pleiotropic effects of growth hormone gene. . .

- Body composition
- Body shape
- Feed-conversion efficiency
- Disease resistance (may be lower)
- Reproduction (fecundity or survival may be lower)
- Tolerance of low oxygen
- Carcass yield
- Predator avoidance ?

Wild and domesticated strain response

Strain effects, epistasis and genetic background

Selection *and* transgenesis not Selection vs Transgenesis

Disadvantages of transgenic fish

- Genetic pollution
- Health concern
- Environmental concern: competition with wild specimens.

Containment....

- The gold fluorescent fish are sterilised through "chromosome manipulation technique" before they go on the market and are "not predatory".
- Says Jan Fan-hua, professor from Taiwan Ocean University, that they would not be able to survive even if they were thrown into rivers.

