

## **Current trends in chicken genome manipulation: transgenesis and biotechnology of reproduction**

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The production of transgenic poultry is highly desirable for several reasons. In the last few years considerable advances have been made in our primary understanding (on molecular level) of many genetic traits important in poultry industry and biopharmacy as well as in developed techniques that can play important role in biotechnology of reproduction, for instance for preservation of poultry genetic resources. Application of this important basic knowledge through the transgenic poultry to final users gives considerable promise to increase profitability and quality of commercial poultry stocks of any breed, including new promises for novel uses such as production of specific recombinant therapeutic proteins in biopharmacy or use of this transgenic technology for preserving of genetic resources. In near future transgenic technology approach in poultry should also give many practical applications which arise from better understanding of avian biology itself.

At the present there exist several procedures (techniques) how to modify chicken genome but not all of them has been successful in the forming of transgenic poultry so far. On the other hand several of them have made considerable progress and are promising for future applications.

The most known techniques used for construction of transgenic poultry are mainly adaptation of procedures developed for mammalian embryos such as sperm mediated gene transfer (Gavora et al., 1991), direct microinjection of DNA into the blastodisc (Love et al., 1994) direct injection of DNA into the testes (Thoraval et al., 2000), use of early blastodermal cells (Petitte et al., 1990) and primordial germ cells (PGCs), (Chang et al., 1995) for gene transfer via forming of chicken germ line chimeras, use of spermatogonial cells for the gene transfer (Trefil et al., 2003).

Use of embryonic chicken chimeras received high attention in the last 15 years.

Applied methods of forming embryonal chimeras are based on the use of early blastodermal cells or primordial germ cells (PGCs) - Petitte et al., 1990, Carsience et al., 1993, Thoraval et al., 1993, Etches et al., 1993, Trefil et al., 1995. The model of chicken chimeras forming is obviously an adaptation of mouse model but taking an advantage of unique feature provided by chicken embryo. PGCs or their precursors in the form of blastodermal cells are at first isolated from the specific tissue and then cultivated. After the transfection with desired genetic information these cells are returned into the recipient embryo hopefully to produce germ line chimeras that contain transgenic gametes. Then these chimeras should produce a transgenic offspring. Recognition of early PGCs in the stadium X epiblast in the area pelucida by using antibodies anti chick germ cell markers SSEA-1 and EMA-1 (Karagenç et al., 1996) plays the key role in this technique. All methods of producing transgenic poultry are related on techniques designed to insert foreign genetic information into the cells that will give rise to germ cells. Most of developed techniques will produce hen with mosaic transgene insertion in the germline (i.e., only several cells in the gonad may carry the transgene) and so efficient targeting of the germ cells will be essential in the production of transgenic poultry. From this point of view present all techniques seems to be related with PGCs. We can say that key target for any modification of the chicken genome is germ cell because only this type of cells is able to deliver the transgene to the next or subsequent generations. Practically it means the modification of mature oocytes, spermatozoa, fertilized egg and PGCs at any stadium of development.

Development of an efficient method for genetic modification of chickens has proved a significant technical challenge (Sang, 1994; Zajchowski & Etches, 2000). The earliest methods developed were based on the use of avian retroviruses: replication-competent vectors derived from avian leucosis virus – ALV (Salter et al., 1987) and replication-defective vectors derived from reticuloendotheliosis virus (Bosselman et al, 1989). Vick et al., (1993) has been successful in construction of transgenic chicken after retroviral infection of germinal crescent. More recently, an ALV replication-defective vector has been used to produce transgenic birds at low frequency. Transgene expression from these vectors has only been detected at low levels (Rapp et al, 2003). An improvement in the frequency of production of germline transgenic birds has been shown using a spleen necrosis virus-based vector, although the germline transmission frequency was still low (Mozdziak et al., 2003). The achievement of transgenic chicken via retrovirus construct depends mainly on very high integration and efficiency. Kwon et al., 2004 demonstrated the successful production of transgenic chickens expressing the enhanced green fluorescence protein (EGFP) gene using replication-defective recombinant retrovirus pantropised with G glycoprotein of vesicular stomatitis virus. This concentrated pseudotyped retrovirus was injected beneath the blastoderm of non-incubated chicken embryos (stage X).

McGrew et al., 2004 described new group of vectors for successful construction of transgenic chicken derived from members of the lentivirus class of retroviruses. These have potential advantages over those derived from oncoretroviruses, including the ability to infect non-dividing cells.

Several non-viral methods for genetic modification of the avian germ line have been described but so far the frequencies obtained transgenic chickens are even lower than those obtained using retroviral vectors (Sang, 1994; Zajchowski & Etches, 2000).

At present we can say that unavailability of any method for efficient production of transgenic chicken decreases exploitation of transgenic technologies and their fast realization in poultry industry - modification of production traits for poultry breeding, biopharmacy (expression of pharmaceutical proteins in eggs), investigation of genes involved in vertebrate development, for which the chick is becoming an increasingly useful model (Brown et al, 2003).

Poultry industry all over the world is multibillion \$ industry with increasing trend of development. Eventually there should be commercial interest in using transgenic technology to improve for instance meat and eggs production. Very interesting and promising is use of transgenic technology influencing of chicken disease resistance, which was successful in transgenic plants recently.

For biopharmaceutical industry hen (*Gallus domesticus*) looks to be an ideal candidate for the efficient production of therapeutic proteins because of:

- Low cost, short generation time, easy keeping of poultry
- Naturally sterile environment of the egg
- Large amount of protein that may be produced per egg
- Large number of eggs that are produced per year

For basic research:

- investigation of genes involved in vertebrate development
- as a model for developmental biology research community

For instance in biopharmacy the demand for glycosylated proteins that are folded in appropriate 3D structures and are of pharmaceutical importance to human is rapidly growing. In comparison to mammals, the glycosylation patterns of some chicken proteins are reported to be more similar to those of humans (Raju et al., 2000).

Many of these proteins with a native conformation are produced mainly from in vitro mammalian cell culture systems. However, low yield and high production cost associated with the recombinant proteins production system have necessitated the use of transgenic

livestock as bioreactors. Mammary gland became a promising bioreactor due to the large milk production capacity and already some pharmaceutical proteins were produced in the milk of several transgenic farm animal species. However, there exist several disadvantages of the mammary gland as a bioreactor including long generation times for domestic mammals and difficulties in purifying recombinant proteins due to the biochemical complexity of milk proteins and fats.

Use of the hen egg overcomes these problems and has additional advantages including shorter generation time (chicken become adult within 20 weeks), lower expenses and high fecundity. Purification of recombinant protein seems to be much easier because egg white proteins are biochemically less complex comparing to milk proteins. Egg albumin fraction consists of around 3,5-4 g of proteins and ovoalbumin forms more than half of these proteins. Egg type of hen is able to lay around 280 eggs per year and for instance 4 hens are theoretically able to produce around 1 kg of recombinant protein per year. There exists real suppose that the price of purified protein should be around 10 USD per gram (Alper, 2003) and that is 100 fold less than the cost of current systems using cultured mammalian cells. There exists also big advantage for possible chicken bioreactor in comparison with mammalian bioreactor - world authorities are familiar with eggs as bioreactors because large amount of vaccines including for instance vaccine against influenza are already produced in chicken eggs although not transgenic ones. The presence of natural protease inhibitors in egg content and also the naturally sterile microenvironment of the egg system provide an ideal environment for stabilizing biological activity of foreign proteins and to allow long term storage of these proteins without lost of biological activity, just in shell of laid egg (Modziak and Petite, 2004)

Although transgenic mice have been available to the research community for more than 20 years, transgenic chicken technology is still not available at all. Despite of many advantages, production of transgenic avian species has been delayed against mammalian models mainly for the specific avian reproductive biology. This suggests that there is a strong need for more intensive basic research in areas such as avian reproductive physiology, for efficient and tissue-specific transgene expression.

The progress achieved during last years in biology of reproduction thanks to transgenic technologies allows us to start the practical discussion about effective preserving of avian genetic resources on the whole world. The interest of preserving genetic resources including local poultry breeds has already been extensively discussed in the framework referred to as COP 7 (Convention of Biological Diversity, Kuala Lumpur, 2004). Biotechnologies of reproduction have long been of practical application in cattle in order to optimise the genetic potential issued from the best sires. Thus, the rapid extension of artificial insemination (A.I.), semen cryopreservation, *in vitro* fertilization and embryo transfer cover broad range of commercial applications and, more generally, have facilitated the transport and extensive use of various genetic materials between continents. In addition, such techniques are now perceived, for obvious sanitary reasons, as a necessary issue to preserve genetic resources in complement to preserve live breeds. Interestingly, large majority of these technologies has, up-to-date, found limited application in avian (including poultry) species, in part due to the difficulties to access the avian oocyte (or embryo) and also due to the relatively limited amount of information available in this field. However, several biotechnologies including artificial insemination, which has been extensively used for the selection and multiplication of turkeys, ducks, guinea-fowl, chickens and geese have already been of interest to poultry specialists. However, recent advances in the field of semen cryopreservation and related biotechnologies are also of interest in purpose of preserving genetic resources for future generations.

## A) BIOTECHNOLOGIES REFERRING DIRECTLY TO SEMEN

### Artificial insemination and short term storage

While AI was originally used as a tool to optimize breeding performances (e.g. progeny testing) in the main poultry species, its success among secondary breeders was at first derived from the possibility to expend genetic potential from highly selected sires to their progeny (see review by Lake, 1995). From a biological standpoint, the economical success of AI in poultry species is based on a) the existence of prolonged sperm storage sites in the oviduct and b) the absence of ovulation induced by the presence of a male (exception: pigeon). Extensive research in the last decades has helped defining optimal conditions of male and female management (see Bakst and Brillard, 1995). For example, the need for optimized conditions of semen preservation and transport and, more generally, the interest to provide maximal flexibility between semen collection and its deposition into the female tract have led to the development of simple protocols to preserve fertilizing potential under *in vitro* conditions up to few hours. Quite surprisingly, while fertility performances obtained with stored semen rapidly improved (e.g. chicken: Lake, 1986; turkey: Sexton, 1982, Van Wambeke and Huyghebaert, 1989) no major breakthrough has been observed in this field since the late 80<sup>ies</sup>. However, the progressive decline of sperm viability during *in vitro* storage remains a severe limiting factor which needs to be addressed. In this prospect, a better knowledge of sperm metabolism and of the protection of its membranes against peroxydation appears necessary.

### Monitoring long term in vitro semen preservation

Since early studies by Shaffner et al (1941) who obtained the first hatched chicken from frozen semen, poultry geneticists have constantly expressed special interest for long term storage of poultry germplasm. However, progress in this field has remained very slow due to the existence of several biological barriers making difficult to maintain some persistence of fertility over 2-3 days following intra-vaginal insemination. Indeed, the first barrier, described by Lake (1967) was due to the fact that, upon thawing and sperm deposition into the vagina, glycerol, the most commonly used cryoprotectant in mammals, becomes highly toxic to chicken sperm at concentrations well below those required for successful freezing. This was circumvented by eliminating glycerol through centrifugation and, also, by proposing (depending on species), working alternatives to glycerol such as dimethylacetamide (DMA), dimethylsulfoxide (DMSO). In addition, at least in chickens, semen freezability shows a high inter-breed and inter-individual variability due to the fact that this trait is inheritant. Despite pre-cited difficulties, several freezing procedures are now accessible to successfully preserve chicken semen (Tselutin *et al.*, 1999; Chalah *et al.*, 1999) with fertility levels sufficient to ensure the preservation of genetic resources in a majority of breeds. In addition, significant progress has also been made to cryopreserve semen in several other poultry species including turkey, duck and goose. Gene banking based on semen cryopreservation is therefore possible. It is already developing in several countries including Ukraine, France and USA.

Persistently low or highly variable fertility rates still observed in several poultry species (e.g. turkey and guinea-fowl) has hampered the extension of these technique towards relevant poultry industries but the inconstancy of results, even unexplainable *per se*, is an indication that semen cryopreservation in the related species is feasible.

## B) BIOTECHNOLOGIES REFERRING TO THE OOCYTE AND *in vitro* FERTILISATION

The first attempts to control ovulation by *in vitro* procedures in avian species were developed in 1937. Since, the technique has been significantly improved and then associated with *in vitro* fertilisation with aim to provide adequate tools to access the early stages of embryo development and, more precisely, embryonic stem cells (ES cells). A working procedure associating *in vitro* ovulation and *in vitro* fertilisation has been recently described in the quail (Olzanska and Stepinska, 2002) and in the chicken (Batellier *et al.*, 2003). The cascade of highly risky events to be handled before obtaining a viable chicken leads to the conclusion that, in avian species, *in vitro* ovulation associated with *in vitro* fertilisation is an alternative method only to *in vivo* fertilisation with aim to control the access to the first stages of embryo development (an interesting premise to embryo transfer and, also, to transgenesis through the introduction of genetic material from ES donor cells into recipient oocytes at a proper stage of maturation).

## C) ROUTES TO ACCESS GENE TRANSFER IN AVIAN SPECIES

The idea of introducing exogenous genes into recipient embryo tissues up to obtaining their transfer into the germ line has led to an incredible amount of approaches ranging from *in vitro* embryo culture to the production of chimeras and from nuclear transfer (cloning) to germ cell transplantation into sterilised testes. Indeed, the challenge of accessing and modifying the avian genome have been of considerable interest due to the virtually unlimited potential of applications of these techniques in a variety of fields such as poultry production *per se* (e.g. resistance to infections, increased growth rate...), animal or human health (e.g. production of proteins of interest) and sex determinism (e.g. controlled production of poult from one or the other sex).

### In vitro culture of the avian embryo

Perry (1988) was the first to propose a viable method for reproducible production of normal hatched chickens from single stage embryos through a complete *in vitro* culture system. The technique itself is remarkable as it provides direct access to each step of embryo development. However, the series of tricky steps to be successfully completed by the embryo (and the lab specialist) during the so called *ex vivo* culture up to hatch, along with the low rates of integration efficiency of transferred genes (through gene constructs) into recipient embryos have, up-to-date, are two major limits of this approach development.

### Chimeric chickens

The difficulties to insert genetic material into recipient germinal cells or into single cell embryos have resulted in the exploration of an alternative route based on the production of germline chimeras issued from the transfer of donor ES cells – preferable PGCs at any stadium of development (generally at Stage X of Eyal-Giladi and Kochav classification, 1976) into recipient embryos at first subjected to gamma irradiation (Pettite, *et al.*, 1990). Despite the relative success of this technique to transfer genetic material into recipient embryos, the necessity of culturing donor cell populations to introduce the gene of interest, along with the difficulties to control the rate and type of chimerism obtained (somatic or germinal) have resulted in sporadic use of this technique (which is also evident from the limited number of relevant articles recently published on this subject).

### Nuclear transfer (cloning)

The interest for transfer of nuclear material from a donor cell into a recipient enucleated cell was in early works expressed with amphibians (Briggs and King, 1952). However, the successful cloning of embryonic nuclei up to a viable progeny was performed quite recently in the sheep (Willadsen, 1986) while the successful cloning of Dolly (issued from a nucleus removed from a cultured cell transferred into a recipient, unfertilised egg) was

obtained in 1997. Since, this technique has been paid considerable attention by both the scientific and non scientific communities. Seen from a mammalian viewpoint, nuclear transfer requires successful accomplishment of three major steps: oocyte enucleation (recipient cell), isolation of donor nucleus and finally transfer into recipient (enucleated) cell. These steps, up-to-date, have not been successfully achieved in avian species. Japanese scientists described possible use of PGC cells, their nucleus fusion with somatic cells with following transplantation into the place of testes or ovary origin.

#### The "male route"

Spermatogonial stem cell transplantation into recipient testes (trivially referred to as "male route") was first successfully achieved in the mouse (Brinster and Avarbock, 1994) but since has been expended to several other species (Schlatt *et al.*, 1999, Trefil *et al.*, 2003). Meanwhile, despite the remarkable progress observed in this field, the number of papers published on this subject in mammals has remained quite sporadic nevertheless it is legion compared to the quasi-absence of interest expressed by poultry physiologists. In our view, this route may at first appear highly risky in species which testes are located in the middle of the body cavity.

At present there is no big problem to store spermatozoa for a long time in liquid nitrogen in species like *Gallus domesticus* and others. If we want to speak about storing of W chromosome we have to think about freezing of blastodermal or primordial germ cells carrying W chromosome (selected before storage by PCR on the presence of W chromosome) and about consequent use of developed technique for chicken chimeras production for reintroduction of these W chromosome into the genome of produced chimeras. There exists several hundred different chicken species all over the world but only several breeds are used for breeding of current hybrids. There is a tremendous demand for establishing gene bank to save genetic material (Z and W chromosome) of specific local chicken breeds. World globalisation in poultry production can reduce populations of these native chicken breeds. It seems to be right time for establishing modern chicken breeds bank for saving native breeds for other generations and exploration.

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