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Review

Prospects for transgenesis in the chick

Helen Sang*

Roslin Institute, Roslin, Midlothian EH25 9PS, UK

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Abstract

Research to develop a useful method for genetic modification of the chick has been on-going since the first demonstrations in the mouse in the 1980s that genetic modification is an invaluable tool for the study of gene function. Manipulation of the chick zygote is possible but inefficient. Considerable progress has been made in developing potentially pluripotent embryo stem cells and their contribution to somatic chimeric birds well-established. Germ line transmission of gametes derived from genetically modified embryo cells has not been described. Transfer of primordial germ cells from a donor embryo to a recipient and production of functional gametes from the donor-derived cells is possible. Genetic modification of primordial germ cells before transfer and their recovery through the germ line has not been achieved. The first transgenic birds described were generated using retroviral vectors. The use of lentiviral vectors may make this approach a feasible method for transgenic production, although there are limitations to the applications of these vectors. It is likely that a method will be developed in the next few years that will enable the use of transgenesis as a tool in the study of development in the chick and for many other applications in basic research and biotechnology.
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1. Introduction

The development of methods to genetically modify the chick has been challenging a small number of researchers since the first successful production of transgenic mice highlighted the value of such a technology for the study of gene function. Much of the published research has described attempts to modify methods first developed in the mouse and apply them to the chick. The chick has posed a significant challenge due to the specific features of the reproductive system and the necessity for embryo development to take place within a shelled egg. These aspects of chick development and the results of the varied approaches to developing a robust method for transgenesis in the chick have been discussed in detail in several reviews (Zajchowski and Etches, 2000; D'Costa et al., 2001; Ivarie, 2003; Mozdziak and Petitte, 2004) and will be outlined below. This review will not consider the many possible applications of transgenesis in the chick.

2. Manipulation of the zygote

An obvious approach to production of transgenic birds would be to replicate the method, developed in mice and successfully applied in several mammalian species, of pronuclear injection of newly fertilised eggs. Although this method does not allow the more sophisticated modifications possible with gene targeting, it has been used in many informative transgenic studies. Simple modification of the method used in mice is not possible because the chick oocyte or newly fertilised zygote is relatively inaccessible and difficult to handle. A laying hen ovulates once a day and the oocyte (the yolk) is immediately taken up by the oviduct and fertilised within approximately 15 min of ovulation. The egg then spends the next approximately 24 h travelling down the oviduct where firstly the albumen is laid down then, in the uterus or shell gland, the eggshell membrane and the egg shell are synthesised (Fig. 1). During this time the embryo develops rapidly, reaching the blastodermal stage of development by the time the egg is laid. The newly fertilised zygote may be recovered from the oviduct by sacrificing a laying hen within two to three hours of the previous oviposition (Sang, 1994). A culture method has been

* Tel.: +131-527-4234; fax: +131-440-0434.
E-mail address: helen.sang@bbsrc.ac.uk

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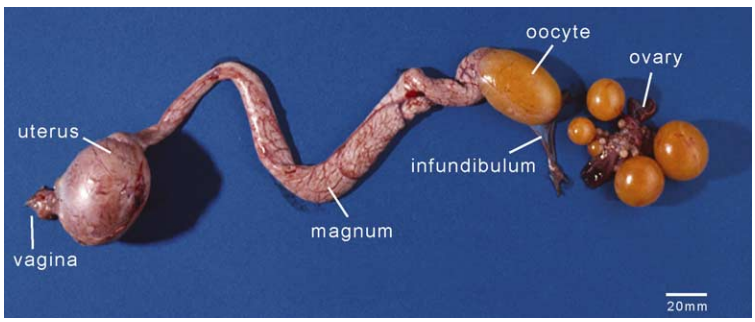

Fig. 1. The oviduct of a laying hen. Developing yellow yolky follicles can be seen in the ovary, on the right. An oocyte has recently been released and is in the infundibulum at the top of the oviduct. The magnum region is where the albumen is synthesised. An egg, just prior to lay, is in the uterus or shell gland. By this stage the embryo will have developed to form a disc of approximately 60,000 cells. Photograph courtesy Roslin Institute.

developed, using host shells and albumen as culture medium, which supports development of zygotes removed from laying hens to hatch, with a hatch rate of 5–10% (Perry, 1988). This method enabled development of a method to produce transgenic chickens by microinjection of gene constructs into the zygote, followed by culture to hatch. The pronuclei of the zygote are found in the germinal disc, the white spot on the surface of the yolk. Visualisation of the pronuclei is not readily possible in live material as they are located approximately 50 μm below the surface of the yolk and the cytoplasm is opaque with white yolk particles (Waddington et al., 1998). Microinjection of linearised gene constructs has been used successfully to produce a germline transgenic cockerel (Love et al., 1994) but the frequency achieved with this method is low and the technique is labour intensive. It may be possible to combine this method with other approaches to promote chromosomal integration of injected DNA. A plasmid carrying the *Drosophila* transposable element *mariner*, that transposes by a cut and paste mechanism, was injected into the cytoplasm of chick zygotes (Sherman et al., 1998). Analysis of surviving embryos and chicks indicated that *mariner* had transposed at high frequency into the chicken genome. Transposition of *mariner* was confirmed by analysis of transgenic offspring bred from a cockerel carrying *mariner* sequences. These results suggest that *mariner* or another transposon, for example the *Sleeping Beauty* element (Isvak and Ivics, 2004), could be used to improve the efficiency of the zygote injection method, although at present transposon vectors are limited in the size of transgene they can carry.

3. Production of chimeras using the ES cell approach

The isolation of pluripotent cells from the inner cell mass of mouse blastodermal cells, embryo stem (ES) cells (Evans and Kaufman, 1981), has led to the development of more sophisticated methods for generation of transgenic mice. Recapitulation of the murine ES cell approach requires isolation of pluripotent cells from the early embryo that can be maintained in culture for a sufficient period to allow genetic modification and preferably selection of modified cells, plus a method for reintroduction of the cells into early embryos where they must contribute to the developing embryo, specifically to the germ line.

Efforts to isolate the chick equivalent of mouse ES cells have focussed on manipulation of cells from the embryos from new laid eggs. The chick embryo in the new laid egg is at a stage of development approximately equivalent to the stage in mouse embryogenesis when ES cells can be isolated from the inner cell mass. At this stage of development, the stage X blastoderm (Eyal-Giladi and Kochav, 1976), is a single layer of cells that consists of two visibly distinct regions: the *area pellucida* or central disc, from which the embryo will develop, and the surrounding *area opaca* that gives rise to the extraembryonic membranes. Identification of cells equivalent to the inner cell mass of the mouse blastodermal embryo has not been possible as the organisation of the early chick embryo differs significantly from that of the mouse. Stern (1990) demonstrated that cells that will go on to form the hypoblast are scattered under the epiblast of the *area pellucida* at stage X. The chick homologue of the *Drosophila* gene *vasa*, that has an essential function in germ cell formation, has been identified (Tsunekawa et al., 2000). The *cvh* protein product is present in approximately 30 cells of the central zone of the *area pellucida*. It is thought that these cells are the precursors of the chick primordial germ cells and therefore, that PGCs are determined before the egg is laid.

Petitte et al. (1990) described the first experiments that suggested that stage X embryo cells could be transferred from one embryo to another and result in development of chimeric birds. Cells from stage X embryos of Barred Plymouth Rock chickens, which have distinctive black feathering, were injected into the subgerminal cavity of stage X embryos of an inbred line of Dwarf White Leghorns, which have white feathers. Feather-colour chimeric embryos were detected in 11.3% of the manipulated eggs and one male chimera hatched that on breeding proved to be a low level germ line chimera. A significant improvement in the proportion of chimeric birds produced by this method, and the level of both somatic and germ line chimerism, was achieved by development of a method that compromised the recipient embryos. Exposure of intact

eggs to 500–700 rads of γ-irradiation from a ^{60}Co source resulted in delayed development of the recipient embryos and over 60% production of somatic chimeras. A high proportion of the birds were also germ line chimeras and in some cases apparently 100% of the offspring of the chimeras were derived from the injected blastodermal cells (Carsience et al., 1993). These experiments have established the method for introduction of blastodermal cells and efficient production of high level chimeras.

In parallel with the development of the method for production of chimeras, methods to culture and transfect the blastodermal cells have been developed. Chick blastodermal cells were initially maintained in culture for a short period, transfected with a *lacZ* reporter construct and injected into recipient embryos (Brazolot et al., 1991). Chimeric embryos were analysed and expression of the reporter gene detected. These experiments were extended to development of culture conditions that supported long term growth, for at least 35 passages, in culture of stage IX–XI blastodermal cells (Pain et al., 1996). The cells were cultured on inactivated mouse STO feeder cells, in the presence of cytokines and growth factors including bFGF, LIF and SCF, plus anti-retinoic acid monoclonal antibody (ARMA). The cells are very similar to murine ES cells, in morphology, cytokine-dependent proliferation, telomerase activity and expression of epitopes specific to murine ES cells. Germ line chimeric birds can be generated using cells maintained in culture for short periods of time (Speksnijder et al., 1999). Although efforts to improve the culture methods and recover genetically modified cells have been continued in several laboratories, no results describing germline transmission from gametes derived from genetically modified cells have been described.

4. Production of germ line chimeras by primordial germ cell transfer

Primordial germ cells in the chick are morphologically distinct and can be easily identified by staining using the periodic-acid Shiff method just after primitive streak formation. By stage 10 (Hamburger and Hamilton, 1951, approximately 18 h of incubation) they are concentrated in the germinal crescent at the anterior of the embryo. As the vascular system develops the PGCs enter the circulation and begin to circulate through the embryo. The number of PGCs in the blood peaks between stages 15–16 of development. By stage 20 the PGCs actively migrate into the developing gonads (Fig. 2). The literature describing the origin and development of primordial germ cells in the chick is reviewed by D'Costa et al. (2001).

The potential for primordial germ cell transfer between individual embryos and their subsequent contribution to the germline of the recipient, was first demonstrated by Reynaud (1976). This easy access to the precursors of the gametes was recognised as a possible

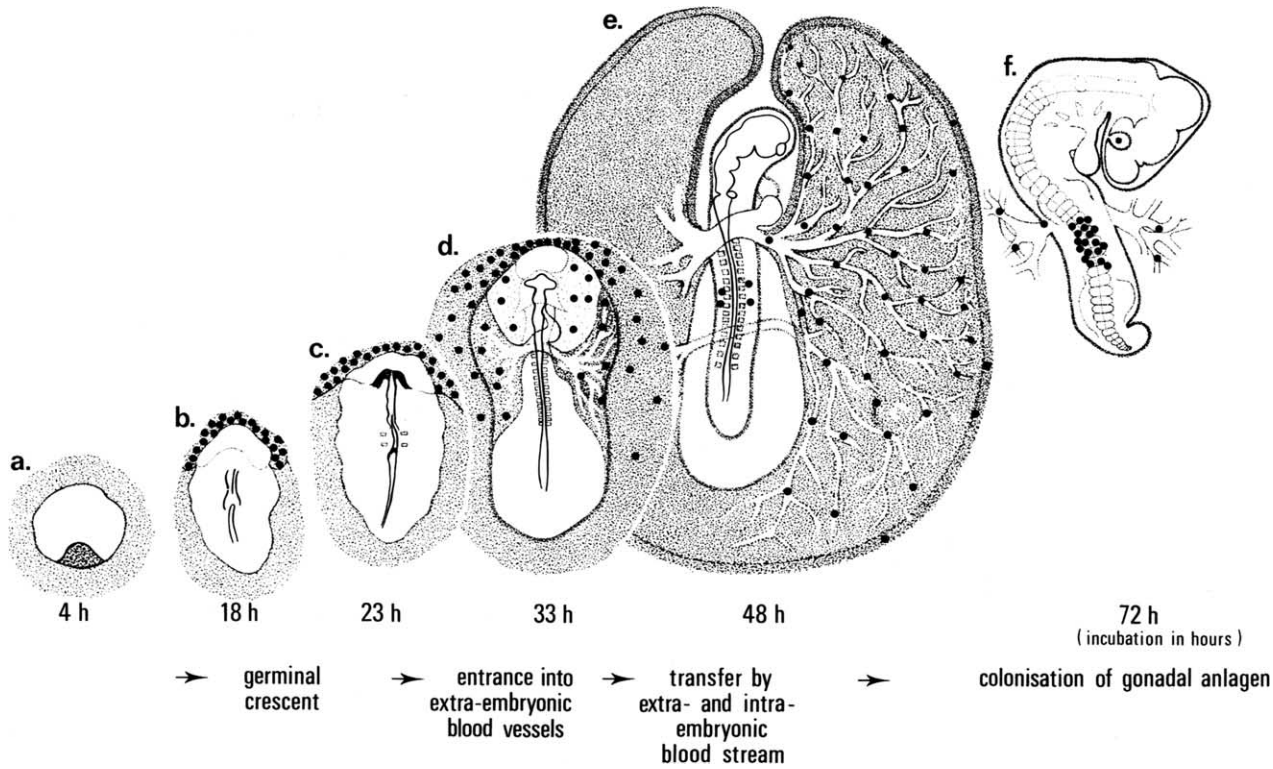

Fig. 2. Migration of PGCs in the chick embryo. (a) Absence of easily identifiable PGCs prior to primitive streak formation (b) and (c) accumulation of PGCs in the germinal crescent (d) penetration of the PGCs into blood islands (e) circulation of PGCs through the vascular system (f) colonisation of the gonadal ridges. Reprinted with permission from Nieuwkoop and Sutasurya (1979).

route to transgenesis: development of this method requires optimisation of the PGC isolation and transfer process, genetic modification of the PGCs before transfer and manipulation of the recipient to allow a greater contribution to the germ line of donor-derived PGCs. PGCs have been isolated from the germinal crescent (Vick et al., 1993a), from the circulatory phase, when they were concentrated on a Ficoll density gradient by centrifugation (Tajima et al., 1993) and from the gonads after colonisation (Han et al., 2002) and transferred to recipient embryos. In all cases the transferred PGCs have been shown to contribute to the germ line by breeding from recipients that survive to maturity and screening for donor-derived progeny using feather colour markers to distinguish them from progeny of the recipients. Several approaches have been taken to achieving partial sterilisation of the recipient embryos. Vick et al. (1993b) injected embryos at approximately 1 day of incubation with the drug busulphan that is toxic to PGCs, followed by transfer of donor PGCs into the circulatory system, just over one day later. This treatment increased the proportion of germ line chimeras and of donor-derived offspring from the chimeras. The other successful approach to sterilisation of recipient embryos is physical removal of PGCs. This has been achieved by either removing the central disc or by withdrawal of blood from the recipient embryos, during the stage at which PGCs are in the circulatory system and prior to injection of donor PGCs. Removal of up to 10 μl of blood from stage 14–15 recipient embryos can increase the proportion of germ line chimeras produced and the frequency of donor-derived offspring. Naito et al. (1994) describe a success rate of chimera production of 95%, with between 2 and 95% of offspring derived from the donor PGCs. Kagami et al. (1997) described a method for removal of approximately by 700 cells from the central disc of embryos in new-laid eggs, by a simple procedure involving sucking the cells out using a micropipette. A high proportion of germ line chimeras were produced after donor PGC injection. These two methods for depletion of recipient PGCs have been combined (Naito et al., 1999), although complete sterilisation of the recipients is not achieved.

The methods described above for isolation and transfer of PGCs are quite robust but the utilisation of these methods for effective generation of transgenic birds has yet to be successful. Preliminary studies have described production of transgenic chickens using genetically modified PGCs (Vick et al., 1993b; Wentworth et al., 1996) but these have not been developed to give a method that can be used to generate transgenic birds at a useful frequency. Naito et al. (1998) described transfection of PGCs by lipofection and detection of expression of the introduced reporter gene construct in germ cells in gonads but germ line transmission of stably modified cells was not shown. Han et al. (2002) have developed a method for long term culture of gonadal PGCs that may form the basis of a method for transfection and selection of stably modified PGCs.

5. Gene transfer using viral vectors

The possibility of using viral vectors to make transgenic birds was recognised very early in the development of transgenic technologies. This was partly because retroviral vectors were being tested for production of transgenic mice and because several avian retroviruses had been studied in detail so that there was significant background knowledge to utilise in modifiying them to make avian retroviral vectors. The ability to transduce chick embryos by injection of viral preparations near the blastodermal embryo in new laid eggs, through a window in the shell that was then resealed, was shown to be a simple procedure (Salter et al. 1987). This method was used to generate transgenic birds using replication-competent vectors derived from avian leucosis virus (ALV) (Salter et al., 1993). Replication defective vectors derived from reticuloendotheliosis virus (REV) and ALV were developed and used to produce transgenic birds (Bosselman et al., 1989; Thoraval et al., 1995). These studies resulted in production of chimeric transgenic birds with the viral vector present at low levels in founder (G_0) birds and very low frequencies of germ line transmission. For example, Bosselman et al. (1989) described injection of REV vector at a titre of approximately 10^4 TU/ml into 2599 eggs, 38% of which hatched. Approximately 8% of the male birds raised contained vector sequences and of those bred to determine germ line transmission all transmitted the vector to between 2 and 8% of their offspring. Although these results were encouraging this approach was not continued for some time, in part because the vectors available were limited in the size of transgene they could carry and because of potential issues related to the use of vectors derived from viruses that are widespread within poultry populations.

The use of vectors derived from avian retroviruses has been revisited. The ALV vectors described by Thoraval et al. (1995) have been used in conjunction with a high-throughput screening method to try and overcome the problem of low germ line transduction (Harvey et al., 2002a). This method was applied to screening for production of transgenic birds transduced with an ALV vector: 546 eggs were injected from which 126 chicks hatched and 10% identified as containing a low but detectable level of the vector. Sperm DNA from 56 roosters was screened by real-time PCR and 3 birds detected as potentially germ line transgenic. Only one of these roosters produced transgenic progeny at a rate of approximately 0.7% (Harvey et al., 2002b). This approach has been used to generate transgenic birds expressing a low level of human interferon α-2b, by incorporation of a transgene including the CMV promoter, again at low frequency (Rapp et al., 2003). These results demonstrate that germ line transgenic birds may be produced and transgene expression detected

but the frequency is such that testing of a range of constructs would be difficult and tissue-specificity of expression has yet to be shown. An improvement in germ line transduction frequency, one of 15 males, has been shown using a spleen necrosis virus-based vector and expression of the reporter gene detected in cultured myoblasts (Mozdziak et al., 2003).

Mizuarai et al. (2001) described the use of a pantropic replication-defective retroviral vector to produce transgenic quail with high frequency. A vector based on Moloney murine leukaemia virus (MoMLV) at a titre of approximately 10^9 TU/ml, pseudotyped with vesicular stomatitis virus G protein (VSV-G), was used to transduce quail embryos, following the method described above. The viral vector sequences were detected in all 37 G_0 chicks hatched and transmission to G_1 was an average of 80%. Expression of a neomycin-resistance reporter gene, under control of the Rous Sarcoma Virus promoter was detected by RT-PCR but expression of GFP, controlled by the MoMLV LTR promoter, was not seen. These results were encouraging, indicating that the combination of a very high titre of vector and VSV-G pseudotyping of the virus could result in high transduction frequencies of the manipulated embryos. The results gave limited indication of the potential for transgene expression or vector capacity in terms of transgene size. It has been suggested that MoMLV-derived vectors may not be widely useful due to problems of silencing of expression of transgenes carried in the vector (Challita and Kohn, 1995; Ikawa et al., 2003).

A new class of vectors are currently being developed, mainly for applications in gene therapy, derived from

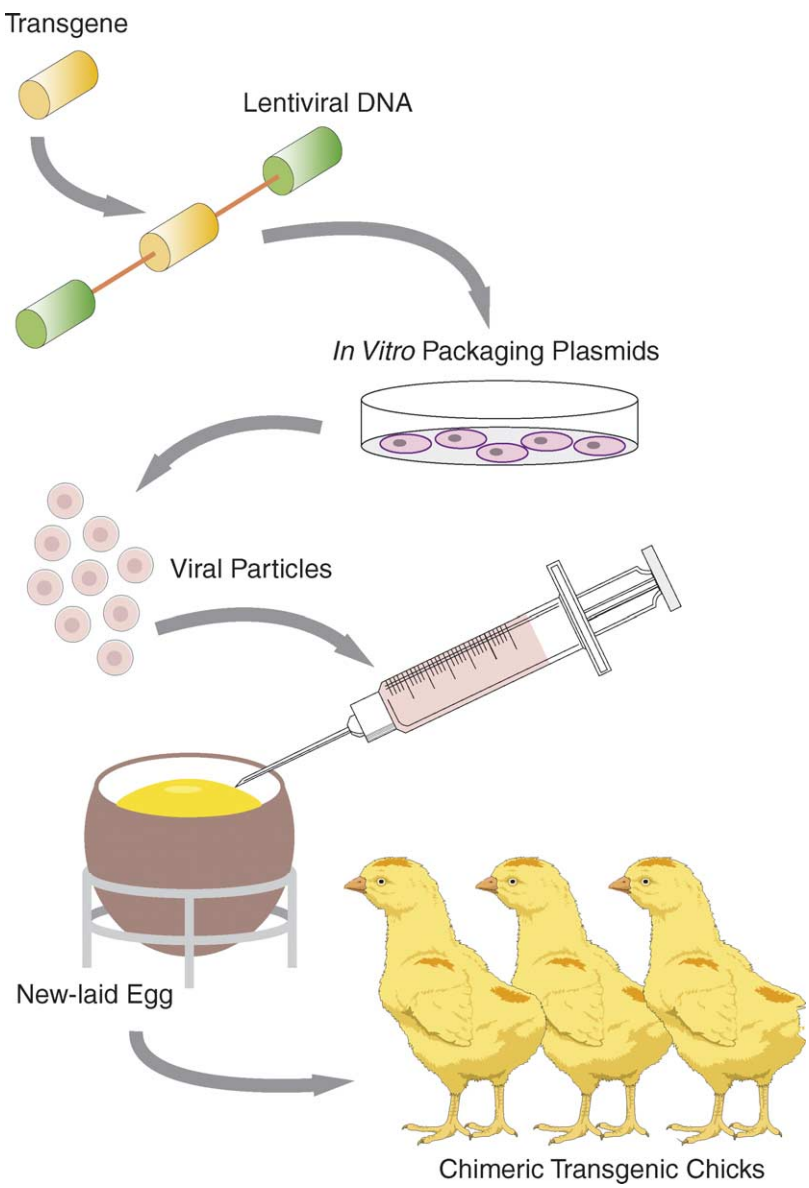

Fig. 3. Preparation and transduction into the chick embryo of lentiviral vectors. A transgene is cloned into an EIAV vector and cotransfected with packaging plasmids into 293T cells. The packaged viral vector is harvested from the medium, concentrated and then injected below the blastodermal embryo in a new laid egg. The embryo is then cultured to hatch and the resulting chimeric chicks screened by PCR to identify G_0 transgenic birds.

members of the lentivirus class of retroviruses. Lentiviral vectors have several advantages over vectors based on oncogenic retroviruses, including the ability to transduce non-dividing cells (Naldini et al., 1996), a transgene capacity of at least 8 kb and, with particular relevance to their use in production of transgenic animals, maintenance of tissue-specific transgene expression after germline transmission. Lois et al. (2002) used vectors derived from HIV-1, pseudotyped with VSV-G and carrying a ubiquitously expressed promoter driving expression of green fluorescent protein (GFP), to transduce single-cell mouse embryos. Production of transgenic mice was efficient (80%) and GFP expression was detected in G_1 offspring. Expression of muscle-specific and T lymphocyte-specific promoters was detected only in appropriate cell types. In another study (Pfeifer et al., 2002), HIV vectors were used to transduce mouse ES cells that were used to make chimeric mice. Embryos derived from crossing these mice expressed the transgene.

The possible advantages of lentiviral vectors over the viral vectors previously tested in avian systems encouraged us to evaluate their use in the production of transgenic chickens (McGrew et al., 2004). Vectors derived from the lentivirus equine infectious anaemia virus (EIAV) pseudotyped with VSV-G, were prepared at titres of between 10^8 and 10^{10} and 1–2 μl of viral suspension injected into the subgerminal cavity of embryos in new laid eggs, that were then cultured to hatch using the method of Perry (1988) (Fig. 3). In this initial study, 12 cockerels were hatched from 73 manipulated eggs and screened by PCR to detect transgenic birds. Eight of the cockerels were identified as chimeric for the viral vector, at levels ranging from 1 to 10%. Interestingly, when DNA from semen was screened all the birds appeared to be transgenic. When ten of these roosters were bred to determine germ line transmission frequency they all produced transgenic offspring, with frequencies ranging from 4 to 45%. The reason for this apparent approximately 10-fold higher transduction of germ cells compared to somatic tissue is not known. It may be because, at the time the virus is injected into the chick embryo, the PGCs are migrating through the subgerminal cavity onto the developing hypoblast below (Urven et al., 1988). Stable transmission of proviral insertions has been shown to the G_2 generation for several lines. Analysis of GFP and *lacZ* reporter genes, driven by the CMV promoter, indicated that the level of transgene expression varied between individual proviral insertions but that the pattern of expression between individuals is very similar. Expression was detected at a low level in most tissues but at a high level in pancreas and skin. Comparison of expression patterns and levels between individual G_1 birds and their G_2 offspring demonstrated that both the pattern of expression and the level of expression in different tissues was conserved after germ line transmission.

These results suggest that the use of lentiviral vectors may be an effective method for production of transgenic birds. We have yet to demonstrate that tissue-specific expression can be achieved using specific enhancer and promoter sequences but evidence from other species is encouraging. Some sequences may not be compatible with lentiviral vectors, because they are too long or because they contain splice sites or polyA addition sites (Lois et al., 2002). We have successfully generated transgenic birds containing the CAGGS enhancer/promoter (Niwa et al., 1991) that contains the chicken β-actin promoter and first intron, driving expression of GFP (Fig. 4). This transgene has been used to promote ubiquitous expression of GFP in transgenic mice and we will analyse the embryos produced by the G_1 birds to determine if the expression is the same in these transgenic birds, a possible useful resource for developmental studies.

Fig. 4. GFP expression in a transgenic chick. The chick on the right is a G_1 transgenic carrying the CAGGS-GFP transgene while the chick on the left is a non-transgenic sib, photographed under fluorescence.

Lentiviral vectors have been used successfully to generate transgenic pigs and bovine oocytes have also been transduced (Hofmann et al., 2003). Some very useful applications of the technology are being developed in the mouse that could be utilised in the chick system. A potential application of lentiviral vector-mediated transgenesis is the expression of small interfering RNAs (siRNAs) for gene knockdown studies. This use of lentiviral vectors has been exemplified in studies in transgenic mice (Tiscornia et al., 2003; Rubinson et al., 2003) and should be readily applicable in the chick.

6. Prospects for transgenesis in the chick

The progress in development of the methods outlined above is very encouraging, although all either still have technical challenges to overcome or have limitations in their application. Manipulation of the oocyte or zygote is possible and may become more useful if it becomes possible to increase the frequency of integration of microinjected gene

constructs. This method could also underpin the development of nuclear transfer in the chick, a method that is used as a route to transgenesis in livestock species (Thomson et al., 2003). This approach does require access to laying hens and is technically demanding and is unlikely to be applied widely if these resources are not available.

The ES cell route to transgenesis is potentially a very useful method as it would enable sophisticated modifications of the cells in culture prior to their recovery through chimeric intermediates. There is no report as yet that chick blastodermal cells that have been maintained in culture for more than a week contribute to the germ line in chimeras, even if they apparently make up approaching 100% of the somatic cells of the chimera. If this problem can be overcome, perhaps by gaining a better understanding the development of germ cells in the chick (see Petitte et al., 2004), then this method would become one of choice for many applications. Significant advances have been made in production of germ line chimeras by transfer of PGCs from one embryo to another. Successful germ line transmission of modified cells has not been reported. It may be possible to for example transduce PGCs with lentiviral vectors prior to transfer but the method could be more laborious than required. It is unlikely that PGCs can be maintained in culture and proliferate for the extended period necessary to identify gene targeting events, without losing their ability to migrate to the developing gonad after transfer.

The first transgenic chickens were produced using retroviral vectors derived from avian viruses and this approach has been continued. The frequency with which transgenic founder birds were generated and the frequency of transgenic offspring from these birds have been generally low. Our recent results using lentiviral vectors pseudotyped with VSV-G suggest that this development of the viral vector approach is highly efficient and may be more widely applied. The preliminary results on transgene expression in several generations of transgenic birds carrying reporter gene constructs indicate that transgene expression will be reproducible and that transgene silencing is not induced. Currently the level of transgenic somatic cells produced is low so transgene analysis must be carried out in G_1 progeny of founder birds, adding a significant time, at least 6 months, before the transgenic embryos or birds can be analysed.

Few research laboratories have access to facilities for raising and breeding birds that will allow them to exploit transgenic technologies fully. Establishment of centres where transgenic birds can be generated and bred could be of benefit to the research community. There is an advantage in the chick system in that many transgene constructs may be analysed by transient expression in embryos by electroporation (Itasaki et al., 1999), enabling selection of key constructs for generation of transgenic lines. It is likely in the next few years that one or more of the methods described in this review will be developed to a stage where it is feasible to apply it to answering many questions, not only in studies of development but in applied areas, for example the study of disease, investigation of quantitative traits and exploiting the potential for production of pharmaceutical proteins in eggs. It is also possible that some of these methods may be successfully combined or that a fresh approach may be taken to develop a technically feasible and efficient method for transgenesis in the chick.

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