Development of an *in vitro* Model for Analysis of Transgene Expression in the Hen Oviduct

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Declaration

I declare that the work presented in this thesis is my own, except where otherwise stated. All experiments were designed by myself, in collaboration with my supervisor Dr. Helen Sang. No part of this thesis has been, or will be, submitted for any other degree, diploma or qualification.
Acknowledgements

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Abstract

A potential application of transgenesis in birds is the production of therapeutic proteins in eggs. It has recently been shown that lentiviral vectors can be used to generate transgenic chickens with high frequency. Expression of therapeutic proteins in the egg white is likely to be achieved by using the regulatory sequences of the egg white protein genes to drive expression of the therapeutic protein. Analysis of transgene expression in egg white will require production of adult transgenic hens, which takes approximately one year. An in vitro method in which to investigate transgene expression in the chicken oviduct prior to the generation of transgenics is desirable. The egg white protein genes are expressed in the tubular gland cells (TGCs) of the oviduct of laying hens. To analyse transgene expression in TGCs the development of a method to culture active cells and achieve effective gene transfer into the cells is required. Culture of TGCs has been described for the study of hormonal regulation of eukaryotic gene transcription, but these cells were isolated from hormone-stimulated sexually immature chicks and only very low transfection efficiencies of these cells were reported.

TGCs were isolated from the magnum region of the oviduct of adult hens and were found to be sufficiently viable in culture for use to investigate transgene expression. Egg white mRNAs and proteins were detected in TGCs maintained in vitro for 72 hours but tests of transfection using several commercially available reagents, electroporation and lentiviral transduction indicated that the frequency of gene transfer was too low to be useful. As an alternative, the use of oviduct tissue explants was investigated. Explants were isolated from the magnum region of the oviduct of
sexually mature laying hens and the cells in the explants were found to be sufficiently viable in culture for use to investigate transgene expression. Egg white mRNAs and proteins were detected in the explants maintained in vitro for 4-5 days. Transfection using reagents was not successful but gene transfer into the explants was successfully achieved through electroporation. Expression of a therapeutic protein was detected in the explants after electroporation with transgene constructs carrying a ubiquitous promoter or the ovalbumin promoter. Gene transfer into the explants was also achieved through transduction with ELAV and HIV vectors pseudotyped with a variety of envelope proteins. The highest levels of transduction were achieved using vectors pseudotyped with the vesicular stomatitis virus G envelope protein. Expression of a therapeutic protein was detected in the explants after transduction with viral vectors carrying a ubiquitous promoter or the ovalbumin promoter. Chicken oviduct explants were identified as suitable for use in vitro to investigate transgene expression in the chicken oviduct.
CHAPTER 1 INTRODUCTION

1.1 Transgenic technology

The generation of transgenic animals requires the introduction of thousands of copies of a transgene DNA construct into an animal, usually at a very early embryonic stage of development, and stable integration of the transgene into the host genome. The ability to routinely produce transgenic mice has allowed the investigation of gene expression and function in vivo. However, the development of reliable and efficient systems for the production of transgenic livestock animals and particularly poultry has proved a challenge, with continued efforts being made towards this objective. Suggested applications of transgenic technology include the production of organs suitable for xenotransplantation into humans and the production of therapeutic proteins.

1.1.1 Production of therapeutic proteins

The production of valuable human therapeutic proteins in transgenic animals was proposed as a high-quantity, low-cost alternative to the use of chemical synthesis or microbial and mammalian cell culture expression systems. Although many pharmaceutical proteins are currently produced in bacterial fermentors, such as Escherichia coli culture systems, bacteria cannot perform the postsynthetic modification reactions required for full biological activity of a number of bioactive proteins. Many potential therapeutic proteins, such as α1-antitrypsin (Clark et al., 1998), require specific posttranslational glycosylation modifications that occur in vivo for their activity. Yeast and filamentous fungi bioreactors can be scaled-up with relative ease and are capable of post-translational modifications, however these
systems are also limited by their ability to duplicate human patterns of protein processing and can yield recombinant products with undesirable properties such as immunogenicity and lack of activity.

Over the past decade there has been considerable progress in mammalian expression systems for high-level recombinant gene expression. Chinese hamster ovary (CHO) and NS0 murine myeloma cell expression systems have been established as the predominant systems of choice for mammalian expression. However, scaling up these systems for mass production purposes is a very expensive procedure. An advantage of using CHO cells is that the glycosylation machinery is similar to that of humans (Stanley et al., 1996). However, there are several minor differences. For example, human immunoglobulin G (IgG) contain α-2,6-linked sialic acid residues, whereas CHO cell derived human IgG contain α-2,3-linked sialic acids (Shah et al., 1998). Although the integrity of polypeptide chains seems to be largely unchanged in the various expression systems and different culture conditions, significant changes in glycosylation have been noticed (Raju, 2003). Glycosylation has been found to vary with the cell line, animal species and with culture conditions (Raju et al., 2001).

Transgenic animals have a potentially large production capacity and lower costs than mammalian cell culture. The basic strategy for targeting expression of a foreign protein to a secretory tissue has been to identify the regulatory sequences of the protein genes required for high-level tissue-specific expression and subsequently linking these to the coding sequences of a therapeutic protein (Lillico et al., 2005). The lactating mammary gland has generally been considered the tissue of choice to
express valuable recombinant proteins due to the fact that milk is easily collected in large volumes.

Milk composition was first altered in lactating mammary glands of mice harbouring transgenes expressed from mammary gland-specific promoters (Simons et al., 1987; Andres et al., 1987). A great deal of effort has since been made to produce transgenic bioreactors with traditional ‘dairy’ species such as cattle (Hyttinen et al., 1994; van Berkel et al., 2002; Thomassen et al., 2005), goats (Denman et al., 1991; Ebert et al., 1991; Zhang et al., 1997) and sheep (Pursel et al., 1987; Clark et al., 1989).

The production of proteins in the milk of domestic livestock is limited by the long interval from birth to the first lactation, the discontinuous nature of the lactation cycle and the substantial time and material investments needed to produce transgenic dairy animals. Transgenic rabbits (Brem et al., 1994; Van den Hout et al., 2004) and pigs (Swanson et al., 1992; Paleyanda et al., 1997; van Cott et al., 1999) expressing foreign proteins in their mammary glands have been made to address this problem, but milk production rates and numbers of animals needed to produce adequate amounts of protein can be limiting (Dyck et al., 2003).

A variety of human proteins have been expressed in the milk of several species of animals, including insulin-like growth factor 1 in rabbits (Brem et al., 1994), α1-antitrypsin in sheep (Wright et al., 1991), antithrombin III in goats (Edmunds et al., 1998), and protein C in pigs (Velander et al., 1992). The first advanced clinical trials are now underway. The enzyme α-glucosidase from the milk of transgenic rabbits has been successfully used for the treatment of Pompe’s disease in infants (Van den Hout et al., 2004). Several biotechnology companies are developing protein therapeutics
produced in milk. GTC Biotherapeutics, Inc. (http://www.transgenics.com) is at the most advanced stage of product development, with recombinant antithrombin III undergoing review for market authorisation in Europe for the treatment of hereditary antithrombin deficiency. Recombinant C1 inhibitor for the treatment of hereditary angioedema, produced in transgenic rabbit milk, is in phase III clinical trials by Pharming Group N.V (http://www.pharming.com).

1.1.2 Producing therapeutic proteins in hens' eggs

The use of transgenic hens as bioreactors for the production of pharmaceutical proteins presents an attractive alternative to mammals. The standard approach for the production of therapeutic proteins in transgenic mammals is to use the regulatory sequence of a gene encoding a major secreted protein to direct the expression of the therapeutic protein to a secretory tissue such as the mammary gland. In transgenic hens, the regulatory sequence of one of the egg white protein genes would be used to direct the expression of the therapeutic protein to the egg white. As discussed in Section 1.3, the egg white proteins are secreted by the tubular gland cells in the magnum region of the oviduct of laying hens.

There are a number of potential advantages to using chickens as transgenic bioreactors for the production of functional, non-immunogenic pharmaceutical proteins in eggs. The egg white is a naturally sterile environment that can be stored for long periods of time. It contains approximately 4 grams of protein per egg and protease inhibitors in the egg white provide an ideal environment for stabilising the biological activity of foreign proteins (Rapp et al., 2003). Egg collection, cleaning, cracking, and separation of whites from yolks are fully automated at enormously high
throughput. With modern layers producing over 300 eggs per year, the egg industry can generate thousands of eggs per day, therefore it is feasible that large quantities of human proteins could be harvested from the eggs of transgenic hens. There is considerable commercial expertise available in the processing of eggs and the purification of some components, for example, lysozyme (Lillico et al., 2005). Current good manufacturing practice (cGMP) production of over 300 million measles vaccines in fertilized eggs for use in humans has laid the regulatory foundation for FDA approval of human biopharmaceuticals in eggs (Peltola et al., 1994).

Many therapeutic proteins, including antibodies, require correct post-translational glycosylation for normal function. A potential advantage of using chickens as bioreactors is that some of the oligosaccharide moieties added to nascent polypeptides in the chicken have greater similarities with the sugars used by humans than those of other mammals (Lillico et al., 2005). A comparison of thirteen species-specific modifications of immunoglobulins found that chicken and human glycosylation had the greatest similarity (Raju, 2003). Therefore, pharmaceutical proteins produced in the eggs of transgenic hens could potentially be functional and non-immunogenic.

Kamihira and colleagues (2005) recently generated transgenic hens that produce a therapeutic protein in their eggs. They used a Moloney murine leukaemia virus (MoMLV)-derived mouse stem cell virus (MSCV)-based retroviral vector to generate transgenic birds through retroviral injection at the later stages of embryonic development. The vector encoded expression of an antiprion single-chain Fab variable (Fv) region fused with the Fab constant (Fc) region of human immunoglobulin G1 (scFv-Fc). Transgenic hens produced scFv-Fc in their serum and eggs at levels of
approximately 5.6mg/ml. First generation (G1) transgenic progeny appeared with a frequency of 3.3%. Five G1 transgenic chickens expressed scFv-Fc at 0.5-1.9mg/ml in their serum, and G1 transgenic hens produced the protein at 0.2-1.5mg/ml in the egg white.

Zhu and colleagues (2005) recently reported tissue-specific expression of a human monoclonal antibody in the egg white of transgenic hens driven by ovalbumin regulatory sequences. They established and transfected lines of chicken embryonic stem (cES) cells and formed chimeras expressing the antibody. Although they observed low-level expression of the ovalbumin transgene in the gut of chimeras, expression was reported to be largely tissue-specific and restricted to the tubular gland cells in the oviduct. Eggs from chimeras contained up to 3mg of monoclonal antibody that possessed enhanced antibody-dependent cellular cytotoxicity, non-antigenic glycosylation, acceptable half-life, excellent antigen recognition and good rates of internalisation. From these results, Zhu and colleagues concluded that the chicken system is an attractive alternative to plant and mammalian transgenic systems for producing human monoclonal antibodies.
1.2. Strategies for genetic modification of hens

Development of an efficient method for genetic modification of hens has proved a significant technical challenge. Procedures that have worked for other animals are difficult, if not impossible, to apply to chickens due in part to their unique reproductive physiology. The chick oocyte or newly fertilised zygote is fragile 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 minutes of ovulation. The egg then spends the next 24 hours travelling down the oviduct where the albumen is laid down followed by the egg shell membrane and egg shell. During this time the embryo develops rapidly and when the egg is laid the embryo has developed to form a disc of approximately 60,000 cells, of which approximately 50 give rise to the primordial germ cells (Karagenc et al., 1996).

1.2.1 Microinjection of DNA

Microinjection of DNA directly into the pronucleus of fertilized eggs is the most widespread method for generating transgenic animals. In the early 1980s, Gordon and colleagues discovered that transgenic mouse embryos could be generated by microinjection of DNA into pronuclei (Gordon et al., 1980). This technique yielded transgenic mice stably harbouring the transgene and demonstrated germline transmission of foreign DNA (Gordon and Ruddle, 1981).

In mammals, pronuclear injection of DNA involves the injection of several hundreds to thousands of copies of a transgene DNA construct directly into one of the two pronuclei of a recently fertilized ovum. The zygotes are then transferred to recipient females and the offspring screened for integration of the transgene. This technique has
also been used to produce transgenic rabbits, sheep and pigs (Hammer et al., 1985), fish (Ozato et al., 1986), cattle (Pursel, 1989; Bondioli et al., 1991) and goats (Denman et al., 1991; Ebert et al., 1991). Although pronuclear injection is considered reliable, the efficiency is low, with only 3-5% of the animals born carrying the transgene (Nottle et al., 2001).

Microinjection is difficult in the hen because the newly fertilized ovum is relatively inaccessible and very fragile. Preliminary attempts to microinject DNA into ova immediately after fertilisation in early embryos cultured in vitro (Perry, 1988) resulted in episomal DNA that persisted only transiently in the developing embryo (Sang and Perry, 1989). However, transgenic chickens were later successfully generated with this technique (Love et al., 1994). Seven chicks, 5.5% of the total number of injected ova, survived to sexual maturity. One of these, a cockerel, transmitted the exogenous DNA to 3.4% of his offspring.

Germline transmission was also achieved by using the *Drosophila* transposable element *mariner* (Sherman et al., 1998). Transgenic hens were generated using microinjection into the cytoplasm of the germinal disc of chick zygotes, followed by culture of the manipulated embryos using the method of Perry (1988). Analysis of embryos that survived for at least 12 days of development indicated that *mariner* had transposed at high frequency into the chicken genome. The transgenic birds analysed in the study were all mosaic, transmitting the introduced gene construct to one to five percent of their offspring. Further development of *mariner* as a vector for transgenesis in hens has not been described.
1.2.2 **Manipulation of chick embryos at the new laid egg stage**

Embryonic stem (ES) cells are derived from the undifferentiated inner mass cells of a blastocyst, which is an early stage embryo. They are pluripotent, meaning that they are able to grow into any of the cell types in the body. In the early 1980s, mouse embryonic stem (ES) cells were isolated (Evans and Kaufman, 1981), which led to the development of 'gene targeting', the introduction of exogeneous DNA to specific sites in the genome by homologous recombination, for the generation of transgenic mice. In mouse transgenic systems sophisticated gene targeting strategies now permit investigators to manipulate the genome.

Efforts to isolate the chick equivalent of mouse ES cells have focussed on manipulation of cells from embryos from newly laid eggs. The chick embryo in the newly 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. Cells equivalent to the inner cell mass of the mouse blastodermal embryo have not been identified in the chick embryo as the physiology differs significantly from that of the mouse.

Petitte and colleagues (1990) described the first experiments that demonstrated that blastodermal cells, isolated from the centre of the area pellucida of the blastoderm from freshly laid fertilised eggs, could be transferred from one embryo to another and result in development of chimeric birds. Cells from freshly laid fertilised eggs of black-feathered Barred Plymouth Rock chickens were injected into the subgerminal cavity of freshly laid fertilised eggs of an inbred line of white-feathered Dwarf White
Leghorns. In 11.3% of the manipulated eggs feather-colour chimeric embryos were detected, with one male chimera proving to be a low level germline chimera.

The exposure of intact eggs to 500-700 rads of γ-irradiation from a $^{60}$Co source resulted in a significant improvement in the proportion of chimeric birds produced by this method and the level of both somatic and germline chimerism (Carsience et al., 1993). Introduction of DNA into blastodermal cells is possible by lipofection (Brazolot et al., 1991; Fraser et al., 1993). Although chimeras bearing transfected, pluripotent blastodermal cells can readily be produced (Carsience et al., 1993), germline transmission from gametes derived from genetically modified cells has not been reported.

Pain and colleagues (1996) reported that the chicken early blastoderm contains cells characterised as putative avian embryonic stem (ES) cells that could be maintained in vitro for up to three weeks. These cells exhibited features similar to those of murine ES cells such as typical morphology, strong reactivity toward specific antibodies, cytokine-dependent extended proliferation and high telomerase activity. These cells were also reported to differentiate in vitro into various cell types including cells from ectodermic, mesodermic and endodermic lineages. Chimeras were produced after injection of the cultivated cells and the evidence for donor-derived contributions from cultured cells was observed at low frequency for the germline in at least two chickens.

As discussed in Section 1.1.2, Zhu and colleagues (2005) established and transfected lines of chicken embryonic stem (cES) cells and formed chimeras expressing a human monoclonal antibody in the egg white driven by ovalbumin regulatory sequences. To
accommodate large regulatory regions, they extended cES cell technology by maintaining the cells indefinitely, developing a system for transfecting the cells with large transgenes of 41-49kb and demonstrating that genetically modified cells retain the ability to make somatic chimeras. Eggs from chimeras were reported to contain up to 3mg of monoclonal antibody.

1.2.3 **Targeting primordial germ cells**

The use of primordial germ cells (PGCs) as a possible route to transgenesis in the chicken requires the optimisation of PGC isolation and transfer, genetic modification of the PGCs prior to transfer and manipulation of the recipient to allow a greater contribution to the germ line of donor-derived PGCs. PGCs 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 approximately 18 hours of incubation the PGCs 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 prior to actively migrating into the developing gonads.

The potential for PGC transfer between individual embryos and their subsequent contribution to the germline of the recipient was first demonstrated in the 1970s by Reynaud (1976). PGCs have been isolated from the germinal crescent (Vick et al., 1993a), the circulatory phase (Tajima et al., 1993) and the gonads after colonisation (Ha et al., 2002) and transferred to recipient embryos. In all cases the transferred PGCs have been shown to contribute to the germline.
Vick and colleagues (1993b) increased the proportion of germline chimeras and of donor-derived offspring by injecting embryos at approximately one day of incubation with the drug Busulphan, which is toxic to PGCs, followed by transfer of donor PGCs into the circulatory system just over one day later. More recently, Song and colleagues (2005) reported that a solublised Busulfan emulsion (SBE) resulted in a more consistent and extensive depletion of PGCs than that observed with Busulfan treatment. Repopulation of SBE-treated embryos with exogenous PGCs resulted in a threefold increase of PGCs in embryos. Subsequently, germline chimeras were produced by the transfer of male gonadal PGCs from Barred Plymouth Rock embryos into untreated and SBE-treated White Leghorn embryos. The frequency of germline chimerism in SBE-treated recipients was reported to increase fivefold when compared to untreated recipients. The number of donor-derived offspring from the germline chimeras was also reported to increase eightfold following SBE-treatment of the recipient embryos.

Another successful approach to sterilisation of recipient embryos is the removal of PGCs, which 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 PGCs in blood. Naito and colleagues (1994) described a success rate of chimera production of 95% by injection of PGCs after withdrawal of blood from recipient embryos. 2-95% of the offspring were derived from the donor PGCs. Kagami and colleagues (1997) described a method for removal of approximately 700 cells from the central disc of embryos in newly laid eggs by a simple procedure involving the removal of the cells using a micropipette. A high proportion of germline chimeras were produced after donor PGC injection.
Preliminary studies have described production of transgenic chickens using genetically modified PGCs (Vick et al., 1993b; Wentworth et al., 1996), but these techniques have not been developed to generate transgenic birds at a useful frequency.

Naito and colleagues (1998) described transfection of PGCs by lipofection and detection of expression of the introduced reporter gene construct in germ cells in the gonads. However, germline transmission of stably modified cells was not shown. More recently, Ha and colleagues (2002) have developed a method for long term culture of gonadal PGCs that could potentially provide the basis for transfection and selection of stably modified PGCs for the generation of transgenic hens.

1.2.4 Retrovirus-mediated gene transfer

Retroviruses are RNA viruses that are packaged into a capsid and a membranous envelope. Due to their efficient mechanism for gaining entry into host cells and integrating into the hosts' chromosomes these viruses can successfully be used as gene transfer vectors. Retroviral life cycle, genome and vectors are discussed in more detail in Section 1.4.

In the early 1980s retroviral vectors were the first vectors used to achieve efficient and stable gene transfer into mammalian cells. Cone and Mulligan (1984) described the use of a Moloney murine leukaemia virus-based vector to efficiently transduce human HeLa cells and human histiocytic lymphoma cells. The transfer of genes into the germ line using retroviral vectors was first demonstrated in the mouse later in the 1980s by Soriano and Jaenisch (1986). The possibility of using retroviral vectors to make transgenic birds was then recognised, as retroviruses could potentially overcome the difficulty in accessing the germline cells in multi-celled chick embryos.
Salter and colleagues reported the generation of transgenic hens by microinjection into the subgerminal cavity using replication-competent retroviral vectors based on two chicken retroviruses, reticuloendotheliosis virus (REV) and avian leucosis virus (ALV) (Salter et al., 1986). Unfortunately, replication-competent retroviral vectors can result in viremia in transgenic birds and many do not survive to maturity due to pathogenic effects. Subsequent research overcame this problem by the development of replication-defective retroviral vectors where the virus is capable of only one round of replication. Bosselman and colleagues (1989) described the generation of transgenic birds using a replication-defective REV-derived vector. Approximately 8% of the male founder birds contained vector sequences and those bred to determine germline transmission all transmitted the vector to 2-8% of their offspring.

To try to overcome the problem of low germline transduction, avian leucosis virus (ALV)-based vectors developed by Thoraval and colleagues (1995) have been used in conjunction with a high-throughput screening method to identify transgenic chicks (Harvey et al., 2002a). 10% of hatched chicks were identified as low level transgenics. Three roosters out of the 56 screened by real-time polymerase chain reaction (PCR) were identified as potentially germline transgenic. One of these roosters was bred from and produced transgenic progeny at a rate of approximately 0.7% (Harvey et al., 2002b). This approach has also been used to generate transgenic birds expressing a low level of human interferon α-2b (Rapp et al., 2003). Only approximately 1% of hatched males proved to be germline transgenic and the transgenic birds transmitted the vector to less than 1% of their offspring. An improvement in the frequency of production of germline transgenic birds to 1 in 15
males has been shown using a spleen necrosis virus (SNV)-based vector, although the germline transmission frequency was still low at 0.9% (Mozdziak et al., 2003).

The use of a replication-defective retroviral vector to produce transgenic quail with high frequency was described by Mizuarai and colleagues (2001). Quail embryos were transduced using a vector based on Moloney murine leukaemia virus (MoMLV) pseudotyped with vesicular stomatitis virus G protein (VSV-G). Transgenic quail were generated with high frequency of over 80% germline transmission. These results were very encouraging as they suggested 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. Unfortunately, transgene expression was not detected in the birds and it has been suggested that MoMLV-derived vectors may not be widely useful due to problems with silencing of transgene expression (Ikawa et al., 2003).

1.2.4.1 Lentiviral vectors

A new type of vector derived from members of the lentivirus class of retroviruses has been developed, mainly for applications in gene therapy but also in transgenesis (for a detailed description of lentiviral vectors see Section 1.4.3.1). These vectors have potential advantages over those derived from oncoretroviruses, for example they are able to transduce non-dividing cells (Naldini et al., 1996) and have a transgene capacity of at least 8kb.

Lois and colleagues (2002) used green fluorescent protein (GFP) expressing vectors derived from human immunodeficiency virus (HIV-1) and pseudotyped with VSV-G
to transduce mouse embryos. This resulted in efficient production of transgenic mice at a frequency of 80% and GFP expression was detected in the first generation offspring. Pfeifer and colleagues (2002) also used HIV-based vectors to transduce mouse ES cells that were used to make chimeric mice, with embryos derived from crossing these mice expressing the transgene. Transgenic pigs (Hofmann et al., 2003, Whitelaw et al., 2004) and cows (Hofmann et al., 2004) have also been successfully generated using lentiviral vectors.

Lentiviral vectors based on equine-infectious anaemia virus (EIAV) have been successfully used to transduce the chicken germline (McGrew et al., 2004). PCR screening identified 8 of the 12 hatched cockerels as chimeric for the viral vector at levels ranging from 1-10%, however screening of the semen showed that all the birds were transgenic. Ten transgenic founder cockerels transmitted the vector to between 4-45% of their offspring and stable transmission to the second generation was demonstrated. Analysis of expression of reporter gene constructs in several transgenic lines showed a conserved expression profile between individuals that was maintained after transmission through the germline. This was the first demonstration of stable transgene transmission through the germline to the second generation using this method. These results suggest that the use of lentiviral vectors is an effective method for producing transgenic birds.

Tissue-specific expression in transgenic chickens generated using lentiviral vectors has yet to be reported. However, tissue-specific expression of green fluorescent protein (GFP) has been observed in oviduct tubular gland cells (TGCs) of lentiviral
transgenic chickens using the ovalbumin gene regulatory sequences to drive expression (Sang and colleagues, unpublished results).
1.3 **Production of therapeutic proteins in the hen oviduct**

The egg white protein gene regulatory sequences are obvious candidates for directing the expression of a therapeutic protein in the oviduct of a transgenic hen with its subsequent deposition into the egg white.

1.3.1 **Hen oviduct physiology**

The left and right ovary and oviduct are present in the embryonic stages of all female birds. The distribution of the primordial germ cells to the ovaries becomes assymetrical by day 4 of incubation and by day 10 regression of the right oviduct is initiated under the influence of Mullerian Inhibiting Substance (Whittow, 2000). The oviduct remains undeveloped until the hen reaches sexual maturity at 16-20 weeks of age when oestrogen stimulates epithelial stem cells to develop into three morphologically distinct cell types: tubular gland cells, ciliated cells and goblet cells. The oviduct then develops rapidly and becomes fully functional just prior to the onset of egg production. The oviduct is subdivided into five functional regions, the infundibulum, magnum, isthmus, uterus, and vagina, discussed in more detail the following Section (see Figure 1.1).
Figure 1.1 Oviduct from a sexually mature adult chicken. (a) U = uterus (shell gland), Is = isthmus, M = magnum, In = infundibulum, O = ovary. White dotted lines represent the boundaries of the magnum. (b) Ovum in the magnum region of the oviduct. Size bar represents 20mm.
1.3.2 **The egg-laying cycle**

The domestic layer hen is a continuous breeder and can produce over 300 eggs per year. Hens begin egg production at approximately 16-20 weeks of age with ovulation occurring once every 24 hours. Subsequent to ovulation, the ovum is engulfed by the infundibulum where it resides for approximately 18 minutes (see Figure 1.1). Fertilization of the ovum occurs in the infundibulum, and it is here that the first layer of albumen is produced. The ovum then passes to the largest portion of the oviduct, the magnum, where it remains for approximately 2-3 hours. This is where the majority of albumen is formed. Both inner and outer shell membranes are formed during the 1-2 hours passage through the isthmus, which is a relatively short section of the oviduct at only around 8cm in length. Prior to calcification, the egg takes up salts and approximately 15g of fluid into the albumen from the tubular glands, a process termed ‘plumping’. The ovum then remains in the shell gland for approximately 18-26 hours where the eggshell is laid down over the membrane. The egg is then laid approximately 24 hours after fertilisation. The vagina is separated from the shell gland by the uterovaginal sphincter muscle and terminates at the cloaca. The vagina has no role in the formation of the egg, but, in coordination with the shell gland, participates in expulsion of the egg (Whittow, 2000).
1.3.3 *Hen oviduct tubular gland cells*

The magnum is the longest part of the oviduct at approximately 30cm long in the adult hen. It is easily distinguished from the infundibulum as it is noticeably wider and has a thicker wall. The epithelium lining of the magnum consists of secretory and ciliated cells. As successive yolks pass down through the oviduct there occurs a cycle of secretion and regeneration in the glandular cells lining the oviduct. This cycle is particularly obvious in the magnum glands where large quantities of albumen are produced for each egg, giving rise to considerable variations in the appearance of the lining epithelium (Hodges, 1974).

The tubular glands are very highly developed in the magnum (see Figure 1.2). The openings of the glands are scattered over the surface of the mucosal folds, being most numerous along the sides and near the bottom of the folds, and consist of small invaginations of the epithelium forming short ducts connecting with the underlying glands. The cells lining the tubular glands are known as the tubular gland cells. They are pyramidal in shape, 10-15μm in height and possess a basal nucleus. From the narrow apical surface they project relatively few microvilli (Hodges, 1974).
Protein accumulates in the gland cells accompanied by a small amount of water. The protein is hydrated after discharge, at which point the outlines of individual glands are easily recognised (Whittow, 2000). The major proteins found in albumen are shown in Table 1.1. The oviduct tubular gland cells synthesize the egg white proteins including ovalbumin, conalbumin, ovomucoid and lysozyme, during the initial phase of the egg-laying cycle. Expression of these proteins is controlled at a transcriptional level by the steroid hormones oestrogen, progesterone and testosterone. Differentiation and proliferation of the tubular gland cells is also under the control of oestrogen (Hauser et al., 1981).
<table>
<thead>
<tr>
<th>Protein</th>
<th>% in albumen</th>
<th>Grams per egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>54</td>
<td>2.2</td>
</tr>
<tr>
<td>Conalbumin</td>
<td>13</td>
<td>0.5</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>11</td>
<td>0.5</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>3.5</td>
<td>0.15</td>
</tr>
</tbody>
</table>

**Table 1.1** The major egg white proteins in chicken albumen (Whittow, 2000).

Transgene constructs containing an egg white protein gene promoter can only be evaluated *in vitro* in oviduct tubular gland cells from sexually mature hens as the egg white proteins are only secreted by these cells. Therefore, cultured oviduct cells or tissue could be used to analyse expression from transgene constructs containing such a promoter. The regulatory sequences of two of the egg white protein genes, ovalbumin and lysozyme, have been characterised in detail at the molecular level and are therefore the most obvious candidate genes to modify to target expression to the oviduct.
1.3.4 **The Ovalbumin Gene**

Ovalbumin is one of the major egg white proteins and makes up over 50% of the albumen protein. It serves as a source of amino acids for the embryo during development and may also play a role in suppressing enzyme activity in the egg (Whittow, 2000). Utilising the ovalbumin gene regulatory sequences to drive transgene expression in the eggs of transgenic hens could potentially result in high levels of target protein in the egg white, compared to the use of other egg white protein gene promoters such as lysozyme.

![Figure 1.3 The chicken ovalbumin gene.](image)

**Figure 1.3 The chicken ovalbumin gene.** The ovalbumin transcription region is 7.6kb and contains 8 exons and 7 introns. An oestrogen-responsive enhancer element, steroid-dependent response element and negative regulatory element are located 5' of the transcription start site.
In the early 1980s, Woo and colleagues defined the entire sequence of the ovalbumin gene. As shown in Figure 1.3, the transcription region is 7564bp in length and contains 8 exons interrupted by 7 introns (Woo et al., 1981). As discussed later in this Section, expression of the ovalbumin gene is controlled by a complex array of 5'-flanking elements that enable the gene to respond to steroid hormones and to restrict its expression to the avian oviduct. Expression is regulated at a transcriptional and post-transcriptional level by four classes of steroid hormones: oestrogens, progestins, glucocorticoids and androgens (Palmiter et al., 1978; LeMeur et al., 1981). Each tubular gland cell in laying hens contains approximately 50 000 copies of ovalbumin mRNA, but only a few copies exist per cell in the absence of steroid hormones (Sensenbaugh and Sanders, 1999).

An oestrogen-responsive enhancer element (OREE) is located at approximately -3814 to -3139. The OREE is responsible for conferring oestrogen inducibility to the ovalbumin gene promoter (Kato et al., 1992). A steroid-dependent regulatory element (SDRE) is located at -892 to -780. Deletion of the SDRE eliminates responsiveness to steroids, and transcriptional activity is at basal levels (Sanders and McKnight, 1988). A negative regulatory element (NRE) is located at -308 to -88. The NRE represses transcription of the ovalbumin gene in the absence of steroid hormones (Sanders and McKnight, 1988). The NRE is a complex regulatory region, consisting of multiple positive and negative sites that contribute to the regulation of the ovalbumin gene (Sensenbaugh and Sanders, 1999). The ovalbumin gene appears to be actively repressed in the absence of steroid hormones by four independently acting elements within the NRE region, at -280 to -252, -237 to -228, -175 to -132 and -132 to -87 (Haecker et al., 1995).
The distal promoter region of the chicken ovalbumin gene contains a duplicate GTCAAA box (GGTGTCAAAAAGGTCAAACT) at -85 to -73 which is essential for efficient and accurate transcription. This repeated sequence was named the chicken ovalbumin upstream promoter (COUP) (Knoll et al., 1983; Pastorcic et al., 1986). Transfection analysis of linker scanning mutants revealed a strong repressor site located between -119 to -111 in the NRE that was designated the COUP-adjacent repressor (CAR) site because of its proximity to the COUP binding site.

Studies indicate that the ovalbumin gene is a secondary response gene (Dean and Sanders, 1996) as the oestrogen receptor does not bind to the proximal 900bp that are required for responsiveness to steroid hormones (Schweers et al., 1990). Also, the synergistic induction of the ovalbumin gene by oestrogen and corticosterone follows a two hour lag (Palmiter et al., 1976) and protein synthesis inhibitors abolish induction (McKnight et al., 1980). Secondary response genes take longer to respond to steroids and do not appear to directly bind the steroid receptors.

The ovalbumin gene is regulated by two oestrogen-inducible primary-response genes, δ-EF1 and Chirp-I (Sensenbaugh and Sanders, 1999). The gene appears to be induced by a transcriptional cascade initiated by steroid hormone-receptor complexes that culminates in the binding of Chirp-I to the SDRE (Dean et al., 1996). Linker-scanning mutants demonstrated that an element in the distal region of the SDRE is critical for induction of the ovalbumin gene by steroid hormones. This element was designated Chirp-I (chicken ovalbumin induced regulatory protein) and is located from -900 to -863 (Dean et al., 1996). In vivo footprinting showed that binding of Chirp-I is completely dependent on treatment with steroid hormones. A minimal site for Chirp-I
from -891 to -878 is well defined, with residues -889 and -885 being critical for binding. It is thought that Chirp-I may alter the topology of the surrounding chromatin, giving rise to an inducible DNaseI-hypersensitive site (Dean et al., 1996).

The chicken transcription factor δ-EF1 (δ-crystallin/E2-box factor) was cloned in a Southwestern screen in the early 1990s by Funahashi and colleagues (1993). Differential display identified δ-EF1 as being regulated by oestrogen in the hen oviduct. The site that binds δ-EF1 is located at -174 to -146 (Chamberlain and Sanders, 1999).

If high levels of transgene expression are to be achieved in vitro in oviduct tubular gland cells or in vivo in transgenic hens it could be important to include the significant ovalbumin regulatory elements, such as the oestrogen-responsive enhancer element, steroid-dependent regulatory element and negative regulatory element, in the transgene construct.
1.4 Retroviral gene transfer

Retroviruses are RNA viruses that infect birds and mammals, where they tend to establish chronic infections. The retroviridae family consists of seven genera: alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus, lentivirus, and spumavirus.

1.4.1 The retroviral life cycle

The first phase of the retroviral life cycle begins with attachment of the virus particle to the host cell (Varmus, 1988) (see Figure 1.4). This adsorption process is mediated via specific interactions between cell surface receptors that recognise the viral envelope glycoprotein located on the surface of the virus particle (virion). Following penetration of the cell and upon entry into the cytoplasm, the viral RNA genome is released, reverse transcribed into linear double-stranded DNA by the viral enzyme reverse transcriptase and circularised. Once it has integrated into random sites in the hosts' genome catalysed by viral integrase the viral DNA is then termed a provirus and becomes a stable part of the hosts' chromosomes.

The second phase of the life cycle involves the expression of the proviral genes and the production of new virions. The provirus contains the coding information for viral proteins and uses the transcriptional and translational system of the host cell to produce genomic viral RNA that is packaged into virions as well as viral RNA that serves as a messenger for production of viral proteins. The final steps in the replicative cycle involve the assembly of viral components into new virus particles which are released from the cell to begin a new round of infection.
Figure 1.4  Retroviral life cycle. Step 1: Retroviral envelope fuses with plasma membrane. Step 2: Viral reverse transcriptase copies the single-stranded RNA genome of the virus into a double-stranded DNA copy. Step 3: Viral DNA copy is transported into nucleus and integrated into the host-cell chromosomal DNA. Step 4: Integrated viral DNA (provirus) is transcribed by the host-cell RNA polymerase, generating mRNAs and genomic RNA molecules. The host-cell machinery translates the viral mRNAs into glycoproteins and nucleocapsid protein. The latter assemble with genomic RNA to form progeny nucleocapsids, which interact with the membrane-bound viral glycoproteins. Step 5: Host-cell membrane buds out and progeny virions are pinched off. (Figure from Lodish, 2000).
1.4.2 The retroviral genome

The RNA genome of retroviruses contains three coding regions: *gag* that encodes the internal structure, assembly and processing proteins of the core virion, *pol* that encodes the reverse transcriptase that is responsible for synthesis and the integrase that catalyses integration of viral DNA, and *env* that encodes the glycoprotein located in the envelope of the virion that is responsible for the virus entering and budding from the cell (see Figure 1.4 and 1.5).

The viral genome has a long terminal repeat (LTR) region located at each end that provides a number of essential functions in reverse transcription, including the viral promoter, enhancer and termination signal and the attachment sites necessary for integration. The *cis*-acting sequences that are needed to complete the virus life cycle include the packaging signal (Ψ) that is required for encapsidation of the viral genome RNA into the virion, a process known as packaging. This sequence is located 3' of the 5' LTR region.
Figure 1.5  (a) Retroviral genome components. The retroviral genome contains three coding regions: \textit{gag} that encodes the internal structure, assembly and processing proteins of the core virion, \textit{pol} that encodes the reverse transcriptase that is responsible for synthesis, the integrase that is responsible for integration of viral DNA and the protease that is responsible for processing the polyproteins, and \textit{env} that encodes the envelope glycoprotein that is responsible for the virus entering and budding from the cell. (b) Replication-defective retroviral vector components. Most of the \textit{gag} coding region and the complete \textit{pol} and \textit{env} coding regions are removed. This leaves a backbone of the 5' and 3' long terminal repeats (LTRs) and the packaging signal (Ψ). The transgene is placed between the LTRs.
1.4.3 **Retroviral vectors**

There are two types of retroviral vector: replication-competent and replication-defective. Replication-competent vectors are basic vectors that contain all the viral sequences needed to complete both phases of the life cycle and produce infectious particles. In the 1980s, replication-competent retroviral vectors were used to transfer genes into the germline of the mouse (Soriano and Jaenisch, 1986). These vectors are useful for transferring genes to cultured cells or to embryos when the presence of the replicating virus does not interfere with development or cause a hazard. These vectors produce high rates of infection, however the main disadvantage is the possibility of pathogenesis from the vector and decreased immune response to retroviral infection (Shuman, 1991).

Replication-defective retroviral vectors, which lack the viral proteins essential for replication, were developed in the late 1980s to overcome the problems associated with replication-competent vectors (Bossleman et al., 1989). The life cycle of replication-defective viruses is decreased to only one round of replication and they cannot produce infectious viral particles. In a replication-defective vector the majority of the *gag* coding region, and the complete coding region for the *pol* and *env* genes, are removed leaving a backbone of the 5' and 3' LTRs and the packaging signal. A transgene can be inserted between the LTRs, and the resulting RNA transcript can be packaged into a virus if all the packaging functions, *gag*, *pol* and *env*, are provided on separate plasmids (see Figure 1.5)

Due to the promoter activity of the LTRs, an important safety concern with retroviral vectors is the possibility of insertional activation of oncogenes by random integration
of the vector into the host genome. Therefore lentiviral vectors were designed in which the self-inactivating (SIN) safety modification of the vectors permanently disables the viral promoter within the viral LTR after integration (Miyoshi et al., 1998; Zufferey et al., 1999). This enables transgene expression in the targeted cells to be controlled solely by internal promoters.

To package retroviral vectors into virions, the viral genome is separated into a packaging construct, which contains the viral sequences encoding proteins required for packaging of the vector genome and its replication. The vector construct contains the transgene and cis-acting sequences that are essential for encapsidation of the vector genome and for viral transduction in the target cells. Retroviral packaging constructs are either transfected transiently into the packaging cells or a cell line is established that stably expresses the viral proteins. The vector and packaging constructs are expressed in the packaging cells, which produce the recombinant viral particles.

### 1.4.3.1 Lentiviral vectors

Lentiviruses are a member of the large family of retroviruses and have been isolated from sheep (visna/maedi virus), goats (caprine arthritis encephalitis virus), cattle (bovine immunodeficiency virus), horses (equine infectious anaemia virus), cats (feline infectious anaemia virus), monkeys (simian immunodeficiency virus) and humans (human immunodeficiency virus) (Pfeifer, 2004).

In addition to the structural genes found in all retroviruses, more complex lentiviral vectors have two additional groups of genes that have essential functions during the
viral life cycle and pathogenesis: the regulatory genes, *tat* and *rev*, and the accessory genes, *vpr*, *vpu*, *vif* and *nef*. The tat protein regulates the promoter activity of the 5' LTR and is necessary for the transcription from the 5' LTR. Rev protein binds to the *rev* response element (RRE) within the viral RNA, allows transport of unspliced RNA out of the nucleus, and is necessary for efficient *gag* and *pol* expression.

Lentiviral vectors are capable of carrying a transgene of up to at least 8kb, can accommodate the use of various internal promoters, either ubiquitous or cell-specific, and are able to transduce non-dividing cells. These vectors were developed mainly for applications in gene therapy but also in transgenesis. The first widely used lentiviral vectors were based on human immunodeficiency virus (HIV-1) (Naldini et al., 1996). HIV-1-based vectors have been used to generate transgenic mice (Lois et al., 2002), pigs (Hofmann et al., 2003) and cows (Hofman et al., 2004) (for more details see Section 1.2.3.1). Vectors derived from nonprimate lentiviruses, such as equine infectious anaemia virus (EIAV), have been developed in the interests of biosafety and to potentially broaden the range of cell types that can be transduced efficiently. Vectors based on equine-infectious anaemia virus (EIAV) have been used to generate transgenic chickens (McGrew et al., 2004) and pigs (Whitelaw et al., 2004).

Viral tropism of lentiviral vectors is determined by specific interactions between the envelope glycoprotein on the vector and the surface receptor molecules on the target cell, which may be expressed in specific populations in differentiated tissue. This interaction is the earliest event in the infection process after gene transfer and establishes the tropism of the lentiviral vector. The replacement of the original envelope protein of the lentiviral vector system with a heterologous envelope protein
is called pseudotyping. Pseudotyping of lentiviral vectors with certain envelope proteins (such as the vesicular stomatitis virus G (VSV-G) protein) can affect the transducing properties of the viral vector and alter the host range of the lentivirus. VSV-G is a fusogenic protein that interacts with membrane phospholipids to facilitate viral entry. Retroviral vectors pseudotyped with VSV-G do not require a cellular receptor for cell entry, which permits a very broad spectrum of infectivity (Yee et al., 1994).
1.5 **Thesis objectives**

A potential application of transgenesis in birds is their use as 'bioreactors' for the production of therapeutic proteins in eggs. Analysis of transgene expression in egg white requires the production of adult transgenic hens, which takes approximately one year. Therefore, an *in vitro* method with which to investigate transgene expression in the hen oviduct prior to the generation of transgenics is desirable, as such a method could be used to assess the effectiveness of transgene constructs in a relatively short period of time. In the production of transgenic 'dairy' species, transgene constructs containing a mammary gland-specific promoter can be evaluated in mice prior to the costly and time-consuming generation of large transgenic animals. Expression of therapeutic proteins in the egg white is likely to be achieved by using the regulatory sequences of the egg white protein genes to drive expression of the therapeutic protein. At present, a system for evaluating constructs containing such promoter sequences prior to their use in transgenic hens does not exist, as the egg white protein genes are only expressed in hen oviduct tubular gland cells (TGCs).

The objectives of the experiments in this thesis were to achieve the development of a method to culture active TGCs and to identify techniques with which to achieve successful gene transfer into this cell type in order to use them for investigating transgene expression. Different hen oviduct culture methods, for example using whole tissue explants, will be assessed as an alternative to TGCs if they are found to be unsuitable for this purpose.
CHAPTER 2 MATERIALS AND METHODS

2.1 General materials

Tris/EDTA (TE) pH 7.4

10mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 1mM ethylenediaminetetraacetic acid (EDTA).

LB-medium

1% Bacto-tryptone (Difco), 0.5% Bacto-yeast extract (Difco) and 0.125M sodium chloride (NaCl).

LB-Agar

1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.125M NaCl and 1.5% agar (Difco).

DNA loading buffer (6x)

0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll type 400 (Pharmacia) in distilled water.
PBS

0.16M NaCl, 0.003M KCl, 0.008M disodium hydrogen phosphate and 0.001M potassium dihydrogen phosphate supplied in tablet form. Reconstituted in distilled water and autoclaved prior to use.

X-gal staining solution

0.025g potassium ferricyanide, 0.032g potassium ferrocyanide, 30μl magnesium chloride (1M) and 150μl of X-gal stock (50mg/ml; Promega) in 15ml PBS.

Blocking solution

5% heat-inactivated donkey serum (Diagnostics Scotland) and 0.05% Tween-20 (polyoxyethylene (20) sorbitan monolaurate) (VWR International) in PBS.

PBT washing solution

0.05% Tween-20 in PBS.

0.12M Phosphate buffer pH 7.2

0.3% monobasic sodium phosphate and 1.3% dibasic sodium phosphate in distilled water.
0.12M Phosphate buffer – 15% sucrose

Dissolve the sucrose in 0.12M phosphate buffer at room temperature.

0.12M Phosphate buffer – 15% sucrose – 7.5% gelatin

Dissolve the gelatin in the 0.12M phosphate buffer – 15% sucrose at 37°C for 1-2 hours.

Paraformaldehyde solution

4% paraformaldehyde dissolved in PBS and pH adjusted to 7.4 with sodium hydroxide (NaOH).

Formulation buffer (pH 7.4)

4.48g Trizma base, 11.68g NaCl, 20.0g sucrose and 20.0g mannitol dissolved in 2 litres of ultrapure water and filtered through a 0.2μm membrane.
2.2  **Cell culture materials**

All materials purchased were of cell culture grade and were purchased from Sigma or Invitrogen unless stated otherwise.

2.2.1  **Hen oviduct tubular gland cell and explant medium**

Hen oviduct cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM):Hams F-12 (1:1 mixture), pH 7.0-7.6, supplemented with 0.1% bovine serum albumin, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin.

2.2.2  **Chicken embryonic fibroblast medium**

Chicken embryonic fibroblasts were cultured in DMEM, pH 7.0-7.6, supplemented with 10% foetal calf serum, 100U/ml penicillin and 100µg/ml streptomycin.

2.2.3  **Hen oviduct tubular gland cell dissociation solution**

25mg protease, 2mg trypsin, 98mg collagenase and 10mg DNaseI dissolved in 100ml DMEM (pH 7.0-7.6).

2.2.4  **Freezing mix**

60% complete cell culture medium, 20% foetal calf serum, and 20% dimethyl sulfoxide (DMSO).
2.3 **Animal Methods**

Each hen was sacrificed by intravenous injection of 1ml pentobarbital sodium (200mg/ml; Merial). Injection was performed by F.Thomson, R.Mitchell or M.Hutchison. The magnum portion of the oviduct was then removed from the sacrificed hen.
2.4 **Cell culture methods**

2.4.1 **Hen oviduct tubular gland cell isolation and maintenance**

The magnum portion of the hen oviduct was removed as described in Section 2.3 and was minced finely with sharp scissors prior to incubation in 50ml dissociation solution (see 2.2.3) at 37°C for 30 minutes to allow enzymatic dissociation. Following incubation the oviduct tissue was mechanically dissociated by pipetting and the medium was replaced with fresh dissociation solution. The incubation and mechanical dissociation procedure was performed twice. The medium was then removed and aliquoted into 15ml centrifuge tubes. Following centrifugation at 100g for 5 minutes, resuspended hen oviduct cells were transferred to tissue culture plates and cultured in complete medium (see 2.2.1). Cells were maintained at 37°C in a gassed incubator with 5% CO₂ and were not passaged due to the lack of cell division.

2.4.2 **Hen oviduct explant isolation and maintenance**

The magnum portion of the chicken oviduct was removed as described in Section 2.3 and the inner lining was dissected into explants of approximately 1-2mm in diameter. The explants were then transferred to tissue-culture plates and cultured in complete medium (see 2.2.1). Explants were maintained at 37°C in a gassed incubator with 5% CO₂.

2.4.3 **Chicken embryonic fibroblast preparation and maintenance**

Freshly laid fertilised eggs were incubated, with rocking, at 37°C for 10 days. Eggshells were broken by tapping above the air space, membranes were cut and the embryo was decapitated with scissors. The carcass was removed to a plastic dish.
where wings, legs and viscera were discarded and was then placed into 30ml of 0.05% trypsin solution in a 50ml polypropylene tube. The carcass was finely minced with scissors, vortexed for 2 minutes and incubated at room temperature for 5 minutes. Approximately 80% of the supernatant was removed and divided between three T25 flasks and the total volume of complete medium (see 2.2.2) was made up to 5ml per flask. The remaining 20% was discarded. Cells were maintained at 37°C in a gassed incubator with 5% CO₂. Cells were passaged when they reached 80-90% confluency, which was approximately every 2-3 days, by washing once with PBS before incubation in trypsin solution. During incubation cells lost their tight connection with the dish and with other cells. Manual agitation of the flask after approximately 4 minutes of incubation in trypsin caused the cells to detach from the dish. Cells were then washed with medium to inactivate the trypsin and were centrifuged at 1000g for 5 minutes. Following resuspension in fresh medium (see 2.2.2) cells were plated out in dishes/flasks.

2.4.4 Freezing cells

Cells were washed once with PBS prior to incubation in trypsin solution. Once cells had detached from the dish/flask they were washed with culture medium to inactivate the trypsin followed by centrifugation at 1000g for 5 minutes. Cells were resuspended in half of the final volume of medium for freezing. An equal volume of freezing mix (see 2.2.4) was added to the resuspended cells and gently mixed. This suspension was aliquoted into 1.5ml cryotubes and stored at -150°C.
2.4.5 Resuscitation of cells

Frozen vials of cells were removed from -150°C storage and placed in a water bath at 37°C to thaw. 5ml of medium was then added to the cells prior to centrifugation at 1000g for 5 minutes. Cells were then resuspended in fresh medium and plated out into dishes/flasks.

2.4.6 Culture of hen tubular gland cells on growth matrices

BD Matrigel Matrix (BD Biosciences), 0.01% poly-L-lysine (Sigma) and 0.1% gelatin (Type B from bovine skin; Sigma) were used to coat a 12-well tissue-culture plate three hours prior to use. The excess material was removed from each dish prior to the addition of the cell suspension.
2.5 Preparing cells and explants for immunohistochemistry

2.5.1 Fixation and permeabilisation of cells

Medium was aspirated from cultured cells and the cells were washed three times with PBS. 4% paraformaldehyde (PFA) was added to the cells prior to incubation at 4°C for 15 minutes. Fixed cells were washed 3 times in PBS for 20 minutes to remove residual PFA. The cells were then incubated with 100% ethanol for one minute to permeabilise followed by washing twice in PBS for 20 minutes. Fixation and permeabilisation of suspension cells was carried out using the same procedure, however the cells were transferred to 1.5ml microfuge tubes prior to fixing and were centrifuged briefly at 1000g to collect the cells at the bottom of the tube before each solution was aspirated.

2.5.2 Fixation of explants

Explants were fixed prior to gelatin-sucrose embedding (see 2.5.3). Medium was aspirated from cultured explants and they were transferred to 1.5ml microfuge tubes and washed once in PBS. The explants were fixed in 4% paraformaldehyde at 4°C for 20 minutes.

2.5.3 Gelatin-sucrose embedding of explants

Following fixation, explants were washed twice in PBT and were incubated in 0.12M phosphate buffer- 15% sucrose (see 2.1) for 30 minutes. A cushion of 0.12M phosphate buffer- 15% sucrose- 7.5% gelatin solution (PSG; see 2.1) was poured into the wells of a 12-well plate and was hardened at 4°C for 30 minutes. During this time the explants were incubated at 37°C in PSG. Each explant was transferred to a well of
the 12-well plate and covered in PSG solution. The explants were then incubated at 4°C for 45 minutes to harden the PSG. Following incubation the explants were cut out using a scalpel, frozen in isopentane, cooled to -65°C in liquid nitrogen and stored at -80°C.

2.5.4 Cryo-sectioning of explants

Embedded explants were sectioned onto microscope slides at -25°C using a cryo-sectioner (Bright). Slides were stored at 4°C.
2.6 Transfection of cells and explants

2.6.1 Lipofectamine2000 transfection of cells and explants

Cells in a 6-well plate at 50% confluency and freshly isolated explants were transfected using Lipofectamine2000 (Invitrogen). Medium was aspirated from the cells/explants and was replaced with 1.5ml serum- and antibiotic-free medium 2 hours prior to transfection. Plasmid DNA was diluted in 250μl medium per 1.5ml microcentrifuge tube. For each ratio of DNA:reagent the appropriate amount of Lipofectamine2000 was diluted in 250μl medium. Samples were incubated at room temperature for 5 minutes. Diluted DNA was added to diluted Lipofectamine2000 and the solution was mixed gently and incubated at room temperature for 20 minutes. The transfection complexes were added drop-wise onto the cells/explants, followed by incubation at 37°C for 4 hours. The medium containing the transfection complexes was then replaced with fresh medium. Gene expression was assayed in the cells/explants 24 hours post-transfection.

2.6.2 FuGENE6 transfection of cell and explants

Cells in a 6-well plate at 50% confluency and freshly isolated explants were transfected using FuGENE6 (Roche). For each ratio of DNA:reagent the appropriate amount of FuGENE6 was diluted into medium. Plasmid DNA was added to the diluted FuGENE6 to a total volume of 100μl. Samples were incubated at room temperature for 15 minutes. The transfection complexes were added drop-wise onto the cells/explants followed by incubation at 37°C. Gene expression was assayed in the cells/explants 24 hours post-transfection.
2.6.3 **GeneJammer transfection of cells and explants**

Cells in a 6-well plate at 50% confluency and freshly isolated explants were transfected using GeneJammer (Stratagene). For each ratio of DNA:reagent the appropriate amount of GeneJammer was diluted into 100µl antibiotic- and serum-free medium. The diluted GeneJammer was incubated at room temperature for 10 minutes. Plasmid DNA was added to the diluted GeneJammer and the samples were incubated at room temperature for 10 minutes. Medium was aspirated from cells/explants and was replaced with 900µl fresh complete medium. The transfection complexes were added drop-wise onto the cells/explants followed by incubation at 37°C. 3 hours post-transfection 1ml of complete medium was added to each well. Gene expression was assayed in the cells/explants 24 hours post-transfection.

2.6.4 **Electroporation of cells**

The appropriate amount of plasmid DNA was diluted in 100µl PBS. Cell pellets from each well of a 24-well plate were resuspended in 700µl PBS. The diluted DNA was mixed with the resuspended cells and each sample was transferred to a 0.4cm electroporation cuvette and incubated at 4°C for 20 minutes. Samples were electroporated in the BioRad Gene Pulsor II followed by incubation at 4°C for 20 minutes (for voltage and capacitance details see 4.3.2). The electroporated cells were transferred to a 100mm tissue-culture dish and 9ml of complete medium was added (see 2.2.1). Cells were incubated at 37°C and gene expression was assayed 24 hours post-electroporation.
2.6.5 **Electroporation of explants**

Plasmid DNA at a concentration of 3-5μg/μl was mixed with 0.1% Fast Green FCF dye (10mg/ml in PBS; Sigma). The plasmid DNA-fast green dye solution was either pipetted or injected into the explants. For pipetting, a small incision approximately 0.5-1mm deep was made on the surface of the explant using a scalpel. The incision was filled with approximately 2μl of the plasmid DNA-fast green dye solution. For injection, a microinjection needle attached with plastic tubing to a 20ml plastic syringe was used. The needle was inserted to a depth of approximately 0.5-1mm under the surface of the explant and the DNA-dye solution was injected.

Three electroporation devices were used in the experiments, the Genetrode (Genetronics, Inc), long EggATrode and short EggATrode (RMR Technologies). The Genetrode consists of two 1mm electrodes 4mm apart. The electrodes were placed on either side of the incision/injection site and 2 x 6 pulses were applied using the ElectroSquare Porator ECM 830 (BTX). Voltage was applied using switch position 1. The EggATrode devices each have 5 electrode wires. The electrodes on the long EggATrode are approximately 10mm long and are insulated. The electrodes on the short EggATrode are approximately 0.5mm long and are uninsulated (see Figure 2.1). The electrodes were placed directly over the incision or injection site and a set of 10 pulses was applied using the ElectroSquare Porator ECM 830 (BTX). The switch positions for the ElectroSquare Porator ECM 830 (BTX) control the electrical current in the EggATrode devices. The current runs from positive to negative electrodes as shown in Figure 2.2. For voltage details see Section 6.3.2.1. Explants were transferred to microfuge tubes containing complete medium (see 2.2.1) immediately after electroporation, and were then transferred to 12-well plates containing complete
medium. Explants were incubated at 37°C and gene expression was assayed 24 hours post-electroporation.

Figure 2.1 Short EggATrode electroporation device (RMR Technologies). The five electrodes are un-insulated and are approximately 0.5mm in length.
<table>
<thead>
<tr>
<th>Switch position</th>
<th>Positive electrodes</th>
<th>Negative electrodes</th>
</tr>
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<tbody>
<tr>
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<tr>
<td>7</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 2.2 Switch settings for ElectroSquare Porator ECM 830 (BTX) (a) Each switch position results in an electrical current running from positive to negative electrodes. (b) Positioning of electrodes on EggAtrode.
2.7 Bacterial methods

2.7.1 Transformation of competent bacteria

XL10-gold or XL2-blue ultracompetent cells (Stratagene) were thawed on ice. 2μl of ligation mix or plasmid DNA was added to 20μl thawed ultracompetent cells. Samples were incubated on ice for 30 minutes followed by incubation at 42°C for 45 seconds. Samples were then incubated on ice for 2 minutes prior to the addition of 100μl of LB medium per tube. Samples were incubated at 37°C for 1 hour and were then plated out on dishes of LB agar containing 0.2% ampicillin.

2.7.2 Small scale preparation of plasmid DNA

DNA was isolated using the QiaPrep Spin Mini-prep kit (Qiagen). A single colony from a fresh LB agar plate containing the appropriate antibiotic was used to inoculate 3ml of LB medium, also containing the antibiotic, in a 15ml polypropylene tube. This culture was incubated for 16-18 hours at 37°C with shaking at 210rpm. Cultures were harvested by centrifugation at 10000g for 5 minutes. The supernatant was discarded and the bacterial pellet was resuspended in 250μl of cell resuspension solution and transferred to a 1.5ml microcentrifuge tube. 250μl of cell lysis solution was added and the samples were gently inverted six times to mix. 350μl of neutralization solution was added and the samples were immediately inverted six times to mix. Samples were centrifuged at 14000g for 10 minutes. The supernatant was transferred to a QIAprep Spin Column by decanting and was centrifuged at 14000g for 1 minute. The flow-through was discarded and the column was washed with 750μl of column wash solution by centrifugation at 14000g for 1 minute. The flow-through was discarded and the samples were centrifuged for an additional 1 minute at 14000g to remove
residual wash buffer. The QIAprep Spin Column was then placed in a clean 1.5ml microcentrifuge tube and DNA was eluted with 50μl of elution buffer by centrifugation at 14,000g for 1 minute.

2.7.3 Large scale preparation of plasmid DNA

DNA was isolated using the Hi-Speed Plasmid Maxi kit (Qiagen) A single colony from a fresh LB agar plate containing the appropriate antibiotic was used to inoculate a 3ml starter culture of LB medium, also containing the antibiotic, in a 25ml pyrex flask. This culture was incubated for 8 hours at 37°C with shaking at 210rpm and was then inoculated into 100ml selective LB medium and grown for 12-16 hours at 37°C with shaking at 210rpm. Cultures were harvested by centrifugation at 6000g for 15 minutes at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 10ml of Buffer P1. 10ml of Buffer P2 was added to the resuspended bacteria and mixed by inverting 6 times. Samples were incubated at room temperature for 5 minutes followed by addition of 10ml of chilled Buffer P3 to the lysate and the samples were mixed immediately by inverting 6 times. The lysate was poured into the barrel of the QIAfilter Cartridge followed by incubation at room temperature for 10 minutes. During this time a HiSpeed Maxi Tip was equilibrated by applying 10ml of Buffer QBT and allowing the column to empty by gravity flow. The plunger was inserted into the QIAfilter Cartridge and the cell lysate was filtered into the previously equilibrated HiSpeed Tip. The cleared lysate was allowed to enter the resin by gravity flow and the HiSpeed Tip was then washed with 60ml Buffer QC. The DNA was eluted with 15ml Buffer QF and was precipitated by the addition of 10.5ml room temperature isopropanol. Samples were mixed and incubated at room temperature for 5 minutes. The DNA-isopropanol mixture was then filtered through the
QIAprecipitator using a 30ml syringe and washed by pressing 2ml of 70% ethanol through the QIAprecipitator. The membrane was dried by attaching the QIAprecipitator to the syringe, inserting the plunger and pressing air through the QIAprecipitator quickly and forcefully. This drying procedure was performed twice.

The DNA was eluted from the QIAprecipitator into a 1.5ml microcentrifuge tube with 1ml of Buffer TE.
2.8 Molecular biology methods

2.8.1 Agarose gel electrophoresis

Agarose powder was dissolved in 1X TAE by heating in a microwave and was allowed to cool. Ethidium bromide was added to a final concentration of 0.5ug/ml before pouring the gel using a horizontal electrophoresis gel kit. DNA samples were mixed with 6X loading buffer (see 2.1) prior to loading onto the gel. Molecular weight marker was loaded into the first lane of the gel. An electrical current was applied at between 100 and 150 volts. Following electrophoresis, DNA was visualised using UV light.

2.8.2 Preparation of DNA from cells

DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen). 20μl of proteinase K was added to the bottom of a 1.5ml microcentrifuge tube for each sample and 200μl of homogenised and filtered explant was added to each tube. 200μl Buffer AL was added to each sample followed by mixing by pulse-vortexing for 15 seconds. The samples were incubated at 56°C for 10 minutes and were briefly centrifuged. 200μl 100% ethanol was added to each sample followed by mixing again by pulse-vortexing for 15 seconds and then briefly centrifuging. The mixture was carefully applied to the QIAamp spin column in a 2ml collection tube followed by centrifugation at 6000g for 1 minute. The QIAamp Spin Column was placed in a clean 2ml collection tube and 500μl Buffer AW1 was added. The samples were centrifuged at 6000g for 1 minute. 500μl Buffer AW2 was added to the QIAamp Spin Column followed by centrifugation at 14,000g for 3 minutes. The QIAamp Spin Column was then placed in a clean 1.5ml microcentrifuge tube and 200μl Buffer AE
was added. Samples were incubated at room temperature for 1 minute and DNA was eluted by centrifugation at 6000g for 1 minute.

2.8.3 Preparation of RNA from cultured cells and explants

The NucleoSpin RNA II kit (BD Biosciences Clontech) was used for isolating RNA from cultured cells and tissue explants. Lysis of cells (approximately 5 x 10⁶ cells per sample) and explants was achieved by the addition of 350μl of RA1 buffer and 3.5μl of β-mercaptoethanol per sample. The cell samples were then lysed by pipetted up and down several times followed by vortexing. The explant samples were lysed by homogenisation using a hand-held homogeniser (Sigma). To reduce the viscosity of the lysed explant sample and to clear the solution, the sample was filtered using a NucleoSpin Filter unit for 1 minute at 11,000g. The RNA isolation procedure was then performed on the flow-through. 350μl of 70% ethanol was added to the lysed samples followed by vortexing to mix. Each sample was washed through a NucleoSpin column by centrifugation at 8000g for 30 seconds. The column was washed with 350μl Buffer MBD at 11,000g for 1 minute. 95μl of DNaseI Reaction Buffer, containing 10μl of reconstituted DNaseI to 90μl of DNaseI Reaction Buffer per isolation, was added to the column followed by incubation at room temperature for 15 minutes. 250μl Buffer RA2 was added to the column followed by centrifugation at 8000g for 30 seconds. The NucleoSpin column was placed into a new 2ml collection tube and was washed with 600μl of Buffer RA3 for 30 seconds at 8000g. A further 250μl of Buffer RA3 was added to the column followed by centrifugation at 11,000g for 2 minutes. The column was then placed into a new 1.5ml microcentrifuge tube and the RNA was eluted by adding 50μl of nuclease-free water.
and centrifuging at 11,000g for 1 minute. The yield was then estimated using UV spectroscopy.

2.8.4 cDNA synthesis

The AMV Reverse Transcriptase System (Promega) was used to synthesize single-stranded cDNA from isolated RNA. A reaction in a total volume of 19.25μl was prepared containing the following components: 4μl magnesium chloride (25mM), 2μl Reverse Transcription 10X Buffer, 2μl dNTP mixture (10mM), 0.5μl Recombinant RNasin Ribonuclease Inhibitor, 1μl random primers (0.5μg), 7.75μl RNA (1-2μg) and nuclease-free water. The reaction was incubated at 42°C for 2 minutes prior to the addition of 0.75μl AMV Reverse Transcriptase (15 units) per sample. The reactions were then placed in a thermocycler (Hybaid) and heated at 42°C for 50 minutes followed by heating at 70°C for 15 minutes. cDNA was resuspended in 80μl nuclease-free water per sample and stored at -20°C.

2.8.5 Reverse-transcriptase polymerase chain reaction (RT-PCR)

AccuPrime Supermix I (Invitrogen) was used for amplification of cDNA templates. The mixture contains anti-Taq DNA polymerase antibodies, thermostable AccuPrime protein, magnesium chloride, deoxyribonucleotide triphosphates, and recombinant Taq DNA polymerase. Each reaction was set up in a volume of 25μl containing the following components: 12.5μl AccuPrime SuperMix I, 0.5μl each primer (0.2μM each, 100-200ng template cDNA and DNase-free water.
Reactions to detect lysozyme and β-actin mRNA were incubated in a thermocycler (Hybaid) at 94°C for 2 minutes to completely denature the template and activate the enzyme. 35 cycles of PCR amplification were performed for detection of lysozyme and 25 cycles were performed for detection of β-actin as follows: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 68°C for 1 minute. Reactions to detect ovalbumin mRNA were incubated in a thermocycler (Hybaid) at 94°C for 2 minutes, followed by 35 cycles of PCR amplification performed as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 68°C for 1 minute. All reactions were maintained at 4°C after cycling and were stored at -20°C until use. Primers were designed against genomic sequences to cover one to two exons and to produce a PCR product of 200-500bp. For primer sequences see Figure 2.3.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>TATGAAGCGTCACGGACTTC</td>
<td>GCTGTTATGTCTGAGCTCAG</td>
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<tr>
<td>β-actin</td>
<td>GGATGATGATATTGCTGCGC</td>
<td>ATGGCTACATACATGGCTGG</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>GCCAAGCTCCGTGGATTCTC</td>
<td>CCTACCACCTCTCTGCTGC</td>
</tr>
</tbody>
</table>

**Figure 2.3 Primers for RT-PCR analysis.** Primers were designed against genomic sequences of the chicken lysozyme gene, chicken β-actin gene and chicken ovalbumin gene to cover 1-2 exons and to produce a PCR product of 200-500bp.
Reactions to detect secreted alkaline phosphatase (SeAP), green fluorescent protein (GFP) and R24 mRNAs were incubated in a thermocycler (Hybaid) at 94°C for 2 minutes to completely denature the template and activate the enzyme. For detection of SeAP and GFP mRNAs 38 cycles of PCR amplification were performed as follows: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 68°C for 1 minute. For detection of R24 mRNA the same cycle conditions were performed for 35 cycles. All reactions were maintained at 4°C after cycling and were stored at -20°C until use. For primer sequences see Figure 2.4.

<table>
<thead>
<tr>
<th></th>
<th>Primer Sequence</th>
<th>Length</th>
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</thead>
<tbody>
<tr>
<td>SeAP F:</td>
<td>CCAAGAACCTCATCATCTTC</td>
<td>520bp</td>
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<tr>
<td>SeAP R:</td>
<td>CGAAACATGTACTTTTCGGCC</td>
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<tr>
<td>GFP F:</td>
<td>CTGAAGTTCATCTGCACCAC</td>
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<td>GFP R:</td>
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<tr>
<td>R24 F:</td>
<td>CAGATCCCTGTACTACTTCG</td>
<td>560bp</td>
</tr>
<tr>
<td>R24 R:</td>
<td>TACCAGTTGAACCTTCACCTC</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.4** Primers for RT-PCR analysis and predicted product sizes. Secreted alkaline phosphatase (SeAP), green fluorescent protein (GFP) and R24. (F = forward; R = reverse).
2.9 Immunological methods

2.9.1 Antibodies

Antibodies were used for immunohistchemistry in Chapters 3 and 5. Primary and secondary antibody dilutions and suppliers are detailed in Section 3.3.5.2.

2.9.2 Immunohistochemistry on cells

Suspension cells were fixed in 1.5ml eppendorf tubes in 4% paraformaldehyde. Fixed cells were washed twice in PBS for 5 minutes followed by incubation in blocking solution (see 2.1) for one hour and 30 minutes at room temperature. Cells were incubated with the primary antibody at the appropriate dilution in blocking solution overnight at 4°C. Unbound primary antibody was removed by washing 3 times in PBT for 20 minutes at room temperature. Cells were then incubated with the secondary antibody at the appropriate dilution in blocking solution for 1 hour at room temperature. Unbound secondary antibody was removed by washing 3 times in PBT for 20 minutes at room temperature and the cells were transferred to a slide and mounted in Hydramount mounting medium (National Diagnostics).

2.9.3 Immunohistochemistry on explant sections

Sectioned explants were washed twice in PBS for 5 minutes at room temperature followed by incubation in blocking solution for 1 hour at room temperature. Slides were incubated with the primary antibody at the appropriate dilution in blocking solution overnight at 4°C. Unbound primary antibody was removed by washing 3
times in PBT for 20 minutes at room temperature. Slides were then incubated with the secondary antibody at the appropriate dilution in blocking solution for 1 hour at room temperature. Unbound secondary antibody was removed by washing 3 times in PBT for 20 minutes at room temperature and the slides were mounted in Hydramount mounting medium (National Diagnostics).

2.9.4 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was performed on medium samples at Viragen Scotland Limited by Dr Robertson. A capture ligand (1/250 dilution of α-human IgG (The Binding Site) in carbonate-bicarbonate buffer, pH 9.6) was coated onto a 96-well ELISA plate. Excess ligand was washed from the plate and R24 minibody (The Binding Site) was added. Doubling dilutions of the antibody or protein solution were made on the plate. The plates were washed after the antibody or protein solution had bound to the coated ligand. Bound antibody was detected with an appropriate anti-antibody horse radish peroxidase (HRP) conjugate (The Binding Site). The amount of HRP bound to the plate was quantified colourimetrically at 490nm and compared with the amount of colouration generated by a standard antibody in the same ELISA.
2.10 Histological methods

2.10.1 Detection of β-galactosidase activity

β-galactosidase activity was detected in fixed suspension cells, adherent cells, and sectioned explants by staining in X-gal solution (see 2.1) for 3 hours at 37°C. Excess staining solution was washed from the cells or slides with PBS for 10 minutes at room temperature.

2.10.2 Detection of alkaline phosphatase activity

1 bottle of Thermo Trace (AlphaLabs) was reconstituted with 20ml of reagent AMP buffer (aminomethylpropanol; supplied with kit) and was heated to 37°C. Samples were added in duplicate to a 96-well plate. 250μl of substrate (paranitrophenyl phosphate) was then added to each sample and the plate was placed inside a Dynatech MR7000 plate-reader for 6 minutes. Alkaline phosphatase activity was determined by measuring the cleavage of paranitrophenyl phosphate at 405nm.

2.10.3 Haematoxylin and Eosin staining

Oviduct explant sections were stained with filtered 0.1% Mayers Haematoxylin (Sigma) for 10 minutes in a glass staining chamber. Sections were rinsed in cold tap water for 5 minutes followed by staining in 0.5% Eosin (Sigma) for 2 minutes. Sections were rinsed in distilled water to remove excess Eosin. Sections were dehydrated with 50% ethanol for 2 minutes followed by 70% ethanol for 2 minutes,
and were equilibrated in 100% ethanol for 1 minute. Sections were then placed in xylene (Sigma) for 1 minute prior to mounting with cytoseal XYL (Fisher Scientific).

2.10.4 **Trypan blue staining**

Dye exclusion with trypan blue (0.4%; Sigma) was used to count viable cells. A 1:1 mixture of trypan blue and cell suspension was mixed thoroughly and left for 5 minutes at room temperature. 10μl of the mixture was transferred to a haemocytometer and the viable (unstained) and non-viable (stained) cells were counted.

2.10.5 **Annexin-V-Fluos staining**

The Annexin-V-Fluos kit (Roche) was used to detect cellular apoptosis. 20μl of labelling reagent was prediluted in 1ml incubation buffer. 20μl propidium iodide solution was added to the diluted labelling reagent. Medium was aspirated from the cells in a chamber slide and the chamber was then removed from the slide. The slide was covered with Annexin-V-Fluos labelling solution (100μl per slide) and a coverslip was added to the slide prior to incubation for 15 minutes at room temperature. The cells were then analysed by fluorescence microscopy.
2.11 Viral Methods

2.11.1 Packaging viral constructs

HEK-293T and D17 cells in 144mm plates at 30-50% confluency were transfected with plasmid packaging mix. For each transfection 1462.7 μl of Optimem and 45.3 μl FuGene (Roche) were mixed together and incubated at room temperature for 5 minutes. 10.2 μg of the plasmid being packaged was mixed with 5.1 μg of GagPol plasmid (pESYNPG), 2.5 μg Rev plasmid (pPLP-2) and 0.2 μg Env plasmid (pVSV-G). The plasmid mix was added to the transfection reagent mix and 1560 μl of the resultant mixture was added dropwise to each 144mm plate of cells. The cells were incubated at 37°C with 5% carbon dioxide. For vectors containing the cytomegalovirus (CMV) enhancer/promoter, 300 μl of sodium butyrate (99%; Sigma) was added to each plate after 24 hours to induce expression from the enhancer/promoter. The medium was changed to fresh complete medium after 6 hours of incubation at 37°C. Approximately 16-18 hours later, the virus-containing medium was aspirated from the plates and was filtered to remove cellular debris using a 150ml filter (0.44 μm; Nalgene). Four 1ml aliquots of unconcentrated virus were taken at this stage and stored at -80°C. The rest of the filtered medium was centrifuged for 18 hours at 7000g in an ultracentrifuge. Each pellet was then resuspended in 5ml of PBS prior to incubation on ice for 30 minutes. Each sample was resuspended by pipetting and was transferred to a 50ml polycarbonate centrifuge tube followed by centrifugation for 2 hours at 20,000g in an ultracentrifuge (Beckman XL70; SW40-TI rotor). The pellet was then resuspended in 50 μl Formulation Buffer (Oxford BioMedica; see 2.1) and was incubated on ice for 30 minutes. The resuspended pellet was then transferred to microcentrifuge tubes followed by centrifugation at 2000g for
1 minute. 3 aliquots of 20μl concentrated virus and 1 aliquot of 10μl concentrated virus were transferred to screw-cap microcentrifuge tubes and stored at -80°C.

2.11.2 Titration of virus

24 hours prior to titring HEK-293T and D17 cells were plated into 12-well plates at a density of 9 x 10^4 and 8 x 10^4 cells per well respectively. The virus dilutions were prepared in complete medium containing 8μg/ml polybrene as shown in Figure 2.5. The medium was aspirated from the cells and 500μl of the appropriate virus dilution was added to each well. The cells were then incubated at 37°C for 3 hours followed by the gentle addition of 1ml of medium to each well. After 48 hours D17 cells were analysed for transgene expression. A colony of 3-5 cells in close proximity showing expression of the transgene was considered to be from the same clone. These clones were counted to give the biological titre of the virus.

On the same day that the D17 cells were assayed for gene expression, the HEK-293T cells were passaged in a 12-well plate. They were then passaged every second day for 6 days. On the sixth day the DNA was extracted from each well using the QIAamp DNA Blood Mini Kit (Qiagen) as described in Section 2.8.2. An integration assay was performed by quantitative polymerase chain reaction (qPCR) on the genomic DNA by Viragen (Scotland) Limited.
**Figure 2.5** Titration packaged virus on HEK-293T and D17 cells in a 24-well plate. Numbers in red represent unconcentrated virus, numbers in green represent concentrated virus, numbers in blue represent the amount taken from each well for the serial dilution and numbers in the wells represent the amount of complete medium the virus was diluted in per well.
2.11.3   **Viral transductions on cells**

Viral transductions were performed on cells in a 96-well plate. A total volume of 100µl of virus diluted in medium containing 8µg/ml polybrene was added to each well. After 3 hours incubation at 37°C with 5% carbon dioxide, 100µl of complete medium (see 2.2.1) was added to each well. Cells were analysed for transgene expression 72 hours post-transduction.

2.11.4   **Viral transductions on explants**

Viral transductions on explants were carried out in 1.5ml eppendorf tubes. Each explant was transduced in a total volume of 20µl of virus diluted in medium containing 8µg/ml polybrene. After 24 hours each explant and the viral solution were transferred to a 12-well plate and 1ml of fresh complete medium (see 2.2.1) was added to each well. 72 hours post-transduction explants were embedded in 0.12M phosphate buffer-sucrose-gelatin solution as described in Section 2.5.3 and were stored at -80°C for at least 24 hours. The embedded explants were then cryosectioned as described in Section 2.5.4 and were analysed for transgene expression.
2.12 Statistical Analysis

2.12.1 Calculation of standard error

Standard deviations between samples were calculated using Microsoft Excel. Average numbers for each sample set were displayed on each graph and standard error bars were added to each data series.
CHAPTER 3  ANALYSIS OF THE PROPERTIES OF CHICKEN TUBULAR GLAND CELLS MAINTAINED IN CULTURE

3.1  Introduction

Tubular gland cells (TGCs) constitute approximately 80% of the cells in the magnum region of the hormone-stimulated hen oviduct (Palmiter, 1972), with the remaining 20% consisting of fibroblasts, smooth muscle cells and epithelial cells. TGCs synthesize the egg white proteins during the initial phase of the egg-laying cycle and expression of the egg white protein genes is controlled at a transcriptional level by steroid hormones (Hauser et al., 1981).

Expression of therapeutic proteins in the egg white of transgenic hens is likely to be achieved by using the regulatory sequences of the egg white protein genes to drive the expression of the therapeutic protein. Evaluation of transgene constructs containing such promoter sequences can only be carried out in hen oviduct TGCs, as the egg white protein genes are only expressed in this cell type. To date, the isolation and culture of TGCs from sexually mature hens has not been described. The use of TGCs from mature hens is desirable over the use of TGCs from hormone-stimulated sexually immature chicks, as mature TGCs may reflect transgene expression in the adult hen oviduct more accurately.

Oviduct cells were first isolated and cultured from hormone-stimulated four day old chicks in the late 1960s (O'Malley and Kohler, 1967). Chick oviduct tubular gland cells (TGCs) have since been used as a model for studying transcriptional control of
egg white protein gene expression by steroid hormones. McKnight’s (1978) protocol for isolating and culturing TGCs was subsequently developed so that the egg white protein genes in the TGCs were induced by the addition of steroid hormones (Sanders and McKnight, 1985). This protocol was used for the culture of TGCs in all subsequently published research (for example, Sanders and McKnight, 1988; Dean et al., 1996; Sensenbaugh and Sanders, 1999). The protocol involves establishing primary cell cultures from the oviduct of sexually immature chicks after oestrogen stimulation and withdrawal. Three to four day old female chicks are stimulated with diethylstilbestrol for approximately ten days, followed by withdrawal for the same period of time, followed by restimulation (Sanders and McKnight, 1988). The addition of oestrogen is reported to prolong the half-life of and stabilise ovalbumin mRNA in cultured TGCs (Arao et al., 1994). To exert its effects on transcription of the ovalbumin gene, oestrogen has been found to require the permissive effects of insulin (Evans and McKnight, 1984). Direct evidence that hormones other than oestrogen elicit their effects on the post-transcriptional levels of ovalbumin is still lacking.

Sanders and McKnight’s protocol for isolating and culturing TGCs from sexually immature chicks was used in the following experiments for the isolation and culture of TGCs from sexually mature laying hens (Sanders and McKnight, 1985). The viability of the cultured TGCs and their ability to express the egg white protein genes is fundamental to their use for investigating transgene expression, therefore these properties of the cultured TGCs were assessed. Analysis of gene expression after transfection with plasmids carrying the appropriate transgenes requires that the plasmids continue to function for at least 24 hours post-transfection, as this is the
minimum time required for expression of any transgenes following transcription and translation. Analysis of gene expression after transduction with lentiviral vectors requires that the explants continue to function for at least 72 hours post-transduction as this is the minimum time required for expression of any transgenes following integration, transcription and translation.

Short-term culture of up to 24 hours of TGCs isolated from sexually immature chicks has been described. Attempts to develop a long-term and/or improved culture method for this cell type have not been reported. Since the 1980s there have been major advances in mammalian cell culture techniques, including the use of growth matrices in order to achieve optimal cell growth and differentiation. The use of BD Matrigel Matrix (BD Biosciences), poly-L-lysine (Sigma) and gelatin (Sigma) in the culture of TGCs from mature laying hens was investigated in this Chapter. Many cell types are now cultured on growth matrices such as these. BD Matrigel Matrix is a soluble extract of basement membrane proteins (Kleinman and Martin, 2005) that is routinely used for the culture of human embryonic stem cells (for example Xu et al., 2001) and its main components are laminin, which promotes cell adhesion, migration, growth and differentiation, and collagen IV, which promotes cell adhesion and extracellular matrix protein binding. Poly-L-lysine promotes cell adhesion and is routinely used for the culture of nerve cells (for example Langui et al., 1988; Nakatsuji and Nagata, 1989). When absorbed to the culture surface, it increases the number of positively charged sites available for cell binding. In the late 1970s Hopkins reported the adherence of rat pituitary cells in culture with the use of poly-L-lysine. Analysis of the secretion of luteinizing-hormone by these cells was reportedly improved by their adherence, as was the cell viability (Hopkins, 1977). Gelatin is commonly used for the
culture of a number of different cell types including mouse embryonic stem cells (for example Evans and Kaufman, 1981) and vascular endothelial cells (for example Minakawa, 1989; Kim and Kim, 1991). It is a mixture of proteins derived through the hydrolysis of collagen, which promotes cell adhesion. Long-term culture of capillary endothelial cells was reported by Folkman and colleagues (1979) after growing the cells on gelatin-coated plates to encourage adherence. Long-term culture of this cell type had previously not been achieved.
3.2 Aims

1. To become familiar with the techniques involved in TGC isolation and to successfully isolate TGCs from sexually mature laying hens.

2. To analyse the properties of cultured TGCs, including their viability and ability to proliferate.

3. To investigate the ability of TGCs to retain their function in culture by assessing expression of the endogenous egg white protein genes.
3.3 Results

3.3.1 Viability of cultured TGCs

To visualise the tubular glands where the TGCs are located, sections from the magnum region of the oviduct of a sexually mature hen were stained with haematoxylin and eosin as described in Section 2.10.4 (see Figure 3.1). The nuclei of the cells in the sections stain blue with haematoxylin whilst the cytoplasm is stained pink with eosin. Using this staining technique the tubular glands were clearly visualised in the magnum sections.

![Figure 3.1 Cultured hen tubular gland cells (TGCs) from the magnum region of the oviduct of a sexually mature hen.](image)

(a) Haematoxylin and eosin staining on sections taken from the magnum region of the oviduct of a sexually mature hen. Arrows point to the tubular glands where the TGCs are located. (b) Cultured TGCs one hour after isolation.
TGCs were isolated from the magnum region of the oviduct as described in Section 2.4.1. TGCs were cultured with and without the addition of $10^{-7}$M oestrogen and $10^{-10}$M insulin and the medium was changed daily (for details see 2.2.1; see Figure 3.1). These concentrations of oestrogen and insulin are routinely added to the medium for culture of TGCs (for example, Sanders and McKnight, 1985; Dean et al., 1996; Sensenbaugh and Sanders, 1999). As discussed in Section 3.1, oestrogen is reported to prolong the half-life of and stabilise ovalbumin mRNA in cultured TGCs and has been found to require the permissive effects of insulin. To assess viability, trypan blue (0.4%; Sigma) staining was performed on TGCs cultured in 6-well plates over a 5 day time period as described in Section 2.10.5. Staining was performed in triplicate on TGCs isolated from three different hens. Viable cells did not absorb the stain and non-viable cells stained blue (see Figure 3.2).

![Figure 3.2 Trypan blue staining of cultured tubular gland cells (TGCs). To assess viability, trypan blue staining was performed on TGCs cultured in 6-well plates over a 5 day time period. Examples of viable cells that did not stain are indicated (A) and examples of non-viable cells that stained blue are indicated (B). Size bar represents 60µm.](image)
The average percentage viability of the cells was calculated for each day (see Figure 3.3). The TGCs were 100% viable immediately after isolation and remained so for one to two days in culture, followed by a slight drop in the percentage of live cells by day three to 94-95%. The proportion of live cells decreased more significantly by day four to 65-70% and had decreased to 61-62% by day five, however the cell viability did not drop below 60% during the time period analysed (see Figure 3.3). No significant difference in the proportion of live cells was observed with the TGCs cultured with or without oestrogen and insulin, suggesting that the addition of these hormones to the cell culture medium did not enhance the viability of the cultured TGCs.

![Viability of cultured tubular gland cells](image)

**Figure 3.3** Percentage viability of tubular gland cells (TGCs) cultured over 5 days. TGCs were cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin. Cell viability was assessed by trypan blue staining and the average percentage viability was calculated for each day.
3.3.2 Analysis of TGC growth in culture

Cells were cultured for seven days in 24-well plates with and without the addition of $10^{-7}$M oestrogen and $10^{-10}$M insulin. Daily cells counts were performed on TGCs isolated from three different hens. An average daily count for TGCs grown with and without hormones was used to generate a growth curve (see Figure 3.4). Proliferation of the cultured TGCs was not observed during the seven day time period and after three days in culture the cell numbers began to decrease. These results indicate that the TGCs did not divide in these culture conditions, and that the addition of oestrogen and insulin to the cultured cells did not promote proliferation of the TGCs.

![Growth curve for cultured TGCs](image)

**Figure 3.4** Tubular gland cells (TGCs) seven day growth curve. TGCs were cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin. Daily cells counts were performed on TGCs isolated from three hens. An average daily count for TGCs grown with and without hormones was used to generate the growth curve.
3.3.3 TGC culture on growth matrices

TGCs maintained *in vitro* did not adhere to tissue-culture dishes. The culture conditions were changed by using growth matrices to encourage adherence. The cells were cultured on BD Matrigel Matrix, poly-L-lysine or gelatin (see 2.4.6). The experiment was performed in triplicate on TGCs isolated from three different hens. After five days in culture no evidence of adherence was observed (see Figure 3.5), indicating that these growth matrices did not promote adherence of the TGCs.

![Figure 3.5 Tubular gland cells (TGCs) cultured on growth matrices.](image)

TGCs were cultured on (a) BD Matrigel Matrix, (b) poly-L-lysine, (c) gelatin and (d) no growth matrix. (Images depict a representative field of view and are representative of each experiment performed in triplicate). Size bar represents 25µm.
3.3.4 **Annexin-V-Fluos staining of TGCs to detect apoptotic cells**

TGCs maintained *in vitro* for four days were analysed to determine if the cells were dying due to apoptosis, programmed cell death, or due to necrosis, the progressive degradative action of enzymes within the cell. Analysis of phosphatidylserine on the outer leaflet of apoptotic cell membranes was performed using the Annexin-V-Fluos staining kit (see 2.10.5). Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine. Annexin-V-Fluos stains apoptotic as well as necrotic cells, therefore propidium iodide was included in the staining protocol as it stains the DNA of leaky necrotic cells only. To enable identification of apoptotic as opposed to necrotic cells, apoptotic cells and cellular debris appear red when visualised under fluorescence and necrotic cells appear green.

TGCs were stained with the Annexin-V-Fluos kit at 24 hour intervals over four days in culture. Staining was performed in triplicate on TGCs isolated from three different hens. Green staining was not observed, which indicated that apoptotic cells were not present during this time period (see Figure 3.6). Stained cellular debris appeared as red dots. A small number of necrotic cells were detected, mostly at day four, and these appeared as red circles or ovals approximately half the size of healthy TGCs (see Figure 3.6). These results indicate that the decrease in cell viability was due to necrosis and not apoptosis.
Figure 3.6  Annexin-V-Fluos staining on tubular gland cells (TGCs) cultured over 4 days. Panel 1 shows cultured TGCs. Panel 2 shows Annexin-V-Fluos staining on TGCs. The lack of green staining, suggests an absence of apoptotic cells. Panel 3 shows propidium iodide staining which stains cellular debris and necrotic cells red. Necrotic cells can be seen by day 4 as small circular cells approximately half the size of healthy cells. Panel 4 shows merged pictures from panels 1 and 3. (Images depict a representative field of view and are representative of the results of the experiment performed in triplicate). Size bar represents 25μm.
3.3.5  **Expression of egg white protein genes in cultured TGCs**

The TGCs in the magnum region of the hen oviduct synthesize the egg white proteins.

The ability of TGCs maintained *in vitro* to express the egg white protein genes was assessed over 72 hours. Experiments were performed to detect the expression of two of the major egg white protein genes, ovalbumin and lysozyme, at the level of mRNA and protein.

### 3.3.5.1  **Detection of ovalbumin and lysozyme mRNAs**

TGCs were cultured with and without the addition of 10^{-7} M oestrogen and 10^{-10} M insulin. Total RNA was isolated from TGCs at 24 hour time points over 72 hours in culture as described in Section 2.8.3. Single-stranded cDNA was synthesized as described in Section 2.8.4. RT-PCR analysis was performed in triplicate to detect ovalbumin, lysozyme and β-actin mRNAs on cDNA from the same cell isolates from three different hens as described in Sections 2.8.5. Detection of β-actin gene expression was used as a positive control. As shown in Figure 3.7, the ovalbumin and lysozyme primers were tested on the ovalbumin cDNA clone ChEST854P20 and the plasmid pIIIiLys, respectively (see appendix for plasmid details). For primer details see Figure 2.3.

![Figure 3.7](image-url)  **Figure 3.7  RT-PCR to test lysozyme, β-actin and ovalbumin primers.** Ovalbumin and lysozyme primers were tested on the ovalbumin cDNA clone ChEST854P20 and the plasmid pIIIiLys respectively using 35 cycles of PCR. β-actin primers were tested on cDNA isolated from hen oviduct tubular gland cells using 25 cycles of PCR. PCR products match the predicted sizes of 250bp for lysozyme, 400bp for β-actin and 350bp for ovalbumin. 1kb+ DNA ladder used for each gel (Invitrogen).
PCR product for β-actin was detected at a consistent level in TGCs cultured for 72 hours as shown in Figure 3.8. The addition of $10^{-7}$M oestrogen and $10^{-10}$M insulin did not affect the levels of PCR product observed for β-actin over this time period.

Ovalbumin and lysozyme mRNAs were detected in TGCs cultured for 72 hours as shown in Figure 3.8. PCR products were detected for ovalbumin and lysozyme from both TGCs cultured with and without hormones, however the PCR products from TGCs cultured with hormones were at slightly higher levels. As the levels of intensity of RT-PCR products can vary slightly, these results did not conclusively demonstrate that the addition of oestrogen and insulin increased the levels of ovalbumin and lysozyme mRNAs in the TGCs. The results did show that the addition of these hormones did not reduce the levels of ovalbumin and lysozyme mRNAs.
Figure 3.8 Detection of lysozyme and ovalbumin mRNAs in cultured tubular gland cells (TGCs). TGCs were cultured with (+) and without (-) $10^{-7}$M oestrogen and $10^{-10}$M insulin. Analysis was performed in triplicate at 24 hour intervals over 72 hours on cells isolated from three hens. Detection of β-actin was used as a positive control. Negative controls were performed without reverse transcriptase (RT). 35 cycles of PCR were performed for detection of ovalbumin and lysozyme and 25 cycles were performed for detection of β-actin. 1kb+ DNA ladder used for each gel (Invitrogen).
3.3.5.2 Detection of ovalbumin and lysozyme proteins

TGCs were cultured with and without the addition of $10^{-7}$M oestrogen and $10^{-10}$M insulin. The cells were analysed for the presence of ovalbumin and lysozyme protein at 24 hour intervals over a 72 hour time period. Cells were fixed and permeabilised as described in Section 2.5.1 and immunohistochemistry was performed as described in Section 2.9.2. Rabbit anti-lysozyme (Biodesign International) primary antibody and anti-rabbit IgG (whole molecule) FITC conjugate (Sigma) secondary antibody were used for the detection of lysozyme protein. Monoclonal anti-chicken Egg Albumin Clone Ova-14 (Mouse Ascites Fluid; Sigma) primary antibody and anti-mouse IgG (whole molecule) FITC conjugate (Sigma) secondary antibody were used for the detection of ovalbumin protein. Primary antibodies were diluted 1/1000 and secondary antibodies were diluted 1/200. Slides were mounted in Hydramount mounting medium (National Diagnostics) and the cells were visualised under fluorescence.

Immunohistochemistry was performed in triplicate on TGCs isolated from three different hens. Ovalbumin and lysozyme were detected in the TGCs cultured with and without hormones over 72 hours as shown in Figures 3.9 and 3.10. The antibody staining of the cells cultured with hormones was slightly stronger, which is consistent with the RT-PCR analysis of mRNA in Section 3.4.2.1. The results suggest that ovalbumin and lysozyme were present in the TGCs for 72 hours in culture and that the addition of oestrogen and insulin to the culture medium may have enhanced the levels of these egg white proteins.
Figure 3.9 Detection of ovalbumin protein in tubular gland cells (TGCs). TGCs were cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin and were analysed for the presence of ovalbumin protein at 24 hour intervals over 72 hours. Immunohistochemistry was performed in triplicate on TGCs isolated from three different hens. Fluorescence indicates the detection of ovalbumin in the TGCs cultured with and without hormones over 72 hours. (Images depict a representative field of view and are representative of the levels of fluorescence observed for each experiment repeated in triplicate). Size bar represents 5mm.
Figure 3.10  Detection of lysozyme protein in tubular gland cells (TGCs). TGCs were cultured with and without 10^{-7}M oestrogen and 10^{-10}M insulin and were analysed for the presence of lysozyme protein at 24 hour intervals over 72 hours. Immunohistochemistry was performed in triplicate on TGCs isolated from three different hens. Fluorescence indicates the detection of lysozyme in the TGCs cultured with and without hormones over 72 hours. (Images depict a representative field of view and are representative of the levels of fluorescence observed for each experiment performed in triplicate). Size bar represents 5mm.
3.4 Discussion

Isolation and culture of tubular gland cells (TGCs) from hormone-stimulated sexually immature chicks has been described since the late 1960s (O’Malley and Kohler, 1967), however long-term culture and viability of TGCs has not been investigated. Isolation and culture of TGCs from sexually mature laying hens has not been described in the literature. In this Chapter, successful isolation of TGCs from sexually mature laying hens was described. The viability of TGCs cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin was found to be 100% until day two to three, when the viability started to slowly decrease. This suggests that the TGCs are sufficiently viable in culture for use to investigate transgene expression. The addition of hormones did not enhance the viability of the TGCs in these experiments.

Investigation of the proliferative capacity of TGCs from either sexually immature chicks or sexually mature laying hens has not been described in the literature. Proliferation of TGCs cultured with and without the addition of $10^{-7}$M oestrogen and $10^{-10}$M insulin was not observed and after 72 hours the cell numbers began to decrease coinciding with the time of viability decrease. The addition of hormones to the TGCs did not promote proliferation. This could be because the cells were isolated from sexually mature laying hens and were therefore fully developed after having been exposed to hormones in vivo.

There are conflicting reports in the literature about the ability of TGCs isolated from immature chicks to adhere in culture. Sanders and McKnight (1985) describe the cells as being well-attached to the culture dishes, but state that they plate poorly onto glass coverslips. Lampard and Gibbins (2002) report that TGCs float freely in suspension.
and are non-adherent. The culture of TGCs from sexually immature chicks or mature hens on growth matrices has not been described. The TGCs isolated from sexually mature hens described here settled onto the tissue-culture dishes after approximately one hour in culture but did not adhere. BD Matrigel Matrix, poly-L-lysine and gelatin were investigated and found not to promote adherence of TGCs.

TGCs were stained with the Annexin-V-Fluos kit to detect apoptotic cells over five days in culture. No evidence of apoptosis was observed, although a small number of necrotic cells were detected. This suggests that the cell death observed in the viability and growth curve experiments in Sections 3.3.1 and 3.3.2 was due to necrosis. TGCs were also stained over a longer period of 10 days (results not included) and all cell death during this time was also due to necrosis.

Ovalbumin and lysozyme mRNAs and protein were detected in the TGCs cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin over 72 hours. Taken together, the RT-PCR and immunohistochemistry results suggest that the addition of oestrogen and insulin to the culture medium may have enhanced the levels of ovalbumin and lysozyme mRNAs and protein in the cultured TGCs. The half life of ovalbumin mRNA is 6 hours in the absence of hormones and is increased to 24 hours in the presence of oestrogen (Arao et al., 1996). In this Chapter, both lysozyme and ovalbumin mRNAs were detected in TGCs in culture for 72 hours, which suggests that active transcription occurred.

The culture method for TGCs isolated from mature hen oviduct described here fulfilled the criteria for their use to investigate transgene expression and were found to
express the egg white protein genes whilst in culture. Methods to achieve successful gene transfer into the TGCs were investigated in the following Chapter.
CHAPTER 4 GENE TRANSFER INTO CHICKEN TUBULAR GLAND CELLS MAINTAINED IN CULTURE

4.1 Introduction

The development of methods to isolate and culture chicken oviduct tubular gland cells (TGCs) from sexually mature hens was described in Chapter 3. In order to investigate transgene expression in the hen oviduct using cultured TGCs it is essential that efficient gene transfer into the cells can be achieved. Possible methods of gene transfer include transfection, electroporation and viral transduction. These methods of gene transfer into cultured TGCs were assessed in the following experiments.

4.1.1 Transfection and electroporation of TGCs

TGCs from sexually immature chicks have been routinely transfected using calcium-phosphate coprecipitation (for example, Knoll et al., 1983; Sanders and McKnight, 1988 and Haecker et al., 1995). The transfection efficiency of TGCs using this method has been consistently low at approximately 0.2% (Sanders and McKnight, 1988). More recently, Lampard and Gibbins (2002) reported that they achieved low transfection efficiencies with lipofection and electroporation, although precise data were not given. The use of reagents to transfect TGCs from immature chicks or mature laying hens has not been described. Lipid-mediated gene transfer into cultured cells was introduced in the 1980s (Felgner et al., 1987). Since then, a constant improvement of available reagents has been achieved by lowering toxicity and increasing the transfection efficiency. Cationic lipids and polyamines are commonly used as the basis for transfection reagents as they function by complexing with DNA,
allowing it to overcome the electrostatic repulsion of the cell membrane and to be taken up by the cell (Dalby et al., 2004).

In the following experiments, transfection of cultured TGCs with Lipofectamine 2000, FuGENE 6 and GeneJammer is described to investigate the efficiency of gene transfer. Due to the fact that low transfection efficiencies of 0.2% were previously described using calcium-phosphate coprecipitation, this method of transfection was not used. Higher transfection efficiencies would be desirable in order to use the TGCs for the investigation of transgene expression.

Lipofectamine 2000 can be used to transfect a wide range of cell types, including cardiomyocytes (for example Djurovic et al., 2004) and primary astrocytes (for example Tinsley et al., 2004). FuGENE 6 can also be used to transfect a wide range of cell types including glioma cells and primary glial cells (for example Wiesenhofer et al., 1999). GeneJammer is a more recently developed polyamine reagent that has been used for gene delivery into various cell types, including zebrafish embryos (Sussman, 2001) and human oral squamous cell carcinoma cells (for example Konopka, 2005).

Electroporation of TGCs from immature chicks or mature laying hens has not been described. In the following experiments electroporation of cultured TGCs is described to investigate the efficiency of gene transfer using this technique. Electroporation involves the application of a direct current pulse to the cell suspension following addition of the plasmid DNA. The pulse temporarily disrupts the membrane and electrophoreses DNA into the cells. Electroporation can be used to introduce DNA into a wide range of cell types including epidermal cells (for example Reiss et al.,
1986), hematopoietic stem cells (for example Toneguzzo and Keating, 1986) and lymphoid cells (for example Toneguzzo et al., 1986).

4.1.2 Transduction of TGCs

Viral transduction of TGCs from sexually immature chicks or mature laying hens has not been described in the literature. In the following experiments TGCs from sexually mature laying hens were transduced with lentiviral vectors derived from equine infectious anaemia virus (EIAV) pseudotyped with different envelope proteins. As discussed in Section 1.4.3.1, the range of cell types that can be infected by lentiviral vectors can be expanded or altered by pseudotyping. VSV-G (Indiana)-pseudotyped vectors will be referred to as VSV-G-pseudotyped and VSV-G (Chandipura)-pseudotyped vectors will be referred to as Chandipura-pseudotyped.

Among the first and still most widely used envelope glycoproteins for pseudotyping lentiviral vectors is the vesicular stomatitis virus G (VSV-G) protein from the Indiana serotype, due to the very broad tropism and stability of the resulting pseudotyped virus. Wong and colleagues (2004) reported that lentiviral vectors pseudotyped with the VSV-G glycoprotein from the Indiana serotype transduced the striatum of adult rats more successfully than vectors pseudotyped with VSV-G from the Chandipura serotype. Ebola-pseudotyped vectors are reported to readily infect a large number of cell types in vitro, including lung epithelial cells (Kobinger et al., 2001), skeletal myocytes and cardiomyocytes (MacKenzie et al., 2002).
4.1.3 Transgenes

The plasmids and viral vectors used in the following experiments carry transgenes containing the cytomegalovirus (CMV) enhancer/promoter driving expression of the jellyfish green fluorescent protein (GFP) or bacterial β-galactosidase (lacZ) reporter genes. The enhancer/promoter of the human CMV gene encoding the major immediate-early protein has been found to drive transgene expression in most cell types in vitro and is referred to as ubiquitous. GFP and lacZ are commonly used reporter genes and their expression is readily detected. GFP expression is observed by visualisation under fluorescence and lacZ expression is detected by X-gal staining (for details see 2.10.1). Plasmids and viral vectors carrying a GFP or lacZ reporter gene controlled by the ubiquitous CMV enhancer/promoter will be used to investigate the efficiency of different methods of gene transfer into chicken TGCs.
4.2 Aims

1. To determine if TGCs can be successfully transfected and/or electroporated with reporter gene constructs and express transgenes.

2. To determine if TGCs can be successfully transduced with lentiviral vectors and express transgenes.
4.3 **Results**

4.3.1 **Transfection of TGCs**

Transfection of TGCs from sexually immature chicks or mature laying hens using a plasmid containing a CMV enhancer/promoter transgene has not been described in the literature. TGCs were transfected in 6-well plates with the reporter plasmid pCMV-GFP using Lipofectamine 2000 (Invitrogen), FuGENE 6 (Roche) and GeneJammer (Stratagene) as described in Section 2.6 (for plasmid details see Appendix). 4 x 10⁵ to 8 x 10⁵ cells per well were transfected with 5 or 10μg of DNA at a ratio of 1 (amount of DNA):1 (amount of transfection reagent) to 1:10. Chicken embryonic fibroblasts (CEF) were used as a positive control for the transfection experiments (for details on CEF culture see 2.4.3). Each transfection was performed in triplicate on TGCs isolated from three different hens. TGCs and CEFs were visualised under fluorescence 24 hours post-transfection.

Reporter gene expression was not observed in the TGCs after transfection with Lipofectamine 2000, FuGENE6 or GeneJammer, although expression was observed in the positive control CEFs (see Figure 4.1). The highest transfection efficiencies for transfected CEFs were visually estimated at approximately 25% with Lipofectamine 2000, 25% with FuGENE 6 and 20% with GeneJammer. The results suggest that the TGCs may have been successfully transfected but that the levels of GFP expression were too low for detection, or that gene transfer into the TGCs was not achieved in these experiments.
Figure 4.1 Transfection of tubular gland cells (TGCs). Cells pictured were transfected with Lipofectamine 2000 (6 x 10^5 cells per well, 1:3 ratio using 5μg of DNA), FuGene 6 (7 x 10^5 cells per well, 1:5 ratio using 5μg of DNA), GeneJammer (6 x 10^5 cells per well, 1:8 ratio using 5μg of DNA) and were electroporated (6 x 10^5 cells per well, 50μg of DNA, 300 volts and 700μF). CEFs were used as positive controls. (Images depict a representative field of view and are representative of each experiment performed in triplicate). Size bars represent 100μm.
4.3.2 Electroporation of TGCs

Electroporation of TGCs from sexually immature chicks or mature laying hens has not been described in the literature. TGCs were electroporated with the reporter plasmid pCMV-GFP using the BioRad Gene Pulsor II as described in Section 2.6.3 (for plasmid details see Appendix). 10, 50, 100 or 150μg of DNA was used for each electroporation with a voltage of 250 or 300 volts and capacitance of 400, 500, 600, 700, 800, or 900 μF. CEFs were used as a positive control and each electroporation was performed in triplicate on TGCs isolated from three different hens. TGCs and CEFs were visualised under fluorescence 24 hours post-electroporation.

Reporter gene expression was not observed in the electroporated TGCs, however expression was observed in the positive control CEFs (see Figure 4.1). The highest electroporation efficiency in the electroporated CEFs was visually estimated at approximately 5%. The results suggest that the TGCs may have been successfully electroporated but that the levels of GFP expression were too low for detection, or that gene transfer into the TGCs was not achieved in these experiments.
4.3.3 Transduction of TGCs with lentiviral vectors

4.3.3.1 Transduction with a VSV-G pseudotyped lentiviral vector

TGCs were transduced with a VSV-G pseudotyped EIAV lentiviral vector carrying a CMV-GFP transgene (for vector details see Appendix). CEFs were used as a positive control and transductions were performed in duplicate on TGCs isolated from three different hens. TGCs were transduced in 96-well plates at a cell density of $6 \times 10^5$ cells per well as described in Section 2.11.3. 10μl of a $10^9$ viral units/ml virus preparation was used per well giving a multiplicity of infection (MOI) of 10. CEFs were used as a positive control for the transduction experiments. TGCs and CEFs were visualised under fluorescence 72 hours post-transduction.

GFP expression was not observed in the transduced TGCs, however expression was observed in the positive control CEFs (see Figure 4.2). The transduction efficiency in the transduced CEFs was visually estimated at approximately 5%. The results suggest that the TGCs may have been successfully transduced but that the levels of GFP expression were too low for detection, or that gene transfer into the TGCs was not achieved using the VSV-G-pseudotyped EIAV vector in these experiments.
Figure 4.2 Transduction of tubular gland cells (TGCs) with a VSV-G pseudotyped CAG-GFP lentiviral vector. CEFs were used as a positive control for the transductions. A multiplicity of infection of 10 was used for each transduction (Images depict a representative field of view and are representative of each transduction performed in triplicate). Size bar represents 100 μm.
TGCs were transduced with EIAV lentiviral vectors carrying a CMV-LacZ transgene. The vectors were pseudotyped with envelope proteins from VSV-G, Chandipura and Ebola (Zaire) (for vector details see Appendix). CEFs were used as a positive control for the transduction. Transductions were performed in duplicate on TGCs isolated from three different hens. TGCs were transduced in 96-well plates at a cell density of 6 x 10^5 cells per well as described in Section 2.11.3. 10μl of a 10^9 viral units/ml virus preparation was used per well giving a multiplicity of infection (MOI) of 10. CEFs were used as a positive control for the transduction experiments. TGCs and CEFs were visualised under fluorescence 72 hours post-transduction.

β-galactosidase expression was not detected in the transduced TGCs, however expression was detected in the positive control CEFs (see Figure 4.3). The average transduction efficiencies in the transduced CEFs were estimated visually at approximately 90% with the Ebola-pseudotyped vector, 40% with the VSV-G-pseudotyped vector and 25% with the Chandipura-pseudotyped vector. The results suggest that the TGCs may have been successfully transduced but that the levels of β-galactosidase expression were too low for detection, or that gene transfer into the TGCs was not achieved using the VSV-G-, Chandipura- or Ebola (Zaire)-pseudotyped EIAV lentiviral vectors in these experiments.
Figure 4.3  **Transduction of tubular gland cells (TGCs) with EIAV vectors carrying CMV-LacZ transgenes.** Vectors were pseudotyped with Chandipura, VSV-G and Ebola envelope proteins. CEFs were used as a positive control and a multiplicity of infection of 10 was used for each transduction. (Images depict a representative field of view and are representative of each transduction performed in triplicate). Size bar represents 100μm.
4.4 Discussion

4.4.1 Transfection and electroporation of TGCs

Previous transfection experiments described in the literature using TGCs from immature chicks were performed by calcium phosphate coprecipitation, although the transfection efficiency using this method is reported to be low at approximately 0.2%. Sanders and McKnight (1988) attempted different gene transfer techniques into TGCs, including microinjection and protoplast fusion, and concluded that calcium phosphate coprecipitation was the most effective method of gene transfer into this cell type. Higher transfection efficiencies than those achieved by calcium phosphate coprecipitation are required for the use of TGCs in investigating transgene expression, therefore other methods of gene transfer were assessed in this Chapter. Studies using TGCs described in the literature involve transfection with constructs containing the ovalbumin gene promoter driving expression of the chloramphenicol acetyltransferase (CAT) reporter gene (for example, Sanders and McKnight, 1988; Arao et al., 1994; Dean et al., 1996). Detection of CAT activity is performed by a highly sensitive CAT assay. The use of other reporter genes in the transfection of TGCs, such as GFP, has not been reported in the literature.

TGCs were transfected with pCMV-GFP using Lipofectamine 2000, FuGENE6 and GeneJammer. Reporter gene expression was demonstrated in the positive control CEFs but was not observed in the TGCs, suggesting that the TGCs may have been successfully transfected but that the levels of GFP expression were too low for detection, or that gene transfer into the TGCs was not achieved in these experiments.
Unsuccessful transfection could be caused by the fact that the cells are non-dividing. Lipofection of nondividing cells is inefficient because much of the transfected DNA is retained in endosomes, and any that escapes to the cytoplasm enters the nucleus at low rates. TGCs were also electroporated with the reporter plasmid pCMV-GFP in this Chapter, and gene expression was demonstrated in the positive control CEFs but was not observed in the TGCs. These results suggest that the TGCs may have been successfully electroporated but that the levels of GFP expression were too low for detection, or that gene transfer into the TGCs was not achieved in these experiments.

4.4.2 Transduction of TGCs

TGCs were transduced with VSV-G-, Chandipura- and Ebola-pseudotyped EIAV vectors carrying either CMV-GFP or CMV-LacZ transgenes. Reporter gene expression was demonstrated in the positive control CEFs but was not observed in the TGCs, suggesting that the TGCs may have been successfully transduced but that the levels of transgene expression were too low for detection, or that gene transfer into the TGCs was not achieved using these VSV-G-, Chandipura- and Ebola-pseudotyped EIAV vectors. Ivarie and colleagues (2000) also reported that chicken oviduct cells cannot be transduced with retroviral vectors and that it is unknown what the block to transduction of this cell type is. The fact that the cells are non-dividing should not hinder lentiviral transduction as lentiviruses transduce non-dividing cells.

The attachment of the virion to the host cell receptor is the first step in gene delivery and is a crucial factor in determining vector tropism. This viral attachment is mediated by specific interactions between the envelope glycoprotein on the virion and one or more surface receptor molecules on the target cell. It is possible that the receptor
molecules for the Ebola envelope protein are absent in the TGCs, or are variant in the binding region, therefore explaining why gene transfer did not occur. VSV-G is a fusogenic protein that interacts with membrane phospholipids to facilitate viral entry (Yee et al., 1994). As the TGCs synthesize the egg white proteins, it is possible that these proteins are coating the cell membrane and are therefore inhibiting fusion of the G glycoprotein with the membrane phospholipids.

In Chapter 3, TGCs were shown to be viable and to synthesize egg white proteins whilst maintained in culture for 72 hours. However, in order to use the TGCs for investigation of transgene expression, it is essential that efficient gene transfer into the cells can be achieved. In this Chapter, gene expression was not observed in transfected, electroporated or transduced TGCs. The use of chicken oviduct whole tissue explants was therefore assessed in the following Chapters as an alternative for investigating transgene expression in vitro prior to the generation of transgenic hens.
CHAPTER 5  CHICKEN OVIDUCT EXPLANT ISOLATION AND ANALYSIS

5.1 Introduction

Gene expression was not observed in transfected, electroporated or transduced tubular gland cells (TGCs) in the experiments described in Chapter 4. In the following Chapters, whole tissue hen oviduct explants were investigated as an alternative to the use of dissociated oviduct TGCs. Explants from the magnum region of the oviduct of sexually mature hens consist predominantly of TGCs, but also contain fibroblasts, smooth muscle cells and epithelial cells. Such explants could be used to investigate transgene expression in vitro prior to the generation of transgenic hens. The viability of the cells in the explants and their ability to express the egg white protein genes whilst maintained in culture is fundamental to their use in investigating transgene expression, therefore these properties of the explants were assessed in the following experiments.

Mammalian tissue explants have been used in culture systems for more than 50 years. For example, investigation of the growth of embryo heart explants in vitro was described in the early 1950s (Stewart and Kirk, 1952). Tissue explants are now commonly used for investigating the efficiency of viral vectors for gene therapy applications. For such applications it is desirable to work with explants which consist of material in which the original tissue architecture is preserved rather than using cell lines. For example, De Gruijl and colleagues (2002) described the use of a human skin explant model to test the transduction potential of CD40-targeted adenoviral vectors in dendritic cells for tumor immunotherapy applications. Marsman and colleagues
(2004) described a human oesophageal explant model to test the transduction potential of adenoviral vectors for gene therapy applications. More recently, Lee and colleagues (2005) reported the use of cultured human sweat glands as a model for testing cystic fibrosis gene therapy vectors.

The isolation and culture of individual oviduct explants from sexually immature chicks or mature laying hens has not been described. However, the culture of finely minced magnum from hormone-stimulated immature chicks was described in the late 1970s by McKnight (1978). In the following experiments a protocol for the isolation and culture of individual explants from the magnum portion of the oviduct from mature hens was devised based on the protocol of McKnight (1978) as described in Section 2.4.2.

To utilise the explants to investigate transgene expression in the hen oviduct they must be able to be efficiently transfected with plasmid DNA and/or transduced with lentiviral vectors. As discussed in Section 3.1, analysis of gene expression after transfection with plasmids carrying the appropriate transgenes requires that the explants continue to function for at least 24 hours post-transfection and analysis of gene expression after transduction with lentiviral vectors requires that the explants continue to function for at least 72 hours post-transduction. In the following experiments the viability of the cells in the explants was assessed over 72 hours in culture.
5.2 Aims

1. To isolate and culture hen oviduct explants from sexually mature laying hens.

2. To assess the viability of hen oviduct explants maintained in culture.

3. To investigate the ability of hen oviduct explants to express the egg white protein genes whilst maintained in culture.
5.3 Results

5.3.1 Viability of cells in cultured explants

Explants were isolated from the inner lining of the magnum region of the oviduct from sexually mature hens that had recently laid or were about to lay an egg as described in Section 2.4.2. The egg white protein synthesizing tubular gland cells are abundant in the lining of the magnum (see 1.3.3). Each explant was approximately 1-2 mm in diameter as shown in Figure 5.1. Explants were cultured with and without the addition of $10^{-7}$ M oestrogen and $10^{-10}$ M insulin and the medium was changed daily (see 2.2.1). As discussed in Section 3.3.1, these concentrations of oestrogen and insulin are routinely added to the medium for culture of chicken oviduct cells to enhance the expression of the egg white protein ovalbumin. The viability of the cells in the cultured explants was assessed using trypan blue (0.4%; Sigma) dye exclusion. Explants were dissociated into single cell suspension prior to trypan blue staining by mechanical and enzymatic dissociation as described in Section 2.4.1. To assess cell viability, trypan blue staining was performed at 24 hour intervals over a five day time period as described in Section 2.10.5. Staining was performed on two explants isolated from the same hen and the experiment was repeated three times on explants isolated from three different hens.
Figure 5.1 Hen oviduct explant. Explants were isolated from the inner lining of the magnum region of the oviduct from sexually mature laying hens that had recently laid or were about to lay an egg. Each cultured explant was approximately 1-2mm in diameter.
The average percentage of live cells in each of the explants was calculated every day (see Figure 5.2). As shown in Figure 3.2, viable cells did not absorb the stain and non-viable cells stained blue. The cells in all of the explants were 100% viable immediately after isolation and this level of viability was observed for two days in culture, followed by a slight drop in percentage of live cells by day three to 97-100%. The proportion of live cells decreased more significantly by day four to 79-83% and continued to decrease to 77-78% by day five, however the cell viability in all the explants did not drop below 70% during this time period (see Figure 5.2). The results indicate that the addition of oestrogen and insulin to the cell culture medium did not enhance the viability of the cultured explants.

![Viability of cells in cultured explants](image.png)

**Figure 5.2** Percentage viability of cells in explants cultured over 5 days. Explants were cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin. Prior to trypan blue staining explants were dissociated into a single cell suspension. The average percentage of live cells was calculated for each day.
5.3.2 Egg white protein expression in cultured explants

Egg white protein gene expression in the cultured explants was assessed at 24 hour intervals over 72 hours. Experiments were performed to detect the expression of two of the major egg white protein genes, ovalbumin and lysozyme, at the level of mRNA and protein.

5.3.2.1 Detection of ovalbumin and lysozyme mRNAs

The explants were cultured with and without the addition of 10⁻⁷M oestrogen and 10⁻¹⁰M insulin. Total RNA was isolated from cultured explants as described in Section 2.8.3. Single-stranded cDNA was synthesized from the isolated RNA as described in Section 2.8.4. AccuPrime Supermix I (Invitrogen) was used for amplification of cDNA templates. Reactions to detect lysozyme, ovalbumin and β-actin mRNAs were incubated in a thermocycler (Hybaid) as described in Section 2.8.5. For primer sequences see Figure 2.3. Detection of β-actin mRNA was used as a positive control for the RT-PCR. Analysis was performed for detection of β-actin, ovalbumin and lysozyme mRNAs on the same explants isolated from one hen. The experiment was repeated three times on explants from three different hens.

PCR product for β-actin was detected at a consistent level over 72 hours in all the cultured explants as shown in Figure 5.3. The addition of 10⁻⁷M oestrogen and 10⁻¹⁰M insulin did not affect the levels of PCR product observed for β-actin over this time period. PCR products for ovalbumin and lysozyme were detected in the explants cultured with and without oestrogen and insulin over 72 hours (see Figure 5.3). The level of PCR products for ovalbumin and lysozyme from the explants cultured with hormones appeared to be slightly higher than the PCR products from explants.
cultured without hormones. As the levels of intensity of RT-PCR products can vary slightly, these results did not conclusively demonstrate that the addition of insulin and oestrogen increased the levels of ovalbumin and lysozyme mRNAs in the cultured explants. The results did show that the addition of these hormones did not decrease the levels of ovalbumin and lysozyme mRNAs.

**Figure 5.3 Detection of lysozyme and ovalbumin mRNAs in cultured explants.** Explants were cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin. RT-PCR was performed at 24 hour time points over 72 hours. Detection of β-actin was used as a positive control. Negative controls were performed without reverse transcriptase (RT). 35 cycles of PCR were performed for detection of ovalbumin and lysozyme and 25 cycles were performed for detection of β-actin. (Figure depicts representative result from each experiment performed in triplicate).
RT-PCR analysis was also performed to detect ovalbumin and lysozyme mRNAs in explants cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin at 24 hour intervals over an extended seven day time period. Analysis was performed for detection of $\beta$-actin, ovalbumin and lysozyme mRNAs on the same explants isolated from one hen. The experiment was repeated three times on explants from three different hens. PCR product for $\beta$-actin was detected over seven days in all the cultured explants as shown in Figure 5.4. The addition of oestrogen and insulin did not affect the levels of PCR product observed for $\beta$-actin over this time period. PCR products for ovalbumin and lysozyme from the explants cultured without hormones were present until day four, and PCR products for ovalbumin and lysozyme from the explants cultured with hormones were present until day five (see Figure 5.4). PCR products for ovalbumin and lysozyme from the explants cultured with hormones were at a slightly higher level than from the explants cultured without hormones after day two. These results were observed with the explants isolated from each of the three hens, suggesting that the addition of oestrogen and insulin to the cultured explants may have enhanced the levels of and prolonged the presence of ovalbumin and lysozyme mRNAs.
Figure 5.4 Detection of lysozyme and ovalbumin mRNAs in explants cultured over 7 days. RT-PCR analysis was performed in triplicate on the same explants to detect ovalbumin, lysozyme and β-actin mRNAs at 24 hour intervals over a 7 day time period. Explants were cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin (indicated by + and - symbols). Detection of β-actin mRNA was used as a positive control. Negative controls were performed without reverse transcriptase (RT). (Figure depicts representative result from each experiment performed in triplicate).
Detection of ovalbumin and lysozyme proteins

The explants were cultured for 72 hours with and without the addition of $10^{-7}$ M oestrogen and $10^{-10}$ M insulin. Explants were analysed for the presence of ovalbumin and lysozyme protein at 24 hour intervals over 72 hours by fixing, embedding and cryo-sectioning the explants as described in Section 2.5. Immunohistochemistry was performed for detection of ovalbumin and lysozyme protein on sections from the same set of explants from one hen as described in Section 2.9.3. The experiment was repeated three times on explants from three different hens. Primary and secondary antibodies used for the detection of ovalbumin and lysozyme are detailed in Section 3.3.5.2.

Ovalbumin and lysozyme proteins were detected in the explants cultured over 72 hours as shown in Figure 5.5. The antibody staining appears slightly stronger on the explants cultured with $10^{-7}$ M oestrogen and $10^{-10}$ M insulin. This was observed with the explants isolated from each of the three hens. These results are consistent with the RT-PCR results for detection of mRNAs in Section 5.3.2.1, and indicate that ovalbumin and lysozyme are present in the explants for 72 hours in culture and that the addition of oestrogen and insulin may enhance the levels of these egg white proteins in the explants.
Figure 5.5 Detection of ovalbumin and lysozyme protein in explants cultured for 72 hours. Explants were cultured with and without the addition of 10^{-7} M oestrogen and 10^{-10} M insulin and were analysed for the presence of ovalbumin and lysozyme protein at 24 hour intervals over 72 hours. Immunohistochemistry was performed on sections from the same explants for detection of ovalbumin and lysozyme protein. (Images depict a representative field of view and are representative of each experiment performed in triplicate). Size bar represents 0.5mm.
5.4 Discussion

The development of a method to isolate and culture whole tissue oviduct explants from the magnum region of the oviduct of sexually mature hens was described in this Chapter. The viability of the cells in oviduct explants was 100% until day three to four when the proportion of live cells started to slowly decrease. This result was similar to the viability of tubular gland cells maintained \textit{in vitro} as described in Section 3.3.1, and indicates that the explants are sufficiently viable in culture for the required time period for investigating expression of transgenes. The addition of hormones to the cultured explants did not appear to increase the proportion of live cells in the explants. Ovalbumin and lysozyme mRNAs were present in the explants cultured without hormones for four days and in the explants cultured with $10^{-7}$M oestrogen and $10^{-10}$M insulin for five days. RT-PCR analysis also indicated that lysozyme and ovalbumin mRNAs were present at slightly higher levels when the explants were cultured with oestrogen and insulin. As described in Section 3.4, the half life of ovalbumin mRNA is 6 hours in the absence of hormones and is reported to increase to 24 hours in the presence of hormones. As lysozyme and ovalbumin mRNAs can be detected in explants cultured for four to five days, this indicates that active transcription was occurring.

Ovalbumin and lysozyme proteins were detected in the explants cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin over 72 hours. Antibody staining was slightly higher in the explants cultured with hormones, which is consistent with the RT-PCR results. The results indicate that in order to stimulate expression of ovalbumin and lysozyme in the cultured explants, $10^{-7}$M oestrogen and $10^{-10}$M insulin should be added to the culture medium.
As the hen oviduct explants maintained in culture were demonstrated to be sufficiently viable for use to investigate transgene expression and were expressing the egg white protein genes whilst maintained in culture, methods for gene transfer into the explants were investigated in the following Chapter.
CHAPTER 6  TRANSGENE EXPRESSION IN CHICKEN

OVIDUCT EXPLANTS DRIVEN BY UBIQUIOUS PROMOTERS

6.1 Introduction

In Chapter 5, the development of a method for the isolation and culture of hen oviduct explants from sexually mature hens that fulfilled the criteria for use to investigate transgene expression in the hen oviduct was described. In order to use cultured explants for this purpose, it is essential that efficient gene transfer into the cells in the explants can be achieved. Possible methods of gene transfer include transfection, electroporation and viral transduction. The assessment of these methods of gene transfer into cultured explants is described here.

6.1.1 Transfection and electroporation of explants

The use of lipid-based transfection or electroporation of oviduct explants from immature chicks or mature hens has not been described. In Chapter 4, gene transfer into tubular gland cells (TGCs) using several transfection reagents and electroporation was not successful. As discussed in Section 4.1.1, the transfection reagents tested and electroporation are commonly used to achieve gene transfer into a wide range of cell types. Reports on the use of transfection reagents or electroporation with cultured tissue explants are limited. For example, Bauer and colleagues (2001) described the use of Lipofectamine 2000 (Invitrogen) to transfect brain tissue explants from 6-12 week old human foetuses. Pu and Young (1990) described the use of electroporation to achieve successful gene transfer into intact chick embryonic retinal explant cultures. More recently, Cardoso and colleagues (2004) reported successful gene transfer into cultured embryonic intercostal muscle using electroporation of intact
pieces of tissue. In the following experiments, transfection and electroporation of hen oviduct explants is described to investigate the efficiency of gene transfer.

6.1.2 Transduction of explants

Viral transduction of hen oviduct explants from immature chicks or mature laying hens has not been described. In Chapter 4, transgene expression was not observed in TGCs transduced with lentiviral vectors derived from equine infectious anaemia virus (EIAV) pseudotyped with several envelope proteins, including vesicular stomatitis virus G protein (VSV-G). In the following experiments, oviduct explants from sexually mature laying hens were transduced with lentiviral vectors derived from EIAV or human immunodeficiency virus (HIV) pseudotyped with VSV-G (Indiana and Chandipura serotypes), Ebola (Zaire serotype), Mokola and leukocytic choriomeningitis virus (LCMV) envelope proteins to investigate the efficiency of gene transfer. VSV-G (Indiana)-pseudotyped vectors will be referred to as VSV-G-pseudotyped and VSV-G (Chandipura)-pseudotyped vectors will be referred to as Chandipura-pseudotyped.

The transduction of cultured explants from various organs has been described for testing the efficiency of viral vectors for purposes such as gene therapy. For example, Lehtonen and colleagues (2002) described transgene expression in cultures of rat ventral mesencephalon explants transduced with an adenoviral vector. Marsman and colleagues (2004) established a human oesophageal explant model to test the transduction of adenoviral vectors ex vivo, reporting limited and localised transduction in the explants. More recently, Hasson and colleagues (2005) described transgene expression in cultures of murine lung-, skin-, spleen- and colon-derived
micro-organs transduced with a Moloney murine leukaemia virus vector, adenoviral vector, vaccinia vector and Herpes Simplex Virus I vector. All four vectors reportedly resulted in infection of the micro-organs, with the adenoviral vector resulting in considerably higher levels of expression. The use of a lentiviral vector to successfully transduce cultures of murine salivary gland explants was also described recently by Shai and colleagues (2005).

As discussed in Section 4.1.2, the VSV-G (Indiana) envelope protein is the most widely used for pseudotyping lentiviral vectors due to its broad tropism, and Ebola-pseudotyped vectors have been reported to readily infect a number of cell types in vitro. Vectors pseudotyped with the lymphocytic choriomeningitis virus (LCMV) envelope protein have also been reported to infect a broad range of cells (Beyer et al., 2002) and Mokola-pseudotyped vectors have been reported to infect different cell types including cells of the mouse central nervous system (Watson et al., 2002).

6.1.3 Transgenes

The plasmids and viral vectors used in the following experiments all carry transgenes containing either the human cytomegalovirus (CMV) enhancer/promoter (see 4.1.3) or the CMV-chicken β-actin (CAG) enhancer/promoter. The CMV enhancer/promoter is described as functioning ubiquitously in many cell types in vitro, however expression from this enhancer/promoter in transgenic mice has been found to vary between tissues and different transgenic lines (Furth et al., 1991). In particular, it has been reported that the CMV enhancer/promoter expresses in most tissue at varying levels but predominantly drives transgene expression in the exocrine pancreas in transgenic mice (Zhan et al., 2000) and transgenic chickens (McGrew et al., 2004).
The CAG enhancer/promoter is a combination of the CMV enhancer and the chicken β-actin promoter and first intron. This enhancer/promoter has been demonstrated to have high activity in cultured cells (Niwa et al., 1991) and to promote high levels of ubiquitous expression in transgenic mice (Ikawa et al., 1995) and birds (Sang et al., unpublished results). The CMV and CAG enhancer/promoters were used in the following experiments to drive expression of green fluorescent protein (GFP), lacZ and secreted alkaline phosphatase (SeAP) reporter genes or the therapeutic protein, R24. As discussed in Section 4.1.3, GFP is detected by visualisation under fluorescence and lacZ expression is detected by X-gal staining. Expression of the SeAP reporter gene results in secretion of alkaline phosphatase by the transfected or transduced cells, which can be detected by a kinetic assay (for details see 2.10.2). The use of plasmids or viral vectors carrying GFP or LacZ transgenes would allow investigation of different methods of gene transfer into cultured chicken oviduct explants. Successful gene transfer into explants using a plasmid or viral vector carrying a SeAP transgene would allow investigation of the expression of a secreted protein by the cells in the explants.

R24 was chosen as a potential target therapeutic protein to exemplify the transgenic chicken system in the oviduct explants. R24 is a mouse-human hybrid minibody that is specific for the cell-surface tumour antigen disialoganglioside GD3. The minibody was constructed from the key functional domains from the original antibody using the sequence of the heavy and light chain variable domains of the R24 antibody and the sequences of human immunoglobulin G1 constant domains of the heavy chain (CH2 and CH3) (see Figure 6.1). Secretion of R24 by transfected or transduced cells can be detected by enzyme-linked immunosorbant assay (ELISA) (for details see 2.9.4).
Successful gene transfer into chicken oviduct explants using a plasmid or viral vector carrying an R24 transgene would allow investigation of the expression of a therapeutic protein by the cells in the explants.

![Figure 6.1 R24 minibody comprising the mouse single chain Fab (antigen binding) variable region and the human immunoglobulin (Ig) G1 Fc (crystallizable) region. VH indicates the variable domain of the heavy chain; VL indicates the variable domain of the light chain.](image-url)
6.2 Aims

1. To determine if hen oviduct explants can be successfully transfected and/or electroporated with reporter gene constructs and express transgenes.

2. To determine if hen oviduct explants can be successfully transduced with lentiviral vectors and express transgenes.
6.3 Results

6.3.1 Transfection of explants

In the following experiments, explants from mature laying hens were isolated as described in Section 2.4.2. Explants were transfected in 6-well tissue-culture plates, using one explant per well, with the reporter plasmid pCMV-GFP (for plasmid details see Appendix). Transfections were performed using Lipofectamine 2000 (Invitrogen), FuGENE6 (Roche) and GeneJammer (Stratagene) as described in Section 2.6. For each transfection 5, 10 or 15μg of DNA was used at a ratio of 1 (amount of DNA):1 (amount of transfection reagent), 1:2, 1:4, 1:6, 1:8, 1:10 or 1:20. Each transfection was performed on two explants isolated from the same hen and the experiment was repeated three times on explants from three different hens. Explants were analysed for GFP expression 24 hours post-transfection.

GFP expression was not observed in any of the explants transfected with Lipofectamine 2000, FuGENE6 or GeneJammer (see Figure 6.2). The results suggest that the explants may have been successfully transfected but that the levels of GFP expression were too low for detection, or that gene transfer into the TGCs was not achieved in these experiments.
Figure 6.2 Transfection of explants with reagents. Explants transfected with the reporter plasmid pCMV-GFP using (a) Lipofectamine 2000, (b) FuGENE 6 and (c) GeneJammer. Size bar represents 250µm. (Images are representative of the expression levels observed in explants from each transfection experiment performed in triplicate).
6.3.2  **Electroporation of explants**

Electroporation experiments were initially performed as described in Section 2.6.5 with the ElectroSquare Porator ECM 830 (BTX) using the Genetrode (Genetronics, Inc.), short EggAtrode and long EggAtrode (RMR Technologies). Explants were electroporated with the reporter plasmid pCAG-GFP using all three electrode devices in order to determine which resulted in the most efficient gene transfer into the explants. Following determination of the most suitable electroporation device and conditions, electroporations were performed with several transgene constructs.

6.3.2.1  **Expression of CAG-GFP in electroporated explants**

Explants were electroporated with the reporter plasmid pCAG-GFP to determine the most effective electroporation device and conditions (see Appendix for plasmid details). Electroporations were performed using the Genetrode, long EggAtrode and short EggAtrode. Plasmid DNA was pipetted into an incision made on the surface of the explant prior to electroporation as described in Section 2.6.5. Each electroporation was performed on two explants isolated from the same hen and the experiment was repeated three times on explants isolated from three different hens. Explants were analysed for GFP expression 24 hours post-electroporation.

Explants were electroporated with the Genetrode as follows: 70-130 volts (increased by 10 volts for each electroporation), 30-60 milliseconds (ms) pulse length (increased by 10ms for each electroporation) and 300-600ms length between pulses (increased by 50ms for each electroporation). Phosphate buffered saline (PBS) was added to the explants prior to electroporation. This resulted in burning of the tissue and GFP expression was not observed. PBS was therefore not added to the explants for
subsequent electroporations with this device. GFP expression was observed in explants electroporated using 120 volts with a 50ms pulse length and 500ms between pulses (see Figure 6.3). GFP expression was visually estimated at less than one percent of the cells in the explants.

Explants were electroporated with the long EggATrode as follows: 80-120 volts for positions 1 and 2 (increased by 10 volts for each electroporation), 60-100 volts for positions 4, 5, 6 and 7 (increased by 10 volts for each electroporation), 5-15ms pulse length (increased by 1ms for each electroporation) and 100-300ms length between pulses (increased by 50ms for each electroporation). The addition of PBS to the explants did not cause burning of the tissue, but did not appear to enhance the electroporations. GFP expression was observed in explants electroporated using 100 volts at positions 1 and 2, 80 volts at positions 4, 5, 6 and 7, 11ms pulse length and 250ms between pulses (see Figure 6.3). GFP expression was visually estimated at less than one percent of the cells in the explants.

Explants were electroporated with the short EggATrode as follows: 80-120 volts for positions 1 and 2 (increased by 10 volts for each electroporation), 60-100 volts for positions 4, 5, 6 and 7 (increased by 10 volts for each electroporation), 5-15ms pulse length (increased by 1ms for each electroporation) and 100-300ms length between pulses (increased by 50ms for each electroporation). The addition of PBS to the explants did not cause burning of the tissue, but did not appear to enhance the electroporations. GFP expression was observed in explants electroporated using 100 volts at positions 1 and 2, 80 volts at positions 4, 5, 6 and 7, 11ms pulse length and
250 ms between pulses (see Figure 6.3). GFP expression was visually estimated at less than one percent of the cells in the explants.

**Figure 6.3 Electroporation of explants with pCAG-GFP.** GFP expression observed in explants electroporated using the Genetrode (120 volts, 50 ms pulse length, 500 ms gap between pulses), long EggATrode (100 volts at positions 1 and 2, 80 volts at positions 4, 5, 6 and 7, 11 ms pulse length, 250 ms gap between pulses) and short EggATrode (100 volts at positions 1 and 2, 80 volts at positions 4, 5, 6 and 7, 11 ms pulse length, 250 ms gap between pulses). Arrow points to a characteristic burn mark made by one of the electrodes. Size bar represents 500 μm. (Images are representative of the expression levels observed in explants from each electroporation experiment performed in triplicate).
Subsequent experiments were performed by injecting the plasmid DNA directly into the explant prior to electroporation as described in Section 2.6.5. Explants were electroporated with the Genetrode, long EggAtrode and short EggAtrode using the conditions described above. Each electroporation was performed on two explants isolated from the same hen and the experiment was repeated three times on explants from three different hens. The same conditions that resulted in GFP expression previously also resulted in the highest levels of GFP expression in these experiments (see Figure 6.4). Higher levels of expression were observed using the injection technique than with the previous technique of pipetting the DNA into an incision. For this reason injection of DNA was used for all subsequent electroporations.
Figure 6.4 Electroporation of explants with pCAG-GFP. GFP expression observed in explants electroporated with and without PBS using the Genetrode (120 volts, 50ms pulse length, 500ms gap between pulses), long EggATrode (100 volts at positions 1 and 2, 80 volts at positions 4, 5, 6 and 7, 11ms pulse length, 250ms gap between pulses) and short EggATrode (100 volts at positions 1 and 2, 80 volts at positions 4, 5, 6 and 7, 11ms pulse length, 250ms gap between pulses). Arrows point to characteristic burn marks made by the electrodes. Size bar represents 500μm. (Images are representative of the expression levels observed in explants from each electroporation experiment performed in triplicate).
GFP expression in explants electroporated with the short EggATrode was visually estimated at approximately 3-10% of the cells in the explants. This was higher than the levels of approximately 1-2% observed in explants electroporated with the Genetrode or long EggATrode. The short EggATrode was therefore used in all subsequent electroporations along with the electroporation conditions that resulted in the highest levels of GFP expression in the explants, as shown in Figure 6.5. These conditions were 100 volts at positions 1 and 2, 80 volts at positions 4, 5, 6 and 7, 11ms pulse length and 250ms between pulses.

![Image of electroporation](image)

**Figure 6.5 Electroporation of explants with pCAG-GFP using the short EggATrode.** GFP expression observed in explants electroporated with and without PBS. (a) 90 volts at positions 1 and 2, 70 volts at positions 4, 5, 6 and 7, 11ms pulse length, 250ms gap between pulses, (b) 100 volts at positions 1 and 2, 80 volts at positions 4, 5, 6 and 7, 11ms pulse length, 250ms gap between pulses, and (c) 110 volts at positions 1 and 2, 90 volts at positions 4, 5, 6 and 7, 11ms pulse length, 250ms gap between pulses. Arrows point to characteristic burn marks made by the electrodes. Size bar represents 500μm. (Images are representative of the expression levels observed in explants from each electroporation experiment performed in triplicate).
6.3.2.2 Expression of CMV-GFP in electroporated explants

Explants were electroporated with the reporter plasmid pCMV-GFP (for plasmid details see Appendix). Plasmid DNA was injected into the explants prior to electroporation with the short EggATrode. The optimum conditions for electroporation with this device were used for each electroporation (see 6.3.2.1). Each electroporation was performed on two explants isolated from the same hen and the experiment was repeated three times on explants from three different hens. The explants were viewed under fluorescence 24 hours post-electroporation. GFP expression was visually estimated at less than one percent of the cells in the electroporated explants (see Figure 6.6), indicating that successful gene transfer into the explants had been achieved. This level of expression was significantly lower than the 3-10% observed in explants electroporated with pCAG-GFP (see Figure 6.5), suggesting that the CMV enhancer/promoter may be less efficient than the CAG enhancer/promoter at driving transgene expression in the explants.

![Image](image_url)  

**Figure 6.6 Electroporation of explants with pCMV-GFP.** GFP expression in explants electroporated with pCMV-GFP. Arrow points to a characteristic burn mark made by an electrode. Size bar represents 500μm. (Image is representative of the expression levels observed in explants from each experiment performed in triplicate).
6.3.2.3 Expression of CAG-SeAP in electroporated explants

Explants were electroporated with the reporter plasmid pCAG-SeAP (for plasmid details see Appendix). Each electroporation was performed as described in Section 6.3.2.1 on two explants isolated from the same hen and the experiment was repeated three times on explants from three separate hens. RT-PCR analysis was performed 24 hours post-electroporation as described in Section 2.8.5 to detect SeAP expression at the mRNA level. Total RNA was isolated from explants as described in Section 2.8.3. Single-stranded cDNA was synthesized from the isolated RNA as described in Section 2.8.4. SeAP primers were tested on the plasmid pONY8.45NCAGSeap2 (see Figure 6.7). SeAP mRNA was detected in the electroporated explants as shown in Figure 6.8. The medium from the electroporated explants was analysed for the presence of alkaline phosphatase as described in Section 2.10.2, however alkaline phosphatase was not detected. These results suggest that although alkaline phosphatase was not detected in the medium, successful gene transfer into the explants was achieved as demonstrated by expression of SeAP at the mRNA level. SeAP mRNAs were not detected using less than 38 cycles of PCR amplification, indicating that the percentage of cells expressing the transgene was low.
Figure 6.7 RT-PCR to test secreted alkaline phosphatase (SeAP), R24 and green fluorescent protein (GFP) primers. SeAP primers were tested on the plasmid pONY8.45NCAGSeAP2, R24 primers were tested on the plasmid pRI57 and GFP primers were tested on the plasmid pONY8.7NCAG. 30 cycles of PCR were performed. PCR products match the predicted sizes of 520bp for SeAP, 560bp for R24 and 400bp for GFP.

Figure 6.8 Electroporation of cultured explants with pCAG-SeAP. 38 cycles of RT-PCR were performed to detect SeAP mRNA in the electroporated explants. Detection of β-actin was used as a positive control (25 cycles). Positive control (+) for SeAP primers was performed on the plasmid pONY8.45NCAGSeAP2 (30 cycles). 520bp SeAP PCR product was detected in all samples. Figure depicts results for explants isolated from three different hens. 1kb+ DNA ladder was used for each gel (Invitrogen).
6.3.2.4 Expression of CAG-R24 in electroporated explants

Explants were electroporated with the plasmid pCAG-R24 (for plasmid details see Appendix). Each electroporation was performed as described in Section 6.3.2.1 on two explants isolated from the same hen. The experiment was repeated three times on explants from three different hens. RT-PCR analysis was performed as described in Section 2.8.5 to detect R24 expression at the mRNA level. R24 primers were tested on the plasmid pRI57 (see Figure 6.7). R24 mRNA was detected in the electroporated explants as shown in Figure 6.9. The medium from the electroporated explants was analysed for the presence of R24 protein by ELISA as described in Section 2.9.4, however R24 was not detected. The results suggest that although R24 was not detected in the medium, successful gene transfer into the explants had been achieved as demonstrated by expression of R24 at the mRNA level.

**Figure 6.9 Expression of R24 in electroporated explants.** 35 cycles of RT-PCR were performed to detect R24 mRNA in the electroporated explants. Detection of β-actin was used as a positive control for the PCR (25 cycles). Positive control (+) for R24 primers was performed on the plasmid pRI57 (30 cycles). 560bp R24 PCR product was detected in all samples. Figure depicts results for explants isolated from three different hens. 1kb+ DNA ladder was used for each gel (Invitrogen).
6.3.3 **Transduction of explants with lentiviral vectors**

Explants were transduced with replication-defective lentiviral vectors derived from equine infectious anaemia (EIAV) or human immunodeficiency virus (HIV) vectors carrying several different transgenes as described in Section 2.11.4 (for vector details see Appendix). 20μl of a $10^9$ viral units/ml virus preparation was used per explant. Each explant was estimated to contain approximately $6 \times 10^6$ cells therefore giving a multiplicity of infection (MOI) of 2. The tropism of lentiviral vectors can be changed by pseudotyping, which involves substitution of the wild type envelope glycoprotein with one from a different virus (see 1.4.3.1). The vectors used in these experiments were pseudotyped with several different envelope proteins.

6.3.3.1 **Expression of CMV-LacZ in transduced explants**

Explants were transduced with EIAV vectors carrying a CMV-LacZ transgene. The vectors were pseudotyped with either VSV-G, Chandipura, Mokola, Ebola (Zaire) or leukocytic choriomeningitis virus (LCMV) envelope proteins (for vector details see Appendix). Each transduction was performed as described in Section 2.11.4 on two explants isolated from the same hen. The experiment was repeated three times on explants from three different hens. Explants were embedded and cryo-sectioned 72 hours post-transduction. Sections were stained for β-galactosidase activity as described in Section 2.9.1. Expression was observed in all the transduced explants as shown in Figure 6.10. The highest level of expression was observed in explants transduced with the VSV-G-pseudotyped vector, with approximately 90-100% of the cells in the sections expressing β-galactosidase. Expression was observed in approximately 30-50% of the cells in the sections from explants transduced with the Ebola- and Chandipura-pseudotyped vectors. The lowest levels of expression were
observed in explants transduced with the Mokola and LCMV-pseudotyped vectors, with approximately 5-20% of the cells in the sections expressing β-galactosidase.

Figure 6.10 Sections from chicken oviduct explants transduced with EIAV vectors carrying CMV-LacZ transgenes. Explants were transduced with vectors pseudotyped with envelope proteins from the following viruses: (a) negative control (no vector) (b) Mokola (c) lymphocytic choriomeningitis virus (LCMV) (d) Ebola (e) Chandipura (f) VSV-G. Size bar represents 500μm. (Images are representative of the levels of expression observed in explants from each transduction experiment performed in triplicate).
These results demonstrate successful gene transfer into chicken oviduct explants by transduction with EIAV vectors pseudotyped with Mokola, LCMV, Ebola, Chandipura and VSV-G envelope proteins. Transduction with the VSV-G-pseudotyped vector resulted in the highest levels of reporter gene expression, suggesting that vectors pseudotyped with this envelope protein infect the cells in the explants particularly well. All subsequent transductions were performed with VSV-G-pseudotyped lentiviral vectors.

6.3.3.2 Expression of CAG-GFP in transduced explants

Explants were transduced with a VSV-G pseudotyped EIAV vector carrying a CAG-GFP transgene (for vector details see Appendix). Each transduction was performed as described in Section 2.11.4 on two explants isolated from the same hen. The experiment was repeated three times on explants from three different hens. Explants were analysed for GFP expression by visualising under fluorescence 72 hours post-transduction. GFP expression was not observed in any of the transduced explants (see Figure 6.11). RT-PCR analysis was performed as described in Section 2.8.5 to detect GFP expression at the mRNA level. GFP primers were tested on the plasmid pONY8.7NCAG (see Figure 6.7). GFP mRNA was detected in the transduced explants as shown in Figure 6.11. The results suggest that although GFP expression was not observed in the explants viewed under fluorescence, successful gene transfer into the explants had been achieved by transduction with this VSV-G pseudotyped EIAV vector as demonstrated by expression of GFP at the mRNA level. GFP mRNAs were not detected using less than 38 cycles of PCR amplification indicating that the percentage of cells in the explants expressing the transgene was low.
Figure 6.11 Transduction of explants with a VSV-G pseudotyped EIAV vector carrying a CAG-GFP transgene. (a) GFP expression in transduced explants. Size bar represents 500μm. (Image is representative of the expression levels observed in the explants in this experiment). (b) 38 cycles of RT-PCR were performed to detect GFP mRNA in the electroporated explants. Detection of β-actin was used as a positive control for the RT-PCR (25 cycles). Positive control (+) for GFP primers was performed on the plasmid pONY8.7NCAG (30 cycles). 400bp GFP PCR product was detected in all samples. Figure depicts results for explants isolated from three different hens. 1kb+ DNA ladder was used for each gel (Invitrogen).
6.3.3.3 Expression of CAG-SeAP in transduced explants

Explants were transduced with a VSV-G pseudotyped EIAV vector carrying a CAG-SeAP transgene (for vector details see Appendix). Each transduction was performed as described in Section 2.11.4 on two explants isolated from the same hen. The experiment was repeated three times on explants from three different hens. RT-PCR analysis was performed as described in Section 2.8.5 to detect SeAP expression at the mRNA level. SeAP mRNA was detected in the transduced explants as shown in Figure 6.12. The medium from the transduced explants was analysed for the presence of alkaline phosphatase 72 hours post-transduction as described in Section 2.10.3, however alkaline phosphatase was not detected. The assay can detect a minimum of 2ng/ml protein. The results suggest that although alkaline phosphatase was not detected in the medium, successful gene transfer into the explants had been achieved by transduction with the VSV-G pseudotyped EIAV vector as demonstrated by expression of SeAP at the mRNA level. SeAP mRNAs were not detected using less than 38 cycles of PCR amplification indicating that percentage of cells in the explants expressing the transgene was low.

Figure 6.12 Transduction of explants with a VSV-G pseudotyped EIAV vector carrying a CAG-SeAP transgene. 38 cycles of RT-PCR were performed to detect SeAP mRNA in the electroporated explants. Detection of \( \beta \)-actin was used as a positive control for the PCR (25 cycles). Positive control (+) for SeAP primers was performed on the plasmid pONY8.45NCAGSeAP2 (30 cycles). 520bp SeAP PCR product was detected in all samples. Figure depicts results for explants isolated from three different hens. 1kb+ DNA ladder was used for each gel (Invitrogen).
6.3.3.4 Expression of CAG-R24 in transduced explants

Explants were transduced with a VSV-G pseudotyped HIV vector carrying a CAG-R24 transgene (for vector details see Appendix). Each transduction was performed as described in Section 2.11.4 on two explants isolated from the same hen. The experiment was repeated three times on explants from three different hens. RT-PCR analysis was performed as described in Section 2.8.5 to detect R24 expression at the mRNA level. R24 mRNA was detected in the transduced explants as shown in Figure 6.13. The medium from the transduced explants was analysed for the presence of R24 72 hours post-transduction by ELISA as described in Section 2.9.4. The ELISA can detect a minimum of 10ng/ml R24. R24 protein was detected in two of the samples as shown in Figure 6.13, although in one of the samples the amount of R24 was too low to quantify. These results demonstrate successful gene transfer into chicken oviduct explants by transduction with a VSV-G pseudotyped HIV vector as demonstrated by expression of R24 at both the mRNA and protein level.
Figure 6.13 Transduction of explants with a VSV-G pseudotyped HIV vector carrying a CAG-R24 transgene. (a) 35 cycles of RT-PCR were performed to detect R24 mRNA in the electroporated explants. Detection of β-actin was used as a positive control for the PCR (25 cycles). Positive control (+) for R24 primers was performed on the plasmid pRI57 (30 cycles). 560bp R24 PCR product was detected in all samples. Figure depicts results for explants isolated from three different hens. 1kb+ DNA ladder was used for each gel (Invitrogen). (b) Detection of R24 protein in the medium from transduced explants by enzyme-linked immunosorbant assay (ELISA). Results are shown for two transduced explants from three different hens, corresponding to the RT-PCR result in (a).
Explants were transfected with pCMV-GFP using Lipofectamine 2000, FuGENE6 and GeneJammer. GFP expression was not observed in the transfected explants, suggesting that the explants may have been successfully transfected but that the levels of GFP expression were too low for detection, or that gene transfer into the TGCs was not achieved in these transfection experiments. In Chapter 4, transgene expression was not observed in dissociated TGCs after transfection with these three reagents, although expression was observed in the transfections of CEFs. Tubular gland cells (TGCs) constitute approximately 80% of the cells in the magnum region of the hormone-stimulated hen oviduct (Palmiter, 1972), which is the region from which the explants were isolated. Unsuccessful transfection could be attributed to the high proportion of TGCs in the explants.

Explants were electroporated with pCAG-GFP using the Genetrode, long EggAtrode and short EggAtrode by pipetting the plasmid DNA into an incision made on the surface of the explant prior to electroporation. The electroporation conditions that resulted in successful gene transfer into the explants were determined. A variety of electroporation conditions were attempted with each device, however reporter gene expression was only observed in an estimated one percent of the cells in the explants. Although the levels of expression were low, this method of gene transfer into the explants was successful. This was encouraging, as transgene expression had not been observed in transfected, electroporated or transduced TGCs in Chapter 4, or in explants transfected with reagents in this Chapter.
Subsequent electroporations were performed by injecting the plasmid DNA directly into the explant prior to electroporation, which resulted in higher levels of GFP expression. It is possible that the DNA pipetted into an incision on the surface of the explant may have moved across the surface of the explant when the voltage was applied, whereas the DNA injected directly into the explant may have remained in place during the electroporation. GFP expression was observed in explants electroporated with the short EggATrode at levels of approximately 3-10% of the cells in the explants. This was higher than the levels of approximately 1-2% observed in explants electroporated with the Genetrode or the long EggATrode. This could be explained by the fact that the electrodes are shorter and closer together in the short EggATrode, facilitating ease of placement of the electrodes directly over the site of DNA injection.

Subsequent electroporations were performed using the short EggATrode along with DNA injection and the electroporation conditions that resulted in the highest levels of GFP expression. Successful gene transfer into the explants was achieved by electroporation with pCMV-GFP as demonstrated by GFP expression observed at levels of less than one percent of the cells in the explants. This level of expression was lower than the 3-10% observed in explants electroporated with pCAG-GFP, suggesting that the CMV enhancer/promoter may be less efficient than the CAG enhancer/promoter at driving transgene expression in the oviduct explants. McGrew and colleagues (2004) generated transgenic birds carrying a CMV-GFP transgene, and observed that expression of GFP was not ubiquitous as it varied between tissues and was highest in the exocrine pancreas. This pattern of expression has also been reported in transgenic mice containing the CMV enhancer/promoter driving transgene
expression (Zhan et al., 2000). Sang and colleagues (2004) generated transgenic chickens containing a CAG-GFP transgene and analysis of the offspring of these birds has shown ubiquitous expression of GFP (unpublished results).

Explants were successfully electroporated with pCAG-SeAP and pCAG-R24 as demonstrated by transgene expression at the mRNA level. Alkaline phosphatase and R24 protein were not detected in the medium from the electroporated explants. It is possible that there were too few cells in the explants secreting alkaline phosphatase or R24, resulting in protein levels too low for the assays to detect. The alkaline phosphatase assay can detect a minimum of 2ng/ml protein, whereas the ELISA can detect a minimum of 10ng/ml R24. The highest levels of transgene expression in electroporated explants were approximately 10% using the pCAG-GFP plasmid, therefore even if 10% of the cells in the explants were synthesizing the proteins it is possible that this was too low an amount to detect using these assays.

Explants were successfully transduced with EIAV vectors carrying a CMV-LacZ transgene pseudotyped with Mokola, LCMV, Ebola, VSV-G and Chandipura envelope proteins. Explants were also transduced with VSV-G-pseudotyped EIAV vectors carrying CAG-GFP or CAG-SeAP transgenes and with a VSV-G-pseudotyped HIV vector carrying a CAG-R24 transgene. Transgene expression at the mRNA level was observed in explants transduced with the VSV-G-pseudotyped EIAV vectors carrying CAG-GFP or CAG-SeAP transgenes, however transgene expression at the protein level was not observed in explants transduced with these vectors. This could be because the number of cells in the explants expressing the
Transgene were too low to produce sufficient quantities for detection as discussed above.

Transgene expression at the protein level was observed in explants transduced with EIAV vectors pseudotyped with Mokola, LCMV, Ebola, VSV-G or Chandipura carrying a CMV-LacZ transgene. The highest levels of expression were observed in explants transduced with the VSV-G-pseudotyped vector, with approximately 90-100% of the cells in the sections expressing β-galactosidase. Expression was observed in approximately 30-50% of the cells in the sections from explants transduced with the Ebola- and Chandipura-pseudotyped vectors. The lowest levels of expression were observed in explants transduced with the Mokola and LCMV-pseudotyped vectors, with approximately 5-20% of the cells in the sections expressing β-galactosidase. Transgene expression was observed at both the mRNA and protein level in explants transduced with a VSV-G pseudotyped HIV vector carrying a CAG-R24 transgene.

These results demonstrate efficient gene transfer into chicken oviduct explants by transduction with EIAV and HIV vectors and synthesis of a therapeutic protein by the cells in the chicken explants. Transduction efficiencies of 90-100% suggest that the VSV-G envelope protein is the most effective pseudotype for infection of the cells in the oviduct explants. The EIAV vectors carrying CAG-GFP or CAG-SeAP transgenes were pseudotyped with VSV-G, therefore it is unclear why transgene expression at the protein level was not observed in the explants transduced with these vectors. The same MOI was used for each transduction.
Successful gene transfer into hen oviduct explants has been demonstrated in this Chapter by electroporation and lentiviral transduction with constructs carrying CMV or CAG transgenes. In the following Chapter these methods of gene transfer were used to investigate expression in explants driven by an oviduct-specific promoter.
CHAPTER 7  TRANSGENE EXPRESSION IN CHICKEN

OVIDUCT EXPLANTS DRIVEN BY AN OVIDUCT-SPECIFIC PROMOTER

7.1 Introduction

In Chapter 6, methods for gene transfer into hen oviduct explants using electroporation and lentiviral transduction were developed. The aim was to develop an oviduct explant culture method in which to investigate transgene expression and to analyse transgene constructs containing oviduct-specific promoters, such as the ovalbumin gene promoter. Transgene expression driven by the ovalbumin gene promoter was investigated in electroporated and transduced explants in the following experiments.

Ovalbumin is one of the major egg white proteins and makes up over 50% of the albumen protein. The characteristics of the ovalbumin gene are discussed in Section 1.3.4. An oestrogen responsive enhancer element is located at -3139 to -3814, a steroid-dependent regulatory element (SDRE) is located at -892 to -780 and a negative regulatory element (NRE) is located at -308 to -88 at the ovalbumin gene locus (Sanders and McKnight, 1988). In the following experiments, the regulatory elements of the gene were assessed for their potential to drive transgene expression in the explants.
7.1.1  **Electroporation of explants**

In Chapter 6, a method was developed for electroporating hen oviduct explants. Transgene expression was demonstrated in explants electroporated with plasmids carrying CMV or CAG transgenes using the short EggAtrode device (see 6.4.2.1). Electroporation was therefore identified as a method for achieving gene transfer into oviduct explants for investigation of transgene expression. In the following experiments, explants were electroporated with plasmids carrying ovalbumin transgenes to investigate expression driven by an oviduct-specific promoter.

7.1.2  **Transduction of explants with lentiviral vectors**

In Chapter 6, a method was developed for lentiviral transduction of hen oviduct explants. Explants were successfully transduced with lentiviral vectors derived from equine infectious anaemia virus (EIAV) and human immunodeficiency virus (HIV). Transgene expression was demonstrated in explants transduced with vectors pseudotyped with vesicular stomatitis virus G (VSV-G), Chandipura, Ebola, Mokola and lymphocytic choriomeningitis virus (LCMV) envelope proteins. High transgene expression levels of 90-100% were observed in explants transduced with a VSV-G-pseudotyped EIAV vector carrying a CMV-LacZ transgene. In the following experiments, oviduct explants from mature laying hens were transduced with VSV-G-pseudotyped EIAV vectors carrying ovalbumin transgenes to investigate expression driven by an oviduct-specific promoter.
7.1.3 Transgenes

The plasmids and viral vectors used in this Chapter carry transgenes containing a 3.5kb section of the ovalbumin gene promoter driving expression of the GFP reporter gene or the therapeutic protein R24. The location of the promoter sequences used are shown in Figure 1.3 and include the oestrogen responsive enhancer element (OREE), steroid-dependent responsive element (SDRE), negative regulatory element (NRE), and exons one and two. If high levels of transgene expression are to be achieved \textit{in vitro} in oviduct explants or \textit{in vivo} in transgenic hens it could be important to include these significant ovalbumin regulatory elements in the transgene construct. Successful gene transfer into chicken oviduct explants using an ovalbumin-GFP transgene would allow investigation of transgene expression driven by an oviduct-specific promoter. Successful gene transfer into chicken oviduct explants using a plasmid or viral vector carrying an R24 transgene would allow investigation of the expression of a therapeutic protein driven by an oviduct-specific promoter.
7.2 Aims

1. To assess the ability of electroporated explants to express ovalbumin transgenes.

2. To assess the ability of transduced explants to express ovalbumin transgenes.
7.3 Results

7.3.1 Electroporation of explants

7.3.1.1 Expression of Ovalbumin-GFP in electroporated explants

Explants were cultured with the addition of $10^{-7}$M oestrogen and $10^{-10}$M insulin to the culture medium. Explants were electroporated with pOVA-GFP (for plasmid details see Appendix). The plasmid DNA was injected into the explant prior to electroporation with the short EggATrode. The electroporation conditions were as follows: 100 volts at positions 1 and 2, 80 volts at positions 4, 5, 6 and 7, 11ms pulse length and 250ms gap between pulses (see 6.3.2.1). Each electroporation was performed on two explants isolated from the same hen and the experiment was repeated three times on explants from three different hens. Explants were analysed for GFP expression 24 hours post-electroporation.

GFP expression was not observed in any of the electroporated explants (see Figure 7.3). RT-PCR analysis was performed as described in Section 2.8.5 to detect GFP expression at the mRNA level, however GFP mRNA was not detected (see Figure 7.1). The results suggest that the explants may have been successfully electroporated but that the levels of GFP expression were too low for detection, or that gene transfer into the TGCs was not achieved in these electroporation experiments using pOVA-GFP.
Figure 7.1 Electroporation of explants with pOVA-GFP. (a) GFP expression in explants electroporated with pOVA-GFP. Arrows point to characteristic burn marks made by electrodes. Size bar represents 500μm. (Images are representative of the expression levels observed in each electroporated explant in this experiment). (b) 38 cycles of RT-PCR were performed to detect GFP mRNA in the electroporated explants. Detection of α-actin was used as a positive control for the PCR (25 cycles). Positive control (+) for GFP primers was performed on the plasmid pONY8.7NCAG (30 cycles). 400bp GFP PCR product was not detected in the samples. Figure depicts results for explants isolated from three different hens. 1kb+ DNA ladder used for each gel (Invitrogen).
To demonstrate that the ovalbumin promoter was inactive in non-oviduct cells pOVA-GFP was used to transfect chicken embryonic fibroblasts (CEFs) as shown in Figure 7.2. CEFs were transfected with FuGene (Roche) as described in Section 2.6.2 at a density of $7 \times 10^5$ cells per well, with a DNA:reagent ratio of 1:5 using 5μg of DNA. Transfection with the reporter plasmid pCMV-GFP was performed as a positive control. GFP expression was not observed in the CEFs transfected with pOVA-GFP, however expression was observed in the positive control CEFs as shown in Figure 7.2.

![Figure 7.2 Transfection of CEFs with pOVA-GFP.](image)

To demonstrate that the ovalbumin promoter was inactive in non-oviduct cells pOVA-GFP was used to transfect CEFs. CEFs were transfected with FuGene (Roche) at a density of $7 \times 10^5$ cells per well, with a DNA:reagent ratio of 1:5 using 5μg of DNA. (a) Transfection with pCMV-GFP as a positive control. (b) Transfection with pOVA-GFP.
7.3.1.2 **Expression of Ovalbumin-R24 in electroporated explants**

Explants were electroporated with pOVA-R24 (for plasmid details see Appendix).

Each electroporation was performed as described in section 6.3.2.1 on two explants isolated from the same hen. The experiment was repeated three times on explants from three different hens. RT-PCR analysis was performed as described in Section 2.8.5 to detect R24 expression at the mRNA level. R24 mRNA was detected in the electroporated explants as shown in Figure 7.3. The medium from the electroporated explants was analysed for the presence of R24 protein by ELISA as described in Section 2.8.4, but R24 was not detected. The results suggest that although R24 was not detected in the medium, transgene expression driven by the ovalbumin regulatory sequences was achieved in the explants as demonstrated by expression of R24 at the mRNA level.

![Figure 7.3 Electroporation of explants with pOVA-R24. 35 cycles of RT-PCR were performed to detect R24 mRNA in the electroporated explants. Detection of β-actin was used as a positive control for the RT-PCR (25 cycles). Positive control (+) for R24 primers was performed on the plasmid pRI57 (30 cycles). 560bp R24 PCR product was detected in all of the samples. Figure depicts results for explants isolated from three different hens. 1kb+ DNA ladder used for each gel (Invitrogen).](image)
To demonstrate that the ovalbumin promoter was inactive in non-oviduct cells pOVA-R24 was used to transfect CEFs as shown in Figure 7.4. CEFs were transfected with FuGene (Roche) as described in Section 2.6.2 at a density of $7 \times 10^5$ cells per well, with a DNA:reagent ratio of 1:5 using 5μg of DNA. Transfection with the plasmid pCAG-R24 was performed as a positive control. 35 cycles of RT-PCR were performed to detect R24 mRNA in the electroporated explants. Detection of β-actin was used as a positive control for the RT-PCR. PCR product for R24 mRNA was detected in CEFs transfected with pCAG-R24 but not in CEFs transfected with pOVA-R24 as shown in Figure 7.4.

**Figure 7.4 Transfection of CEFs with pOVA-R24.** To demonstrate that the ovalbumin promoter was inactive in non-oviduct cells pOVA-R24 was used to transfect CEFs. CEFs were transfected with FuGene (Roche) at a density of $7 \times 10^5$ cells per well, with a DNA:reagent ratio of 1:5 using 5μg of DNA. (a) Transfection with pCAG-R24 as a positive control. (b) Transfection with pOVA-R24. 35 cycles of RT-PCR were performed to detect R24 mRNA in the transfected explants. Detection of β-actin was used as a positive control for the RT-PCR (25 cycles). Positive control (+) for R24 primers was performed on the plasmid pRl57 (30 cycles). 560bp R24 PCR product was detected in CEFs transfected with pCAG-R24 but not in CEFs transfected with pOVA-R24. 1kb+ DNA ladder used for each gel (Invitrogen).
7.3.2 Transduction of explants with lentiviral vectors

7.3.2.1 Expression of Ovalbumin-GFP in transduced explants

Explants were cultured with the addition of $10^{-7}$M oestrogen and $10^{-10}$M insulin to the culture medium. Explants were transduced with a VSV-G pseudotyped EIAV vector carrying an ovalbumin-GFP transgene (for vector details see Appendix). Each transduction was performed as described in Section 2.11.4 on two explants isolated from the same hen. The experiment was repeated three times on explants from three different hens. 20μl of a $10^7$ viral units/ml virus preparation was used per explant giving an estimated MOI of 0.02. Explants were analysed for GFP expression 72 hours post-transduction.

GFP expression was not observed in any of the transduced explants (see Figure 7.5). RT-PCR analysis was performed as described in Section 2.8.5 to detect GFP expression at the mRNA level. GFP mRNA was detected in the transduced explants as shown in Figure 7.5. This suggests that although GFP expression was not observed in the explants viewed under fluorescence, transgene expression driven by ovalbumin regulatory sequences was achieved by transduction of explants with a VSV-G pseudotyped EIAV vector as demonstrated by expression of GFP at the mRNA level.
Figure 7.5 Transduction of explants with a VSV-G pseudotyped EIAV vector carrying an ovalbumin-GFP transgene. (a) GFP expression in transduced explants. Size bar represents 500μm. (Image is representative of the expression levels observed in each electroporated explant in this experiment). (b) 38 cycles of RT-PCR were performed to detect GFP mRNA in the transduced explants. Detection of β-actin was used as a positive control for the PCR (25 cycles). Positive control (+) for GFP primers was performed on the plasmid pONY8.7NCAG (30 cycles). 400bp GFP PCR product was detected in all the samples. Figure depicts results for explants isolated from three different hens. 1kb+ DNA ladder was used for each gel (Invitrogen).
7.3.2.2 **Expression of Ovalbumin-R24 in transduced explants**

Explants were transduced with a VSV-G pseudotyped HIV vector carrying an ovalbumin-R24 transgene (for vector details see Appendix). Each transduction was performed as described in Section 2.11.4 on two explants isolated from the same hen. The experiment was repeated three times on explants from three different hens. RT-PCR analysis was performed as described in Section 2.8.5 to detect R24 expression at the mRNA level. R24 mRNA was detected in the transduced explants as shown in Figure 7.6. The medium from the transduced explants was analysed for the presence of R24 by ELISA 72 hours post-transduction as described in Section 2.9.4. R24 protein was detected in two of the samples as shown in Figure 7.6. These results demonstrate synthesis of a therapeutic protein in chicken oviduct explants driven by ovalbumin regulatory sequences by transduction with a VSV-G pseudotyped HIV vector as demonstrated by expression of R24 mRNA and protein.
Figure 7.6 Transduction of cultured explants with a VSV-G pseudotyped EIAV vector carrying an ovalbumin-R24 transgene. (a) 35 cycles of RT-PCR were performed to detect R24 mRNA in the electroporated explants. Detection of β-actin was used as a positive control for the PCR (25 cycles). Positive control (+) for R24 primers was performed on the plasmid pRI57 (30 cycles). 560bp R24 PCR product was detected in all samples. Figure depicts results for explants isolated from three different hens. 1kb+ DNA ladder was used for each gel (Invitrogen). (b) Detection of R24 protein in the medium from transduced explants by enzyme-linked immunosorbant assay (ELISA). Results are shown for two transduced explants from three different hens, corresponding to the RT-PCR result in (a).
In Chapter 6, methods for gene transfer into chicken oviduct explants using electroporation and lentiviral transduction were developed. In this Chapter, these methods were used to assess the ability of the explants to express transgenes controlled by an oviduct-specific promoter.

Following electroporation with pOVA-GFP, transgene expression was not observed, however expression was observed at the mRNA level in explants electroporated with pOVA-R24. Transduction of explants with a VSV-G pseudotyped EIAV vector carrying an OVA-GFP transgene resulted in expression at the mRNA level. Transgene expression at both the mRNA and protein level was observed following transduction with a VSV-G pseudotyped EIAV vector carrying an OVA-R24 transgene. These results demonstrate transgene expression in chicken oviduct explants successfully driven by an oviduct-specific promoter following both electroporation and lentiviral transduction. This shows that the ovalbumin promoter was active in the tubular gland cells in the explants for up to 72 hours in culture and was able to drive transgene expression during this time. The synthesis of R24 following transduction demonstrates that the cultured explants are capable of expressing a therapeutic protein driven by ovalbumin regulatory sequences.

These results, along with the results described in Chapters 5 and 6, suggest that hen oviduct explants are suitable for use in vitro to investigate transgene expression in the hen oviduct. However, consistently high levels of transgene expression were not observed in either electroporated or transduced explants. In order to use the explants
for the investigation of transgene expression from different plasmids or viral vectors, consistently high levels of transgene expression are required in order to reliably compare between different plasmids or viral vectors and their components. Therefore, further investigation into methods for enhancing expression is required, and such methods are discussed in the following Chapter.
CHAPTER 8 DISCUSSION

8.1 Summary of Results

The objective of the experiments described in this thesis was to develop an in vitro method for the investigation of transgene expression in hen oviduct cells prior to the generation of transgenic hens. Analysis of transgene expression in egg white requires the production of adult transgenic hens, which takes approximately one year. Therefore, an in vitro method with which to investigate transgene expression in the hen oviduct prior to the generation of transgenics would be very useful, as such a method could be used to assess the effectiveness of transgene constructs in a relatively short period of time.

Tubular gland cells (TGCs) were isolated from the magnum region of the oviduct of sexually mature hens and were maintained in vitro for up to five days. The cells were found to be non-proliferating, non-adherent and 100% viable for up to three days in culture. Ovalbumin and lysozyme mRNAs and protein were detected in TGCs cultured with and without $10^{-7}$M oestrogen and $10^{-10}$M insulin over 72 hours, indicating that the cells retained the ability to synthesize egg white proteins whilst maintained in vitro for this time period. Methods to achieve gene transfer into the cultured TGCs were subsequently investigated using transfection with several commercially available reagents, electroporation, and lentiviral transduction with replication-defective EIAV vectors pseudotyped with different envelope proteins. Transgene expression was not observed in the TGCs, indicating that the frequency of gene transfer was too low for the cells to be used for investigating transgene
expression. As an alternative, the use of whole tissue oviduct explants was investigated.

Whole tissue oviduct explants were isolated from the magnum region of the oviduct of sexually mature hens and were maintained in vitro for up to seven days. The explants were found to be 100% viable for up to four days in culture. Ovalbumin and lysozyme mRNAs were detected in the explants cultured without hormones for four days and in the explants cultured with $10^{-7}$M oestrogen and $10^{-10}$M insulin for five days. Ovalbumin and lysozyme proteins were detected in the explants cultured with and without these hormones for 72 hours, indicating that the tubular gland cells in the explants retained their ability to synthesize egg white proteins. Gene transfer into the oviduct explants was investigated using transfection with several commercially available reagents, electroporation, and lentiviral transduction with replication-defective EIAV vectors pseudotyped with several different envelope proteins. Transfection using reagents was not successful but gene transfer into the explants was successfully achieved through electroporation. The highest expression levels, approximately ten percent of the cells in the explants, were observed in explants electroporated using the short EggATrode device with the reporter plasmid pCAG-GFP. Transgene expression at lower levels was also observed following electroporation with the short EggATrode using several transgene constructs, in some cases only at the mRNA level.

Transgene expression was observed in explants following transduction with EIAV vectors pseudotyped with Mokola, LCMV, Ebola, VSV-G and Chandipura envelope proteins. Expression levels of approximately 90-100% of the cells in the explants
were observed using a high titre VSV-G-pseudotyped vector carrying a CMV-LacZ transgene. Transgene expression at lower levels was also observed in explants following transduction with VSV-G pseudotyped EIAV vectors carrying CAG-GFP and CAG-SeAP transgenes. These results demonstrate that the cells in the explants were capable of functioning in terms of gene expression whilst maintained in vitro for 72 hours.

Gene transfer into the chicken oviduct explants was therefore achieved using electroporation and lentiviral transduction as demonstrated by reporter gene expression. Experiments were subsequently performed to assess the ability of the electroporated and transduced explants to synthesise and secrete a therapeutic protein. Following electroporation with the plasmid pCAG-R24, expression of the anti-melanoma minibody R24 was observed at the mRNA level. Transduction with a VSV-G-pseudotyped HIV vector carrying a CAG-R24 transgene resulted in detectable synthesis of R24 at both the mRNA and protein level. These results demonstrate that the chicken oviduct explants were capable of synthesising a human/mouse therapeutic protein following lentiviral transduction, which is essential if explants are to be used for investigation of transgene expression prior to the generation of transgenic hens.

Following the identification of suitable methods of gene transfer into the explants, and the demonstration of secretion of a therapeutic protein by the explants, transgene expression controlled by ovalbumin gene regulatory sequences was then analysed in both electroporated and transduced explants. Expression was not observed in explants electroporated with the plasmid pOVA-GFP, however expression was observed at the
mRNA level in explants transduced with a VSV-G-pseudotyped EIAV vector carrying an OVA-GFP transgene. This result demonstrated transgene expression in transduced explants using an oviduct-specific promoter, and showed that the ovalbumin regulatory sequences were active in the explants after 72 hours in culture. Following electroporation with the plasmid pOVA-R24, expression of R24 was observed at the mRNA level, demonstrating transgene expression in electroporated explants driven by an oviduct-specific promoter. Transduction with a VSV-G pseudotyped EIAV vector carrying an OVA-R24 transgene resulted in detectable synthesis of R24 at both the mRNA and protein levels. This showed that the cultured chicken explants were able to synthesize a therapeutic protein driven by an oviduct-specific promoter. This property of the explants is highly desirable if they are to be used for investigation of transgene expression prior to the generation of transgenic hens.

8.2 Enhancing transgene expression

The highest level of reporter gene expression observed in electroporated explants was approximately 10% of the cells in the explants, whereas the highest levels observed in transduced explants was approximately 90-100%. This level of efficiency of gene transfer was reflected in the expression of a therapeutic protein, where expression was only detected at the mRNA level in electroporated explants and was detected at both the mRNA and protein level in transduced explants. Gene transfer using electroporation was found not to be quantitative. Due to the low and variable levels of transgene expression observed following electroporation, constructs could not be reliably compared using this technique. Gene transfer by transduction using high viral titres was visually quantitated, and constructs could be compared as demonstrated by
transduction with EIAV vectors carrying CMV-LacZ transgenes pseudotyped with different envelope proteins. The use of a VSV-G-pseudotyped vector resulted in the highest levels of expression at 90-100% of the cells in the explants. However, the use of visual estimation and reverse-transcriptase polymerase chain reaction (RT-PCR) to quantify transgene expression following gene transfer into explants was not truly quantitative, and therefore these methods are of limited use for accurately assessing levels of transgene expression. The percentage of positive cells could be more accurately assessed using methods such as real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). qRT-PCR could potentially be used to measure levels of transgene expression in order to provide quantitative data for comparison of different constructs. For example, it would be useful to compare transgene expression controlled by different ovalbumin regulatory elements in order to identify the elements required for maximum expression. The transduction efficiency of lentiviral vectors pseudotyped with different envelope proteins or containing different components, such as the central polypurine tract (cPPT) or the woodchuck hepatitis post-transcriptional regulatory element (WPRE), could also be compared using a technique such as qRT-PCR. In HIV-derived and simian immunodeficiency virus (SIV)-derived lentiviral vectors the inclusion of a cPPT has been shown to enhance transduction efficacy (Follenzi et al., 2000). Incorporation of a WPRE in HIV-derived vectors was found to increase transgene expression five- to eight-fold after transduction of 293T cells (Zufferey et al., 1999).

By improving the explant culture method it is possible that the levels of transgene expression achieved following electroporation could be enhanced. Such improvements could include, for example, culturing the explants in rotary shakers as
described by McKnight (1978) or varying the length of time the explants are cultured for prior to electroporation. Bauer and colleagues (2001) found that transfections performed on human ventral mesencephalic tissue explants after four days in culture resulted in significantly increased levels of transgene expression compared to transfections performed on freshly isolated explants or after two or eight days in culture.

Viral transduction appears to be a more efficient way of achieving gene transfer into the explants, although high levels of transgene expression were not observed following transduction with every viral vector used. The highest expression levels were observed following transduction with a VSV-G-pseudotyped viral vector with a titre of $10^9$ tu/ml, using a multiplicity of infection (MOI) of 2. The constructs that gave lower expression levels were pseudotyped with different envelope proteins or had lower titres of $10^6$ to $10^7$ tu/ml, using an MOI of 0.02. The use of such a low MOI is clearly unsuitable for achieving efficient transduction of chicken oviduct explants. Therefore, for reliable comparison of lentiviral vectors and their components, transductions should be performed using an MOI of at least 2.

### 8.3 Conclusion

The objective of the experiments described in this thesis was to develop an *in vitro* method in which to investigate transgene expression in the hen oviduct prior to the generation of transgenic hens. A method to culture oviduct explants from sexually mature hens was developed, and effective gene transfer into the cells in the explants was achieved through electroporation and lentiviral transduction with EIAV and HIV
vectors. Lentiviral transduction appears to be more efficient than electroporation for achieving gene transfer into explants. One reason for this could be the fact that following transduction the transgene construct becomes integrated into each cell's genome, whereas following electroporation the transgene construct is maintained episomally and does not integrate. Further investigation into methods for enhancing expression levels following electroporation is required in order to make this technique useful for investigating transgene expression.

High levels of transgene expression were achieved using an EIAV vector pseudotyped with the vesicular stomatitis virus G (VSV-G) envelope protein. Secretion of a therapeutic protein was demonstrated by explants transduced with viral vectors driven by both ubiquitous promoters and an oviduct-specific promoter. Viral titre was determined to be an important factor in the efficiency of gene transfer, with titres of $10^8$ vu/ml and above being desirable for maximum efficiency. Therefore, in order to achieve consistent high transduction frequencies in hen oviduct explants using lentiviral vectors, the use of a VSV-G pseudotype in conjunction with a high viral titre is required.

In conclusion, a method to culture chicken oviduct explants that contain active TGCs was developed, and effective gene transfer into the cells in the explants was achieved as demonstrated by transgene expression controlled by both ubiquitous promoters and an oviduct-specific promoter. Chicken oviduct explants were therefore identified as suitable for use in vitro to investigate transgene expression in the chicken oviduct, and a patent application has been filed by Viragen (Scotland) Limited on the use of this explant culture system.
Bibliography


Appendix

1. EIAV vector carrying a CMV-GFP transgene (un-packaged plasmid referred to as pCMV-GFP)

2. EIAV vector carrying a CMV-LacZ transgene (un-packaged plasmid referred to as pCMV-LacZ)
3. EIAV vector carrying a CAG-GFP transgene (un-packaged plasmid referred to as pCAG-GFP)

4. EIAV vector carrying a CAG-SeAP transgene (un-packaged plasmid referred to as pCAG-SeAP)
5. HIV vector carrying a CAG-R24 transgene (un-packaged plasmid referred to as pCAG-R24)
6. EIAV vector carrying an OVA-GFP transgene (un-packaged plasmid referred to as pOVA-GFP)

7. EIAV vector carrying an OVA-R24 transgene (un-packaged plasmid referred to as pOVA-R24)