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In Vivo Gene Transfer in the Female Bovine: Potential Applications for Biomedical Research in Reproductive Sciences

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1. Introduction

"We still share genes around, and the resemblance of the enzymes of grasses to those of whales is a family resemblance" (Lewis Thomas, The lives of a cell, 1974).

Gene transfer technology is an invaluable research tool to study gene function and its regulation. *In vitro* transfer of exogenous nucleic acids into mammalian cells has been of pivotal importance for the characterization of gene functions (Hampton & Kinnaird, 2010). Likewise, *in vivo* transfer of functionally active foreign genes into target mammalian somatic tissues or organs has played a critical role in the development of effective gene therapy strategies (Yang, 1992) that has escalated into clinical application for the therapeutic treatment of inherited and acquired diseases (Mountain, 2000). In addition, the genetic engineering of model organisms became possible (Niemann & Kues, 2003). Most of the gene transfer research has been conducted on several non-reproductive topics including blood diseases (Nienhuis, 2008), neurological dysfunctions (Manfredsson & Mandel, 2010), cancer (Pei et al., 2010), lung diseases (Geiger et al., 2010), bone healing (Evans, 2010), skin diseases (Long et al., 2009) and heart failure (Poller et al., 2010). To a lesser extent gene transfer research in gynecological diseases (Raki et al., 2006; Hassan et al., 2009) and reproductive medicine (Stribley et al., 2002; Daftary & Taylor, 2003; Yoshimura et al., 2010) has been undertaken using the mouse as model species. Although the mouse model possesses several advantages (*e.g.* short generation interval, large litter size), some large animals (*e.g.* non-human primates, dogs, pigs, sheep, cattle and horses) are considered relevant model species in biomedical research. For instance, pig and sheep species have been used as models for cardiovascular disease (Ishii et al., 2006), wound repair (Graham et al., 2000), respiratory disease (Scheerlinck et al., 2008), cancer (Du et al., 2007), diabetes (Dyson et al., 2006), ophthalmological disorders (Klassen et al., 2008) and neurological dysfunctions (Kragh et al., 2009). The physiology, organ size, genome organization, life span and pathology of farm animal species reflect the human situation much better than rodent models (Casal & Haskins 2006; Habermann et al., 2007; Jacobsen et al., 2010; Muschler et al., 2010).

Implementation of *in vivo* gene transfer technology in relevant large animal models is pivotal to elucidate molecular pathways involved in reproductive processes such as ovarian

follicular development, fertilization, and early embryo development. This research will allow the generation of safer and more efficient strategies in human reproductive medicine for infertility treatment and contraception.

A large animal model for gene transfer studies in reproduction should resemble human reproductive features as close as possible. In this regard, the bovine model is increasingly accepted as an alternative model species to generate conceptual models of relevance for human reproduction (Adams & Pierson, 1995; Ménézo & Hérubel, 2002; Baumann et al., 2007; Velazquez, 2008). Both species are monovulatory and displayed similarities regarding ovarian folliculogenesis, gene expression profile during early embryogenesis, and gestation length period (Campbell et al., 2003; Adjaye et al., 2007; Mihm & Evans, 2008; Kues et al., 2008; Xie et al., 2010). Furthermore, several gynaecological procedures are performed virtually in the same fashion as in women (Velazquez et al., 2009b). The aim of this chapter is to highlight the methods than could potentially be applied for *in vivo* gene delivering in the reproductive tract of female bovine species in order to address topics of reproductive relevance for both humans and cattle.

2. Basics of gene transfer technology

2.1 Gene transfer

Broadly speaking, gene transfer technology involves the transfer of exogenous nucleic acids (deoxyribonucleic acid [DNA] or ribonucleic acid [RNA]) into target cells either to produce a biologically active protein or to inhibit protein synthesis. The classic concept of gene transfer involves the insertion of DNA encoding the desire gene as complementary DNA (cDNA) into the nucleus of target cells, followed by transcription into messenger RNA, which is then exported to the cytoplasm and translated into its encoding protein (Grigsby & Leong, 2010). The introduced cDNA may restore a lost gene function, interfere with gene's function or initiate a new function (Stribley et al., 2002). However, progress in gene transfer technology now allows the deliver of large fragments of genomic DNA containing the whole locus of the desired gene (Lufino et al., 2008). Furthermore, with the increasing understanding of the non-coding DNA functions the use of non-coding regulatory RNAs is becoming an important tool for gene transfer technology (Poller et al., 2010). Several RNA-based technologies have been used to down-regulate gene expression in loss-of-function studies including small interfering RNA (siRNA), short hairpin RNA (shRNA) and micro RNA (miRNA) (Guo et al., 2010; Khurana et al., 2010; Poller et al., 2010). Although RNA interference (RNAi) technology (via siRNAs and shRNAs) is only used for gene silencing, miRNA interventions can also be applied for up-regulation of protein expression (Poller et al., 2010). Gene transfer can be targeted to either somatic (somatic gene transfer) or germ-line (*i.e.* oocyte, spermatozoa, and preimplantation embryos) cells (Stribley et al., 2002).

2.2 Gene therapy

Gene transfer studies using *in vitro* cellular assays have been critical to unravel basic features of gene function (Hampton & Kinnaird, 2010). However, gene transfer technology has been put forward for clinical use as a therapeutic tool (*i.e.* gene therapy) (Mountain, 2000). Gene transfer for therapeutic purposes can be aimed at correcting a genetic defect in target cells (*i.e.* correcting gene therapy) or to destroy target cells using a cytotoxic pathway (*i.e.* cytotoxic gene therapy) (Stribley et al., 2002). Gene therapy can be carried out either *ex vivo* or *in vivo* (Yang, 1992; Stribley et al., 2002; Gardlík et al., 2005). The e*x vivo* approach

involves the *in vitro* transfer of exogenous genetic material into cells followed by the *in vivo* delivery of the genetically modified cell into the target tissue (Yang, 1992; Stribley et al., 2002; Gardlík et al., 2005). *In vivo* gene therapy makes reference to the direct transfer of nucleic acids into target cells (Yang, 1992; Stribley et al., 2002; Gardlík et al., 2005).

2.3 *In vivo* gene delivery

The delivery of nucleic acids to the nuclei of target cells requires the use of carrier vehicles called vectors. After systemic or topical administration, the vector carrying the transgene has to cross the plasma membrane and move through the cytosol before delivering the transgene into the nucleus target cell (Ziello et al., 2010). In order to achieve efficient gene transfer the vector has to avoid degradation from components in the extracellular matrix (*e.g.* exonucleases) and the cytoplasm (*e.g.* endonucleases) and effectively release the transgene for nuclear uptake and transcriptional processing (Escoffre et al., 2010; Parra-Guillén et al., 2010). Gene transfer vectors can be classified into viral and non-viral (Table 1) (Niidome & Huang, 2002; Niemann & Kues, 2003; Gardlík et al., 2005; Young et al., 2006; Vassaux et al., 2006; Lufino et al., 2008; Al-Dosari & Gao, 2009; Tros de llarduya et al., 2010).

Viral vectors	**Non-viral vectors**
Retrovirus/Lentivirus	Naked plasmid DNA
Adenovirus	Lipoplexes
Adeno-associated virus	Polyplexes
Herpes simplex virus	Inorganic nanoparticles
Alphavirus	Artificial chromosomes
Poxvirus	Peptides
Vaccinia virus	Bacteria
Simian virus 40	Minicircle DNA
Moloney murine leukemia virus	Transposon

Table 1. Common viral and non-viral vectors used for gene transfer technology

Viral vector-mediated gene transfer is based on the innate capacity of viruses to infect cells. Recombinant viruses without the ability to replicate have to be synthesized in order to avoid infectious diseases in the host. This requires the deletion of essential genes for viral replication and the insertion of the gene of interest into the viral genome (Kay et al., 2001). Viral vectors enter target cells via receptor-mediated endocytosis (Ziello et al., 2010). Following endocytosis viral vectors are released from endosomes and travel along the microtubules towards the nucleus where they deliver the transgene through nuclear pores (Fig. 1) (Dinh et al., 2005; Yea et al., 2007). However, other viral vectors (*e.g.* retroviruses) deliver the transgene in the nucleus during mitotic-nuclear-envelope breakdown (Kay et al., 2001) or do not depend on microtubule-mediated transport. Instead, they do not escape endosomes soon after cellular uptake and reach the nucleus in a diffuse motion where they are released from late endosomes or lysosomes (Dinh et al., 2005; Akita et al., 2010).

The majority of non-viral vectors rely on plasmid DNA as the primary carrier of the transgene (Schleef & Blaesen, 2009; Escoffre et al., 2010). Injection of naked plasmid DNA is the simplest gene delivery system, but transgene expression (*i.e.* transfection) is usually low due to its rapid degradation after delivery, especially under systemic administration (Parra-Guillén et al., 2010). This problem has been addressed with the use of chemical vectors, which act as protective complexes (*i.e.* DNA-complexes) that facilitate cellular uptake and

intracellular delivery (Tros de llarduya et al., 2010). It has been suggested that DNA complexes can enter the cytosol by fusion with the plasma membrane, but most of the experimental evidence indicates that the main entrance route of non-viral DNA-complexes currently used in gene transfer research is receptor-mediated endocytosis (Medina-Kauwe et al., 2005; Khalil et al., 2006; Ziello et al., 2010). Nevertheless, within a vector line, the origin of the vector could determine its cellular uptake pathway. Accordingly, chemically derived gold nanoparticles enter the cytoplasm using an endocytic pathway, but gold nanoparticles produced by laser ablation can enter cultured bovine immortalized cells by passive diffusion (Taylor et al., 2010).

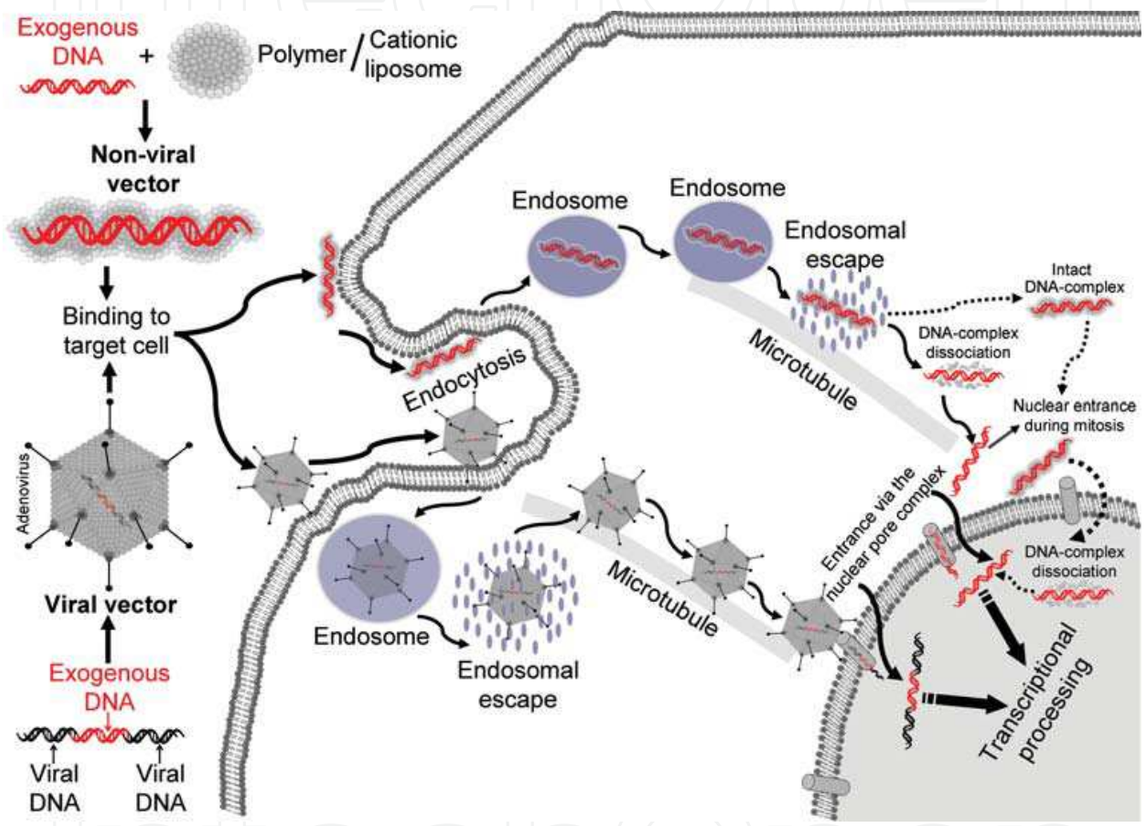

Fig. 1. General pathway of vector cellular uptake via receptor-mediated endocytosis followed by microtubule-mediated transport in viral (*e.g.* adenovirus) and non-viral (*e.g.* polyplexes and lipoplexes) gene delivery systems. Non-viral DNA-complex dissociation can occur in the cytosol or in the nucleus, after nuclear entry of the DNA-complex during mitosis, when the nuclear envelope disassembles. Dissociated exogenous DNA can enter the nucleus either through the nuclear pore complex or during mitosis.

The most accepted model of cellular uptake of non-viral vectors is based in polyplexes (*i.e.* polymer-based transfection agents) and lipoplexes (*i.e.* cationic liposome-based transfection agents). Following endosomal entrapment non-viral vectors undergo microtubule-facilitated trafficking and accumulate within close proximity to the nucleus (Fig. 1) (Vaughan & Dean, 2006; Doyle & Chan, 2007). The site of release of the transgene seems to depend on the type of non-viral DNA-complex. Endosomal release of polyplexes in the cytoplasm can occur without release of DNA from the polymer and the polyplexes may enter the nucleus intact,

where they subsequently release the transgene (Cohen et al., 2009). Polyplexes are released from the endosome by a proton-sponge mechanism in which the polyplex exacerbates proton accumulation in the endosome, resulting in passive chloride influx followed by osmotic swelling and endosomal rupture (Medina-Kauwe et al., 2005; Midoux et al., 2009). It has been hypothesized that the nuclear entry of polyplexes takes place at the time of nuclear membrane breakage during mitosis (O'Rorke et al., 2010), but there is evidence that nuclear proteins, such as nucleolin, can incorporate non-viral vectors into the nucleus in an endocytosis-independent manner (Chen et al., 2008b).

Nevertheless, it has been demonstrated that some polyplexes can release their DNA cargo before entering the nucleus via ion exchange with RNA present in the cytoplasm (Huth et al., 2006). Presumably, DNA release from polyplexes in the nucleus could be caused by ion exchange with chromosomal DNA (Schaffer et al., 2000) or by polymerases through stripping of histone proteins during DNA replication (Thomas & Klibanov, 2003a). Release of DNA from polyplexes is affected by polymer degree of deacetylation (DDA) and molecular weight (MW), as high DDA and MW reduced DNA dissociation, whereas intermediate values of these polymer characteristics are associated with efficient DNA dissociation rate (Thibault et al., 2010).

Experimental evidence has shown that lipoplexes are more likely to release the DNA in the cytosol during endosomal escape (Pollard et al., 1998; Cohen et al., 2009). Current evidence indicates that liposomes fuse with endosomal membranes leading to a neutralization of cationic lipids in the lipoplexes by anionic membrane lipids. This process causes endosome destabilization and displaces the DNA from the cationic lipids into the cytoplasm (Tarahovsky et al., 2004; Medina-Kauwe et al., 2005; Caracciolo et al., 2007). Recent evidence suggests that the capacity of lipoplexes to escape the endosome is strongly influenced by its formulation. For instance, multicomponent lipoplexes (*i.e.* incorporation of three to six lipid species simultaneously) displayed an enhanced ability to destabilized endosomes compared to binary complexes (Caracciolo et al., 2009).

Due to their significant size, DNA constructs are unable to cross the nuclear membrane by passive diffusion (Lukacs et al., 2000). Investigations on the mechanism of nuclear translocation of plasmid DNA have documented that nuclear uptake of exogenous DNA occurred during mitosis, when the nuclear envelope breaks down and the permeability barrier to the nucleus is lost (Mortimer et al., 1999; Brunner et al., 2000; Cohen et al., 2009). However, this cytoplasmic to nuclear translocation mechanism does not operate in differentiated non-dividing cells (Dean et al., 2005, Khalil et al., 2006). The other documented form of entry of foreign DNA into the nucleus is through the nuclear pore complex, mediated by nuclear localization signals (NLSs) (Dowty et al., 1995; Dean, 1997; Boulikas, 1998; Dean et al., 2005). Alternatively, non-viral vectors can release the transgene into the nucleus after fusion with the nuclear membrane (Kamiya et al., 2002).

There are several physical methods that can increase the efficiency of vector delivery (Table 2) (Russ & Wagner, 2007; Al-Dosari & Gao, 2009; Escoffre et al., 2010; Wells, 2010). These physical techniques can be applied alone or in combination, and are mainly used in *in vitro* settings (Escoffre et al., 2010). Nonetheless, some of these physical methods (*e.g.* electroporation and ultrasound) have shown to work efficiently under *in vivo* conditions, enhancing nucleic acid delivery at a specific location (Huber & Pfisterer, 2000; Saito & Nakatsuji, 2001; Sato et al., 2003; Brown et al., 2004). Although the precise mechanisms by

Method	Putative Mechanism
Needle injection	Physical damage caused by needle insertion generates pores in the cell membrane
Jet injection	High-speed ultrafine stream generates pores in the cell membrane
Electroporation	Electrical field pulses generate pores in the cell membrane
ltrasound	Ultrasonic waves induce pores in the cell membrane by acoustic cavitation
Hydrodynamic injection	The high pressure of a rapid injection of a large volume of vector solution generates cell membrane pores
Laser irradiation	A short exposure to a laser beam generates cell membrane pores
Photochemical internalization	Illumination induces photochemical damage and rupture of endosomal membranes
Plasma	Ion deposition on the cell surface by direct current plasma induces membrane permeability
Hyperthermia	Cells lose their cytoskeletal structure and contract, causing widening of intercellular gap junctions
Gene gun	Heavy metal macroparticles are impacted at high velocity allowing direct penetration through the plasma membrane into the cytoplasm and even the nucleus
Magnetofection	Magnetic forces accelerate accumulation of vectors (superparamagnetic nanoparticles) on the cell surface followed by endocytosis

Table 2. Physical methods for gene delivery

which these physical methods operate are not totally understood, most of them allow direct entrance of vectors into the cytosol by generating a transient membrane permeabilization, avoiding in this way the endocytic pathway (Escoffre et al., 2010; Wells, 2010).

Alternative strategies to improve transgene delivery include the use of the hybrid vectors. For instance, coating of adenovirus with polymers or liposomes allows the production of "stealth" viruses that can avoid recognition by the host's antibodies and permits targeting of desired receptors following linkage of ligands to the chemical coating (Han et al., 2010; Kim et al., 2010; Zhong et al., 2010). Other hybrid vector combinations include polymer-artificial chromosome (Magin-Lachmann et al., 2004); polymer-gold nanoparticle (Thomas & Klibanov, 2003b); liposome-peptide-artificial chromosome (White et al., 2003); polymer-peptide (Huang et al., 2010), liposome-polymer (Schäfer et al. ,2010) and viral vector combinations such as adenovirus-Epstein-Barr virus (Gardlík et al., 2005).

The general consensus is that viral gene deliver systems achieve stable *in vivo* transgene expression more efficiently than non-viral systems (Gardlík et al., 2005; Hassam et al., 2009; Escoffre et al., 2010; Grigsby & Leong, 2010). The lower efficiency of non-viral vectors seems not to be associated with their capacity to reach the vicinity of the nucleus, but with their ability to cross the nuclear envelope (Dean et al., 2005). Accordingly, Hama et al. (2006)

reported lower efficient transgene expression with lipoplexes than with adenoviral vectors, which was attributed to differences in nuclear transcription efficiency rather than to differences in intracellular trafficking. Nevertheless, advances with artificial chromosomes and transposons could offer an efficient *in vivo* non-viral gene delivery system (Lufino et al., 2008; Wilson & George, 2010). Non-viral vectors offer appealing advantages over viral vectors including higher insert capacity for DNA cargo size, low host immunogenicity, easy manufacturing and potential for repeated administration (Niidome & Huang, 2002; Al-Dosari & Gao, 2009; Grigsby & Leong, 2010).

2.4 Transgene expression

Once in the nucleus, expression vectors (*i.e.* expression cassette in a viral or non-viral vector) may integrate into the host genome or remain as extrachromosomal genetic elements (*i.e.* episomal vector) (Lufino et al., 2008). Nuclear uptake of the expression vector can result in either permanent or transient transgene expression (Ehrhardt et al., 2008; Lufino et al., 2008; Voigt et al., 2008; Romano et al., 2009). Although some viruses can provide transient expression (*e.g.* adenovirus, vaccinia virus), most of the viral gene transfer systems result in permanent transgene expression (*e.g.* retrovirus, adeno-associated virus) due to their capacity to achieve chromosomal integration (Young et al., 2006). Nevertheless, some integrating viruses are prone to epigenetic silencing and provide a transient burst of transgene expression. Lentiviruses have been known to escape epigenetic silencing and are currently the viral vector with most faithful expression (Park, 2009). In contrast, with the exception of transposons (Hackett et al., 2005; Wilson & George, 2010) non-viral vectors so far investigated do not integrate into the host genome (Gardlík et al., 2005). Non-integrated plasmid vectors from non-viral vectors usually produce a transient transgene expression (Gardlík et al., 2005), except when they are combined with viral replicons, which facilitates extrachromosomal replication, the presence of the vector as a stable episome and high efficiency of transfer (Gardlík et al., 2005). Furthermore, advances in extrachromosomal vector technology have allowed the creation of high capacity non-viral episomal vectors with the ability to achieve stable transgene expression (Lufino et al., 2008).

When an expression vector is not integrated into the host genome of dividing cells the extrachromosomally expression vector may not be segregated to all daughter cells during cellular division (Fig. 2) (Ehrhardt et al., 2008; Lufino et al., 2008). This transgene dilution due to a reduction in the number of copies of extrachromosomal DNA in each cell cycle is partially responsible for the transient levels of gene expression (Gardlík et al., 2005). When stable chromosomal integration takes place, the transgene will be inherited to both daughter cells after each cell cycle (Fig. 2). Chromosomal integration of viral vectors and transposons is usually random, which bring the risk of disrupting host gene expression (*i.e.* insertational mutagenesis) (Baum et al., 2006; Hackett et al., 2007). Activation of host cell oncogenes and inactivation of tumor suppressors are among the genetic consequences of insertational mutagenesis (Baum et al., 2006; Hackett et al., 2007). However, whereas retro- and lentiviruses preferentially integrate into promoter and exonic regions of transcribed genes, most transposons integrate into intergenic regions (Yant et al., 2005; Hackett et al., 2005). Thus, transposons represent probably the safest method currently available for genetic engineering (Grabundzija et al., 2010; Kues et al., 2010).

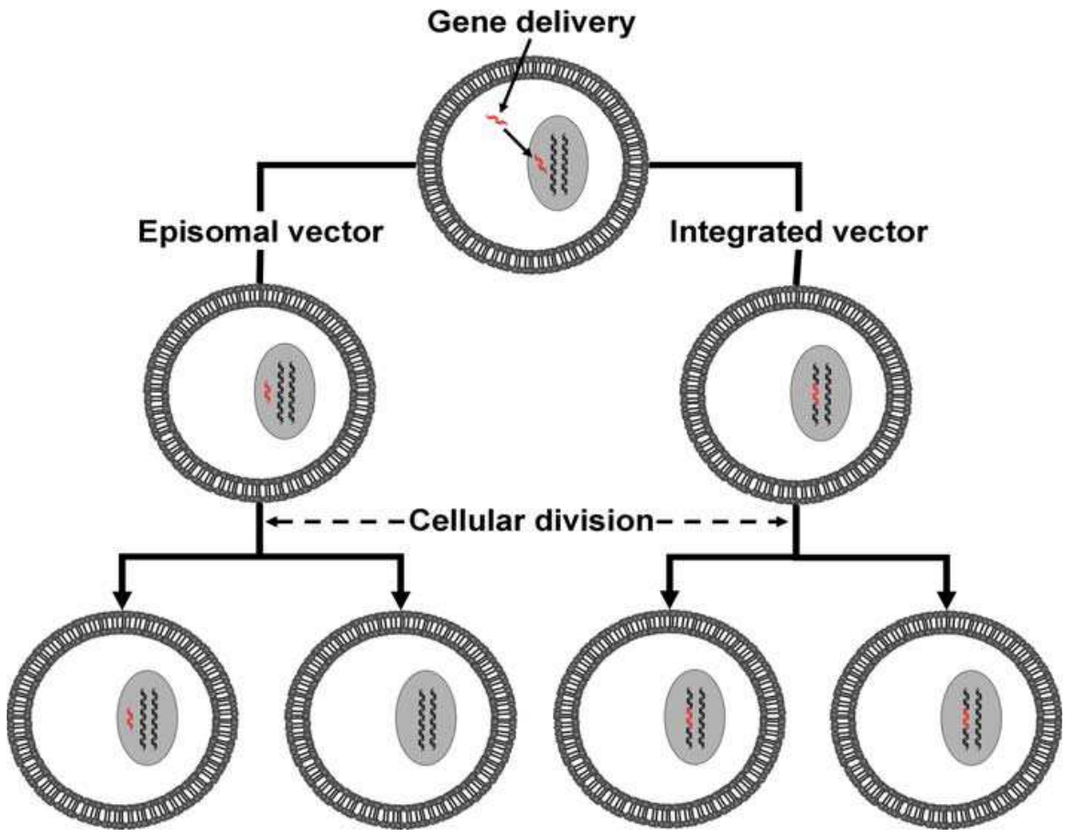

Fig. 2. Transgene segregation in extrachromosomal positioning (episomal) or chromosomal integration of expression vectors after gene delivery.

Regardless of the expression vector position in the nucleus (*i.e.* episomal or chromosomal), transcriptional activity is not always efficient, which can result in ectopic, weak or even undetectable transgene expression (Giraldo & Montoliu, 2001). Possible causes of transgene expression variability include differences in the number of integrations, transgene inactivation, or different genome integration sites of the vector. Transgene expression critically depends on the number of plasmids entering the nucleus (James & Giorgio, 2000; Glover et al., 2010), which not only depends on the ability of vectors to deliver intact DNA but also on the type of cell, as some cells translocate plasmid DNA from the cytoplasm to the nucleus more efficiently than others (James & Giorgio, 2000). However, high copy numbers of the transgene can increase methylation pattern of the promoter and thereby inducing transgene silencing (Garrick et al., 1998). In addition to promoter hypermethylation, transgene silencing may also involve chromatin modifications such as chromatin condensation caused by histone tail deacetylation and histone code switch (He et al., 2005). In some cases formation of repressive heterochromatin on the plasmid DNA backbone without methylation of the promoter can also cause transcriptional silencing of the transgene (Chen et al., 2008a). Moreover, integration into a transcriptionally inactive region of DNA, such as constitutive heterochromatin, will also result in transgene inactivation (Hackett et al., 2007). It is common to observe the transferred gene undergoing a brief period of expression followed by a decline to undetectable levels even though the vector DNA concentration remains constant in cells (Bestor, 2000).

3. Current gene transfer models in bovine species

Current uses of gene transfer models in bovine species include the production of transgenic cattle via germ-line gene transfer to produce valuable proteins in milk and serum of cattle for therapeutic purposes in humans (Table 3).

Protein	Possible therapeutic application	Reference
Human lactoferrin	Infectious complications	Krimpenfort et al., 1991
Human α-lactalbumin	Phenylketonuria	Eyestone et al., 1998
Human serum albumin	Blood volume restoration	Behboodi et al., 2001
Human bile salt-stimulated lipase	Pancreatic insufficiency	Chen et al., 2002
Human immunoglobulin	Immuno-related diseases	Kuroiwa et a., 2002
Human growth hormone	Growth-related disorders	Salamone et al 2006
Human myelin basic protein	Multiple sclerosis	Al-Ghobashy et al., 2009

Table 3. Examples of human proteins produced in transgenic cattle and their possible therapeutic application

Other applications of bovine transgenesis include the production of recombinant antibodies for tumor cell killing therapy (Grosse-Hovest et al., 2004) and the creation of cattle resistant to diseases (*e.g.* mastitis) (Wall et al. ,2005) or with enhanced milk composition (*e.g.* higher levels of casein) (Brophy et al., 2003).

Methodologies to produce transgenic cattle include microinjection of exogenous DNA into the pronuclei of zygotes, sperm-mediated gene transfer (via intracytoplasmic injection), injection (in the perivitelline space) of oocytes with viral vectors, and somatic cell nuclear transfer (SCNT) (Niemann & Kues, 2003; Velazquez, 2008). Currently the most common method to produce transgenic bovine offspring is SCNT. This approach involves the *in vitro* transfer of the foreign DNA into somatic cells followed by the insertion of positive transgenic cells into enucleated oocytes which develop to the blastocyst stage and are subsequently transferred to recipients (Fig. 3).

The use of *in vivo* gene therapy has been reported in a neonatal bovine model of citrullinemia (Lee et al., 1999), a urea-cycle disorder causing hyperammonemia due to the lack of argininosuccinate synthetase (ASS) (Marquis-Nicholson et al., 2010). In this study, two calves diagnosed with citrullinemia were supplemented with arginine and sodium benzoate from 24 hrs after birth onwards to avoid death. At day 10 after birth a single application of a viral vector carrying human ASS cDNA into the external jugular vein caused selective transduction of hepatocytes and resulted in decreased levels of glutamine (an indication of *de novo* synthesis of urea), which lasted until day 18 posttreatment. Since the treatment just restored ASS activity in liver and not in the kidneys, arginine therapy had to be continued. This partial enzymatic correction was lost 3 weeks after vector application (Lee et al., 1999).

In a different *in vivo* gene transfer model, Brown et al. (2004) injected a single dose of plasmid DNA carrying cDNA for growth hormone-releasing hormone (GHRH) in the trapezius muscle of dairy heifers followed by electroporation. Treated animals displayed an increase in haemoglobin, red blood cells, peripheral blood mono-nuclear cells and insulin-like growth factor-1 (IGF-1) compared to control animals at 300 days posttreatment. These physiological effects were observed without effect on concentrations of glucose and insulin.

The treatment was associated with increased body condition score, reduced hoof pathology and decreased mortality (Brown et al., 2004). The same group tested this gene delivery system during heat stress in pregnant heifers (Brown et al., 2009). They reported that calves from treated cows showed lower mortality and a significant improvement of survival from birth to 260 days, along with increased daily weight gain. In dams, milk production and prolactin concentrations were increased. Furthermore, the second pregnancy rate was improved in cows receiving the plasmid-based GHRH (Brown et al., 2009).

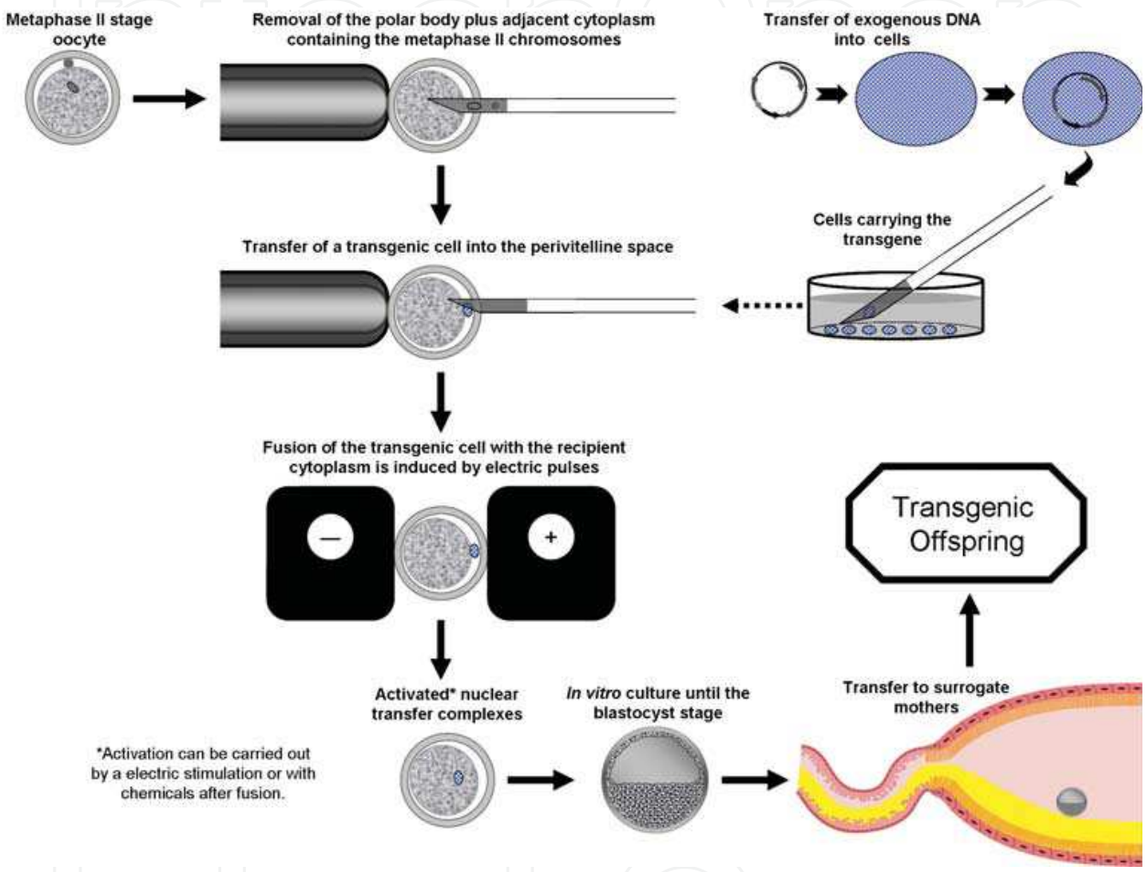

Fig. 3. Main steps in the production of transgenic bovine offspring using conventional somatic cell nuclear transfer.

Previous research has shown that growth hormone and IGF-1 play a significant role on ovarian follicular development and preimplantation embryo development (Kaiser et al., 2001; Sirotkin, 2005; Velazquez et al., 2009b). It will be interesting to test whether the plasmid-based GHRH treatment could serve as therapy to increase the superovulatory response of low responder animals (De Roover et al., 2005) or to increase pregnancy rates in cows with infertility problems (Thatcher et al., 2006).

Bovine models with RNA interference (RNAi) technology have been also implemented in loss-of-function studies to address gene function on ovarian follicular development and development of oocytes and preimplantation embryos (Table 4). Although the effects of gene knockdown on embryo development have been analyzed mostly *in vitro*, approaches using *in vivo* embryo culture have been reported (Tesfaye et al., 2010).

Gene	RNAi	Parameter analyzed	Reference
Cyclin B1	dsRNA	Oocyte maturation	Paradis et al., 2005
C-mos	dsRNA	Oocyte maturation	Nganvongpanit et al., 2006
Oct-4	dsRNA	Embryo development	Nganvongpanit et al., 2006
JY-1	siRNA	Embryo development	Bettegowda et al., 2007
p66Shc	shRNA	Embryo development	Favetta et al., 2007
Survivin	dsRNA	Embryo development	Park et al., 2007
Connexin 43	dsRNA	Embryo development	Tesfaye et al., 2007
E-cadherin	dsRNA	Embryo development	Tesfaye et al., 2007
FIBP	siRNA	Ovarian steroidogenesis	Forde et al., 2008
Betaglycan	siRNA	Ovarian steroidogenesis	Forde et al., 2008
Follistatin	siRNA	Embryo development	Lee et al. ,2009
KPNA7	siRNA	Embryo development	Tejomurtula et al., 2009
CENPF	dsRNA	Embryo development	Toralová et al., 2009
KRT18	dsRNA	Embryo development	Goossens et al., 2010
BIRC6	ds/shRNA	Embryo development	Salilew-Wondim et al., 2010
MSX1	ds/siRNA	Embryo development	Tesfaye et al., 2010

Table 4. Examples of use of RNAi in bovine models. dsRNA=double-stranded RNA, siRNA=small interfering RNA, shRNA=short hairpin RNA.

4. Methods for *in vivo* gene delivery in the reproductive tract of female bovine species

4.1 *In vivo* gene delivery to the ovaries

Since its first reported use as a tool to aspirate bovine oocytes *in vivo* (Pieterse et al., 1988), ovarian transvaginal ultrasonography (OTU) has been used in ovum-pick programs for the *in vitro* production of bovine preimplantation embryos world-wide for commercial purposes (van Wagtendonk-de Leeuw, 2006). Bovine OTU is performed in virtually the same way as in humans, with the advantage that the bovine ovary can be fixed to the probe more precisely via rectal palpation. OTU is considered a non-invasive technique that has played a pivotal role in the elucidation of mechanism involved in the control of follicular growth and developmental capacity of the oocyte in both humans(Revelli et al., 2009) and cattle (Beg & Ginther 2006; Leroy et al., 2008). This has been mainly accomplished with the analysis of aspirated oocytes and ovarian follicular fluid samples. Other possible *in vivo* procedures with OTU include injections in individual follicles (Beg & Ginther 2006), ovarian stroma (Oropeza et al., 2004) and corpus luteum (CL) (Yamashita et al., 2008). Ovarian biopsies can also be performed with OTU for the collection of CL (Kot et al., 1999) and ovarian cortical samples (Aerts et al., 2005). Moreover, OTU allows the *in vivo* transfer of oocytes from one ovarian follicle to another (*i.e.* interfollicular oocyte transfer) (Bergfelt et al., 1998).

Bovine OTU could afford the possibility of *in vivo* delivering of vectors directly to the ovaries without the necessity of surgical procedures. The feasibility of this model is partially supported by the transient transfection of murine ovarian cells achieved after direct *in vivo* intraovarian (IOI) injection of circle plasmid DNA followed by electroporation (Sato et al., 2003). Likewise, production of transgenic mice expressing green fluorescent protein has been achieved after direct IOI of plasmid DNA without subsequent electroporation (Yang et al., 2007). The necessity of surgical exposure of ovaries in these murine models hinders the

opportunity for repeated administration of vectors producing transient transgene expression. This could be easily circumvented with the bovine model as intraovarian injections could be carried out at least twice per week (Velazquez et al., 2009a). Advances in ultrasound-triggered targeted gene delivery vehicles such as echogenic liposomes (Smith et al., 2010) could provide an efficient OTU *in vivo* vector delivery system in cattle. Following gene delivery, integration of the transgene could be analyzed in primordial and preantral follicles (Aerts et al., 2005), oocytes from antral follicles (Zaraza et al., 2010) and granulosa cells (Wells et al., 1999) with minimal discomfort for the carrier animal. Furthermore, since sheep ovaries can be imaged *in situ* (*i.e.* ovaries are exteriorized through a mid-ventral laparotomy) with fibered confocal fluorescence microscopy (Al-Gubory, 2005), the possibility of analyzing *in vivo* transgene integration with fluorescent reporter genes such as green fluorescent protein (Zizzi et al., 2010) is plausible (Fig. 4).

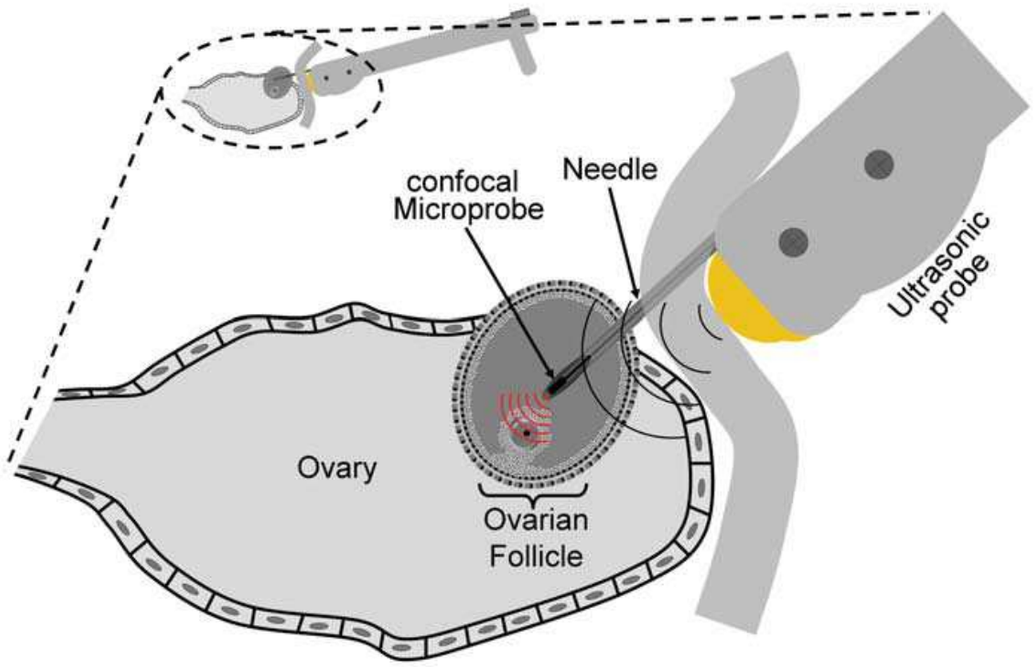

Fig. 4. Hypothetical *in vivo* monitoring of ovarian transgene integration with fluorescent reporter genes using fibered confocal fluorescence microscopy in cattle.

Since human and cattle ovaries are similar in size (Kagawa et al., 2009), the bovine OTU model could be useful to investigate the effect of silencing or overexpressing oocyte-specific genes known to be expressed also in humans such as bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) (Simpson, 2008). Experiments with these genes in a bovine OTU model aimed at controlling granulosa cell proliferation and follicle-stimulating hormone (FSH) responsiveness (Shimasaki et al., 2003) could substantially contribute to the development of therapeutic strategies for conditions such as premature ovarian failure (Simpson, 2008) or ovarian hyperstimulation syndrome (Mikkelsen, 2005). This latter disorder could be also addressed in the bovine OTU model with gain- or loss-of-function studies with anti-müllerian hormone in superovulated cows, as in both species anti-müllerian hormone is associated with the number of antral follicles responding to ovarian superstimulation (Broer et al., 2010; Monniaux et al., 2010).

The bovine OTU model will be an important tool for *in vivo* silencing of candidate genes involved in the regulation of cattle dominant follicle selection recently identified by genomic approaches (Mihm & Evans, 2008). Monitoring of ovarian follicular development with transrectal ultrasonography is a routine procedure in the cattle industry that was the based for the characterization of the follicular wave pattern in women (Baerwald, 2009). Data generated with the bovine model could provide valuable information for humans, especially when using modified cow models (*i.e.* FSH-suppressed cows during the follicular phase) that mimic the time of follicular recruitment and development of the dominant follicle in a highly analogous manner to women (Campbell et al., 2003).

4.2 In vivo gene delivery to the oviduct

In vivo transfection of oviductal epithelium has been reported in rodents via deposition of naked plasmid DNA or lipoplexes in the lumen of the infundibulum (Relloso & Esponda, 1998; 2000; Rios et al., 2002). Access to the fallopian tubes in this laboratory animal model requires invasive surgery. In cattle, a minimal invasive technique based on transvaginal endoscopy has been developed that allows *in vivo* access to the oviducts (Besenfelder et al., 2001). This endoscopy-mediated transvaginal access to the fallopian tubes has made possible the *in vivo* recovery and transfer of embryos from the zygote to the 8-16 cell stage in standing cows without the necessity of general anaesthesia (Besenfelder et al., 2010). The use of this transvaginal endoscopic procedure was pivotal for the generation of gene expression profiles of *in vivo* preimplantation embryos (Kues et al., 2008). The importance of using this technique to recover early stages embryos relies in the fact that postmortem recovery of *in vivo*-produced embryos can alter embryo gene expression (Knijn et al., 2005). This is especially relevant when studying the effects of gene silencing on preimplantation embryo development *in vivo* (Tesfaye et al. 2010).

With this technique it will be possible to infuse vectors into the lumen of the oviduct repeatedly (Besenfelder et al., 2008) and the efficiency of transfection could be improved with the combined use of transrectal ultrasonography, as ultrasound application can improve *in vivo* vector cellular uptake in the reproductive tract (Maruyama et al., 2004). Transgene integration in the oviductal epithelium with fluorescent reporter genes could be also monitored *in vivo* with flexible fibered confocal fluorescence microscopy microprobes. This is supported by the used of this technology to carry out *in vivo* imaging of fluorochrome-labelled ram spermatozoa to analyze *in situ* sperm motility in the ewe genital tract after surgical positioning of the microprobe (Druart et al., 2009). With the endoscopic approach the positioning of the confocal microprobe into lumen of the oviducts may be feasible (Fig. 5).

Silencing (or overexpression) of genes in the oviduct thought to play similar roles in oviductal biology in humans and cattle (identified during comparison of data from microarray analysis from the two species [Bauersachs et al., 2004]), could provide clues for the development of therapies for human contraception and for the formulation of enhanced embryo culture medium. Suggested candidate genes of bovine embryo developmental competence (El-sayed et al., 2006) could be tested. This may be achieved with an *ex vivo* approach of embryo gene silencing such as the one developed by Tesfaye et. al. (2010). Data from the bovine model could be useful for humans, as global transcription profiles during the maternal-zygotic transition are similar in both species (Xie et al. 2010). The model could be particularly relevant for genes showing high homology between the two species such as HMGN3a and SMARCAL I, which are known to play a critical role in chromatin remodelling during early embryo development (Uzun et al., 2009).

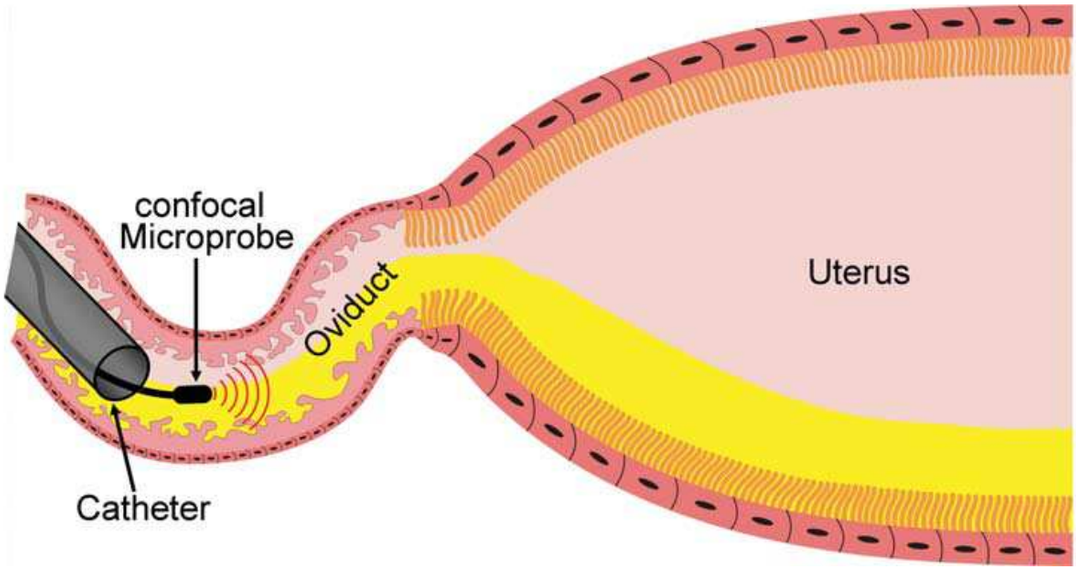

Fig. 5. Hypothetical *in vivo* monitoring of oviductal transgene integration with fluorescent reporter genes using fibered confocal fluorescence microscopy in cattle.

4.3 In vivo gene delivery to the uterus

Non-invasive access to the uterus is a standard procedure broadly used for artificial insemination (AI) and embryo transfer in cattle herds (Velazquez, 2008) that could be applied for repeated *in vivo* gene transfer in the bovine uterus. Uterine *in vivo* gene transfer has been demonstrated in mice (Charnock-Jones et al., 1997; Kimura et al., 2005; Rodde et al., 2008) and rabbits (Laurema et al., 2007). However, accurate access to the lumen of uterus in small animals requires invasive surgical procedures (Ngô-Muller & Muneoka, 2010). As with ovaries and oviducts, transrectal ultrasonography could improve vector cellular uptake via sonoporation (Maruyama et al., 2004). *In vivo* transgene tracking in the uterus with fibered confocal fluorescence microscopy, as previously reported in transgenic rabbits (Al-Gubory and Houdebine, 2006), could be performed in a non-invasive way with transcervical endoscopy (Fig. 6). Transcervical endoscopy is a fairly established technique in cattle used to evaluate uterine involution and its association with uterine diseases (Mordak et al., 2007; Madoz et al., 2010). In addition, confocal laser endomicroscopy technology is already available (Buchner et al., 2010).

Genes with possible roles in uterine biology in humans and cattle, identified during comparison of data from microarray analysis from the two species (Bauersachs et al., 2008), could be silenced (or overexpressed) in order to develop therapies for human contraception and for the formulation of enhanced embryo culture medium. The development of models of uterine cancer in superovulated cows (Velazquez et al. 2009b), will be particularly relevant to test the therapeutic usefulness of tumor suppressor induction (*e.g.* TP53) or silencing of growth factor receptors (*e.g.* IGF-1R). Testing (*i.e.* silencing or overexpression) of candidate genes of bovine embryo developmental competence (El-sayed et al., 2006) can be carried out with the use of embryo transfer, a technique well established in the cattle industry (Velazquez, 2008). Information generated with the bovine embryo transfer model could be useful to human assisted reproduction, as gene expression profiles in blastocysts of both species are to a large extent identical (Adjaye et al., 2007).

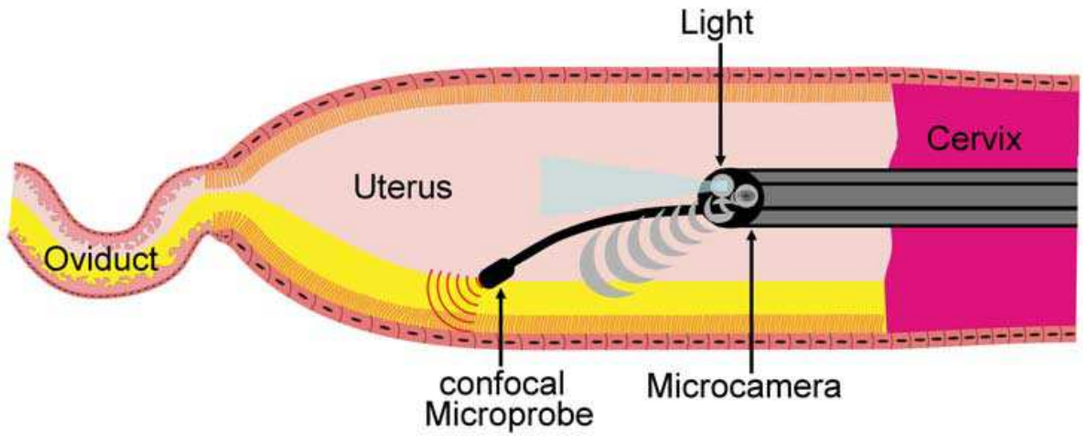

Fig. 6. Hypothetical *in vivo* monitoring of uterine transgene integration with fluorescent reporter genes using fibered confocal fluorescence microscopy in cattle.

5. Animal welfare considerations

All of the techniques mentioned above require special training and should be carried out by professionals that have proper understanding of bovine physiology and anatomy. In the hands of professionals this techniques are safe and cause minimal disturbance to the animal. Nervous cows or those sensitive to rectal palpation (*i.e.* excessive rectal bleeding during exploratory palpation) should be indentified to avoid unnecessary suffering. Environmental enrichment (*e.g.* music or visual effects) should be implemented whenever possible to provide comfort to the animal during the procedure. Health status should be monitored closely after gene delivery to identify and treat ill animals. Euthanasia must be implemented immediately when required.

6. Conclusions

The female bovine could provide a useful model for *in vivo* gene transfer in the reproductive tract. The bovine model may not only offer easiness in the delivering of transgenes in reproductive tract, but also long-term monitoring. This chapter has provided just a handful of the possible scenarios that could be addressed in the bovine model with relevance for human reproductive medicine. The strong similarities in some reproductive characteristics between the two species open the possibility of using the female bovine as a pre-clinical model in reproductive sciences. It is interesting to note that procedures with proved capacity to increase the superovulatory response of cows (*i.e.* aspiration of the dominant follicle) (Bungartz & Niemann, 1994) developed more than a decade ago, are just recently being proposed for application in women as a means to increase the efficiency of assisted reproduction (Bianchi et al. 2010). Perhaps it is time for human reproductive scientists to pay close attention to reproductive large animal models.

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This book is addressed to scientists and professionals working in the wide area of biomedical engineering, from biochemistry and pharmacy to medicine and clinical engineering. The panorama of problems presented in this volume may be of special interest for young scientists, looking for innovative technologies and new trends in biomedical engineering.

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