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Reproduction, Fertility and Development, 2010, **22**, 1049–1056

Embryo biotechnology in the dog: a review

Sylvie Chastant-Maillard[A,B,C,E], *Martine Chebrout*[B,C], *Sandra Thoumire*[B,C], *Marie Saint-Dizier*[B,C,D], *Marc Chodkiewicz*[A,B] *and Karine Reynaud*[B,C]

[A]Department of Reproduction, Ecole Nationale Vétérinaire d'Alfort, 7 Avenue du Général De Gaulle, 94700 Maisons-Alfort, France.

[B]ENVA, UMR 1198 Biologie du Développement et Reproduction, 7 Avenue du Général De Gaulle, 94700 Maisons-Alfort, France.

[C]INRA, UMR 1198 Biologie du Développement et Reproduction, F-78350 Jouy en Josas, France.

[D]Genetic Breeding and Reproduction, AgroParisTech, 16 Rue Claude Bernard, 75005 Paris, France.

[E]Corresponding author. Email: schastant@vet-alfort.fr

Abstract. Canine embryos are a scarce biological material because of difficulties in collecting *in vivo*-produced embryos and the inability, to date, to produce canine embryos *in vitro*. The procedure for the transfer of *in vivo*-produced embryos has not been developed adequately, with only six attempts reported in the literature that have resulted in the birth of 45 puppies. *In vitro*, the fertilisation rate is particularly low (~10%) and the incidence of polyspermy particularly high. So far, no puppy has been obtained from an *in vitro*-produced embryo. In contrast, cloning of somatic cells has been used successfully over the past 4 years, with the birth of 41 puppies reported in the literature, a yield that is comparable to that for other mammalian species. Over the same period, canine embryonic stem sells and transgenic cloned dogs have been obtained. Thus, the latest reproductive technologies are further advanced than *in vitro* embryo production. The lack of fundamental studies on the specific features of reproductive physiology and developmental biology in the canine is regrettable in view of the increasing role of dogs in our society and of the current demand for new biological models in biomedical technology.

Additional keywords: biology, development, fertilisation, *in vitro*, *in vivo*, techniques.

Introduction

In a large number of domestic species, advances in the development of reproductive biotechnology have involved oocyte and embryo manipulation, with subsequent *in vitro* production of embryos, embryo transfer, cloning and transgenesis. In the canine, although sperm technologies are as efficient as for other mammalian species, only a few laboratories have made any progress in terms of oocyte and/or embryo manipulation. This gap reflects not only the peculiarities of reproductive physiology in the dog, but also socioeconomic considerations. The potential gain associated with the development of embryo biotechnology in the dog is indeed far lower than that expected in cattle or horses. It may sound strange to try and develop advanced reproduction techniques for the dog while most countries are experiencing an overpopulation of dogs, if anything, and while natural reproduction is regarded as sufficient or excessive (Zawistowski *et al.* 1998; McNeil and Constandy 2006; Purswell and Kolster 2006). Finally, although the biological material required for experimental purposes is plentiful for meat-producing species because vast numbers of ovaries can be collected in slaughterhouses, similar material for dogs is scarce.

In view of those difficulties, it is hardly surprising that relatively few research teams have become involved in the development of reproductive biotechnologies in the dog. Conversely, the growing importance of pets in urban societies, as well as the current concern for the preservation of endangered species, have provided new impetus to the development of the canine as a biological model.

The dog as a relevant model for human diseases and therapeutics

In many instances, the dog turns out to be a relevant model for human diseases and therapeutics owing to its size, longevity and way of life, in addition to being physiologically similar to humans and exhibiting similar reactivity to drugs and radiation. For example, dogs suffer from an age-related syndrome of cognitive dysfunction that reproduces the key aspects of Alzheimer's disease, with an enzymatic machinery for processing Aβ precursor protein that is identical to that in humans (Sarasa and Pesini 2009). But, above all, the dog appears to be a good genetic model. Of the nearly 400 known hereditary diseases described in the dog, more than half (224) have a

10.1071/RD09270 1031-3613/10/071049

counterpart in the human (e.g. cardiomyopathy, muscular dystrophy or prostate cancer; see http://omnia.angis.org.au, accessed October 2009). Recent sequencing of the dog genome (Kirkness *et al.* 2003; Lindblad-Toh *et al.* 2005) positions the dog as a model for the study of the genetic basis of diseases. This species also has three assets that are particularly desirable for genetic studies: (1) potential access to large families (much larger than in humans); (2) the possibility of initiating informative crossings between genetically characterised males and females (impossible in humans); and (3) a heterogeneous genetic background (compared with highly inbred mouse lines). Moreover, dogs are exposed to the same environmental factors as humans, which is of great importance because numerous common inherited human diseases (e.g. asthma, diabetes, epilepsy and various types of cancer) involve complex interactions between genes and the environment (Schneider *et al.* 2007, 2008). Thus, the dog should be regarded not only as a pet, but also in some way as the modern mouse, which creates a definite need for more embryo biotechnology in the species.

Embryo development in the dog

Oocyte and embryo biology in the dog is quite different to that in other mammals and is still largely unknown. In the bitch, between six and 12 oocytes are delivered at each cycle (Tsutsui 1975; Lee *et al.* 2005; Reynaud *et al.* 2006), with ovulations being spread over 24 and even 36 h (Boyd *et al.* 1993; Marseloo *et al.* 2004). The lack of synchronisation in ovulations may account, in part, for the diversity of embryonic stages observed within a single embryo cohort (Bysted *et al.* 2001; Kim *et al.* 2002; Reynaud *et al.* 2005). Although in most mammalian females ovulation delivers haploid oocytes that can be readily fertilised, the oocytes delivered in the bitch are still blocked at Stage I of meiosis prophase. Following ovulation, oocytes require maturation for 54–60 h in the oviduct to reach the MII stage and become fertilisable (Tsutsui 1989; Reynaud *et al.* 2005).

Thereafter, fertilisation occurs in the oviducts 48–83 h after ovulation. Some investigators have suggested that the penetration of spermatozoa into the canine oocyte could occur at immature stages of meiosis (Van der Stricht 1923; Farstad *et al.* 1993). Although such an atypical fertilisation can, indeed, be obtained *in vitro* with oocytes collected during anoestrus (Saint-Dizier *et al.* 2001), examination by confocal microscopy of oocytes collected *in vivo* has demonstrated that this phenomenon occurs only very exceptionally *in vivo*. Of 112 immature oocytes collected *in vivo* issued from 30 inseminated bitches, only three from a single bitch were found to be fertilised (Reynaud *et al.* 2005). *In vivo*, sperm penetration does not take place unless the canine oocyte has reached the MII stage, as in other mammals.

Embryos at the two pronuclei stage are found 72–124 h (3–5 days) after ovulation, whereas the two-cell stage is observed 96–168 h (4–7 days) after ovulation (Reynaud *et al.* 2006). Major activation of the embryonic genome seems to take place at the eight-cell stage, which occurs between 122 and 288 h (4.5–12 days; Fig. 1) after ovulation (Bysted *et al.* 2001; Chastant-Maillard *et al.* 2009). Between 8.5 and 10 days after ovulation, embryos start to reach the morula stage and begin to slip into the uterus (Reynaud *et al.* 2006). Thus, compared with other mammals, dog embryos spend a relatively long time in the oviduct: approximately 9 days in a pregnancy that lasts 63 days altogether, compared with only 4 of a total of 280 days in the cow (Betteridge 1995).

The blastocysts, which appear around Days 10–12 after ovulation (Fig. 2), hatch between Days 16 and 20 and measure approximately 2.5 mm. Implantation takes place shortly thereafter, between Days 18 and 21 after ovulation (Holst and Phemister 1971; Concannon *et al.* 2001; Reynaud *et al.* 2005), which is later than in other species studied; for example, in the cow, embryo implantation takes place between Days 16 and 19 of a pregnancy that lasts 280 days (Betteridge 1995).

Although in the bitch, passage of the embryos into the uterus and implantation occur relatively late compared with other female mammals, embryo development itself is by no means slow. This common misconception ignores the 48–72-h delay between ovulation and fertilisation. Once embryonic development is calculated starting with fertilisation, the kinetics are largely comparable with those in other mammalian species. Nevertheless, canine embryo cohorts are characterised by considerable heterogeneity: non-degenerating embryos at the two-cell stage can be observed together with eight-cell embryos (Bysted *et al.* 2001; Kim *et al.* 2002; Reynaud *et al.* 2005). Some of this developmental heterogeneity may be explained by the spread of ovulation over 24–36 h (Boyd *et al.* 1993; Marseloo *et al.* 2004).

In vivo production of embryos

In vivo production of embryos consists of the collection of embryos by flushing the genital tract of a female after (super) ovulation and insemination. Subsequent fertilisation takes place *in vivo*. The collected embryos are then transferred into recipient females whose cycles are synchronous with that of the donor(s) spontaneously or after cycle induction.

Several difficulties are encountered when this technique, which is commonly used in cattle, is applied to dogs. Whereas superovulation in the donor female, intended to increase the number of embryos to be collected, can be routinely obtained by appropriate treatment in several species (cow, ewe, human, cat, etc.), the female dog does not respond adequately to common combinations used to induce superovulation (e.g. equine chorionic gonadotrophin (eCG) + human chorionic gonadotrophin (hCG); Archbald *et al.* 1980; Yamada *et al.* 1992; Kutzler 2007). Following natural oestrus, without superovulation, an average of six to eight good-quality embryos can be collected from each female dog.

The technique used for embryo collection in other species must be adapted to anatomical and embryo biology constraints. Until Day 9 after ovulation, the collection of canine embryos requires flushing of the oviducts and thus calls for surgery, an invasive procedure. For embryos at a later stage, one could imagine washing the uterine horns non-surgically by using a catheter inserted through the cervical canal. Nevertheless, even with a surgical approach that involves the one-way movement of liquid from the apex of one horn towards the uterine body, these

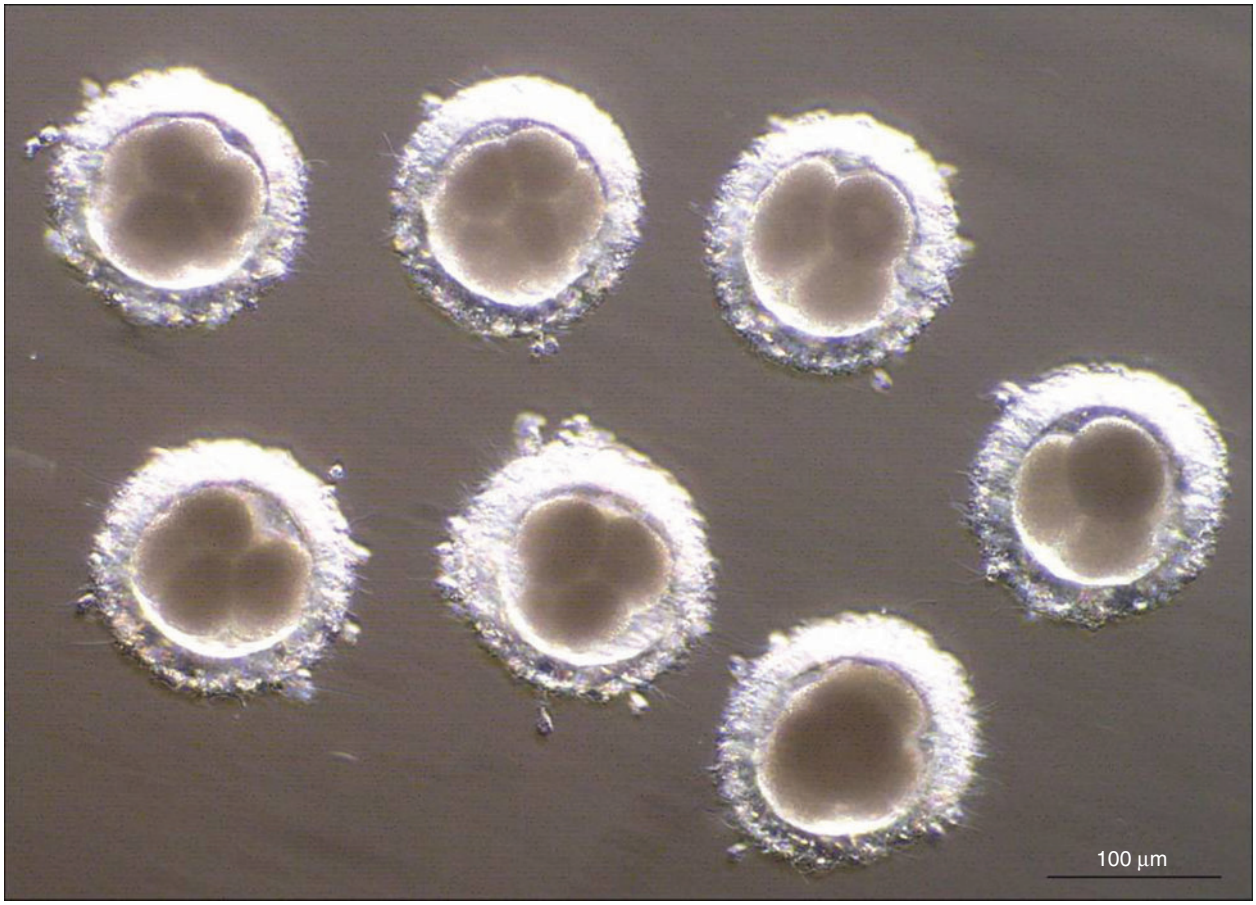

Fig. 1. Embryonic cohort of canine embryos (three- to four-cell and eight-cell stages) on Day 6.5 after ovulation.

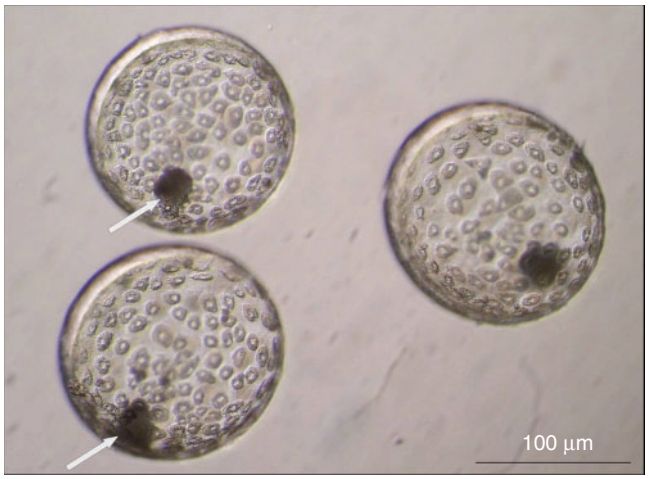

Fig. 2. Canine blastocysts collected 11.5 days after ovulation. Arrows indicate the inner cell mass.

procedures collect only 30–40% of embryos expected from the number of corpora lutea (Archbald *et al.* 1980; Tsutsui *et al.* 2001*b*). This poor recovery rate may result from the considerable endometrial hypertrophy associated with oestrus in the bitch, which means that embryos could remain trapped in the deep folds of the uterine mucosa. The recovery rate can definitely be improved by flushing the oviducts and the uterus *ex vivo* after surgical removal, but this option is of little practical interest (Tsutsui *et al.* 1989, 2001*a*, 2001*b*).

Of the small number of embryos collected from the bitch, 80–90% are viable, at least on the basis of morphological characteristics (Tsutsui 1975; Tsutsui and Ejima 1988; Shimizu *et al.* 1990; Tsutsui *et al.* 2006).

Short of increasing the number of embryos by way of superovulation, which cannot be achieved at this time, one could obtain more embryos from the bitch by shortening the interval between the periods when the bitch is in heat, which is particularly long (approximately 6 months). As reviewed by Kutzler (2005, 2007), attempts to use induction protocols similar to those that work in other species (including progestogens, oestrogens and gonadotropins) have proved of limited or variable success in dogs. Treatments with dopamine agonists are of long duration, cost prohibitive and their efficiency may depend on the stage of anestrus. Yet, the most promising compounds have been found to be gonadotrophin-releasing hormone (GnRH) agonists, primarily deslorelin (Kutzler 2005; Kutzler *et al.* 2009). Such protocols, once developed, could also be used for cycle control in recipient females. Indeed, the recruitment of recipient

females remains problematic as long as no treatment protocol is available to ensure synchronisation. Synchronisation based on natural cycles requires keeping a very large number of females in the hope that one or more will ovulate at the same time as the donor female. No more than 1 or 2 days should elapse between ovulation in the donor and recipient females to obtain a pregnancy (Tsutsui *et al.* 2001*a*, 2001*b*).

Freezing of embryos may represent an alternative to controlling the oestrus cycle. In cattle and swine, the high amount of lipids in the cytoplasm of embryos is known to interfere with the ability of embryos to survive deep-freezing (Nagashima *et al.* 1995; Diez *et al.* 2001). Because the cytoplasm of canine embryos is particularly lipid rich (Fig. 1), the chances of successful deep-freezing appear rather slim. A single attempt was reported in the literature, with no birth obtained after the transfer of eight frozen blastocysts (collected on Day 13 after coitus; Kim *et al.* 2002). One solution could come from vitrification and the open pulled straw (OPS) technique, which has proven successful for porcine embryos (Vajta *et al.* 1998; Somfai *et al.* 2009).

Whatever the technique, the transfer of *in vivo*-produced embryos in the dog remains inefficient, with low rates of success and surgery required for both embryo collection and subsequent transfer. A review of the literature on this subject has found no more than five reports of attempts with fresh embryos (transfers of embryos obtained by nuclear transfer excluded; see below). Altogether, the transfers reported involved 57 recipient females with no more than 45 puppies born (Kinney *et al.* 1979; Tsutsui *et al.* 1989, 2001*a*, 2001*b*, 2006; Kim *et al.* 2002). This figure compares with some 100 000 bovine embryos transferred per year in Europe alone, with pregnancy rates of approximately 60% (http://www.aete.eu, accessed July 2009; Scherzer *et al.* 2008). Because of the poor recovery rate following embryo collection by uterine flushing and because of the difficulties associated with the transfer of embryos into oviducts, Tsutsui *et al.* (2001*a*) have attempted to transfer 52 embryos at tubal stages (zygote to eight-cell stage) into the uterus of 13 recipient females. Four pregnancies were obtained, giving a total of six puppies. This approach of intrauterine transfer of embryos at oviducal stages is a common and efficient practice in women after *in vitro* embryo production.

Development of *in vivo*-produced embryo transfer would make it possible to increase the progeny of genetically valuable females, as well as of females unfit for carrying a pregnancy to full term. Embryo transfer would be applied to females with a history of embryo and/or fetal mortality, especially around term due to obstetric complications. For example, embryos could be collected from female English Bulldogs, in which Caesarean sections are often required, and transferred to Beagle bitches, in which whelping is relatively easier. This situation would also provide an opportunity to evaluate the epigenetic effects of exposure of the fetus to a particular maternal environment during pregnancy and nursing.

Embryo transfer may also contribute to the eradication of some genetic defects following preimplantation diagnosis. In dogs, mutations responsible for numerous hereditary defects have already been identified, including blindness, deafness, muscular dystrophy, nephropathies and various neurological conditions. Because the molecular identification of such defects is possible, embryos free of the unwanted genes could be identified before transfer into a recipient female. Finally, if procedures for freezing canine embryos are to be developed, the exchange and conservation of genetic material could become feasible. In some breeds, molecular selection on the basis of phenotypic traits could also be possible (e.g. fur colour in the Labrador and Newfoundland; fur length in the Alaskan Malamute and Corgi; or the presence of a short tail in the Australian Shepherd and Jack Russell terrier; http://www.antagene.com, accessed July 2009).

In vitro production of embryos

Oocytes are most commonly harvested from anoestrous ovaries, with very low MII rates (10–20%) after 72–96 h culture (Luvoni *et al.* 2005; Songsasen and Wildt 2007). When oocytes are punctured from preovulatory follicles, the maturation rate only reaches approximately 30% (Yamada *et al.* 1993). After the maturation period, IVF is performed by bringing the spermatozoa in contact with cumulus–oocyte complexes. As indicated above, the rate of *in vivo* fertilisation is excellent and *in vivo* polyspermy has not been described. In contrast, *in vitro*, spermatozoa penetrate only 10–50% of oocytes, with normal fertilisation (i.e. the formation of two pronuclei) in only 4–10% of all oocytes subjected to maturation and fertilisation procedures (Mahi and Yanagimachi 1976; Saint-Dizier *et al.* 2001; Hatoya *et al.* 2006*a*; De los Reyes *et al.* 2009). This compares with a normal fertilisation rate of 80–90% in cattle (Galli *et al.* 2001). Furthermore, a dramatically high rate of polyspermy is observed *in vitro*: of 60 oocytes fertilised, 47% were found to have been penetrated with between two and 12 spermatozoa per oocyte, with an average of 3.3 spermatozoa per oocyte (Saint-Dizier *et al.* 2001; Hatoya *et al.* 2006*a*).

Thus, *in vitro*-produced embryos remain exceptional. Blockage occurs around the four- to eight-cell stage (Yamada *et al.* 1992; Hori and Tsutsui 2003). Only a few morula and blastocysts have been reported, with development rates of approximately 0.5% (Otoi *et al.* 2000; Hatoya *et al.* 2006*a*). To date, only one pregnancy has been obtained (but aborted at Day 36) with *in vitro*-produced embryos after the transfer of 90 Day 2 embryos (England *et al.* 2001).

Intracytoplasmic sperm injection (ICSI) may provide a solution to the dual problem of a low fertilisation rate and the high rate of polyspermy. In dogs, a single study on the use of ICSI has been published (Fulton *et al.* 1998). In that study, two pronuclei formed in no more than 8% of microinjected oocytes, but it is of note that only 38 oocytes of unknown nuclear stage (most probably at prophase I) were microinjected.

Whatever the technique (IVF or ICSI), the results are limited by the very low maturation rates and by the low developmental quality of *in vitro*-produced MII oocytes (Viaris de Lesegno *et al.* 2008).

Cloning

As an alternative to fertilisation, embryos can be obtained *in vitro* by nuclear transfer (cloning): a donor cell, obtained from an animal of genetic interest, is fused with the cytoplast

(obtained by extraction of the MII plate) of a mature oocyte. This procedure is made easier in many mammalian species other than the dog because of the relatively large number of MII oocytes that can be obtained after IVM. A further obstacle to cloning in the bitch is the inability, to date, to cultivate or freeze reconstructed embryos. The unavailability of workable synchronisation procedures for recipient females (see above) is another obstacle.

The first cloned puppy (a male Afghan puppy named Snuppy) was born in 2005 after nuclear transfer from adult fibroblasts (Lee *et al.* 2005). From that birth onwards, canine clones have been obtained from a variety of donor cells: male and female, adult and fetal fibroblasts, young and aged donor dogs, small and large breeds, and even from genetically modified cells (Jang *et al.* 2007, 2008*a*, 2008*b*; Hong *et al.* 2009*a*; Hossein *et al.* 2009*a*, 2009*b*; Jang *et al.* 2009). Recently, six transgenic puppies were born following nuclear transfer from fetal canine fibroblasts transfected with the red fluorescent protein (*RFP*) gene (Hong *et al.* 2009*b*).

At the time of writing, a total of 39 cloned puppies have been reported in the literature; to those are to be added four 'commercial' puppies born from family dogs (http://www.bestfriends-again.com, accessed June 2009). The latest, named 'Lancelot Encore', was sold in South Florida for US$120 000.

Compared with protocols used for other mammals, dog cloning is currently performed with *in vivo*-produced oocytes. Reconstructed embryos are transferred after a very short time in culture (<4 h after activation) into spontaneously synchronous recipients. The optimal number of embryos to be transferred seems to be in the range 11–25 (Jang *et al.* 2008*a*, 2008*b*; Hossein *et al.* 2009*a*). The reconstruction process is well controlled (with high fusion rates of $\sim$80%), but the total efficiency of the procedure (no. puppies born/no. embryos transferred) ranges between 0.4% and 4% and is lower than that reported for other mammals (Lagutina *et al.* 2007). Based on reports in the literature, 'abnormal offspring syndrome' (AOS) (formerly called 'large offspring syndrome'; Young *et al.* 1998) seems to occur very rarely. No defects, such as placental hyperdevelopment, excessive fetal growth, anasarc or abnormalities of the immune system, have been reported. This may be due to the good quality of the recipient oocytes (collected *in vivo* and not produced *in vitro* as for other species) and to the immediate transfer of the embryos into surrogate females without culture, which is suspected to induce gene dysregulation. Furthermore, the canine may be more robust than the bovine, as are horses and pigs (i.e. the embryos are more able to cope with errors in gene reprogramming or gene dysregulation during culture). Nevertheless, the occurrence of AOS in the canine cannot be ruled out. For example, neonatal infections have been described (Lee *et al.* 2005). Furthermore, late abortion and the death of clones during the neonatal period until weaning, which are part of the syndrome, are not so rare after somatic cloning in the dog: the abortion rate has been reported to be as high as 23% and 13% of puppies die within 2 months of birth (cf. an abortion rate of 33–43% and death rates of 35% within 2 months and 48% before adulthood reported for the bovine; Cibelli *et al.* 2002; Heyman *et al.* 2002; Chavatte-Palmer *et al.* 2004). Because mortality until adulthood may be increased, further follow up of already born cloned puppies is of considerable importance. At least the fertility of both male and female clones appears to be normal (Park *et al.* 2009).

Because it turned out to be so difficult to obtain canine oocytes at the MII stage, heterospecific nuclear transfer was attempted. Cells from adult dogs were transferred into the caryoplasm of bovine IVM oocytes. These represent an easily accessible (i.e. from the slaughterhouse) and plentiful biological material, the IVM of which is well controlled. For these interspecific transfers, cleavage rates were excellent (74–81%), but few morula (0–1.3%) or blastocysts (0–0.4%; with the exception of 14.5% for Sugimura *et al.* 2009) were obtained, with no development to term after transfer (Westhusin *et al.* 2001, 2003; Murakami *et al.* 2005; Lee *et al.* 2008). Similar results have been obtained using donor cells from other species (pigs, sheep and macaques) transferred into bovine recipient oocytes, yet without any development to term (Dominko *et al.* 1999). Nevertheless, some offspring could be obtained if more closely related species were used as the donor and recipient (Tecirlioglu *et al.* 2006). Because wolf oocytes are even scarcer than dog oocytes, interspecies somatic cell nuclear transfer was attempted with grey wolf fibroblasts transferred into canine recipient oocytes. Even with donor cells collected post partum, the overall efficiency was similar to that of canine cell transfer, with the birth of six wolf puppies (Kim *et al.* 2007; Oh *et al.* 2008).

The major application of cloning in Canidae is the preservation of genotypes of interest. Using cloning, one could obtain a living replicate of an animal, before its death and even post mortem, to avoid genetic loss. This could be applied to endangered canine breeds (like Sapsaree; Jang *et al.* 2009) or endangered canid species (as done for the grey wolf; Kim *et al.* 2007; Oh *et al.* 2008), to mountain rescue dogs, police dogs that detect explosives and guide dogs, all of which are neutered very early in life before their ability becomes apparent. Professional breeders are interested in cloning show champions and some private owners are interested in the cloning of their beloved pets. Cloning could also be used to produce groups of genetically identical dogs for biomedical research, for example as models for specific human diseases. Nevertheless, those who interested in obtaining canine clones and expect a perfect phenotypic copy should be made aware of the diversity of phenotypes that may occur in animals obtained from donor cells with the same genotype. Marked differences in anatomy, coat characteristics, behaviour and performance have been reported among animals obtained from the same cellular source in cattle, horses, cats and pigs (Smith and Murphy 2004; Wilmut 2006).

Embryonic stem cells

Controlling canine embryo biology and related techniques (production, culture and transfer) may give access to a relevant experimental model for setting up embryonic stem (ES) cell technology in humans. Control of ES cell technology is the first step towards cell therapy, a major challenge in the treatment of numerous human diseases in the coming years. The ES cells are obtained from the inner cell masses dissected from blastocysts. Under appropriate conditions, these cells can proliferate

indefinitely *in vitro* while maintaining their pluripotency. They can be transfected and driven to differentiate into numerous cell types. ES cell technology could conceivably be combined with nuclear transfer: for example, fibroblasts collected from a patient suffering from genetic muscular dystrophy could be used as donor cells to produce blastocysts, from which patient-specific ES cells may be derived. After transfection for correction of genetic defects, these ES cells could be reinjected into the patient, colonise the muscles and differentiate into non-defective myocytes without any risk of graft-versus-host disease.

Given the ethical and legal limitations of such experiments in humans, the canine may prove to be a key model in this field (Schneider *et al.* 2008). To date, five characterisations of canine ES cells have been reported, the first being published in 2006 (Hatoya *et al.* 2006*b*; Schneider *et al.* 2007; Hayes *et al.* 2008; Vaags *et al.* 2009; Wilcox *et al.* 2009). In comparison, murine ES cells were isolated from mice in 1981 and from humans in 1998. Blastocysts collected 8–9 days after fertilisation seem to be the ideal developmental stage for ES cell production. The canine ES cells have been reported to differentiate successfully into various cell types, including neurons, epithelial cells, fibroblasts, myocardial cells and haematopoietic progenitors (Hatoya *et al.* 2006*b*; Schneider *et al.* 2007). Nevertheless, the ability of canine embryo-derived cell lines to differentiate *in vivo* remains to be determined, together with an evaluation of their potential to generate unwanted tumours.

The ES cells are also a potent tool with which transgenic animals can be obtained: thanks to canine genome sequencing, such cells could be modified genetically by homologous recombination before reinjection (e.g. after insertion of a normal dystrophin gene to correct Duchenne's muscular dystrophy; Sampaolesi *et al.* 2006). Such transgenic ES cells microinjected into a morula or a blastocyst allow the formation of chimeras that, in some cases, exhibit germinal expression of the transgene.

Conclusion

The specificities of dog reproductive physiology require numerous adaptations to the assisted reproduction procedures that are used efficiently in other species. Even among carnivorous mammals, techniques that have proved efficient in cats result in low production rates when applied to dogs. Dog embryo biotechnologies have developed markedly over the past 5 years, jumping directly to the most advanced procedures, such as somatic cell nuclear transfer, transgenesis and ES cells. Despite a lack of fundamental knowledge in bitch reproductive physiology, considerable progress has been made in parallel with advances in other fields, such as genetics, cell therapy and *in situ* wildlife preservation. The needs arising from these fields will probably act as a potent impetus to progress reproductive biotechnologies in the dog.

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Manuscript received 4 November 2009, accepted 3 March 2010