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Canine embryo-derived stem cells and models for human diseases

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Embryonic stem (ES) cells are pluripotent and permanent cell lines which can differentiate into cell types of all the three germ layers. These features imply multiple opportunities for clinical applications in tissue engineering and regenerative medicine. Most of our knowledge on the biology and technology of ES cells is derived from studies with mouse ES cells. While appropriate for proof-of-principle studies, the mouse model has limitations in its application in translational, pre-clinical studies. This is particularly true for studies evaluating the safety and efficacy of stem cell therapies. For this purpose, large animal models more closely mimicking important aspects of human anatomy, physiology and pathology than mouse models are urgently needed. In this context, the dog is an excellent candidate: the plethora of different dog breeds offer a large phenotypic and genetic variability, which can be exploited increasingly well due to the advanced status of the dog genome project and the rapidly growing box of genomic tools. Recently, the first pluripotent canine embryo-derived stem cells have been described, further increasing the potential of the dog as a model system for regenerative medicine. Although these cells express alkaline phosphatase, *NANOG* and *OCT4*, and can be differentiated *in vitro* towards endoderm-, mesoderm- and ectoderm-lineages (typical features of human and mouse ES cells), their *in vivo* differentiation capability, i.e. formation of teratomas in immunodeficient mice or contribution to chimeric animals, remains to be demonstrated. Here, we discuss the features of reported canine embryo-derived cells and their potential applications in basic and translational biomedical research.

INTRODUCTION

Embryonic stem (ES) cells, pluripotent cells from the embryonic epiblast, were first isolated more than 25 years ago from the inner cells mass of mouse embryos (1,2). Under appropriate conditions, these cells can proliferate indefinitely *in vitro* while maintaining their pluripotent phenotype (self-renewal). Even after long-term culture and genetic manipulation, these cells can contribute to all embryonic tissues after injection into early stage embryos. This property greatly facilitated the introduction of specific genetic modifications into the mouse germ line and revolutionized functional genomics in the 1980s (3). In addition, these cells represent a particularly useful tool for studying the molecular mechanisms controlling differentiation and lineage commitment.

The derivation of human ES cell lines was reported in 1998 (4). Despite significant differences regarding their morphology, their culture requirements and the pathways that regulate their self-renewal and differentiation (5), mouse and human ES cells share the ability to spontaneously differentiate into tissues representing the three embryonic germ layers both *in vitro* (within so-called embryoid bodies) and *in vivo* (in the form of teratomas growing from ES cells injected into immunodeficient mice).

With the availability of human ES cells, a further application gained considerable importance: their differentiation

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in vitro for the use in regenerative medicine and tissue engineering. However, before human diseases can be treated with ES cell-derived tissues, two major problems have to be managed. First, until the reprogramming of the patient's own somatic cells becomes reality, immunological rejection of the tissue graft by the recipient must be suppressed. Secondly, the production of transplantable material from human ES cells must be refined in order to result in highly specific cell populations and to avoid the risk of ES cell-derived tumours. For obvious reasons, *in vivo* studies along these lines have been limited to mouse xenogenic models. While certainly useful for providing proof-of-principle results, the mouse model bears significant anatomical and physiological limitations. In particular, only short-term experiments can be performed in mice. In contrast, large animals live reasonably long, allowing for longitudinal studies. Thus, a large animal model will be necessary to evaluate the efficacy and safety of cells and tissues derived from ES cells before this approach becomes applicable to patients.

DOG AS A MODEL FOR STUDYING HUMAN DISEASES

The reasons why the dog represents a suitable model for the study of human diseases have been excellently reviewed (6). For example, the physiology, disease presentation and clinical responses of the dog are much more similar to man when compared with various other traditional model organisms. Of the ~400 known hereditary canine diseases, more than half have an equivalent human disease, including cardiomyopathies, muscular dystrophy and prostate cancer. Many common inherited human diseases including asthma, diabetes, epilepsy and cancer are due to complex interactions between multiple genes and environmental factors. Importantly, the latter are shared by both dog and man (6).

The sequencing of the dog genome (7,8) and related genomic resources position the dog as an important model for understanding the genetic basis of disease. Investigation of canine disease genes has increased the understanding of the interaction between genes and how such interactions affect disease. The canine system may have the power to map and clone disease genes which are difficult to approach by studies on human families, and it may also facilitate the identification of novel disease mechanisms (9–11).

The dog was the most prevalently used species in early transplantation research (12). In stem cell transplantation, the dog has been used for more than 30 years. The dog is biologically more comparable with humans with respect to stem cell kinetics, haematopoietic demand and responsiveness to cytokines (13). For example, due to the small size and short life span, a mouse produces as many erythrocytes in its lifetime as a human produces in 1 day or a dog in two-and-half days (14). Thus, compared with large animals and humans, mice have a relatively limited proliferative demand on haematopoietic stem cell and progenitor compartments (15). Treatment of donor cells with L-leucyl-L-leucine methyl ester prevented lethal graft versus host disease (GVHD) in mice (16) but induced failure of canine marrow allografts (17). While treatment of donor marrow with recombinant GM-CSF and IL-1

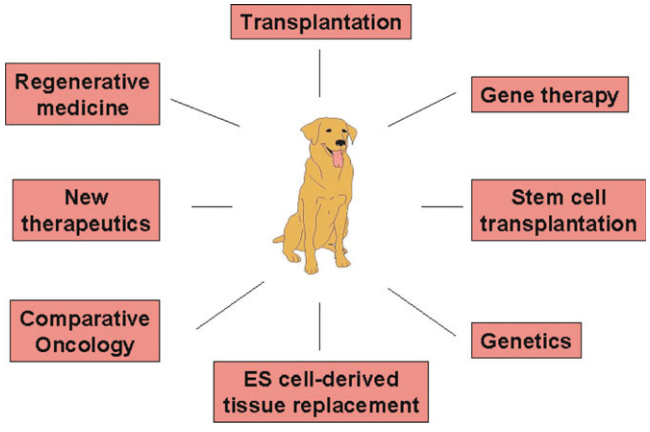

Figure 1. The dog as a model for human diseases.

improved engraftment in mice (18,19), these observations could not be confirmed in dogs (20). Major information has been gained from canine studies with regard to the conditioning for transplantation, selection of the donor by histocompatibility typing, prevention of GVHD by depletion of T cells and adoptive immunotherapy in mixed chimeras (21). In addition, a variety of disease models are available in the dog, including haemolytic anaemias, granulocytic disorders, storage diseases and immunodeficiencies (13). Taken together, the dog represents a promising model for the study of human diseases (Fig. 1).

CANINE REPRODUCTIVE PHYSIOLOGY AND BIOTECHNOLOGY

Studies using dog embryos can currently only be performed after *ex vivo* collection of embryos. In contrast, embryos in cattle and a number of other domestic animals can be produced *in vitro* using *in vitro* matured oocytes and epididymal or ejaculated spermatozoa, with the resulting zygotes cultured *in vitro* up to the blastocyst stage. For the dog, comparable techniques are currently not available, mainly due to problems with *in vitro* maturation. The problems associated with the development of efficient systems for *in vitro* maturation of canine oocytes and the potential underlying mechanism have been recently reviewed (22). Briefly, a pre-ovulatory increase of progesterone and ovulation of immature oocytes are peculiar features of dog reproduction which most probably are the main obstacles to adapt *in vitro* maturation systems from other species (23,24).

Since the first report on *in vitro* maturation of dog oocytes (25), many factors have been studied which are known to influence oocyte maturation in other species, e.g. protein or hormonal supplementation of culture media or co-culture systems. Nevertheless, maturation rates reported in studies with canine immature oocytes are still well below 40% (22). A single blastocyst has been produced from IVM/IVF dog oocytes (26,27), and transfer of potential zygotes or two-cell embryos from *in vitro* matured oocytes did not result in a viable pregnancy (28).

Data about early embryonic development in dogs are very limited and restricted to studies on *ex vivo* collected

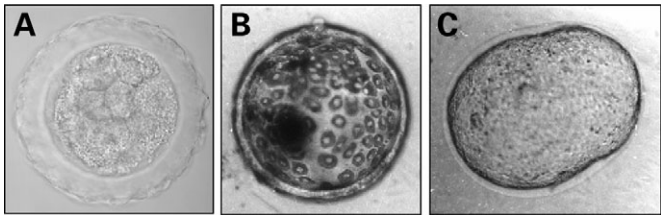

Figure 2. Morphology of canine embryos at different developmental stages: (**A**) morula collected at day 8 post ovulation, ~200 μm diameter; (**B**) blastocyst collected at day 12 post ovulation, ~300 μm diameter; (**C**) expanded blastocyst collected at day 16 post ovulation, ~800 μm diameter.

embryos. A considerable variability in the timing of early embryonic divisions has been reported (29), which may be attributed to a variable length of maturation and time of penetration. Canine morulae and blastocysts (Fig. 2) can be found in the uterus starting from days 8 to 10 after ovulation (23), and implantation is believed to occur around day 20 (30,31).

CANINE EMBRYO-DERIVED STEM CELLS

For the reasons mentioned above, the availability of canine embryo-derived stem cells would be of great value for the development of new therapies, especially in haematology. Since nuclear transfer from canine somatic cells has—albeit with very low efficiency—been demonstrated (32), the derivation of canine embryo-derived stem cells would also offer the possibility to evaluate the concept of 'therapeutic cloning' in a clinically relevant animal model. In terms of haematology, ES cells provide a number of advantages over conventional sources of transplantable material: (i) they can be expanded indefinitely *in vitro*, and, more importantly, (ii) they can either be obtained from a bank representing major haplotype combinations (33), or may even be derived by reprogramming of somatic cells from individual patients. Proof-of-principle for this 'therapeutic cloning' concept has been provided in the mouse (34) but translation of ES cell-based therapeutic strategies to clinical application requires larger animal models for predictive efficacy and safety studies. This holds also true for the development of clinical stem cell gene therapy protocols (13).

Until now, there have been three reports of attempts to establish canine embryo-derived cell lines. Hatoya *et al*. (35) described the isolation of two cell lines from canine blastocysts showing characteristic ES-like morphology and expression of pluripotency markers such as OCT4 (POU5F1), stage-specific embryonic antigen-1 (SSEA-1) and alkaline phosphatase activity. Importantly, the cells formed embryoid bodies in suspension culture, which differentiated into various cell types, including neuron-like, epithelium-like, fibroblast-like, and myocardium-like cells, demonstrating that these cells are indeed pluripotent. Unfortunately, it was not possible to maintain the undifferentiated phenotype of the cell lines beyond passage 8.

Studies from our group confirmed the possibility to establish canine embryo-derived cell lines and, more importantly, demonstrated for the first time that these cells can be differentiated into haematopoietic stem cells (36). Our ES-like cells exhibited alkaline phosphatase activity and expressed transcripts for NANOG, OCT4 (POU5F1) and SOX2, the most important pluripotency-associated transcription factors for mouse and human ES-cells. Furthermore, in agreement with the report by Hatoya *et al*. (35), the canine ES-like cells showed expression of SSEA-1 but where negative for SSEA-4 (36). Differentiation of canine embryo-derived stem cells into haematopoietic progenitor cells was achieved by co-culture with irradiated OP9 murine bone marrow stroma cells. After 6 or 9 days of co-culture, ~50% of the cells stained positive for CD34 as determined by fluorescence-activated cell sorting (FACS) analysis. RT–PCR analysis revealed that the expression of CD34 and GATA2 (a transcription factor specific for haematopoietic progenitor cells) increased substantially after 6 and 9 days of co-culture, further demonstrating differentiation towards the haematopoietic lineage. In one experiment, cells from day 9 of co-culture were able to grow as colonies in colony forming units, while cells from day 0 of co-culture did not grow into colonies. In another experiment, cells harvested from day 6 of co-culture proliferated in response to a mixture of haematopoietic growth factors known to support the growth of canine haematopoietic progenitor cells (37), whereas much less proliferation was observed with cells harvested from day 0 of co-culture. While our *in vitro* differentiation results were obtained using cells at passages 10–12, thereby representing a step forward as compared with the results by Hatoya *et al*. (35), the maintenance of the cells in an undifferentiated, proliferative state after further passaging became increasingly difficult.

Very recently, the generation of several canine blastocyst-derived cell lines satisfying most of the criteria for ES cells has been described by a third group (38). One of these lines (FHDO-7) was maintained through passage 34 and characterized in further detail. The authors showed that these cells expressed the standard pluripotency markers OCT4, NANOG, telomerase, and a cluster of pluripotency-associated microRNAs characteristic of human and mouse ES cells. *In vitro* differentiation towards ectodermal, endodermal, and mesodermal cells confirmed the pluripotency of these cells (38). Thus, the lines established by Hayes *et al*. represent a clear step forward in our common goal of routine generation and *in vitro* differentiation of canine stem cells for translational studies.

Useful lessons can be obtained by comparing the features of the cell lines described by these three groups. Hatoya *et al*. (35) reported encouraging rates of ES cell derivation from blastocysts (25.6%) and hatched blastocysts (67.9%) but failed to isolate ES cells from morulae. This is in agreement with our own results, since our cell line was derived from an expanded blastocyst (36). Similar results were reported by Hayes *et al*. (38). Under their conditions, blastocysts collected at day 12 or beyond day 15 after the presumed surge in circulating levels of luteinizing hormone were unsuitable for isolating ES cell lines, while day 13–14 embryos gave rise to pluripotent cell lines. Thus, there appears to be an ideal time point for collecting the canine embryo for isolating ES cells.

Although *in vitro* differentiation has been shown in all three reports, the ability of the canine embryo-derived cell lines to differentiate *in vivo* remains to be demonstrated. The cell lines described by Hatoya *et al*. (35) were lost at an early stage, probably before *in vivo* differentiation experiments could be attempted. We performed two independent experiments to

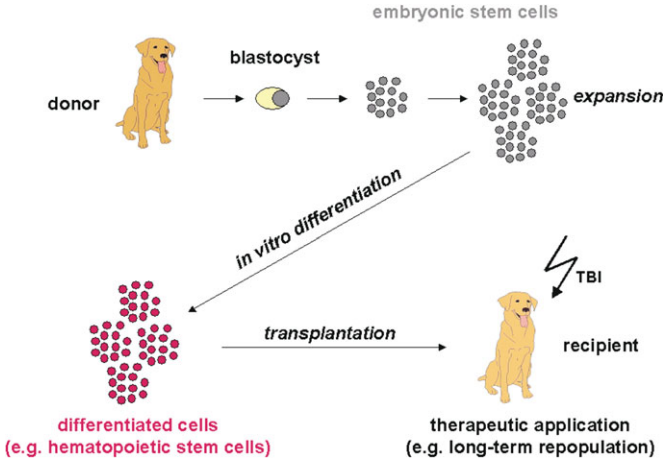

Figure 3. Generation of ES cell-derived transplantable precursors of a specific lineage and their potential use. Blastocysts are obtained from a donor dog, and embryonic stem cells are established which can be indefinitely expanded. Then, they will be differentiated into the tissue of choice and used for tissue replacement therapy. In the example illustrated, the embryonic stem cells are differentiated into haematopoietic stem cells and used for stem cell transplantation to repopulate a dog, which was conditioned by total body irradiation (TBI).

differentiate our canine ES-like cells *in vivo* by injecting varying amounts of cells subcutaneously into severe combined immunodeficient mice. No tumour growth was observed (unpublished data). This negative result has been confirmed by Hayes *et al*. (38), whose massive attempts to grow canine ES cell teratomas in different immunodeficient mouse lines also failed. Thus, the growth of canine ES-derived teratomas in immunocompromized mice is particularly challenging. Further studies involving the generation of chimeric dogs by injecting these cells into canine blastocysts, or the growth of teratomas in immunodeficient dogs are therefore urgently needed.

CONCLUSIONS AND OUTLOOK

The work described in this review provides a launching point for efforts to generate canine ES cells and to differentiate them into transplantable tissue for therapeutic purposes. The availability of canine embryonic stem cells will allow establishing the dog as a large animal model for testing the safety and efficacy of ES cell-derived tissue replacement therapy. Major emphasis is given to the generation of canine ES cell-derived haematopoietic stem cells since stem cell therapies are most advanced in haematology. The ultimate goal will be to create genetically defined (animal-specific) ES cells which could be expanded *in vitro*, differentiated into haematopoietic stem cells and used to repopulate a lethally irradiated host or to treat haematopoietic diseases (Fig. 3). Thus, translation of ES cell-based therapeutic strategies to clinical application, which has recently been proposed for man (39,40), could be provided in a large, clinically relevant animal model.

Encouraging developments in the reprogramming of somatic cells towards a pluripotent phenotype have been recently reported. Takahashi and Yamanaka (41) demonstrated that the forced expression of four factors (OCT4, SOX2, C-MYC and KLF4) can induce mouse fibroblasts to form pluripotent cells almost indistinguishable from ES cells. These exciting findings have been reproduced and expanded in further studies (42–44). The same quartet revealed to be successful for the reprogramming of human somatic cells (45), fuelling the hopes of researchers that the ethical concerns linked to the use of human ES cells will become obsolete. Recently, Mitalipov and colleagues (46) reported the derivation of rhesus macaque ES cell lines from blastocysts produced by somatic cell nuclear transfer using adult skin fibroblasts. These approaches, if successful with canine cells, would certainly boost the interest in the use of dogs as a model for regenerative medicine.

Large animal models, like the dog, are invaluable for working out the practicalities of a therapeutic regimen in a complex system and for verifying established mechanistic theories (12). For example, the small size of the mouse limits the proliferative demand placed on transplanted tissue, and the short life span of the mouse prevents long-term follow-up. The latter is critical for assessing genetic or epigenetic stability of transplanted tissue and clearly needs a preclinical large animal model. Furthermore, the unique evolutionary history of domestic dogs is particularly well suited to analysis of genetic factors underlying complex diseases by genome-wide association studies (47). Research on large animal models has already altered our thinking about therapies, doses and toxicities. For example, the preparative regimen by which chimerism was originally achieved in mice is far too toxic for humans (48). Mice irradiated with 10 Gy delivered at 1 Gy/min showed irreversible damage of the lympho-haematopoietic system only. In contrast, administration of this dose and dose rate to humans and large animals led to irreparable damage in other organs including gut and lungs. Consequently, less-toxic ablative regimens have been employed in patients (49). Findings in dogs have set the stage for clinical studies in patients with certain immunodeficiency diseases without pretransplantation immunosuppression (50). While T-cell-depleted marrow engrafted readily in lethally irradiated mice even across major histocompatibility complex (MHC) barriers (51), similar depletions of human bone marrow resulted in an increased risk of engraftment failure (52). Administration of monoclonal antibodies at doses sufficient to induce T-cell depletion in humans to the same extent as in mice has not been achieved. Thus, before attempting to use such approaches in humans, they need to be applied to large animal models (48). Dogs have been important for the development of numerous conditioning and immunosuppressive regimens (15,53). For example, the combination of methotrexate and cyclosporine was found in dogs to be effective in preventing GVHD (54). Given that humans have a widespread, well mixed gene pool, it is not surprising that random-bred species such as the dog are particularly suitable animal models for preclinical studies (15,53).

Therefore, canine embryonic stem cells present the potential for unique and exciting biological opportunities.

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Conflict of Interest statement. The authors indicate no potential conflict of interest.

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