

Canine Pluripotent Stem Cells: Preclinical Model for Stem Cell Based Regenerative Medicine

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Abstract

Due to the unique similarity to human physiology, biochemistry, and pathology for many diseases, canine breeds have been applied to preclinical research into human medicine for several decades. Since 2006, the discovery of induced pluripotent stem cell technology set the stage for the patient specific stem cell-based therapy. To evaluate the safety and efficacy of this potential therapeutic strategy, a growing body of studies on the generation and differentiation of canine induced pluripotent stem cells (ciPSCs) have been reported. In this review, the current progress from the ciPSCs studies will be described. Additionally, the unique regulatory mechanism(s) for pluripotency maintenance with in canine pluripotent stem cells will be discussed and compared with those in mouse and human.

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Regenerative medicine

During the past decade, the birth and exponential growth of the regenerative medicine research field has been witnessed^[1]. Defined by the National Institutes of Health (NIH), regenerative medicine is aiming at “replace, repair, and regenerate cells, tissues and organs in order to restore biological function that has been halted or compromised by injury or disease”^[2]. One of the basic requirements to achieve this goal is the access to an unlimited source of somatic cell of all differentiated types. Pluripotent stem cells (PSCs), holding the capacity of self-renewal and three-germ-layer differentiation, appears to be the most suitable for this mission. The application of PSCs is believed to promote the development of specific cell culture and differentiation techniques, and will eventually push the regenerative medicine field to move forward. The versatility of PSCs is such that they can potentially generate functional tissues or even organs *in vitro*. This extraordinary progress has set the stage for conducting preclinical evaluation of PSC-related regenerative medicine.

One well-characterized type of the PSCs is embryonic stem cells (ESC). These cells were initially isolated from mouse and human preimplantation embryos^[3,4]. ESCs provide the basis upon which therapies for diseases, especially those previously thought to be incurable, are developed. However, among the hurdles that must be solved before this therapy is applied in

the clinic, is the problem of tissue immune-compatibility^[3,5-8]. Response to minor histocompatibility can be ameliorated by immunosuppressive therapy, but it was reported that in human interleukin antigen (HLA)-identical sibling bone marrow transplantation, combinations of multiple minor histocompatibility resource were a significant cause of graft-versus-host disease (GVHD), which occurs in approximately 40% of total patients^[9]. Examples of minor histocompatibility antigens that trigger hematopoietic stem cell transplant rejection and GVHD include peptides derived from HA-1, and the male H-Y gene product which can be recognized by female immune cells as foreign, as well as mitochondrial gene products^[9]. Therefore, the optimum transplanted cells remain to be the autologous cell resource derived from the patient^[10]. However, with the breakthrough of induced pluripotent stem cell (iPSC) in 2006, mouse and human immuno-compatible PSCs can be produced. An iPSC is a PSC generated by over expressing a set of pluripotency-related transcription factors, (e.g. OCT-3/4, SOX2, KLF4 and c-MYC; OKSM or Yamanaka factors), in somatic cells^[10]. iPSCs are similar to ESCs in morphology, marker expression profile and differentiation potential even though the subtle discrepancies were reported in terms of mutational load and epigenetic profiles^[11,12]. To date, successful generation of iPSCs has been reported in species such as mouse, human, rat, rhesus monkey, pig, cow and

dog^[10,13-18].

Advantages of canine model for preclinical study

The governmental regulatory agencies, such as the Food and Drug Administration (FDA) in the USA, are requiring stringent preclinical inspection for the safety and efficacy of PSC-based therapies. It is anticipated that when iPSC-derived cells are contemplated for use in human, animal species – especially large animals in addition to rodents – may be considered as preclinical animal models^[19]. Experience from canine medicine for more than five decades has extraordinary potential to inform our understanding of diseases in human counter parts and eventually uncover new therapeutic avenues. Compared to small animals like rodents, dogs have larger body size, longer life span, similar relative organ positions, diverse gene pool, and present biochemical and pathological similarities to humans^[20]. Numerous preclinical studies have been conducted in dog models successfully. Research in dogs provided fundamental knowledge for human medicine, particularly in bone marrow transplantation, metabolic diseases, neurological disorders, cancers and heart failure^[20-22]. For example, during the 1950's Dr. Norman Shumway developed heart transplantation techniques that are today's standard surgical practice in humans based on his seminal studies performed in dogs^[23]. Moreover, canine heart mirrors the human's time course of irreversible myocardial injury following ischemia, and it has facilitated the development of rescue treatments such as thrombolytic reperfusion^[24].

Moreover, modern canine breeds show large variation in size, appearance, and behavior, which is a consequence of thousands of years of selected breeding with human intervention, and eventually provides a great animal model to study specific genetic disorders. Over 400 types of genetic diseases have been identified in various canine breeds, and half of them resemble those in humans including cardiomyopathies, muscular dystrophy and prostate cancer^[25,26]. An example is the canine model of spinal cord injury (SCI). Approximately 2% of the dogs in the clinic display SCI, and 77% of them present intervertebral disc diseases^[27,28]. Chondrodystrophic canine breeds like Dachshunds or Beagles are highly susceptible, suffering from SCI following spinal hyperesthesia, non-ambulatory hind limb paraparesis, and complete hind limb paralysis^[27-30]. It is anticipated that this canine model of SCI will facilitate cell therapy to cure SCI with a variety of donor cell types, including those derived from iPSCs^[29,31]. The application of stem cells to treat conditions in the dog for which there are few, if any effective therapies, and that would ordinarily lead to life-long disability, or a significant impact on quality of life, would not only tremendously benefit the animal recipient, but also provide knowledge to develop parallel treatments in human patients.

Pluripotency and stem cells

Pluripotency is an essential feature of embryonic stem cells derived from the inner cell mass (ICM) of blastocyst^[32]. During 1960s, Peices, et al. firstly reported the isolation of embryonic carcinoma cells (ECC) from mouse testicular teratocarcinoma^[33]. ECCs could be induced to differentiate spontaneously into multiple somatic cell lineages *in vitro*, and when injected into a host embryo, contribute to several tissues in the chimeric pups. However, defects such as karyotypic abnormalities and loss of differentiation capability limited their practical

applications^[33-36]. In 1981, two independent reports described the isolation of mouse ESCs (mESCs) using mouse embryonic fibroblasts (MEFs) as feeder layers or ECC-conditioned medium^[4,37]. These cells displayed similar characteristics of ECCs in colonial morphology, self-renewability, gene expression profiles and capability of differentiation. Additionally, ESCs presented a normal karyotype and improved contribution to chimera development, rapidly becoming an ideal model of cell differentiation research that also contributed to the establishment of gene knock-out animal models for studying gene function^[4,38]. Another breakthrough is the derivation of human ESCs from human blastocysts in 1998 by James Thomson's group^[3]. Almost in parallel, several groups began to explore the possibility of using human ESCs (hESCs) in the context of regenerative medicine^[3,39].

Embryonic stem cells: Mouse ESCs are derived from the inner cell mass of pre-implantation blastocysts^[4]. Under *in vitro* culture conditions, colonial mESCs form three-dimensional and dome-shaped structures, a characteristic that set them apart from mouse ECCs. In terms of cell surface markers, mESCs display a specific glycosphingolipids, Stage-Specific Embryonic Antigens-1 (SSEA-1), originally identified in mouse preimplantation embryos^[40]. At the gene and protein expression levels, mESCs express the essential core of pluripotency-associated transcription factors including OCT4, SOX2 and NANOG^[41]. The leukemia inhibitory factor (LIF), is one of the specifically required growth factors to sustain the expression of these core transcription factors^[42].

Human ESC lines were first isolated using similar procedures used in generating mESCs. However, hESCs have unique characteristics that make them different from mESCs^[3]. Morphologically, hESCs resemble cells from the epiblast in post-implantation mouse blastocysts, unlike the mouse ES cells that are more ICM-like. hESCs grow in a tightly adherent, flattened monolayer, instead of the typical mouse ESC dome-shape colony^[3]. Similar to mESCs, hESCs express the same core of pluripotency-associated transcription factors, OCT4, SOX2 and NANOG. However, at the global transcription level, only 13% to 55% of the genes present similar expression levels when compared to their own housekeeping genes between hESCs and mESCs, which indicated that mouse and human ESCs exhibit distinct transcription signatures^[43]. In terms of pluripotency markers, hESCs express SSEA-3, SSEA-4 as well as tumor rejection antigens including TRA-1-60 and TRA-1-81, but not SSEA-1^[44,45]. Perhaps the most striking difference between mouse and hESCs is that the later require basic fibroblast growth factor (bFGF) and the associated downstream signaling pathways instead of LIF as the main growth factor for pluripotency maintenance^[3].

Induced pluripotent stem cells: Mouse iPSCs (miPSCs) were initially derived in 2006 by Shinya Yamanaka's group by over-expressing exogenous OCT4, SOX2, KLF4 and c-MYC (OSKM) in embryonic and adult fibroblasts^[10]. The first report for human iPSC was published in 2007 by the same group, almost at the same time with Dr. James Thomson's group that reported human iPSCs (hiPSCs) using different set of factors, i.e. OCT4, SOX2, LIN28 and NANOG^[46,47]. However, due to the over expression of oncogene such as c-MYC, iPSCs generated by the conventional way based on retrovirus-mediated reprogramming and

Yamanaka factors were observed to be tumorigenic *in vivo*^[10]. Modifications have been introduced to the original protocol to minimize the tumorigenesis, i.e. the proto-oncogene c-MYC can be replaced by v-MYC and subsequently discarded from the reprogramming cocktail^[48]. KLF4 and SOX2 were also demonstrated to be dispensable when specific donor cell types are utilized^[48,49]. A vast body of subsequent literature was published, describing new genes and reprogramming methods, including the use of retrovirus, lentivirus, adenovirus, transposons, episomal vectors, mRNAs, and microRNAs^[10,46,47,50-63]. The addition of small molecules inhibitors was also demonstrated to synergistically enhance the reprogramming efficiency. These include inhibitors targeting certain trophic pathways (MEK inhibitor PD0325901 or glycogen synthase kinase3 inhibitor CHIR99021) or epigenetic modifiers (DNA methyl transferase inhibitor 5-Aza-2-deoxycytidine, histone deacetylase inhibitor valproic acid or trichostatin A)^[64-66]. One of the methods developed thus far for by passing the need for any exogenous recombinant DNA or RNAs is the application of small molecules-only/transgene-free reprogramming. Mouse fibroblasts can be reprogrammed into iPSCs by simply exposing the cells to a cocktail of small molecule inhibitors^[67]. The essential inhibitor in this cocktail is DZNep, which blocks histone methyl transferase EZH2 and significantly enhances OCT4 expression in mouse fibroblasts. A variety of mouse and human donor cell types have been verified for the capacity to be reprogrammed. These donor cells include embryonic and adult fibroblasts, neural stem cells, adipose-derived cells, cord blood cells, mesenchymal stem cells, B and T cells, and keratinocytes^[10,46,47,49,58,68-71].

Pluripotency-associated growth factors and signaling pathways

In 1988, Austin Smith's group reported the essential role of LIF for maintenance of mouse ESC's self-renewal^[42]. LIF is a member of IL-6 family proteins. Its receptor is a heteromeric complex composed of two transmembrane proteins, the LIF receptor (LIFR) and the gp130. LIF can bind to LIFR, and the receptor-associated tyrosine kinase Janus kinase (JAK) phosphorylates the intracellular domains of gp130 and LIFR that recruit and phosphorylate the signal transducer and activator of transcription 3 (STAT3)^[72]. Phosphorylated STAT3 promotes the expression of genes associated with pluripotency and survival including c-MYC and KLF4 in mESCs^[46]. LIF can also activate the PI3 kinase/AKT pathway. Phosphorylation of AKT proteins can modulate the function of numerous substrates including the mammalian target of mTOR and elicit proliferation and suppression of cell death^[72]. LIF is also able to robustly activate the Ras/ERK1/2 canonical signaling cascade triggering the phosphorylation of a series of early transcription factors including c-Jun and c-Fos, which are critical for maintaining viability and proliferation^[72,73].

Striking differences exist in signaling pathways involved in pluripotency maintenance between the mouse and human PSCs^[74]. The mouse PSCs resemble the naïve pluripotent stem cells from inner cell mass within blastocyst, while the human PSCs are more close to the epiblast stem cells with primed pluripotency^[74]. The activation of STAT3 is unnecessary for hESCs' pluripotency, but instead they require bFGF^[75]. bFGF not only exerts its role on human PSCs directly, but indirectly through interactions with the feeder layer cells, which

are typically mitotically-inactivated mouse embryonic fibroblasts (MEFs), stimulating the release of activin-A (ActA) that in turn binds to the TGF-beta receptors in hESCs triggering the activation of intracellular SMAD2/3 pathway^[76]. Phosphorylated SMAD2/3 can exclusively trigger NANOG transcription in hESCs to maintain pluripotency^[76]. bFGF binds to fibroblast growth factor receptors (FGFR) and leads to activation of PI3K/AKT and Ras/ERK1/2 signaling cascades, enhancing survival of hESCs^[72,76]. The pro-survival role of bFGF via activating AKT and ERK1/2 pathways ubiquitously exists throughout all kinds of cell types^[72].

Canine embryonic stem cells: The derivation of canine ESCs (cESCs) was relatively more difficult than human or mouse, and only a few groups succeeded in establishing cESC or ESC-like cell lines from canine blastocysts^[77-81]. All reports characterized canine ESC's pluripotency using molecular markers and *in vitro* differentiation^[77-81]. One particular work by Vaags, et al. showed convincing *in vivo* differentiation results in teratoma formation^[79]. In their study, cESCs displayed mixed morphology of three-dimensional dome-shape and monolayer-like colonies. The cells expressed the core pluripotency markers including OCT4, SOX2 and NANOG, and the surface markers SSEA-3, SSEA-4, and TRA-1-60 but without SSEA-1 expression, similar to the markers expressed by hESCs. The cESCs were capable of differentiation toward three-germ layer cells *in vitro*, and they could differentiate *in vivo* when injected into the kidney capsule of immune deficient mice. cESCs have also been differentiated into specific cell lineages including endothelial cells, cardiac cells, hepatocytes, neural stem cells, and endodermal cells. A unique feature of cESCs that sets them apart from human and mESCs is the dual requirement of growth factors, LIF and bFGF, to maintain pluripotency. While the signaling pathways of LIF and bFGF in mouse and human have been respectively characterized, little is known about the functions of the two factors applied simultaneously on cells *in vitro* for pluripotency and survival maintenance. In addition, a recent study reported the successful conversion of cESCs from primed-like status toward naïve like cells^[82]. The bFGF-dependent cESCs with primed pluripotency were converted to naïve pluripotency by using LIF and two small molecules cocktail via inhibiting glycogen synthase kinase 3 β and mitogen-activated protein kinase 1/2 associated pathways. The authors also indicated that the LIF-dependent cESCs might present more capacity in generating mature teratoma *in vivo* than the bFGF-dependent ones^[82].

Canine induced pluripotent stem cells: The generation, differentiation and transplantation of canine iPSCs (ciPSCs) would eventually allow us to preclinically evaluate the safety and efficacy of autologous iPSCs. But considering the difficulty in deriving cESCs and the low rates of reported teratoma formation from these cESCs, the generation of canine iPSCs (ciPSCs) attracted more attention as an alternative way to derived canine PSCs^[77,78,80]. To date, ciPSC were reported by several groups^[18,83-85]. The first report describing ciPSC generation was published in 2010 by Shimada, et al.^[83]. In 2011, two more groups respectively reported their research on establishing the ciPSC cell lines with relatively more comprehensive characterization^[18,84]. The studies showed that ciPSC lines display pluripotency markers and differentiation capacity similar to cESCs.

It was found that both LIF and bFGF are essential for ciPSC pluripotency maintenance, and LIF but not bFGF is critical for viability of ciPSCs^[18,83,84]. However, the LIF-only dependent ciPSCs were also reported and the addition of bFGF induced differentiation of ciPSCs toward fibroblastic cells^[86]. Reports were also published for differentiation of ciPSCs to specific somatic cell types such as platelet and mesenchymal stem cells^[87,88].

Due to the unlimited resource of cells, ciPSCs also provide an optimal model to study the regulatory system of pluripotency of canine PSCs. It has been recently indicated that bFGF manages ciPSC's pluripotency through a mechanism highly similar to that for primed embryonic stem cells^[17]. ciPSCs rapidly decreased the expression of NANOG, but not OCT4 or SOX2, and the similar expression profile could be observed when SMAD2/3 pathway was inhibited. The inactivation of the SMAD2/3 pathway promotes the spontaneous differentiation of ciPSCs toward neuroectoderm and mesoderm. Moreover, activin-A can replace bFGF to maintain NANOG expression of ciPSCs when cultured in the presence of feeder cells. The association between bFGF, activin-A, SMAD2/3 activation, and ciPSC's pluripotency is in accordance with that reported in primed hESCs^[76]. Interestingly, the simultaneous withdrawal of LIF and bFGF failed to inactivate ERK1/2 and AKT, which may be due to the autocrine/paracrine effect by ciPSCs or feeder cells based on other growth factor such as insulin growth factor or epidermal growth factor^[72]. These results indicate that the ciPSCs may stand close to epiblast stem cells on the pluripotency map. To further determine the pluripotency stage of ciPSCs, other critical properties that distinguish naïve and primed pluripotency apart should be evaluated, such as the level of H3K27me3 on developmental regulators, X chromosome activation/inactivation status and dependence of metabolism on oxidative phosphorylation and/or glycolysis^[74].

It has been demonstrated that ciPSCs seem to have an increased susceptibility to programmed cell death with escalated activation of caspase-3 when LIF is removed from the culture medium^[17]. PSCs display this vulnerability when undergoing spontaneous differentiation *in vitro*: when cultured under regular self-renewal-favoring conditions, hESCs undergo apoptosis at a rate of 30% and increasing up to 40% when allowed to spontaneously differentiate in normoxic conditions without LIF^[89,90]. In contrast with differentiated cells, both mouse embryos and mESCs cultured *in vitro* display hyposensitivity to the DNA damage^[91,92]. These observations indicate that pluripotent cells in general seem to have low tolerance for cellular stress and ultimately undergo cell death. Using drugs known to inhibit specific components of the LIF-associated signaling cascades, it was found that the withdrawal of LIF led to inactivation of JAK-STAT3 pathway, but had negligible impact on two other known LIF-associated signaling pathways, the AKT and ERK1/2 pathways^[17]. In addition, as with ciPSCs maintained without LIF, inhibition of the LIF-JAK-STAT3 pathway in ciPSCs triggered caspase-3 activation, DNA damage, and eventual cell death by apoptosis^[17,18]. A number of publications indicated that the JAK-STAT3 pathway is protective against multiple stressors, suggesting that activation of JAK-STAT3 may compensate unknown stress within the ciPSC culture environment which may be responsible for the cell loss during differentiation^[93].

Future direction

Due to the unique features of canine preclinical model and the advantage of iPSC technologies, the ciPSC model is promising to lead to the development of new treatment options for a variety of diseases and injuries, such as spinal cord injury. The results from ciPSC research suggest that different species are likely to display their own unique set of properties in their PSCs, such as the dependency on specific levels or types of growth factors. A recent study on naïve human ESCs has shown that their metabolism is distinct from that of naïve mouse ESCs, which indicates that the metabolic signatures, and possibly pluripotency signaling pathways of PSCs from different species may vary significantly^[94]. Therefore, it may be important to understand the similarity and difference between canine and human PSC's metabolism profiles, which may eventually favor the establishment of optimal culture conditions specifically supporting canine PSCs. A typical example is the porcine induced pluripotent stem cell which requires the continual activation of exogenous reprogramming factors to maintain its pluripotency, and this phenomenon was also reported in equine pluripotent stem cells^[13,95]. It is likely that the application of reprogrammed pluripotent stem cells in the preclinical evaluation or veterinary clinic will be more sophisticated than mouse or human counterpart. Despite these hurdles, the potential benefits by the application of these cell types to treat animals, and in turn offer valuable knowledge for the future application of iPSC-based cell therapy for human patients, will more than justify these laborious studies.

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