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The road less travelled: The efficacy of canine pluripotent stem cells

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ABSTRACT

The path-breaking discovery of induced pluripotent stem cells has fuelled the scientific advancements of stem cells. Nevertheless, the need to ensure the safety of stem cell therapy at translational level is still at large, prompting scientists to use animal models which are genetically and anatomically homologous to that of humans. Dogs, being genomically and physiologically more similar to humans serve as better models in mimicking human diseases as compared to rodents. The heterogeneity in canine breeds offers an excellent opportunity to comprehend the complexities of many genetic diseases, making them exceptional tools for stem cell therapies. Various canine gene therapy models have paved the foundation for strategizing therapies for humans. But a similar progress is lacking in utilizing canine stem cells for stem cell-based therapies in both dogs and humans. This review attempts to bridge the gap, by articulating the key differences in canine pluripotency pathways, based on the recent derivation of canine embryonic stem cells (cESCs) and canine induced pluripotent stem cells (ciPSCs), thereby attempting to position dog in the reprogramming landscape. The potential clinical application of canine iPSCs also offers great hope to canine patients and might lead to significant contributions in veterinary medicine.

1. Introduction

Pluripotent stem cells (PSCs) have garnered a lot of attention recently with their promising aspects for therapies in regenerative medicine. A lot of stem cell research is carried out in rodent models which are less expensive and easier to handle, but there are many limitations in the understanding of the mechanisms of genetic diseases which are not naturally occurring. Large animal models such as dogs, being closest to the human system and easier to maintain, can hold a key position in translational research [1]. The distinctive evolution of domestic dogs is predominantly suitable for causal genetic factor analysis of complicated diseases by genome-wide association studies. Dogs, like humans, originated from a common ancestor 36,900–41,500 years ago and diversified across the globe [2]. The domestic dog evolved from grey wolf, their origin and divergence being supported by molecular phylogeny studies. The biology of some canine cancers is analogous to that of human cancers [3] as they also share food, environment and carcinogenic load [4] with greater similarities in disease progression.

ciPSCs have been derived by various reprogramming approaches with different media conditions. This review aims to explore the possibilities of canine PSCs in disease modeling and to understand the molecular cascades involved in canine reprogramming. Understanding the evolutionary link of ciPSCs to that of human and mice is essential for planning and optimizing reprogramming strategies and culture conditions.

1.1. Dog as an alternate model

Dog has been the favorite companion of humans for centuries. They are more physiologically and histologically similar to humans compared to rodents [5] which might provide an added advantage of translation from lab to clinics. The size of dogs makes them more amenable for serial blood and bone marrow sampling and intravenous (IV) infusions than that of mice [6]. Among the 450 types of genetic

Abbreviations: cESCs, canine embryonic stem cells; ciPSCs, canine induced pluripotent stem cells; PSCS, Pluripotent stem cells; mESCs, mouse embryonic stem cells; hESCs, human embryonic stem cells; ICM, inner cell mass; LIF, leukemia inhibitory factor; bFGF, basic fibroblast growth factor; DMD, Duchenne muscular dystrophy; AD, Alzheimer's disease; SCI, spinal cord injury; iMEF, inactivated mouse embryonic fibroblasts; EB, embryoid body; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; KSR, knock out serum replacement; MEF, mouse embryonic fibroblasts; STAT3, Signal Transducer and Activator 3; XEN, eXtra embryonic Endoderm

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diseases amid the diverse canine breeds, 360 of the canine diseases are related to particular human genetic diseases, considerably higher than in other domestic animals [7].

The canine genome project, a 30 million dollar mission by National Human Genome Research Institute (NHGRI) was completed in 2004, at National Institute of Health (NIH), by whole-genome sequencing of Tasha, a female boxer [8]. Reciprocal chromosome painting identified 68 evolutionarily conserved segments between human and dog karyotypes, suggesting the possibility of selecting markers of genome screening for specific evolutionarily conserved disease genes [9]. Canine genome map offers a prospect to comprehend the genetic complexities underlying common genetic diseases as well as rare genetic disorders in human populations [10,11]. Various congenital malformations, cardiovascular diseases, diabetes mellitus, muscular dystrophy, epilepsy [12], retinal atrophy, Alzheimer's disease(AD) [13], and several forms of cancers [3] are among them. The pathology and clinical phenotype of canine Duchenne muscular dystrophy (DMD) closely mimic that of human [14]. Mice DMD models show very few clinical symptoms and severe phenotypes like heart failure and muscle wasting occurs only in older mice. The disease severity is more pronounced in canine models. The most preferred animal model for muscular dystrophy regenerative treatments is Golden retriever MD dogs [15].

Gene therapy studies in canines have been pivotal in establishing therapy strategies for Hemophilia A and B [16,17]. Canine tumors are spontaneous and naturally similar to that of humans, as in canine mammary tumor and human breast cancer [18]; in chronic myelogenous leukemia, the genetic translocations are the same in both dogs and humans [9].

Canine models can be effectively used for accurate modeling of many human diseases, which is not possible in mice. One such scenario is in the study of neurodegenerative diseases. Dogs exhibit a natural manifestation of age-related decline in cognitive functions [19]. The pathology and clinical relations to that of the human AD make the dog a good model to attempt therapeutic strategies. A detailed citation of the common diseases in human and dog is given in the Table 1.

1.2. Embryonic development in dog

A clear knowledge of embryonic development in canines is essential for enhancing the use of embryo technologies, genetic manipulations and stem cell therapy. Embryonic and fetal development in dog occurs in shorter durations than in other animals [54]. Key features in the reproductive physiology of dog are given in Table 2.

Many features of canine reproductive physiology and development are markedly different from other mammalian species [55]. Assisted reproductive technologies such as in vitro fertilization (IVF), in vitro culture (IVC) and in vitro maturation (IVM) have been conventionally used for the canine in vitro embryo development. But low efficiency has been detected for all these techniques which may be due to the variances in canine development, high polyspermy and decreased fertilization capacity [56].

1.2.1. Canine pluripotent stem cells (cPSCs)

1.2.1.1. Canine embryonic stem cells(cESCs). mESCs exhibit small, three-dimensional, dome-shaped, tightly packed colonies and hESC colonies have distinct borders and a flattened appearance. Interestingly, cESCs exhibit both types of morphology [6,58–61]. There exists two different states for ESCs, naive and prime. mESCs, derived from the inner cell mass(ICM) of pre-implantation embryos characterizes the naive state, while hESCs obtained from the post-implantation epiblast represents the primed state [62].

Generation of germ-line competent ESCs from dogs is still considered to be an uphill task as a species-wide difference exists in the culture and development of embryos. In comparison with that of human and mouse, fewer reports are available on the development of canine

Table 1
Shared features of diseases in dog and human.

Diseases	Defective gene/ protein	References
Cardiovascular diseases		
Juvenile dilated cardiomyopathy	*PDK4, STRN*	[20] [21]
Tricuspid valve dysplasia	*CTVM*	[22]
Neuro muscular system diseases		
Epilepsy	*ADAM23*	[23]
X-linked muscular dystrophy	*DMD*	[24]
Canine hip dysplasia (CHD)	*CFA01*	[25]
Neuronal ceroid lipofuscinoses	*CLN*	[26]
Myotonia congenita	*CLCN1*	[27]
Cerebral amyloid angiopathy	*PS1,PS2*	[13]
Immuno/hematological diseases		
Hemophilia B	*FIX*	[28]
Hemophilia A (Factor VIII deficiency	*F8*	[29]
X-linked severe combined Immunodeficiency	*IL2RG*	[30]
Cystinuria	*SLC3A1*	[31]
Hereditary nephropathy (HN)	*COL4A4*	[32]
Narcolepsy	*Hcrtr − 2*	[33]
Progressive retinal atrophy	*PRCD*	[34]
Oculoskeletal dysplasia (OSD)	*Col9A1, Col9A2*	[35]
Osteogenesis imperfecta(OI)	*SERPINH1*	[36]
Cancer		[36]
Mammary tumor	*ESR1*	[37]
Gastric cancer	*IL − 2R gamma, EGFR, HER − 2*	[38]
Transitional cell carcinoma (TCC) of the bladder	*EGFR,CDKN2B, PIK3CA,*	[39,40]
Non-Hodgkin's lymphoma	*TRAF3,NIK*	[41,42]
Osteosarcoma	*Rb, RECQL4, PTEN*	[43]
Oral melanoma	*NRAS,PTEN*	[44,45]
Head and Neck Squamous cell carcinoma(HNSCC)	*AKT1,TWIST1, SNAI1*	[43,46]
Fucosidosis	*FUCA1*	[47]
Globoid cell leukodystrophy	*GALC*	[48]
Dermatological diseases		
Fragility syndrome	*PKP1*	[49]
X-linked ectodermal dysplasia	*EDA*	[49]
Epidermolysis bullosa	*PLEC1*	[50]
Exfoliative cutaneous lupus erythematous	*SIPA1*	[51]
Ichthyosis	*NIPAL4*	[52]
Lethal acrodermatitis	*MKLN1*	[53]

Table 2
Key features in the reproductive physiology and development in the dog [57].

Features	Dog
Time of ovulation	1–2 days after onset of oestrus
First primordial follicles	11 days after birth
Duration of oestrus	9 days
Oestrus cycle length	Monocyclic up to 2 months
Time of maternal recognition of pregnancy	Poorly understood
Passage into the uterus	8 days after ovulation
Time of blastocyst formation (days after ovulation)	8
Major genome activation	Fourth cell cycle (8-cell embryo)
Type of placenta	Endotheliochorial
Gestational length	63 (58–68 days)
Size of the embryo proper/foetus (length or CRL)	> 160 mm
Average number of offspring	6–12
Chromosome number	78

Table 3
Summary of derivation of cESCs.

References	Culture conditions	Growth factors	In vitro Differentiation	Teratoma formation	Naive/Prime (SSEA expression)
cESCs					
[59]	DMEM/F12, 20% FBS	mLIF	EB formation	ND	SSEA1
[60]	DMEM/F12, 15% FBS	mLIF hLIF	EB formation, directed differentiation	ND	SSEA1
[58]	DMEM/F12, 20% FBS	hLIF	EB formation, directed differentiation	Unsuccessful	ND
[6]	DMEM/F12, 20% FBS	hLIF, bFGF bFGF	In vitro differentiation	Teratomas	SSEA3,SSEA4, low levels of SSEA1 SSEA1
[61]	DMEM/F12, 20% FBS	hLIF, bFGF	EB formation	No overt teratomas	SSEA3

Abbreviations: DMEM- Dulbecco's Modified Eagle's Medium; FBS- fetal bovine serum; bFGF,-basic fibroblast growth factor; LIF-leukemia inhibitory factor; ND-Not detected; NT-Not tested.

ESC lines (Table 3).

Hatoya et al. [59] first reported the derivation of canine ESCs. They isolated 80 embryos from 15 dogs at morulae, blastocyst and hatched blastocyst stage. The latter stage produced a maximum number of colonies in culture. Efficient colonies were derived by mechanical disaggregation. These colonies were dome-shaped and had high nuclear to cytoplasmic ratio as shown in Fig. 1. The cells were grown on inactivated mouse embryonic fibroblasts (iMEF) feeder layer and failed to grow in gelatin, even in the presence of LIF. These cells were positive for alkaline phosphatase activity, Oct4 pluripotency marker expression, formed EBs. Simple EBs could differentiate into various cell types like myocardial-like cells, neuron like cells, fibroblast like cells, epithelial like cells without LIF and feeder layer in tissue culture plates [59].

Schneider et al. derived cES-like cells from blastocyst stage canine embryo. cES- like cells were co-cultured with OP9 cells(mouse bone marrow stroma cell line) and differentiated into hematopoietic cells [60].

Hayes et al. [58] collected 67 embryos from 10 pregnant dogs at 12–16 days and derived cESC lines from blastocyst stage embryos, with 13–14 day embryos expressing mature blastocyst morphology. cESCs were cultured in inactivated MEFs for three passages and subsequently maintained in SNL 6/7 feeder cells; a mouse cell line transformed with murine LIF. Cells cultured in 0.5% FBS/Activin A/BMP4 showed positive *AFP* expression; cells cultured in 10% knockout serum were positive for *β3tubulin* and γ-*enolase* genes. cESCs did not show expression of SSEA and TRA markers but exhibited positive pluripotency marker expression and alkaline phosphatase activity. Positive expression of three ESC specific microRNAs; miR 302-b, miR 302-c and miR 367, along with constitutively expressed miR16, all homologous to that of human were observed in canine ESC lines. The latter but not the former micro RNA expression was observed in canine adult tissue. Chromosomal aberration, trisomy 8 was observed upon single-cell passaging with TrpLE. Four different attempts of teratoma formation were done by injecting canine ES cells into the testis, kidney capsule, heart and also subcutaneously in NOD/SCID mice but all were unsuccessful [58].

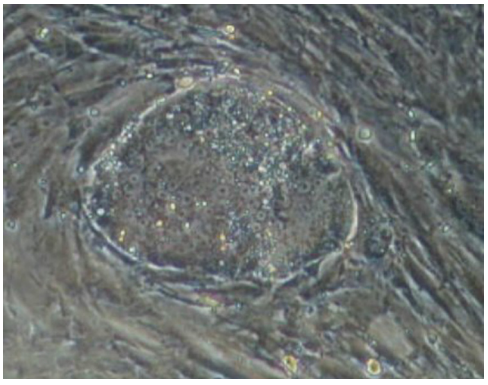

Fig. 1. Morphology of canine embryonic stem cell at passage 3 (mechanically passaged). Image adapted with author [59] permission.

Vaags et al. [6] isolated 122 embryos from 16 bitches at varying stages from 16 celled to the hatched embryo. Different culture conditions were assessed to check the efficient ESC in vitro growth medium based on the hatching, maintenance capability, and maturation in vitro. Embryos were allowed to grow in DMEM F12 with 15% FBS or with 15% KOSR with the former condition more efficient in the spontaneous hatching of the embryos. These cESCs expressed SSEA3, SSEA4, TRA-1–60, and TRA-1–80 similar to human ESCs. When cultured in the absence of hLIF, cESCs spontaneously differentiated, with reduced pluripotent and SSEA4 marker expression [6].

Wilcox et al. [61]. isolated 238 canine embryos at morulae and blastocyst stages from 33 bitches. Two morphologically diverse cESC lines were established; one set by embryo explants (OVC.EX) and the second set by immunodissection of ICM (OVC.ID). The former yielded pure ESC colonies with a surrounding layer of autologous fibroblast-like cells and the latter yielded mixed colonies with hypoblast like feeder cells (cHF). Colony morphology of OVC.EX line was dome-shaped and resembled mouse ESCs while that of OVC.ID lines were flat, similar to hESCs. Both lines were stable and exhibited all the ESC characteristics. cESCs differentiated into fibroblast-like cells upon LIF removal. Cells were inactivated with mitomycin C and used as a feeder layer for further experiments. OVC-EX derived cES cells appeared to differentiate to fibroblast-like on the addition of bFGF. 1×10^6 cESC cells were injected into the testis capsule of NOD mice but no teratomas were formed [61].

In a further report by the same authors, the derived OVC.EX line was further differentiated into healthy neural progenitors and mature, synaptically active neurons by lineage selection with Noggin and EGF. A sufficient amount of these progenitor populations can be used in cell therapy for spinal cord injury (SCI) in canine patients [63].

Similar to mouse ESCs [64], canine ESCs could consist of subpopulations of cells that might resemble different developmental phases or grades of cell specification [61]. Mouse ESC cultures comprise a heterogeneous population of cells corresponding to epiblast, ICM and primitive ectoderm depending on the manner and point of embryo derivation [64]. The colony morphology of cESCs isolated by Vaags et al., are described as heterogeneous, few resembling that of mouse ESCs with a 3D appearance and few of human ESCs with a flat appearance [6]. While one report suggests similarity of cESCs to that of mink ESCs [58], another group reported that the cells exhibit limited

self-renewal and fail to go beyond 8–10 passages [59,60]. Teratoma formation was reported by only one group [6].

Canine ES cells have been differentiated into different cell types such as myocardial-like cells, neuron like cells, fibroblast like cells, epithelial like cells [59], hematopoietic progenitors [60].

1.2.2. Canine iPSCs

Although embryonic stem cells have several advantages, there are serious concerns regarding their use, including ethical reasons, accessibility, teratoma formation and immunological hurdles. The search for alternate forms of cells with similar ESC properties led to numerous studies in a specific line of thought, resulting in the development of iPSCs by Shinya Yamanaka and Takahashi in 2006. Yamanaka group demonstrated that reprogramming somatic cells into a pluripotent state was indeed possible by the over-expression of four transcription factors, Oct4, Sox2, Klf4, Cmyc(OKSM) [65]. These iPSCs exhibited unlimited proliferative potential, expressed ESC specific genes, formed embryoid bodies (EBs), teratomas and contributed to chimeric embryos, all significant features of ESCs. There has been an exponential growth in iPSC research within a short period since the original publication by Yamanaka et.al and iPSCs have been derived from many mammals including the dog. Much literature is available on the mouse and human iPSCs, but less number of reports has been published on iPSC production from dogs. A summary of reports on cESC and ciPSC derivation is given in Tables 3 and 4 respectively.

ciPSCs has been generated from various cell sources like canine embryonic fibroblasts, dermal fibroblasts, testicular fibroblasts, adipose mesenchymal stem cells(MSCs) and adipose stromal cells. Shimada et al. [66] first reported canine iPSC production from canine embryonic fibroblasts using lentiviral transfection with canine OSKM factors.

ciPSCs has been derived from both pure and mixed [67,68] dog breeds like beagle [69,70], poodle [71], weimaraner [72] and German shorthair pointers [73]. Whether the genetic diversity of dog breeds may also affect the reprogramming has to be further analysed [72].

Nishimura et al. reported a feeder-free culture of ciPSCs in a doxycycline-inducible system [69]. Alternately, others reports used inactivated mouse embryonic fibroblasts (MEFs) as the feeder layer.

1.2.3. Reprogramming strategies in ciPSCs

Researchers have been expending various transcription factors in the reprogramming cocktail for developing quality iPS cells which are therapeutically safe. Though the initial protocols were based on lentiviral and retroviral-based reprogramming; further strategies have been employed for iPSC generation, primarily due to integration issues. Reprogramming by Sendai viruses and episomal vectors are also possible albeit with varying reprogramming efficiencies [71]. Use of transfected mRNA, miRNA are non-integrative approaches with high efficiency but repeated transfections are required and the procedure is expensive. Small molecules have been used for reprogramming which are inhibitors of histone deacetylase and histone methyltransferase, such as valproic acid,BIX01294, Vitamin C and anti-p53 specific siRNA [74].

ciPSCs have been generated by retroviral, lentiviral and by sendai virus methods. Hatoya et al. reported integration-free derivation of ciPSCs by the sendai virus method, using SeVdp (KOSM)302 L [75]. Chow et al. derived ciPSCs by sendai virus reprogramming kit [71], but with low efficiency as, only one viable colony was formed even after multiple transfections.

Human or mouse reprogramming factors are commonly used for iPSC derivation in animals. Shimada et al. [66] derived ciPSCs by canine OSKM but most groups used human reprogramming factors [67,72,73,76,77]. Koh et al. and Nishimura et al. [69,70] used mouse OSKM factors for reprogramming. Goncalves et al. generated ciPSCs by murine and human OSKM factors separately and in combination, using lentiviral method. ciPSC lines reprogrammed by mOSKM showed exogenous expression as being in control, while complete silencing was

Table 4
Summary of derivation of ciPSCs.

References	Reprogramming factors	Culture conditions	Growth factors	In vitro Differentiation	Teratoma formation	Naive/Prime (SSEA expression)	Epigenetic validation
[66]	Retrovirus canine OSKM	Primate ES medium	bFGF, hLIF + 3i VPA	Embryoid bodies	NT	NT	NT
[73]	Lentivirus hOSKM	DMEM/F12, 15%KSR	hbFGF, hLIF	Embryoid bodies	ND	SSEA4	De methylation of Nanog promoter
[74]	Lentivirus hOSKM	KO DMEM, 20% FBS	hbFGF,hLIF	Embryoid bodies	Teratomas	SSEA4	NT
[67]	Lentivirus(human OSKMLN)	KO-DMEM/F12, 20% KSR	mLIF + 3i	Embryoid bodies	Germ cell-like tumor	SSEA4,low levels of SSEA1	X chromosome reactivation
[75]	Lentivirus hOS	DMEM/F12, 20% KSR	hbFGF,hLIF	NT	NT	NT	NT
[70]	Retrovirus mOSKM	DMEM/F12, 20% KSR	hLIF + 2i,hbFGF	Embryoid bodies	Teratomas	SSEA1	NT
[68]	Lentivirus hOSKM	DMEM/F12, 20% KSR	hLIF,hbFGF	Embryoid bodies	NT	SSEA4	NT
[72]	Retrovirus hOSKM	DMEM/F12, 15% FCS	bFGF, LIF	Embryoid bodies	NT	SSEA4	X chromosome reactivation
[76]	Lentivirus mouse and human OSKM	KO-DMEM/F12, 20% KSR	bFGF	Embryoid bodies	Teratomas	NT	NT
[69]	Lentivirus mOSKM	Serum free N2B27	hbFGF	Embryoid bodies	ND	SSEA4	NT
[71]	iPS reprogramming kit (sendai virus, hKOS)	KO-DMEM, 20% FBS	hLIF	Embryoid bodies	Teratoma	NT	NT
[78]	Sendai Virus(SeVdp KOSM)	DMEM/F12, 20% KSR	LIF, bFGF	Embryoid bodies	Teratoma	SSEA1	NT
Unpublished	Retrovirus hOSKM	DMEM/F12,20% FBS 20% KSR DMEM/F12, 20% FBS	LIF	Embryoid bodies	ND	SSEA1	NT

Abbreviations: DMEM- Dulbecco's Modified Eagle's Medium; FBS- fetal bovine serum; bFGF,-basic fibroblast growth factor; KSR- knockout serum replacement; LIF- leukemia inhibitory factor; MEF- mouse embryonic fibroblasts, ND- Not detected; NT- Not tested.

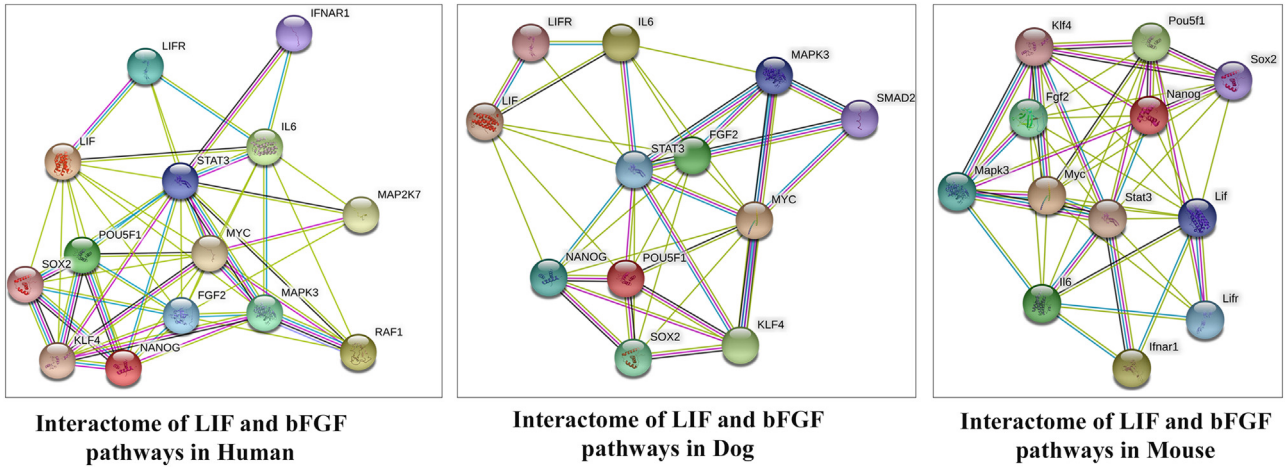

Fig. 2. Interactome of LIF and bFGF pathways in Human,Dog and Mouse respectively.

observed in hOSKM derived lines [78]. It is not yet clear whether species difference in reprogramming factors has any effect on canine iPSC derivation.

1.3. Pathways in pluripotency

Various pathways interact with each other to enable the efficient maintenance of ESCs and iPSCs, and extensive research work is being done in LIF/ Signal Transducer and Activator 3 (STAT3), TGFβ, BMP/ SMAD, MEK/ERK, FGF pathways thereby demonstrating the necessity of two essential growth factors, LIF, and bFGF in the growth medium. hESCs,respond to Fibroblast Growth Factor-2 (bFGF/FGF2) which activates the transforming growth factor beta signaling and regulates the MEK/ERK pathway [79]. mESCs respond to LIF which along with BMP proteins regulate JAK-STAT pathway for self-renewal [62]. While ICM derived mESCs are dependent only on LIF, a distinct population of pluripotent stem cells derived from the mouse postimplantation epiblast, named EpiSCs, seem to require only bFGF. Due to a reduced amount of LIF receptor expression, LIF does not maintain pluripotency of primed hESCs. bFGF promotes differentiation in mESCs, by activating ERK1/2.

Interestingly, the majority of derived ciPSCs favour twin-factor culture of both LIF and bFGF [66,68,70,72,73,76,77]. Whitworth et.al, reported LIF [67] only; Nishimura et al. [69] and Gonçalves et al. [78] reported bFGF only for proliferation. All reported cESCS were derived in the presence of LIF. Absence of LIF showed spontaneous differentiation [6].

The influence of STAT3 on pluripotency maintenance in ciPSCs might be different from that in naive-mESCs. In order to get mechanistic insights into pluripotency of ciPSCs, Luo et al. cultured the cells in the presence and absence of LIF and bFGF and found that the removal of both LIF and bFGF ($LIF^-/bFGF^-$) caused the inactivation of STAT3 straightaway on day 1. The removal of LIF caused STAT3 dephosphorylation from day 2 whereas AKT and ERK1/2 were constantly activated in all. Further, LIF withdrawal or the inhibition of JAK-STAT3 pathway induced apoptosis, thereby implying that LIF is essential for survival [73]. In a later report, Luo proposed that bFGF regulates pluripotency in ciPSCs which indicates similarity to primed cells. Significant repression of NANOG was observed in the absence of bFGF / inhibition of SMAD2/3 pathway and ciPSCs were driven to spontaneously differentiate into neuroectoderm and mesoderm in both conditions. The addition of Activin A showed the reversal of the NANOG expression, which indicates the similarity to primed cells. Conversely, LIF^-/inhibition of JAK showed significant upregulation of NANOG, FGF5, and the cell cycle inhibitor P21, but no significant change in differentiation-related genes. Interestingly, ERK1/2 or AKT activation was not impacted by LIF^- or $bFGF^-$ which might be due to the presence of feeder cells [80].

Koh et al. also reported a loss of pluripotency and reduced alkaline phosphatase staining upon the withdrawal of LIF or bFGF, but did not observe any apoptosis in the absence of LIF [70]. ciPSCs derived by Lee et al. showed the loss of colony morphology in absence of both LIF and bFGF and the loss of expression of Oct-4 and TRA-1–60. A loss of colony morphology was observed upon the withdrawal of MEF as well [76]. LIF-dependent ciPSC colonies, derived by Whitworth et al. differentiated into fibroblast cells in the presence of LIF and bFGF, similar to cESCs derived by Wilcox et al. [61,67].

But the FGF-only ciPSCs derived by Goncalves et al. exhibited no change in maintenance or proliferation, with or without LIF [78].

It is necessary to understand the state of ciPSCs for their efficient culture and maintenance. Whitworth et al. reported the expression of Rex1, which is not expressed in EpiSCs but is ICM specific, and claimed that their ciPSCs belonged to a more naïve state [67]. This can also be due to the use of six reprogramming factors, in place of the usual four Yamanaka factors and 2iL medium (GSK3b and MEK inhibitors with LIF).

The companionship of LIF and bFGF may be essential for activating the functional framework of endogenous pluripotency genes in ciPSCs for their long-term maintenance and genetic stability. The necessity of LIF and bFGF2 addition to ciPSC culture also indicates the probability of an intermediate state between naïve and prime. The existence of various pluripotent states of ciPSCs has to be scrutinized further for a thorough understanding of the iPSC characteristics [81].

String database [82] was used to understand the protein interactions of LIF and bFGF in human, dog and mouse, using a combination of association methods such as gene fusion, neighbourhood, co-occurrence, experiments and databases with a medium confidence score of 0.4. Protein-protein interaction network was constructed by selecting LIF and FGF as the query proteins for human, dog and mouse, as selected organisms. The interactome of LIF and bFGF in human, dog, and mouse is given in Fig. 2.

In the network, a protein (gene) is represented by a node, and protein interactions are represented by lines. Evidence in the network edges were selected where the line colour indicated the evidence for the type of interactions. Different colours of interaction lines showed different molecular actions; green line represents gene neighbourhood, blue line for gene co-occurrence, yellow line for text mining, red line for gene fusions, black line for coexpression and purple line for experimentally determined associations. A tight protein network was observed in the interactomes indicating the role of individual proteins in stem cell maintenance and development. The string interactomes also gave a preliminary indication regarding the interacting partners of LIF

and bFGF. This pointed to the basic differences that may exist in the pathways which can be further analysed using functional proteomics experiments.

A more detailed analysis might point to the difference in the pluripotency maintenance in dog and human, thereby indicating the consistency in genetic stability of canine iPSCs. More detailed functional analysis of LIF, bFGF interactome is required for an evidence-based study.

1.3.1. iPSC characterization

iPSC characterization can be done by colony morphology, pluripotency marker expression, alkaline phosphatase staining, embryoid body formation, lineage gene analysis and in vivo by teratoma formation. Different colony morphologies of ciPSCs have been reported by various groups. Prime type cells, characterized by flat colony morphology, similar to hESCs were reported with a high nuclear to cytoplasmic ratio [66,68,70,72,73,76]. Naive type cells, characterized by dome-shaped colony morphology were reported by other groups [67,69]

Chow et al. reported long-term passaging up to 100 for six months of culturing [71]. Nishimura et al. reported long-term culturing of ciPSCs beyond passage 50 with normal karyotypes and pluripotency marker expression [69]. Two ciPSC lines derived by Hatoya et al. were maintained for 40 and 30 passages [75]. Canine iPS cells reported by Luo et al. [73] showed normal karyotypes, expressed pluripotency markers and were devoid of transgene expression. Baird et al. derived ciPSCs from aneuploid adipose-derived MSCs and reported that aneuploid karyotype was maintained, though further aberrations were not analysed [72]. Goncalves et al. reported the absence of morphological or karyotypical changes for 15 passages. But this has not been true in other cases.

Koh et al. analysed the notch of chromosomal instability in ciPSCs for the first time by comparative genomic hybridization (aCGH), combined with fluorescent in situ hybridization (FISH) [70]. Though the iPSC lines derived have been reported to be normal, later passages have shown chromosomal aberrations [83] on particular chromosomes 4, 8, 13 and 16, probably acquired after passages. This can be due to an integration of viral genes, prior mutations in the cell source itself or due to stress in expansion and differentiation.

Whitworth et al. observed complete transgene silencing in ciPSCs after passage 26 [67], whereas transgene expression was observed in the derived ciPSCs reported by few groups [68,69,72,73,77,78].

Complete teratomas were reported by five groups [70,71,75,76,78]. Whitworth et al. developed germ cell-like tumors [67]. In comparison to the undifferentiated iPSCs, tumors reported by Koh et al. demonstrated high transgene expression, leading the authors to propose that high exogenous expression in cells may have a proliferative advantage in formation of teratomas [70]. But though there was a continuous doxycycline-inducible exogenous expression, Nishimura et al. failed to produce teratomas with bFGF–only ciPSCs [69] wherein the presence of both LIF and bFGF for teratomas was suggested. However, Goncalves et al. reported teratoma with bFGF–only cultures. The authors derived stable ciPSCs from canine dermal fibroblasts with LIF alone but could not form teratomas in immunodeficient mice (unpublished data as in Table 4). There is still some uncertainty in teratoma formation regarding the species specificity of growth factors, as human and mouse reprogramming factors are currently being used for ciPSC derivation and propagation. Also, the efficacy of teratoma as a golden standard for reprogramming has been under debate [84].

Stage-Specific Embryonic Antigens (SSEAs) expression indicates a naive or prime pluripotency state; mouse PSCs express high levels of SSEA-1 and human PSCs express SSEA-3 and SSEA-4. Peculiarly, SSEA-4 expression was reported by six groups [67–69,72,73,76]and two groups reported SSEA-1 expression [70,75]. Interestingly, in cESCs, two groups reported SSEA-1 expression [59,60]and Vaags et al. reported both SSEA-3 and SSEA-4 similar to that of hESCs and low levels of SSEA-1 [6].

X chromosome reactivation is one of the important epigenetic modifications that occur in iPSC reprogramming from differentiated somatic cells, by removal of trimethylation of H3K27; H3K27me3 is a characteristic feature of X chromosome inactivation. X chromosome reactivation is clearly defined in female mouse iPS cells [85] and limited to ICM derived mESCs [86]. X chromosome reactivation was observed in ciPSCs derived by two groups characterized by the absence of H3K27me3 staining [67,72].

1.4. Applications of canine pluripotent stem cells

Clinical applications of iPS cells in humans have been highlighted recently. iPS derived retinal pigmental epithelial(RPE) cells has been transplanted into macular degeneration patients [87]. The first clinical study for the transplantation of iPS based cells for subacute SCI is under trials in Japan from Yamanaka group [88]. Sendai viruses encoding Yamanaka factors has been used to reprogram human melanocytes and melanoma cell lines [89].

Due to greater parallels in disease progression, canine models can be used in molecular and gene therapies, for both canine and human diseases and also in diagnostic studies. Veterinarians have been using stem cell therapies for orthopedic cases, degenerative spinal cord disc degeneration and in canine cancer patients using mesenchymal stem cells [90–92]. Autologous olfactory glial cells were transplanted in severe SCI canine patients [93]. SCI Canine models are used for the purpose of cell-based therapies for cardiac diseases and these can predict the precise outcome in humans [1]. Accurate reproducibility of naturally occurring diseases in dogs makes them more efficient models than mice.

ciPSCs can be derived from easily accessed tissues such as skin or blood, unlike cESCs. Though there have been many canine models of genetic diseases as shown in Table 1, only a few reports are available on the application of ciPSCs for therapy and also as disease models.

The potential of ciPSCs to be used as a model system was first demonstrated by Baird et al. They derived ciPSCs from aneuploid adipose-derived MSCs and reported that aneuploid karyotype was maintained [72]. Autologous transplantation of iPSCs in a large animal model was first reported by Lee et al. They derived endothelial cells from the ciPSCs which were used to treat immune-deficient murine models of myocardial infarction and hind limb ischemia [76].

ciPSCs have been differentiated to platelets and mesenchymal stem cells [67,68,71,94].

Whitworth et al. reported that along with the expression of various mesenchymal markers, ciPSC-MSCs exhibited efficient differentiation into osteo, chondro and adipogenic cells in respective induction media, plastic adherence and also a histological similarity to adult canine adipose-derived MSCs. They also showed that ciPSC-MSCs can be maintained in hydrogel and enhances chondrogenesis being functionalized with pentosan polysulfate. This can serve as cell therapy in osteoarthritis in dogs and also as an effective model for degenerative joint disease in humans [94].

Canine iMSCs derived by Chow et al. showed rapid proliferation and immune modulatory characteristics, similar to that of canine Ad-MSCs and BM-MSCs. The safety of canine MSCs was evaluated by systemic injection to immune-deficient mice and to adult dogs for teratoma and tumor formation respectively. Dogs were monitored up to 15 months and were found to be healthy. No evidence of teratoma was seen in mice during the 6 months of observation [71].

Brevini et al. reported epigenetic conversion of canine fibroblasts into insulin-producing cells (EpiCC), and suggested its use in translational studies [95].

Nishimura et al. reported the derivation of canine induced XEN (ciXEN) like cells by transgene induction in serum-free N2B27 medium with LIF and bFGF supplementation. These cells were maintained for over 50 passages with inhibitors of TGFβ and MAPK p38. ciXEN exhibited features similar to that of eXtra embryonic Endoderm (XEN)

cells and could be differentiated into hepatocytes [96].

ciPSCs can be an ideal model system for neurological and neurodegenerative disorders. Validation studies in canine models are also more efficient than those in mice. The relatively short lifespan of mice limits their use in progressive diseases like Alzheimer's and Parkinson's [97]. The genetic predisposition to some types of cancer in certain canine breeds can be utilized to decipher the associated genetic aberrations. Prospective applications of ciPSCs also include their effective usage in screening and validation of novel compounds for toxicity studies, pharmacological and biomarker screening and drug discovery thereby establishing it as a competent model.

2. Conclusions

Dog is a highly suited biomedical model for the development of stem cell therapies and for long-term experiments, as dogs can be maintained with relative ease whereas preclinical trials of primate models can be done only in controlled environments with proper animal care facilities. Though there are less ethical constraints for use of new therapies in dogs, only limited studies have been reported till date. Dog can be used as model systems for human diseases that don't occur in any other mammalian systems. The shared environment, food sources and similar disease predispositions to that of humans make the dog an ideal model. Canine iPSC derived disease models will provide an efficient platform for pathophysiological assessments, in vitro toxicity studies and for treatment validation. Recent advances in gene editing like CRISPR-CAS9 technology is an efficient tool to generate ciPSC models. A translational approach of ciPSC derived cells in dogs will reduce the ethical constraints around cESCs and will also reduce the possibilities of immune rejection.

Several signaling pathways are associated with the reprogramming and maintenance of canine pluripotent stem cells, but there is an ambiguity in the complete depiction. The pluripotency pathways of canine may differ from that of mouse and humans and a clear understanding of the pluripotent signatures can aid in instituting optimal culture conditions for canine stem cells.

There is an urgent requirement for more research on basic genetic mechanisms in canine models for the assessment of the efficacy and safety of stem cell therapy in dogs and humans. "One Health" initiative involving physicians, veterinarians, and other scientific professionals and the liberal and conducive regulatory settings for animal model therapies can encourage the efficient translation of therapies in human and veterinary medicine. This will ensure that unmet medical needs are addressed.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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