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Induced pluripotent stem (iPS) cells in domestic animals − recent achievements − a review*

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Pluripotent cells show the unlimited self-renewal capacity and the potential to differentiate into the cell lineages originating from three germ layers. To date real pluripotent stem cell lines, able to generate germ line chimeras, have been established in rodents. Large domestic animals, especially pig, due to their considerable anatomical and physiological similarities with humans are thought to be convenient model to test potential of pluripotent cells in cell replacement therapy before their clinical use. However, creating pluripotent cells in domestic animals until now has met the limitations due to lack of evidence for germ line contribution. Therefore they can not be considered real pluripotent cells but rather putative embryonic or embryonic-like stem cells. Recently, much attention has been drawn to the reprogramming technologies which enable redirection of differentiated somatic cells to the pluripotent status. The aim of this review is to present the recent achievements in generating of induced pluripotent stem (iPS) cells in domestic animals.

KEY WORDS: differentiation / iPS cells / pluripotency / proliferation / regenerative medicine / reprograming

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Introduction

The establishment of embryonic stem (ES) cell lines obtained from mouse [Evans and Kaufmann 1981, Martin 1981] and human [Thomson *et al.* 1998, Shamblott *et al*. 1998] embryos revolutionized cell and developmental biology. Since then the idea of cell therapies based on an unlimited self-renew potent source of cells have become an attractive concept of regenerative medicine [Wobus and Boheler 2005]. However, isolation of pluripotent cells from inner cell mass (ICM) of early embryos in humans faces serious ethical concerns.To circumvent this, much effort has been done into generation of pluripotent cells *via* reprogramming of somatic differentiated cells. To achieve this, the following methods have been used: somatic cell nuclear transfer (SCNT) [Wilmut *et al*. 1997], culture of somatic cells with cell extracts isolated from embryonic stem cells [Xu *et al*. 2009] or oocytes [Miyamoto *et al*. 2009], fusion of somatic cell with pluripotent cell [Silva *et al*. 2006] and generation of induced pluripotent stem cells by overexpression of defined transcription factors (Fig. 1).

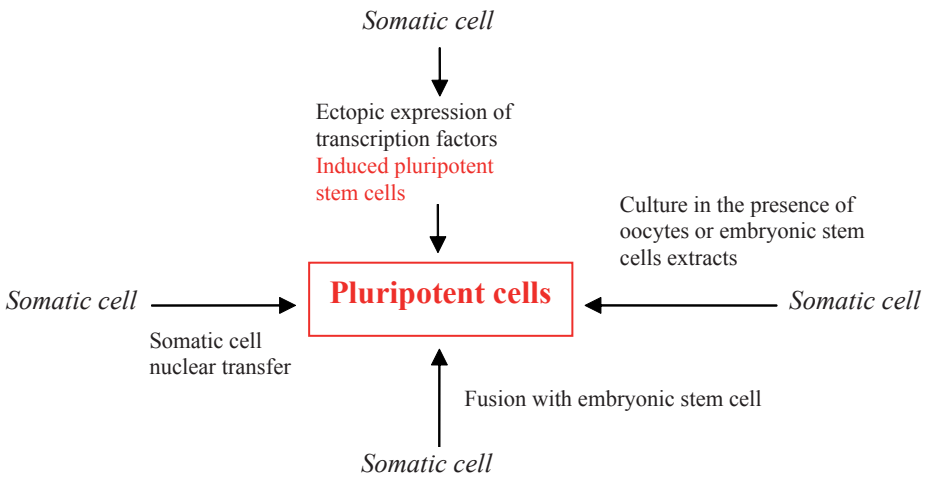

Fig. 1. Current strategies for pluripotent cells generation using somatic cells as a source.

Induced pluripotent stem cells have been generated from mouse somatic cells by overexpression of four transcription factors: c-Myc, Klf4, Oct4 and SOX1 [Takahashi and Yamanaka 2006]. The resultant iPS cells resemble embryonic stem cells at the molecular level and with respect to their differentiation potential *in vitro* and *in vivo*. The use of oncogenes such as c-Myc in the process of iPS cells generation may lead to the formation of tumors in chimeras and in offsprings derived from these iPS cells [Okita *et al*. 2008]. Therefore, the generation of iPS cells must avoid the use of the c-Myc and Klf4 oncogenes to make iPS cells suitable for clinical use. Recent achievements on this area made it possible to generate iPS cells without the use of c-Myc [Kim *et al*. 2009a, 2009b].

Before the reprogrammed cells or tissue can find their application in human patients their safety, integration and lack of tumorigenicity has to be proved in appriopriate animal models bearing significant similarities to humans [Nowak-Imialek *et al*. 2011a, Brevini 2008]. The pig is convenient model for preclinical studies because it is similar in size to humans and has a longer lifespan than mice, which enable to conduct long-term studies of disease progression with age. Because pig and human organ size and physiology are similar, the pig is considered the most probable source of cells and organs for xenotransplantation [West and Stice 2011].

Until now, only putative but not real embryonic stem cells in domestic animals have been obtained. Despite showing some typical features of stem cells such as ability to generate cells derived from all three germ layers, normal karyotype or expression of typical markers, these cells were not able to contribute to the germ line *in vivo* [Nowak-Imialek *et al*. 2011a].

The goal of this review is to present the current state of art on induced pluripotent stem cells in domestic species and their potential further application in biomedicine.

Strategy of iPS cells generation

The revolution in stem and developmental biology caused by induced pluripotent stem cells generation took place quite recently. The iPS cells were first generated from mouse and human fibroblasts in year 2006 [Takahashi and Yamanaka] and 2007 [Takahashi *et al*.], respectively. Complete reprogramming to the pluripotent status was possible by ectopic expression of the defined set of transcription factors (c-Myc, Klf4, Oct4 (also called POU5F1) and SOX2) [Takahashi and Yamanaka 2006]. Factors responsible for reprogramming are usually introduced to the host somatic cells using retroviral or lentiviral vectors [Takahashi and Yamanaka 2006]. However, now it is clear that viral integration to the host genome causes the serious risk of insertional mutagenesis [Wu and Dunbar 2011]. What is more, the set of oncogenes used for iPS cells generation practically excludes the reprogrammed cells from their use in clinical approach [Nowak-Imialek *et al*. 2011a]. To circumvent these obstacles alternative methods for generation of iPS cells from somatic cells were used such as adenoviral vectors [Stadfeld *et al*. 2008], plasmids [Yu *et al*. 2009], recombinant proteins [Zhou *et al*. 2009] and small molecule compounds [Shi *et al*.2008] − Figure 2. However, the efficiency of reprogramming using these alternative methods (without viral integration) was demonstrated to be lower [Nowak-Imialek *et al*. 2011 a]. It was reported that a specific glycogen synthase kinase 3 (GSK-3) inhibitor − CHIR99021 − can induce the reprogramming of mouse embryonic fibroblasts and human primary keratinocytes transduced by only two factors − Oct4 and Klf4 [Li *et al*. 2009]. Also, it was demonstrated that Oct4 alone is sufficient to reprogram directly adult mouse neural stem cells to iPS cells (one-factor (1F) human NiPS cells) − [Kim *et al*. 2009a]. One-factor human NiPS cells resemble human embryonic stem cells in global gene expression profiles, epigenetic status, and pluripotency *in vitro* as well as *in vivo*.

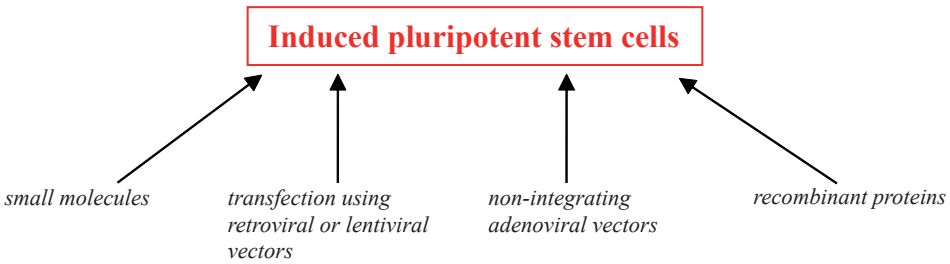

Fig. 2. Schematic illustration of different approaches leading to induced pluripotent stem cells generation.

Recently it was shown that Bmi1 leads to the transdifferentiation of mouse fibroblasts into neural stem cell-like cells, and, in combination with Oct4, can replace Sox2, Klf4 and c-Myc during the reprogramming of fibroblasts into iPS cells [Moon *et al*. 2011].

Zeuschner *et al*. [2010] compared iPS cells derived from mouse foetal fibroblasts with the four factors to embryonic stem cells using electron microscopy. Both cell types were almost indistinguishable at the ultrastructural level, providing further evidence for the similarity of embryonic stem cells to iPS cells populations.

Regarding stoichiometric requirements of the individual reprogramming factors for efficient reprogramming and the effect of this stoichiometry on the quality of derived iPS cells Tiemann *et al*. [2011] suggested that elevated Oct4 levels opposite to modest ones for Sox2 and Klf4 are required for satisfying reprogramming efficiencies and that these stoichiometries are also highly beneficial for achieving a stable pluripotent state independent of ectopic reprogramming factors expression.

The efficiency of reprogramming of somatic or adult stem cells into iPS cells needs to be verified by appriopriate *in vitro* and *in vivo* tests. To confirm epigenetic remodelling in reprogrammed cells, Kim *et al*. [2009a] performed bisulphite sequencing analysis to determine the rate of DNA methylation of the OCT4 and NANOG promoters. Similar to human ES cells, both promoter regions were demethylated in iPS cells relative to the donor human neural stem cells (NSCs). Thus, reprogrammed human cells were very similar to human ES cells at the molecular level. Pluripotency of human neural iPS cells was verified *in vitro* by embryoid body (EB) formation, spontaneous differentiation and directed differentiation. During sppontaneous EB differentiation, human neural iPS cells readily differentiated into vesicular structures with a variety of cell types present in the hanging drops including endoderm (AFP), mesoderm (a-SMA) and ectoderm (TUJ1). To enhance and direct the differentiation towards all three germ layers, human neural iPS cells were cultured following established differentiation protocols [Kim *et al*. 2009a, 2009b]. The expression of markers for all three germ layers was performed immonocytochemically and by qRT-PCR analysis. To evaluate *in vivo* pluripotency of these human iPS cells, they were transplanted into severe combined immunodeficient mice. Induced pluripotent stem

cells gave rise to teratomas containing derivatives of all three germ layers, including respiratory epithelium, skeletal muscle, cartilage and neural epithelium. These results confirm the significant similarities in pluripotential both *in vitro* and *in vivo* between embryonic stem cells and iPS cells. Global gene expression analysis also revealed similar expression profiles between embryonic stem cells and induced pluripotent stem cells [Kim *et al*. 2009a].

Until now, in mouse and human iPS cells have been succesfully obtained from different cell types including blood cells [Ohmine *et al*. 2011], cord blood cells [Meng *et al*. 2012], neural stem cells [Kim *et al*. 2009a], stomach cells [Aoi *et al*. 2008] [reviewed by Nowak-Imialek *et al*. 2011a].

Recently, Hayashi *et al*. [2012] demonstrated that female (XX) embryonic stem cells and induced pluripotent stem cells in mice are induced into primordial germ cell-like cells which exhibit meiotic potential. Upon transplantation under mouse ovarian bursa, primordial germ cell-like cells mature into germinal vesicle-stage oocytes, which then contribute to fertile offspring after in vitro maturation and fertilization.

Induced pluripotent stem cells in domestic species

Induced pluripotent stem cells can not be applied in human therapy before their safety (tumorigenicity) is tested in other species. Domestic species such as domestic pig (*Sus scrofa*), seem to be especially suitable for such evaluation taking into account their similarities to humans in anatomical and physiological aspects. However, in contrast to mouse and human, establishment of pluripotent cell lines in pig [Hall 2008], sheep [Dattena *et al*. 2006], goat [Pawar *et al*. 2009], bovine [Gong *et al*. 2010] and horse [Saito *et al*. 2002] still has low success rate.

Esteban *et al*. [2009] generated iPS cells from fibroblasts of Tibetan miniature pig . Produced iPS cells had normal karyotype, stained positive for alkaline phosphatase, expressed high levels of ESC-like markers (Nanog, Rex1, Lin28, and SSEA4), and could differentiate into teratomas comprising three germ layers. Using as a somatic cell source porcine fetal fibroblasts, Ezaschi *et al*. [2009] generated iPS cells by lentiviral transduction of four human genes − hOCT4, hSOX2, hKLF4, and hc-MYC. The programming efficiency was at the level of 0.1% and resulting cells expressed porcine OCT4, NANOG, and SOX2 and had high telomerase activity, but also continued to express the four human transgenes. Cells were positive for SSEA-1, but negative for SSEA-3 and -4. Reprogramming efficiency was supported by transcriptional profiling and real time RT-PCR. Using drug-inducible expression of defined factors, Wu *et al*. [2009] created porcine induced pluripotent stem cells which expressed alkaline phosphatase, SSEA3, SSEA4, Tra-1-60, Tra-1-81, Oct3/4, Nanog, Sox2, Rex1 and CDH1. These iPS cells expressed high levels of telomerase activity, showed normal karyotypes and could differentiate into cell types of all three germ layers *in vitro* and *in vivo* (teratomas). West *et al*. [2010] showed that porcine mesenchymal stem cells transduced with six human reprogramming factors (POU5F1, SOX2, NANOG, KLF4,

LIN28, and C-MYC) injected into preimplantation-stage embryos contributed to tissue types belonging to three germ layers. Based on skin and tail biopsies chimerism rate was found at the level of 85.3%. This important study supported the possibility to generate porcine iPS cells able to generate chimeric offspring. All other studies in pigs did not clearly reported the germ line contribution of porcine iPS cells suggesting that these cells still are not real pluripotent cells [Nowak-Imialek *et al*. 2011a]. Recently, production of transgenic pigs carrying an 18 kb genomic sequence of the murine Oct4 gene fused to the enhanced green fluorescent protein (EGFP) cDNA was demonstrated [Nowak-Imialek *et al*. 2011b]. This construct allows identification of pluripotent cells by monitoring Oct4 expression by EGFP fluorescence making genetically transformed pigs a reliable tool for monitoring reprogramming and the induction and maintenance of pluripotency in porcine cells. This might significantly accelerate the success in establishment of pluripotent stem cell lines including induced pluripotent stem cells in pigs.

In cattle, cell lines from bovine embryonic fibroblast cells by the transduction of six bovine transcription factors were obtained [Han *et al*. 2011]. The resulting cells showed a mouse embryonic stem cell-like morphology and were positive for alkaline phosphatase and expressed pluripotent markers such as SSEA1, SOX2, and NANOG. Karyotyping analysis demonstrated that bovine iPS cells showed a normal chromosome number and were able to differentiate to three germ layers *in vitro* and *in vivo*.

In sheep iPS cells were generated from fibroblasts reprogrammed to pluripotency by defined factors using drug-inducible system. Derived iPS cells had a normal karyotype, showed morphological features similar to human embryonic stem cells and expressed alkaline phosphatase, Oct4, Sox2, Nanog and the surface marker SSEA-4. Their pluripotency was confirmed by embryonic bodies formation *in vitro* and teratomas generation *in vivo* where cells from three germ layers were observed [Li *et al*. 2011]. Bao *et al*. [2011] reported that sheep somatic cells can be directly reprogrammed to induced pluripotent stem cells using defined factors (Oct4, Sox2, c-Myc, Klf4, Nanog, Lin28, SV40 large T and hTERT). Resulting sheep iPS cells expressed embryonic stem cell markers, including alkaline phosphatase, Oct4, Nanog, Sox2, Rex1, stage-specific embryonic antigen-1, TRA-1-60, TRA-1-81 and E-cadherin. They also exhibited normal karyotypes and were able to differentiate into all three germ layers both *in vitro* and in teratomas. Recently, Sartori *et al*. [2012] demonstrated that ovine iPS cells produced applying strategies established for the derivation of murine − induced pluripotent stem cells can contribute to live-born chimeric lambs.

In goat, iPS cells were successfully generated from primary ear fibroblasts showing a morphology similar to mouse embryonic stem cells and expressed SSEA1, Tra-1-60 and Tra-1-81, but were negative for SSEA-3 and SSEA-4 [Ren *et al*. 2011].

Induced pluripotent stem cells were generated from equine fibroblasts using a piggyBac (PB) transposon-based method to deliver transgenes containing the reprogramming factors Oct4, Sox2, Klf4 and c-Myc, expressed in regulated fashion. Resulting iPS cell lines expressed set of pluripotency markers, had a stable karyotype

even during long-term culture, and formed teratomas containing all three embryonic germ layer − derived tissues upon *in vivo* transplantation into immunocompromised mice [Nagy *et al*. 2011]. Generation of equine iPS cells would be beneficial not only for human medicine as another model for preclinical studies, but also for veterinary medicine with respect to treatment of injuries to muscles, joints, ligaments and tendons. Since up to now these problems in horse can not be efficiently treated using standard veterinary approach, much hope and attention is bound to the regenerative therapies, including potential use of iPS cells.

For over three decades dog has been used as a model for human diseases, especially with the respect to hereditary diseases. However, establishent of canine embryonic stem cells lines still meets limitations as it is a case in other domestic species. Recently, Luo *et al*. [2011] reported the generation of canine − induced pluripotent stem cells from adult fibroblasts, which were obtained by introducing human OCT4, SOX2, c-MYC and KLF4. The resulting iPS cells expressed critical pluripotency markers and showed evidence of silencing the viral vectors and normal karyotypes. Under culture conditions favoring differentiation, canine iPS cells could form cell derivatives from the ectoderm, mesoderm, and endoderm. What is more, canine iPS cells required leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF) to survive, proliferate, and maintain pluripotency. On the contrary, Whitworth *et al*. [2012] noticed that generated from adult fibroblasts canine iPS cells were dependent only on LIF and not both LIF and bFGF to maintain their pluripotency. As a conclusion authors suggest that canine iPS cells closely resemble mouse embryonic stem cells derived from the inner cell mass of preimplantation embryos, whereas the canine iPS cells described by Luo *et al*. [2011] appear to be more representative of cells from the epiblast of mouse postimplantation embryos.

Perspectives

Embryonic stem cells are considered as the gold standard for potential use in regenerative medicine because of their pluripotent nature which means the unlimited self-renewal abilities in culture and keeping the capacity to differentiate into any cell type in the body. Embryonic stem cells can only be derived from early-stage embryos which excludes the possibility to establish of autologous cell lines for patients. The other major concerns rose around moral and ethical dilemma surrounding the need of blastocysts to generate patient-specific pluripotent stem cell lines. For these reasons much effort has been put into understanding of the mechanisms underlying pluripotency with the vision of the potential application of this knowledge to the other types of the partially or fully differentiated cells. In this respect identification of the set of transcription factors necessary for reprogramming of differentiated cells to the pluripotent status by the group of Shinya Yamanaka [2006 Cell 126, 663–676] seems to be enermous success. Neurodegenerative diseases are the type of disorders where therapeutic potential of iPS cells is especially interesting. This

disease affects more and more people in aging populations. It was demonstrated that iPS cells could be differentiated into dopaminergic neurons *in vitro* and upon the transplantation into rat. Parkinson's diseases model tyrosine hydroxylase positive iPS-derived cells were found to have engrafted, expressed dopamine transporters and eventually alleviated behavioral symptoms [Wernig *et al*. 2008, Lengner 2010]. Particularly interesting are the induced pluripotent stem cells obtained from patients suffering from neurodegenerative disease. These cells can be differentiated *in vitro* into the disease-affected type of neurons, generating for the first time a model for neurodegenerative disorders which can be screened *in vitro* for all the disease mechanisms and the effect of environmental factors to the phenotype [Lengner, 2010]. Attempts to generate human patient-specific iPS cells also from individuals suffering from different disorders, such as juvenile *diabetes mellitus* or Huntington's disease have already started [Lengner 2010]. This quick progress in iPS cell research and the successful application of the results in the human theraphy still is dependent of the generation of large animal models for the preclinical tests. Therefore, the elucidation of the mechanism underlying pluripotency and establishment of the conditions for the pluripotent stem cell culture in domestic animals is one of the most important directions in developmental and stem cell biology today.

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