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REVIEW

The past, present and future of bovine pluripotent stem cells: a brief overview

Xiuchun TIAN (✉)

Department of Animal Science, University of Connecticut/UCONN Stem Cell Institute, Storrs, CT 06268-4163, USA

Abstract Although the pursuit of bovine embryonic stem cells started more than 26 years ago for the purpose of gene-targeting, true pluripotent stem cells in this economically important species are still elusive. With the rapid advances in genome-editing and cloning using homologously recombined somatic cells, the need for pluripotent stem cells for precise genetic modification in any species became questionable. With the pig being the better model for human regenerative biology, the identification of the commonalities and uniqueness of the pluripotency circuitry across mammalian species may be the main objective for studying pluripotent stem cells in the bovine.

Keywords bovine, embryonic, induced, pluripotent stem cells

1 Introduction

Generation of pluripotent stem cells (PSCs), including embryonic (ESCs) and induced PSCs, from cattle has been the most challenging of all mammalian species. From the first report of bovine ESCs more than 26 years ago[1], true bovine stem cells capable of germline-chimera formation are still not available while such cells have been produced for rats and pigs[2,3] following the initial mouse work[4]. The lack of major breakthroughs may relate to the lack of abundant funding, sufficient research effort and more likely because cattle are ruminant species. Had research on cattle been given as much funding/manpower as has been the case for human PSCs, the bovine challenge would have been solved long ago. This review will provide a summary of major efforts and advances in the pursuit of bovine PSCs.

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Correspondence: xiuchun.tian@uconn.edu

2 Naive and primed pluripotency and induced pluripotent stem cells (iPSCs)

PSCs are defined as those that can differentiate into all cell types of the three germ layers of a mammalian organism. The gold-standard mouse ESCs are capable of *in vitro* differentiation into embryoid bodies as well as *in vivo* differentiation into teratoma and formation of germline chimeras[4]. It is unethical, however, to produce germline chimeras in humans, therefore the demonstration of human pluripotency has had to stop at the stage of teratoma formation. After the establishment of the putative human ESCs, it was clearly realized that the dome-shaped ESCs of the mouse were in fact an exception rather than a rule in mammals[5]. The term, primed pluripotency[6], was then coined to describe human ESCs and mouse epiblast stem cells[7,8]. These cells have flat, monolayered colonies, rely on basic fibroblast growth factor (bFGF)/activin A signaling for self-renewal, and are unable to form colonies from single cells[9].This contrasts with naive pluripotency, which is used to describe mouse ESCs that have dome-shaped compact colonies, depend on leukemia inhibitory factor signaling for self-renewal and are capable of forming colonies from single-cells, a feature important in gene-targeting.

Induced PSCs are ESC-like, pluripotent cells reprogrammed from differentiated somatic cells by forced expression of transgenes of *OCT4* (or *POU5F1*), *SOX2*, *KLF4*, and *c-MYC* (*OSKM*), or *OCT4*, *SOX2*, *NANOG*, and *LIN28*[10,11]. iPSCs or iPSC-like cells have been reported in many mammalian species including cattle. Consistent with earlier observations on ESCs, in general iPSCs from the mouse and other species including humans exhibit naive and primed states, respectively[10,11]. The ultimate goal of naive human iPSCs has been eagerly pursued and recently reported[12–14]. These cells exhibit more differentiation potential than their primed counterparts. Using an interspecies chimera approach, human "naive" iPSCs formed chimeras when injected into murine, porcine and bovine blastocysts[15] while primed human iPSCs failed to do so[16].

3 Current status of bovine ESCs and iPSCs

Considerable efforts have been expended to generate bovine ESCs starting more than 28 years ago[1]. In general, the reported putative bovine PSCs resembled primed human ESCs/iPSCs. However, most reports did not include data that demonstrated true pluripotency. Also, equally problematic was that the cells differentiated within a few replication cycles. Although a line of putative bovine ESCs that were sub-cultured more than 30 times was reported after knocking out the *CDX2* gene, no data on chimeras were included[17]. To date, only one report documented possible germline chimeras from bovine ESCs[18]. The oocytes of a five-month chimeric calf were reported to test positive for the transgene used as a marker for the injected ESCs. The failure to maintain pluripotency and short-lived nature of putative bovine ESCs may be caused by the unsuitable culture conditions, which mainly mimicked the mouse ESCs media. To overcome the culture problem, new lines of bovine ESCs were recently reported from *in vitro* produced blastocysts. Using a modified human PSC culture medium without growth factors but supplemented with bFGF and IWR1, a WNT signaling inhibitor[19], putative bovine ESCs showed (1) capacity of long-term culture (> 50 cycles) by enzymatic dissociation, (2) high percentages (> 70%) of normal karyotype, (3) expression of pluripotent genes, (4) possession of epigenetic landscape more similar to primed human ESCs than naive mouse ESCs, (5) production of blastocysts when used in cloning by somatic cell nuclear transfer, and (6) formation of teratoma in SKID mice. The major advance of this study compared to the earlier ones is the stability of the putative bovine ESCs, possibility due to the use of a medium developed for primed state stem cells. Without embryonic germline chimeras, these bovine ESCs are still one major step away from declaration of complete pluripotency.

Bovine iPSCs have been reported from bovine fetal or adult cells using reprogramming factors similar to those used in mice or humans[20–24]. Like bovine ESCs, bovine iPSCs generally have primed stem cell morphology. Also, like most reported bovine ESCs, bovine iPSCs are short-lived in culture. More problematic than bovine ESCs, however, is the failure of bovine iPSCs to turn off exogenous transgenes. The reported naive-like bovine iPSCs[25–28] did not seem to fare better in either activation of pluripotent circuitry (i.e., they were dependent on continuous transgene expression), passage capacity or differentiation potential (i.e., they failed to form teratomas in nude mice)[28]. Furthermore, using the reprogramming conditions for iPSCs generation, two research teams independently obtained bovine trophectoderm cells[29,30]. These observations suggest that the reported, presumptive bovine iPSCs are either incompletely reprogrammed or are of trophectoderm-lineage while expressing some pluripotent features, as was also reported in pigs[31].

4 Attempts to produce bovine germline chimeras and pluripotency testing

While there are many tests for the pluripotency of stem cells, such as specific markers, embryoid body formation and *in vitro* random differentiation, as well as *in vivo* differentiation by teratoma formation, the ultimate test for pluripotency is germline chimera formation. The technology and procedures for bovine chimera generation have been well established. Chimeric embryos and/or live-born bovine chimeric calves have been reported by microinjecting or aggregating embryonic cells of one breed/subspecies to the diploid (2N) host embryos of another breed/subspecies[33–36]. Relatively high efficiency of chimeric embryo generation (12%–86%) has been reported. Although the purpose of these studies was not to test the pluripotency of stem cells, they were instrumental in fine-tuning the parameters/procedures for bovine chimera generation.

There have been five reports in which bovine germline chimeras were used to test the pluripotency of bovine stem cells[18,28,37–39]. All reported success in producing non-germline bovine chimeras with low levels of chimerism in at least one tissue. For example, Furusawa et al.[39] injected GFP-labeled, naive ESC-like cells into 2N IVF host embryos. One live fetus was obtained at 62 days of gestation. The fetal tissues showed no GFP florescence but very low levels of GFP was found by PCR in a few tissues. These data demonstrated the feasibility of using chimeras to test stem cell pluripotency but also showed that the stem cells used were not pluripotent. To avoid low integration, Iwasaki et al.[38] injected primed ESC-like cells derived from a Holstein embryo into 4N IVF host embryos of Japanese Black cattle. Surprisingly, the resulting six calves were all Japanese Black, not Holstein which meant that the calves were unintentionally derived from the 4N host embryos. Among them only two calves had a few Holstein cells in some tissues. Their data showed that the host embryos were not fused properly and 2N host embryos were unintentionally used and developed to term. In addition, their ESC-like cells were not pluripotent. As mentioned earlier, the closest report of bovine germline chimera was published by Cibelli et al.[18] in which transgenic ESC-like cells were injected into IVF host embryos. Nine live calves had at least one chimeric tissue. One of them at five months of age was reported to test positive for the transgene in oocytes, potentially a germline chimera. However, there has been no follow-up on offspring from these putative germline chimeras. Moreover, there have been no reports repeating such results for more than 20 years.

To date, the only study that attempted to test the pluripotency of bovine iPSCs was published by Kawaguchi et al.[28] who injected GFP-labeled naive-like bovine iPSCs into 2N IVF embryos. Although chimeric 90-day-old fetuses showed GFP signal in the gonads,

definitive evidence was not given as to whether the GFP$^+$ cells were indeed germ cells. Additionally the bovine iPSCs used did not form teratomas, a clear sign for the lack of pluripotency. In summary, while the technology for bovine chimera production is readily available, definitive, replicable bovine germline chimeras from bovine ESCs/iPSCs have yet to be reliably generated.

5 Future directions of bovine PSCs research

Bovine inner cell masses from which the bovine ESCs are derived are definitely pluripotent. Bovine iPSCs are reprogrammed[20–24] in a similar fashion to that used in mice where full pluripotency is regained. It is highly likely that these reprogramming conditions are sufficient to fully reprogram bovine somatic cells to pluripotency. When such bovine ICM or reprogrammed somatic cells are placed in culture, however, pluripotency quickly disappears. The lack of appropriate culture conditions is likely the main problem. Like ESCs of other non-mouse species, bovine ESCs culture conditions initially duplicated those used for mouse cells and more recently those used for human cells. However, cattle, being ruminants, have completely different endogenous milieu and nutrient requirements. The long-practiced trial and error approach to PSC culture over the past 26 years has not been successful for cattle. It may take collaboration between cell culture biologists and ruminant nutritionists to finally and systematically solve the mystery of bovine PSC culture requirements, with the use of the bovine inner cell mass cells as the study model. Before the proper culture conditions are established, any work on bovine iPSCs may be premature.

6 Applications of bovine PSCs

Lastly a few words on the purpose of bovine PSCs. Before nuclear transfer using homologously recombined somatic cells became successful, bovine PSCs were necessary for precise genetic modifications by gene-targeting, mainly for the purpose of specialty trait generation/breeding, disease control and to a lesser extent, pharmaceutical protein production. Cloning using gene-targeted somatic cells did not completely eliminate the need for bovine PSCs because somatic targeting and cloning are both extremely inefficient and technically challenging compared to ESC targeting and blastocyst injection. The advances in genome-editing combined with pronuclear injection or somatic cell nuclear transfer, however, made the use of true PSCs in any species for the purpose of gene-targeting obsolete. It is true that PSCs have enormous potential in regenerative medicine, but this may only be applicable in human medicine. The cattle industry has historically been a low profit business and will likely continue to be one in the future. It is therefore unlikely that large scaled tissue replacements will be used to treat bovine diseases. Although bovine PSCs may have some roles in serving as models in diseases common to cattle and humans, such as leukocyte adhesion deficiency[40], or those that have no other large animal models such as citrullinemia[41], such examples are relatively rare. In contrast, pigs serve as much better models for human regenerative medicine because they are cheaper, more prolific and more closely resemble human physiology and anatomy. Although suggestions have been made for applications of bovine PSCs in genetic selection and artificial gametes, the well-developed cattle breeding industry will likely continue the traditional bull selection process by combining assisted reproductive technologies and genomic/progeny testing. The main need for bovine PSCs, therefore, may reside in basic research; the discovery of differences/commonalities among different mammalian species in reprogramming, mechanisms/regulatory circuitries involved in pluripotency and development/differentiation.

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Compliance with ethics guidelines Xiuchun Tian declare that he has no conflicts of interest or financial conflicts to disclose.

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