Somatic Cell Nuclear Transfer

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Somatic cell nuclear transfer (SCNT) is a technique for generating embryos with genomic information identical to that of donor cells. SCNT has presented various insights into the process of de-differentiation by cellular reprogramming.

Now authors are focusing on the challenge of understanding the potential of pluripotent stem cells and the safe use. Moreover, stem cells from livestock have provided many potential to the academic field as well as medical and industrial applications.

Keywords: Bovine ; embryonic stem cells ; induced pluripotent stem cells ; extended pluripotent stem cells

1. Introduction

Somatic cell nuclear transfer (SCNT) is a technique for generating embryos with genomic information identical to that of donor cells. This technique was first reported using frog somatic cells in 1962, and the first successful cloning in mammals was achieved by SCNT in 1997, creating Dolly the sheep [1][2]. Over the last five decades, clones have been successfully produced in many species, including the non-human primate species of macaque monkeys [3][4][5][6][7][8].

Although there are minor differences, the basic concept of SCNT is similar between species [9]. The first step of SCNT is the removal of the haploid chromosomes, including the meiotic spindle complex from a metaphase II stage oocyte, called enucleation. Then, a diploid donor cell is transferred into an enucleated oocyte, and electrical cell fusion is carried out to expose the nucleus to the ooplasm. Finally, artificial activation is performed by electric pulses or chemical stimulation, resulting in induction of the early development stage of the embryo.

In bovine, SCNT embryos and cloned offspring have also been reported [10][11][12]. Subsequently, the SCNT technique was used to make transgenic cattle. SCNT technology with transgenic cells providing a donor nucleus is one of the most efficient techniques for generating transgenic animals. Initially, the advantage of SCNT clones with transgenic cells is to provide increasingly good livestock products for human consumption.

Transgenic cattle are also considered as bioreactors for recombinant proteins. Many attempts have been made to generate cloned cows producing different human proteins, such as α-lactalbumin [13], lysozyme [14], granulocyte colony-stimulating factor [15], and lactoferrin [16]. Although the production efficiency is still low, many researchers have recently succeeded in producing higher concentrations (up to 13.6 g/L) of human proteins from cloned cows [17]. In addition, recombinant human myelin basic protein produced by transgenic cows shows a protective effect as a vaccine against multiple sclerosis [18]. These results show the potential for various commercial applications of proteins produced by transgenic cows.

Since stem cell research has expanded, SCNT technology has entered a new era. Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the blastocyst, the pre-implantation embryo, and they have two distinct properties: unlimited self-renewal and the ability to differentiate into all kinds of cells.

Since ESCs were established in mice [19], several ESCs have been reported in various animals, including humans [20][21][22]. Two different types of representative ESC have now been identified, naïve and primed states, reflecting the cellular characteristics of pre- and post-implantation embryos, respectively [23]. Although these two states present very similar features, they also differ from each other in terms of some features, including their morphology, dependent signals for maintaining pluripotency, and their contribution to chimera formation [24]. In large animals such as cattle, substantial efforts have been made to establish ESCs. However, it is still challenging to establish genuine ESCs, which might be related to species-specific characteristics of their reproduction and development.

2. Overview of Pluripotent Stem Cells

Since two types of PSC in naïve and primed states have been characterized in mice [23], the naïve and primed states are considered key criteria for genuine PSCs. In a broad sense, naïve and primed PSCs share core characteristics of pluripotency. They show unlimited self-renewal and the ability to differentiate into three germ layers [19][24]. However, they are not the same. In general, mouse ESCs (mESCs) from pre-implanted embryos are considered to be in a naïve state, and they show distinct characteristics, including small dome-shaped morphology, DNA hypomethylation, and two activated X chromosomes in females. To maintain their pluripotency, mESCs rely on leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP4) signals [25]. Since they can be recolonized through single-cell passaging, chimeras can be produced and show germline transmission. On the other hand, epiblast stem cells (EpiSCs) from post-implanted mouse embryos are considered to be in a primed state [24]. These cells show unique characteristics, different from the naïve state, such as large and flattened shapes, DNA hypermethylation, and X inactivation status in females. Moreover, EpiSCs retain their pluripotency under basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β)-related Activin/Nodal signaling [20]. Compared with the naïve state, mouse EpiSCs lack single-cell proliferation ability and, thus, cannot form chimeras. The transcription of genes of primed PSCs also differs from that of their naïve counterparts. The core pluripotent genes, OCT4, SOX2, and KLF4, are expressed in both; however, in naïve mESCs, STELLA and REX1 are expressed, while FGF5, T, and LEFTY, which are generally associated with differentiation, are expressed only in EpiSCs. Human ESCs (hESCs) are considered to be in a primed state, showing flat morphology and retaining their pluripotency under bFGF and TGF-β signaling. However, hESCs are not identical to mEpiSCs. For example, the pattern of transcription differs markedly, including FGF5, E-CADHERIN, and NANOG [26]. Moreover, some naïve state markers are expressed in hESCs, including PRDM14 and REX1 [27]. These results show that there are differences among species even though they are in a similar pluripotent state, and it is necessary to consider the species specificity to understand the mechanisms of the maintenance of their pluripotency.

3. Bovine Embryonic Stem Cells

After ESCs were successfully established in mice and humans, authentic ESCs were generated in several species [21][22]. There have also been attempts to establish ESCs in large animals, including cattle (Table 1). However, it is still challenging to establish bESCs that meet the criteria of true ESCs.

Table 1. Various characteristics of bovine embryo-derived stem cells.

Medium	Morphology	Pluripotency	Differentiation	Special	Reference
FCS, heparin, LIF	mES-like cells	X	Epithelial, fibroblastic, neuron-type cells	With trophoblastic cell	[28]
FCS	Low cytoplasmic/nuclear ratio	X	in vitro differentiation	Trophecoderm-like cells	[29]
FBS, LIF	Monolayer cells	X	X	Tetraploid embryos test Contributing to liver, placenta, and hair roots in chimera	[30]
FBS	Small cytoplasmic/nuclear volume ratio	SSEA-1(+), SSEA-3(+), SSEA-4(+)	in vitro differentiation	Long term culture Cystic form observed like TE	[31]
FBS, LIF, EGF	Small cells compact colony	AP(+), SSEA-1(+), STAT3(+), OCT4(+) SSEA-3(−), SSEA-4(−)	in vitro differentiation	Chimeric test Contributing to both lineages	[32]
FCS, ITS, LIF, bFGF, EGF, 5-azacytidine	Heterogenetic morphology	REX1(+), OCT4(+), SSEA-4(+)	in vitro differentiation	5-azacytidine improved pluripotency and ability to differentiate	[33]
FBS, bFGF, SCF	Bubble-like or TE-like cell	OCT4(+), SSEA-1(+), SSEA4(+), AP(+)	in vitro differentiation	Stem cell factor (SCF), a cytokine that binds to the c-Kit receptor	[34]
PD0325901, CHIR99021	Flat-shaped	Naïve state markers(+) Primed state markers(−)	in vitro differentiation	GATA6 and CDX2 expression	[35]

Medium	Morphology	Pluripotency	Differentiation	Special	Reference
bFGF, LIF, KSR	Dome-like (early passages) Flat-shaped (late passages)	OCT4(+), SOX2(+), NANOG(+), E-CAD(+), SSEA1(+), SSEA4(+)	in vitro and in vivo differentiation	TE related genes still expressed in CDX2-KD lines	[36]
PD18435, SU5402, CHIR99021	Heterogenetic morphology mixed with TE	Naïve state markers(+) Primed state markers (−)	in vitro and in vivo differentiation	OCT4 or Nanog positive cells without CDX2 negative	[37]
BSA, bFGF, IWR1	Flat-shaped	Primed state markers(+)	in vitro and in vivo differentiation	X	[38]

FCS: fetal calf serum; LIF: leukemia inhibitory factor; FBS: fetal bovine serum; EGF: Epidermal growth factor; ITS: insulin-transferrin-selenium; bFGF: basic fibroblast growth factor; SCF: stem cell factor; KSR: knockout serum replacement; BSA: bovine serum albumin; IWR1: a Wnt/β-catenin inhibitor.

Representative stem cells show two different morphologies: a dome shape for the naïve state and a flattened shape for the primed state. Although some reports have depicted that the morphology of putative bESCs is similar to that of mESCs or hESCs, most shapes of colonies from bovine embryonic cells are heterogeneous, incompact, and irregular with an ambiguous description [29][33][31]. It was also reported that putative bESCs contain trophectoderm (TE). These cells sometimes show unexpected cystic cavities [39][37][36]. Our group attempted to generate putative bESCs from in vitro production (IVP), parthenogenesis (PA), and SCNT embryos [37][40]. There were no major differences in morphology among them. However, these putative stem cells were morphologically different from conventional ESCs. The colony showed two different parts: a central multilayer (CMt) and a peripheral monolayer (PMn). Mainly, the CMt part existed inside a colony and the cells were small and compacted in a clump, whereas the PMn part existed near the boundary and contained large and flattened cells. Unlike PMn, the CMt part was recolonized as a new colony after passaging, suggesting that only the CMt part may include pluripotent cells. The unstable appearance of bESCs means that authentic PSCs have not yet been established.

Like other PSCs, pluripotency marker genes were shown to be strongly expressed in putative bESCs. In particular, it was reported that genes related to naïve state are expressed in putative bESCs [37]. However, the expression of CDX2, a caudal-type homeodomain TF, was also reported in many putative bESCs [39][37][36]. Generally, Cdx2 expression was shown to be inhibited by Oct4 in mESCs [41]. Upon the overexpression of Cdx2, ESCs can differentiate into the trophoblast lineage, which does not happen spontaneously [41][42]. Although this heterogeneous population in bovine species cannot be fully explained, several reports that can help our understanding based on the knowledge of embryogenesis have been published. In early mouse development, the first cell fate is committed after the compacted morula stage, resulting in segregation into two different cell lineages: ICM, which will be the embryo, and TE, which will be the placenta. Cdx2 is an essential gene for TE fate determination, which acts by inhibiting Oct4 and triggering genes required for placental differentiation [43]. In addition, the Cdx2 mutant was shown to exhibit failure of embryo implantation [41]. In cattle, several studies on the transcriptome of early-stage embryos have been performed to find clues about the mechanism of early development. According to previous studies, TE-related markers, CDX2, TEAD4, and GATA4, were expressed in the ICM of bovine blastocyst, but not in the murine counterpart [44][45]. Moreover, there is little difference in CDX2 expression between ICM and TE in bovine blastocysts [36][43][46][47]. Furthermore, bovine embryos exhibit delayed implantation compared with murine and human embryos [36][48]. These findings suggest that this delayed expression of CDX2 may contribute to retard the first cell fate decision, resulting in an incomplete blastocyst stage and allowing TE to partially remain pluripotent. A report has described that cells derived from TE can contribute to ICM when injected into the early embryo [49]. This uncommitted TE may be considered to be able to easily contaminate ICM-derived cells during in vitro cultivation, resulting in some putative bESCs, including TE-derived cells.

Since the expression of CDX2 may impede bESCs in exhibiting true pluripotency, a CDX2-knockdown embryo (CDX2-KD) model was studied [36]. To understand the role of CDX2 in bovine pluripotency, SCNT embryos with CDX2-KD were generated. Unlike in mice, the CDX2-KD embryo not only formed an extended blastocyst stage, but had no significant effect on pluripotency marker gene expression. Embryo-derived stem cells were established from CDX2-KD and showed the expression of various pluripotency markers as well as in vitro and in vivo differentiation capacity. In terms of the shape, a human-like flattened shape was shown. Interestingly, despite the inhibition of CDX2, the expression of TE differentiation markers was increased when they differentiated spontaneously. This implies that the characteristics of putative bESCs differ from those of hESCs and mESCs.

To understand pluripotency, several transcriptome studies have also been conducted in cultured cells from bovine blastocysts. With various blastocyst-derived cells, microarray results suggested signaling pathways for bovine pluripotency [50]. Three different putative bESCs from IVP, NT, and PA embryos were used, with the findings revealing common increases in TGF-β, Wnt, and LIF signaling pathways related to pluripotency, implying that these pathways may be pivotal for capturing authentic pluripotency in bovines. Epigenetic patterns have also been reported to understand bovine pluripotency [38]. This paper confirmed that 62% of the H3K4me3-only bovine gene is shared by hESCs and mESCs. Interestingly, the patterns of H3K4me3 and bivalent genes were similar to those of hESCs rather than mESCs. In addition, with RNA-seq, the expression of primed-specific genes was found to be higher than that of naïve genes. These results suggest that bovine pluripotency may be close to that of hESCs. These reports indicate that transcriptome analysis might be useful for understanding bovine pluripotency, leading to the establishment of true ESCs.

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