

Research Article

Stem Cell Technology- A Perspective on Promises and Challenges for applications in livestock health and production

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Abstract

Stem cells technology has undergone remarkable transformation and offers ample opportunities in livestock production and studies of lineage commitment and molecular developmental dynamics. Innovations in cell culturing and preservation, genomics, cellular metabolomics, nanotechnology, and high throughput screening are expected to solve the major problems currently associated with stem cell technology. Also, reports are available on development of cell lines in some species of domestic animals"

Key words: Stem cells, Pluripotency, Livestock health, Reproduction biotechnology

1. Introduction

Since the beginning of 20th century, scientists have come to understand that all of the different types of blood and tissue cells in the body develop from what have come to be referred to as "stem cells." According to the National Institute of Health (NIH), stem cells have the "remarkable potential to develop into many different cell types in the body". Serving as a sort of repair system for the body, these cells in most multi-cellular organisms can theoretically divide without limit to replenish other cells as long as an animal is alive. The recent upsurge in publication on animal stem cells can be taken as reflection of the interest aroused by these cells due to unique biological properties and numerous applications (Munoz *et al.*, 2009). Historically, the stem cells research started in 1981 with the milestone publications reporting the establishment of murine embryonic stem cells (Evans and Kaufman, 1981; Martin, 1981).

The stem cells are capable of maintaining the ability to multiply meiotically and differentiate into a diverse range of specialized cells types. The stem cells are categorized into two broad areas namely, embryonic stem cells and adult stem cells. In addition, the stem cells exhibiting stem cell-like characteristics can also be derived from the fetus, cord blood and amniotic fluid of the mammalian species.

Embryonic stem (ES) cells are derived from the preimplantation hatching embryos, and are characterized by essentially unlimited self-renewal, and exhibit the ability to differentiate into all cell types in an individual. It is at this point that first step of differentiation takes place during mammalian embryonic development. The individual blastomeres now start to organize themselves into three distinct locations, namely, trophectoderm, the primitive endoderm. ES cells originate from primitive endoderm, which is a transiently existing group of cells in the embryo. *In vitro* produced blastocysts are commonly used as the starting material for ES cell lines.

Characteristics of embryonic stem cells

ES cells are the pluripotent cells derived from inner cell mass (ICM) cells (Fig. 1) of pre-implantation embryo. They can be cultured and exhibit the capacity for long-term propagation and broad differentiation plasticity (Fig. 2). In addition to the ability to self-renew and differentiate, another important characteristic of stem cells is the ability to replenish their niche. Chemicals and media. The ES cells were first established as cell lines from the inner cell mass (ICM) of mouse blastocysts in early 1980s. These cells are characterized by their unique functional feature that upon combination with a host embryo (chimera production) they can contribute to all tissues and organs, including germ cells of the resulting chimeric

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mouse (Talbot and Blomberg, 2008). As these cells are competent to form all cell types including extraembryonic placental tissues, they are considered totipotent or pluripotent (Fig. 2) depending on the particular cell line or in environmental context. Furthermore, they can be clonally propagated and

maintained in culture indefinitely. These characteristics have made them an invaluable genetic engineering tool for studying functional mammalian genetics, mammalian developmental biology, and for producing animal models of human diseases.

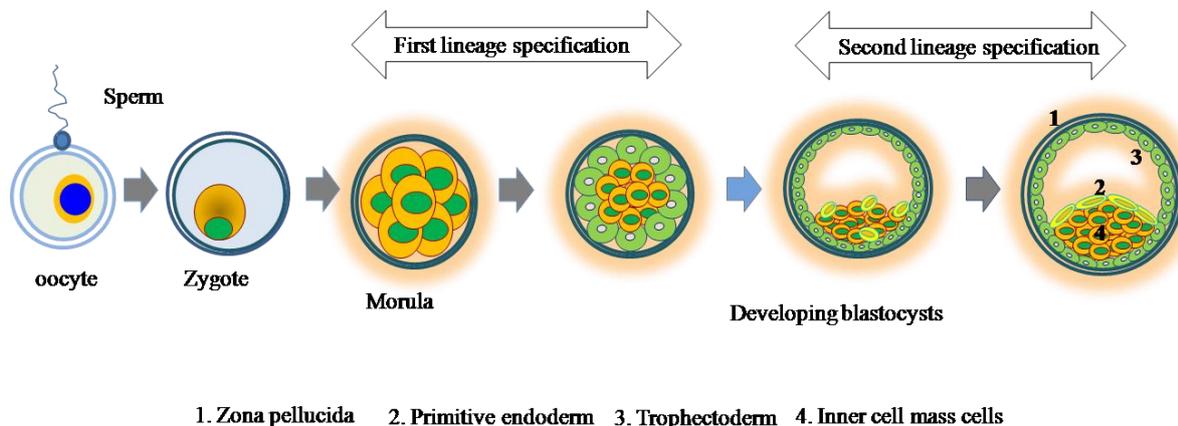


Fig.1 Cellular differentiation and formation of ICM cells during pre-implantation embryo development. ES cells are derived from ICM cells of embryos

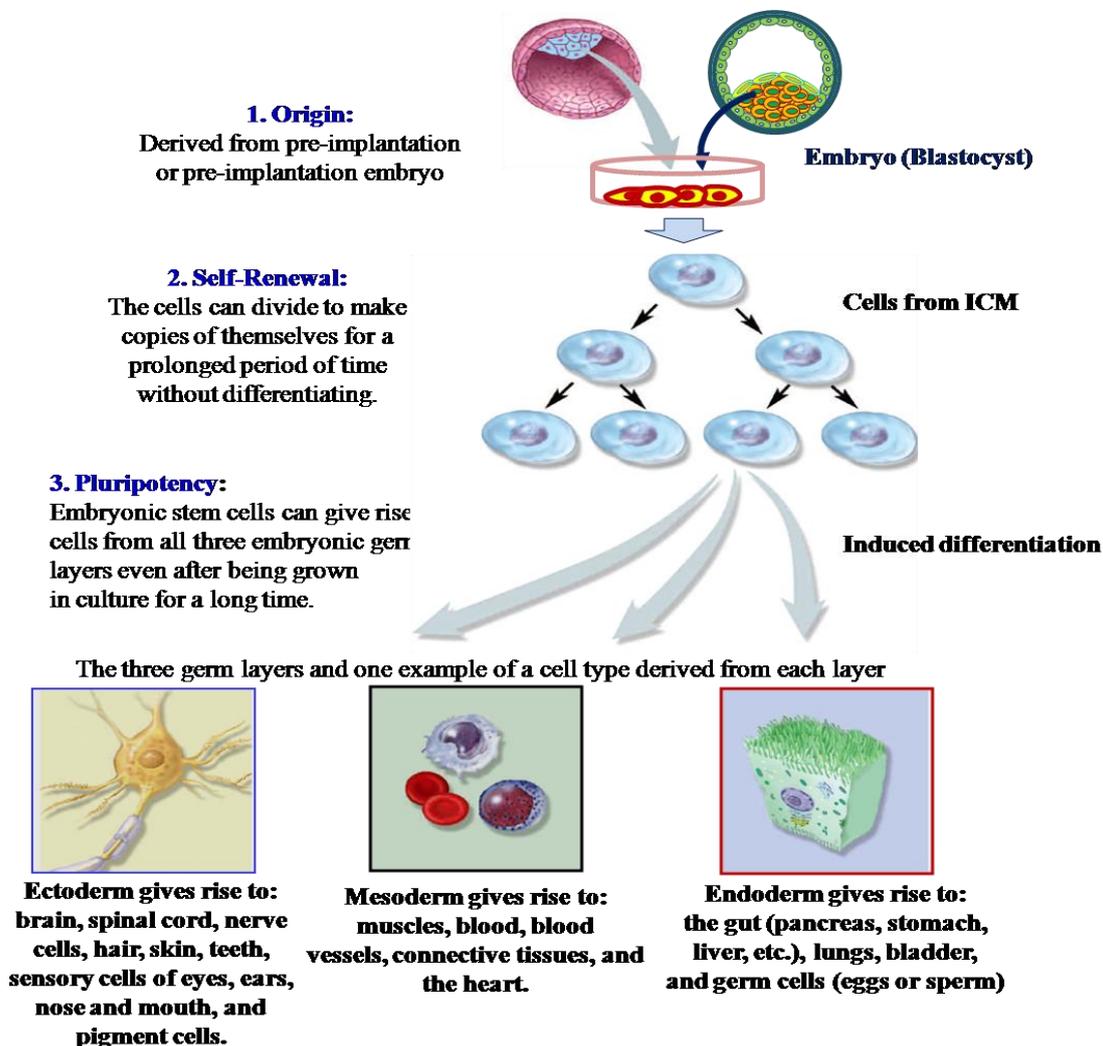


Fig. 2 Important properties of pluripotent stem cells. Prolonged self-renewal, cellular pluripotency and ability to be differentiated into any cell lineage are the characteristic features.

Different techniques, viz. mechanical separation, immunosurgery, and laser-assisted isolation of the ICM cells are employed for isolation of these cells. ES cell lines have similar cell culture properties regardless of the species of origin or the tissue of origin i.e., derivation from morula stage embryos, the ICM of the blastocyst, primordial germ (PG) cells of the embryonic genital ridge, or the early post-implantation epiblast (Brons *et al.*, 2007; Tesar *et al.*, 2007.) The murine ES cells typically grow in compact colonial groups, or 'nests' of cells that often have a convex 3-D shape and a distinct, glistening edge that meets with the flatter feeder cells that the ES cells. The ES cells generally grow on top of or in between the feeder cells. Mouse ES (mES) cell colonies grow quickly to contain hundreds if not thousands of cells per colony, and the colonies will eventually fuse with one another to form monolayers if there are sufficient colonies in close proximity. If left undisturbed the ES cells tend to spontaneously differentiate at the periphery of the colony with the formation of flatter, larger, and irregularly cuboidal visceral endoderm. Primate ES cell colony morphology is different from mES cells in that human ES cells are generally flatter in appearance and spontaneous differentiation tends to begin in the centre of colonies if they are left undisturbed for a week or more without passage (Thomson and Marshall, 1998; Thomson *et al.*, 1998).

Types of stem cells

Like human beings and other mammalian species, the sources for pluripotent stem cells having differentiation potential to all the three germ layers, are either embryos or germ line (Fig. 3). Postnatal sources of pluripotent stem cells include bone marrow, blood, and skeletal muscles, adipose tissue, skin, fetal somatic explants, umbilical cord, placental tissue, amniotic fluid, etc. Adult stem cells are present in specific tissue groups and can both renew themselves and differentiate into the specialized cell type in which they reside. For example, hematopoietic stem cells are the cells that can differentiate into any of the different blood cells, while mesenchymal stem cells can do the same for the cells of skeleton. In addition, the adult stem cells may also differentiate into the cells of other tissue types, a phenomenon known as trans-differentiation or plasticity. Spermatogonial stem cells originate from the primordial germ cells (PG) cells, the progenitor cells for both the male and female germ line which, in turn, is derived from the epiblast cells. PG cells are the single cells that can under certain culture conditions form colonies of cells, which morphologically resemble undifferentiated ES cells. The spermatogonial stem cells are the only adult stem cells that donate genetic material to the progeny. In bovines, the spermatogonial stem cells can be extracted, cultured *in vitro* and used for IVF or ICSI for producing the embryos. Another broader area of the applications of these cells is their application in developing the transgenic animals.

Leydig cells

Leydig cells are the testosterone-producing cells of the testis. The adult Leydig cell population ultimately develops from undifferentiated mesenchymal-like stem cells present in the interstitial compartment of the neonatal testis. Four distinct stages of adult Leydig cell development have been identified and characterized: stem Leydig cells, progenitor Leydig cells, immature Leydig cells and adult Leydig cells. The stem Leydig cells are undifferentiated cells that are capable of indefinite self-renewal, differentiation, and replenishment of the Leydig cell niche. Progenitor Leydig cells are derived from the stem Leydig cells. These spindle-shaped cells are luteinizing hormone (LH) receptor positive, have high mitotic activity, and produce little testosterone but rather testosterone metabolites.

The progenitor Leydig cells give rise to immature Leydig cells which are round, contain large amounts of smooth endoplasmic reticulum, and produce some testosterone but also very high levels of testosterone metabolites. A single division of these cells produces adult Leydig cells, which are terminally differentiated cells that produce high levels of testosterone (Chen *et al.*, 2009).

Testosterone, produced by Leydig cells of the mammalian testis, is important throughout the lifetime of the male. High levels of testosterone are produced by the fetal testis, exerting organizational effects on the morphogenesis of specific organs and programming effects on

neural functions and enzyme activities that are expressed later in life (Forest, 1983; Scott *et al.*, 2009).

Trophoblast stem cells

Trophoblast stem cells (TS) cells are the precursors of the differentiated cells of the placenta. TSC can be derived from outgrowths of either blastocyst polar trophoblast (TE) or extraembryonic ectoderm (ExE), which originates from polar TE after implantation. The mouse TS cell niche appears to be located within the ExE adjacent to the epiblast, on which it depends for essential growth factors, but whether this cellular architecture is the same in other species remains to be determined.

Besides human, TS cell lines have been reported from a few model experimental species including mice and rhesus monkeys. Among ungulates TS cells have been reported in cattle, sheep and horses.

Novel epiblast stem cells

Recently, another class of novel stem cell type- epistem cells (epiSCs) originally reported by (Brons *et al.*, (2007) and Tesar *et al.*, (2007) are also in news. It is observed that ICM cells grown in chemically defined medium does not give rise to pluripotent cell lines. When late epiblast layers were dissected from pre-gastulation stage embryos and cultured in CDM, small

colonies with morphological characteristics of pluripotent stem cells formed. Colonies subsequently picked and passaged were shown to express the pluripotency markers oct4, nanog and SSEA1. Interestingly, epiblast lines could not be derived in the presence of LIF or BMP4, conditions necessary for the derivation of mouse ES cells. There are also observable differences distinguishing this new stem cell type from germ cells. For example, epiSCs do not express alkaline phosphatase (AP) activity or blimp1 and *stella*, demonstrating this cell type is not derived from the (<http://www.abcam.com/index.html?pageconfig=resource&rid=11242&pid=10039>, Feb. 25, 2011) Under the present status of research, epiSCs derivation in farm animals may prove easier than true ES cell derivation, and could represent a significant step forwards for identifying the factors required for maintaining pluripotency in cultured ICM cells of farm animal embryos (Galli and Lazzari 2008).

Nevertheless, the key aspects of animals ES cells, specifically the identification of species-specific ES cell-markers need to be elucidated. It has been pointed out that currently used human or murine ES cell-specific molecular markers are not specific for bovine ES cells, hence, further research is warranted on identification of valid ES cell markers in bovines. It was also suggested that until validated pluripotent bovine stem cell markers are identified, it might be advisable to combine the use of epiblast and trophoblast-specific markers to rule out the presence of trophectoderm cells in ES cell cultures (Munoz *et al.*, 2008).

Mammary stem cells

Adult mammary stem cells are multipotent cells which are committed to give rise to cells with a specific function. These cells produce a lineage of daughter cells with a unidirectional terminal differentiation process. Mammary gland adult stem cells are slow cycling cells with the ability to respond to environmental cues and propagate additional stem cells or differentiate along a specific cell lineage. Once the stem cell commits to differentiation, it enters a brief period of rapid proliferation. Asymmetrical cell division generates one daughter stem cell and one progenitor cell. This process allows for the maintenance of a stem cell pool for future growth while simultaneously generating a differentiated cell population. On the other hand, symmetrical cell division produces 2 daughter stem cells. This enlarges the stem pool from one cell to many. In order to develop therapies that target these cancer stem cells, it is essential to determine the molecular mechanisms that regulate the growth and differentiation of these cells and their normal counterparts (Stingl, 2009).

The murine mammary stem cells (MaSC) have been isolated using methods as employed to identify the hematopoietic stem cells. The isolation methods involve the depletion of hematopoietic and endothelial cells from freshly dissociated cell preparations and differentiation of mammary cell subpopulations based

on their cell-surface expression of CD24 (heat-stable antigen), CD29 (α -1-integrin) or CD49f (α -6-integrin) molecules. Interestingly, these integrins are likely to form a functional α 6- β 1 integrin heterodimeric complex that mediates interaction between epithelial cells and the mammary stroma.

Cord blood and amniotic stem cells

In the late 1980s, umbilical cord blood (UCB) was recognized as an important clinical source of HSCs. Blood from the placenta and umbilical cord is a rich source of hematopoietic stem cells (Abdulrazzak *et al.*, 2110), and these cells are typically discarded after the birth. Increasingly, UCB is harvested, frozen, and stored in cord blood banks either as an individual resource (donor-specific source) or as a general resource, directly available when needed. Advantages of cord blood include its availability, ease of harvest, and the reduced risk of graft-versus-host disease (GVHD). In addition, the cord blood HSCs have a greater proliferative capacity than adult HSCs. *Ex vivo* expansion in tissue culture, to which cord blood cells are more amenable than adult cells, is another approach under active investigation. Umbilical cord matrix cells can also be a good source of fetal stem cells. These cells can be isolated and cultured in animals. For this umbilical cord (UC) is collected from new born calves immediately after birth and processed for deriving hematopoietic cells from umbilical cord vein, and mesenchymal cells from Wharton's Jelly (WJ) (Fig. 4) by using standard cell culture protocols.

Amniotic fluid and cord blood, long considered a waste product, offer considerable therapeutic utility in human beings, but in livestock these stem cells offer a remarkable potential in the genetic engineering of the animals. Umbilical cord blood is extensively rich in hematopoietic stem cells. The amniotic fluid contains the cells which exhibit stem cells like characteristics. The initial studies on culturing and partial characterization of the buffalo amniotic cord blood and amniotic fluid cells (Fig. 4), have raised the hopes of authentic characterization and derivation of somatic cells from these cells.

Reprogrammed and induced pluripotent stem cells

At the 2006 meeting of the International Society for Stem Cell Research in Toronto, Ontario, Canada, Shinya Yamanaka announced the startling discovery that adult skin cells could be directly reprogrammed to pluripotency using a combination of only four genes. The project began by making a list of 24 known pluripotency-associated genes, predominantly those expressed in ES cells. Various combinations of these genes were introduced to adult mouse skin fibroblasts via engineered retroviruses in order to screen for gene combinations that could reprogramme the skin cells to pluripotency. Yamanaka was able to define a rather 'simple' cocktail of just four, Oct4, Sox2, c-Myc and Klf4, which were capable of reprogramming skin cells to a

pattern of gene expression (Takahashi and Yamanaka, 2006). This method was termed ‘direct reprogramming’ as it relied on primary genetic modulation to force reprogramming instead of NT. The resulting cells were dubbed ‘induced pluripotent stem’ or iPS cells. Greater understanding of the molecular

mechanisms that mediate reprogramming will ultimately enable the production of safe human iPSCs and multipotential stem cells for use in clinical applications (Tada, 2008). This aspect has been discussed in chapter on cellular reprogramming.

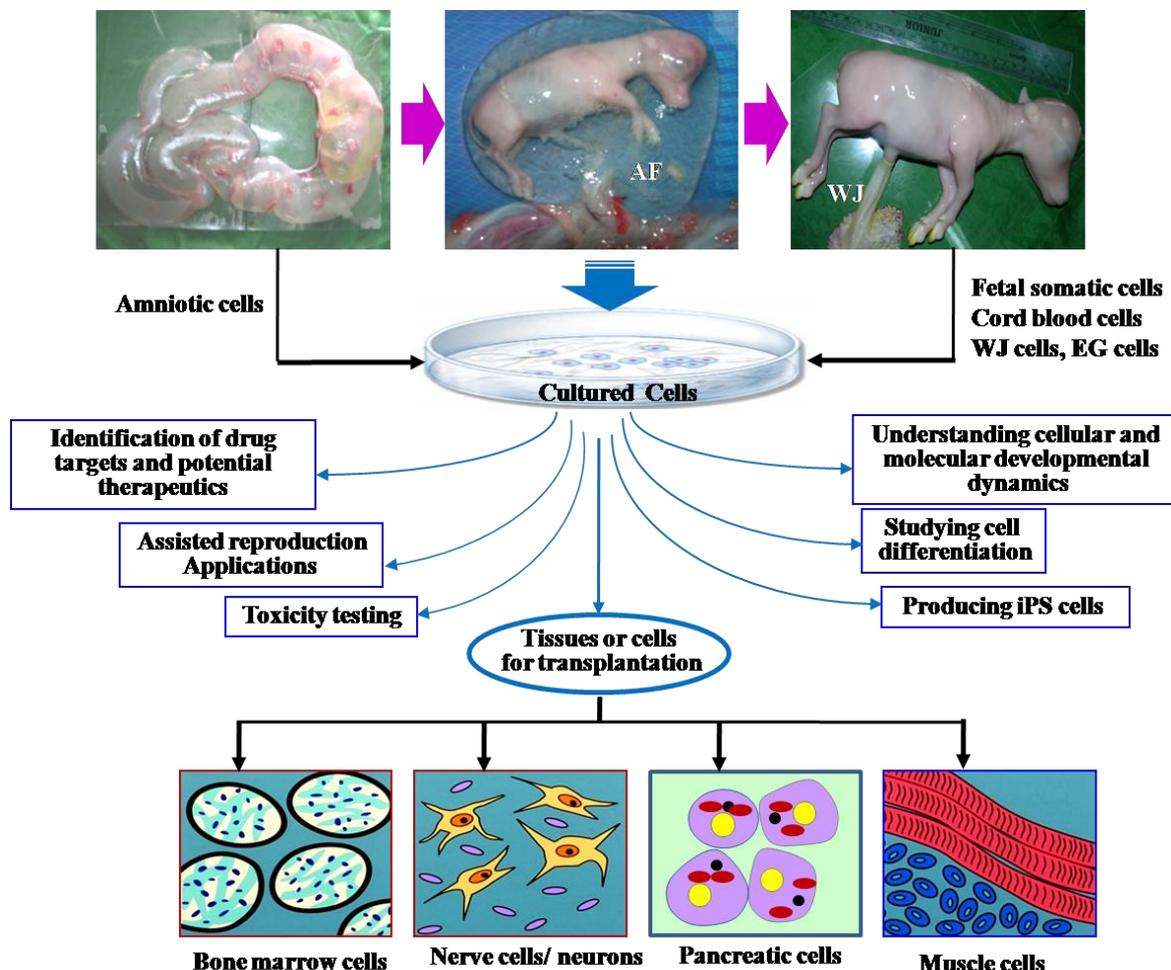


Fig. 4 Alternative sources of pluripotent stem cells in animals. The fetal stem cells could be exploited as alternative sources of stem cells in livestock. The fetal stem cells may have applications in veterinary therapeutic and livestock assisted reproduction

ES-cell research in livestock species

There is considerable interest in pursuing stem cell research in domestic animal species of economic importance. Ever since the first report on establishment of putative ES cell lines in pig (Evans et al. 1990), the stem technology has achieved new horizons. The field of adult stem cell research in livestock species is increasing exponentially and encompasses a wide range of topics- from deepening our understanding of cellular development to applying these findings to repair and create organs. Fundamental to the use of any stem cell therapy is a clear understanding of the immunology for allotransplantation as well as autotransplantation for malignancies and immunologically mediated diseases. Scientists and academic centers for stem cell research

are using adult stem cells to develop therapies for cancer, stroke, spinal cord injury, autoimmune diseases, and regeneration of bone, cartilage, and other tissues.

There are multiple interests in obtaining pluripotent cells in animals with main expectations in the field of genetic engineering, either to produce cloned animals or transgenic animals (Table-1).

Isolation of pure ICM cells for obtaining the ES cells is one of the major technical constraints in establishing the ES cell cultures in the species, though some innovative approaches like “separate and seed” (Cao et al., 2009) are suggested to improve the technology. The bovine embryonic stem cells lines reported till date vary in morphology and marker expression, such as alkaline phosphatase, stage-specific embryonic antigen-4

(SSEA-4), Nanog, and octamer-binding transcription factor (Oct-4), that normally are associated with the undifferentiated pluripotent state. The bovine ES cells can grow in large, multicellular colonies resembling mouse ES cells and EG cells as well as human EG cells.

Not all of the stem cell research conducted in animals and using animal product is purely anthropocentric; some experiments are aimed at improving the lives of animals. The ES cells offer enormous possibilities of producing large number of offspring by nuclear transfer and performing genetic manipulation for production of cloned transgenic animals. In addition, the ES cells have applications in basic research, clinical research and in livestock production improvement.

The potential improvements in stem cell biology would come in the form of reduction in use of animal for research, better therapeutics for veterinary applications, and conservation of endangered fauna. Establishing the ES cell lines in farm animals like, buffalo, goat and cattle is of interest for similar reasons to those of mouse and human ES cell lines. This includes basic research such as, comparative embryology and the cell biology of ungulate stem cell maintenance and differentiation. Also, the ES cells may be useful for generating transgenic animals or developing disease-resistant cloned livestock herds, as these cells have a self-renewable capacity more prolonged than normal somatic cell cultures, thus increasing their applications as donor nuclei (Brevini *et al.*, 2008). In addition, the genetic engineering with ES cells may result in synergistic gains in the ability to precisely induce and study genetic alterations in animals.

Stem cells and *in vitro* derivation of gametes
Advances in stem cell research have opened new perspectives for regenerative and reproductive medicine. Stem cells can differentiate under appropriate *in vitro* and *in vivo* conditions into different cell types. *In vitro* derivation of germ cells and viable gametes is a recent emerging field and a promising area of mammalian assisted reproduction. While pluripotency of ES cells is well established, it came as revelation that murine ES cells could develop into primordial germ cells (PGC) *in vitro*, and that extended culture could occasionally form early spermatids or oogonia (Hubner *et al.*, 2003; Nagano, 2007; Ko and Scholer, 2006). Production of gamete precursor cells was achieved initially through allowing the pluripotent stem cell lines to differentiate more or less randomly. The research continues on identifying the precise mechanisms by which differentiation of pluripotent stem cells into gametes can be controlled. Currently, the strategies to create artificial gametes *in vitro* include converting diploid somatic cells from mitotic division to meiotic division directly (somatic cell haploidization), or dedifferentiating somatic cells into pluripotent stem cells (induced pluripotency) and re-differentiating these cells into gametes, or extracting adult stem cells and re-differentiating them into gametes (Nagy *et al.*, 2008). It has been speculated that

revolutionary studies showing differentiation of ES cells into germ cells *in vitro*, and potential oocyte generation *in vivo* from the terminally differentiated cells not only challenge the long-held dogma, but also herald a promising future in the area of mammalian assisted reproduction

(Oktem and Oktay, 2008). Childs *et al.* (2008) have highlighted the recent developments in stem cell-derived gametes in humans as well as experimental animal models. While evidences are there that cultured ES cells can produce PGC and germ cells, there remains a question as to whether the germ cells can be derived from adult stem cells residing outside the gonads? Germ cells have been derived from the bone marrow (Nyernia *et al.*, 2006) and fetal skin cells *in vitro* in some experimental models (Dyce *et al.*, 2006).

Because the developmentally competent oocytes are restricted in number and accessibility, a robust system to derive oocytes from stem cells would enable a thorough investigation of the genetic, epigenetic, and environmental factors affecting buffalo oocyte development. Also, the technology may offer ample opportunities for derivation of *in vitro* produced oocytes for procedural refinement of genetic engineering tools in buffaloes, progress in which is hampered severely due to use of abattoir-derived oocytes.

Differentiation of germ cells from stem cells has the potential of becoming a future source of gametes for research use, although further investigation is needed to understand and develop the appropriate niches and culture conditions. Additionally, if genetic and epigenetic methodological limitations could be solved, therapeutic opportunities could be also be considered (Marques-Mari *et al.*, 2009). Concerning livestock applications, maintenance and proliferation of various germline cells derived from ES cells along with establishing protocols for their transplantation may offer new opportunities to treat the reproductive failures in high merit genotypes.

Status of stem cell research in livestock

Currently, a number of robust techniques are in use for isolation and *in vitro* culture and characterization of stem cells/ stem cell-like cells in animals other than mice and primate experimental models. The field of adult stem cell research in livestock species is increasing exponentially and encompasses a wide range of topics- from deepening our understanding of cellular development to applying these findings to repair and create the organs. Fundamental to the use of any stem cell therapy is a clear understanding of the immunology for allo transplantation as well as autotransplantation for malignancies and immunologically mediated diseases. Scientists and academic centres are using adult stem cells to develop therapies for cancer, stroke, spinal cord injury, autoimmune diseases, and regeneration of bone, cartilage, and other tissues.

Table 1 Prospects of stem cell technology in livestock

1. Improvement in male fertility through spermatogonial stem cell transplantation.
2. Development of stem cell-based novel therapies to treat animal diseases.
3. Improving cloning efficiency through use of stem cells as donor nuclei.
4. Enhancing efficiency of transgenesis in ungulates.
5. Using spermatogonial stem cells for production of transgenic chimera and animals.
6. Gaining insights into basic cellular and molecular biological aspects of livestock species .
7. In vitro meat production from the muscle and adipose stem cells.
8. To resolve problems related to the livestock therapies e.g. regeneration of mammary tissue damaged by mastitis.
9. Regeneration of damaged ligaments in species like equine or camels.
10. Application of pluripotent stem cells in veterinary and human drug testing and screening.
11. Overcoming the current limitations in efficient gene transfer by providing an abundance of stem cells to be genetically manipulated by using conventional recombinant DNA techniques.
12. Overcoming the use of live animals for use in drug testing and toxicological studies. ES cell-derived different types of cells can be used for the purpose.

Not all of the stem cell research conducted in animals and using animal product is purely anthropocentric; some experiments are aimed at improving the lives of animals. There are multiple interests in obtaining pluripotent cells in domestic ungulates with main expectations in the field of genetic engineering, either to produce cloned animals or cloned transgenic animals (Singh *et al.*, 2009) (Table-1).

The animal ES cells offer enormous possibilities of producing offspring by NT and performing genetic manipulation for production of cloned transgenic animals. The potential improvements in stem cells biology would come in the form of reduction in animal research, better therapeutics for veterinary applications, and conservation of endangered fauna through assisted reproduction. Establishing the ES cell lines in farm animals like, buffalo, goat and cattle is of interest for similar reasons to those of mouse and human ES cell lines. This includes basic research such as, comparative embryology and the cell biology of ungulate stem cell maintenance and differentiation. Also, the ES cells may be useful for generating transgenic animals or developing disease-resistance cloned livestock herds, as pluripotent stem cells have a remarkable self-renewable capacity, more prolonged life than normal somatic cell cultures, thus, exhibit easier genomic reprogramming when used as donor nuclei. In addition, the genetic engineering with ES cells may result in synergistic gains in the ability to precisely induce and study genetic alterations in animals.

Isolation of pure ICM cells for obtaining the ES cells is one of the major technical constraints in establishing ES cell cultures in animals. Lack of defined culture conditions and authentic molecular markers for their characterization is another important hurdle in

advancing animal stem cell research. Among livestock species significant progress has been made in obtaining pluripotent stem cells from bovine, sheep and porcine, and the technology has been applied to derive and establish stem cells in other animal species (Table-2).

Table-2 A summary of remarkable milestones stem cell technology in livestock Species Salient findings/ recommendations (references)

Pig: First report on primitive ES cells (Evans <i>et al.</i> , 1990), derivation of pES cells from the culture of ICM obtained by immunosurgery of day 7-8 in vivo blastocysts (Piedrahita <i>et al.</i> , 1990), isolation of pluripotent stem cells from cultured porcine primordial germ cells (Shim <i>et al.</i> , 1997), derivation and characterization of pluripotent cell lines from pig embryos of different origin (Li <i>et al.</i> , 2003; Brevini <i>et al.</i> , 2007), therapeutic use of mesenchymal stem cells in a porcine model (Dutton <i>et al.</i> , 2010)
Horse: First reports on ES like cells (Saito <i>et al.</i> , 2002), homozygous stem cells from metaphase II oocyte (Lin <i>et al.</i> , 2003), ES cells lines from the proliferation of ICM cells (Li <i>et al.</i> , 2006)
Cattle: Established ES cell-like cells in cattle (Stice <i>et al.</i> , 1996), generation of cloned calves and transgenic chimeric embryos from bovine ES-like cells (Cibelli <i>et al.</i> , 1998; Iwasaki <i>et al.</i> , 2000; Saito <i>et al.</i> , 2003), elucidation of instability of Oct4 expression in bovine ES cell cultures (Kurosaka <i>et al.</i> , 2004; Yadav <i>et al.</i> , 2005); relative evaluation of expression of pluripotency-related genes during bovine ICM explant culture (Pant and Keefer, 2009), isolation and culture of primary bovine embryonic stem cell colonies by a novel method (Cao <i>et al.</i> , 2009); culture of mammary epithelial stem cells (Li <i>et al.</i> , 2009), isolation of parthenogenetic ES cell (bpES) cell lines from in vitro produced parthenotes (Pashaiasi <i>et al.</i> , 2010), derivation of germ cells from developing testis (Fujihara <i>et al.</i> , 2011)
Goat: Production of caprine chimera by injection of EG cells into a blastocyst (Jia <i>et al.</i> , 2008); isolation and cultivation of goat ES-like cells (Yan <i>et al.</i> , 2008; Pawar <i>et al.</i> , 2009); development of enrichment protocols for muscle stem cells (Tripathi <i>et al.</i> , 2010)
Sheep: Isolation, culture, and characterization of embryonic cell lines from ovine and caprine embryos (Meinecke-Tillmann and Meinecke, 1996), vitrified sheep blastocysts (Dattena <i>et al.</i> , 2006), characterization of ES cell-like cells from <i>in vitro</i> -produced blastocysts, recommendation of Oct4 as stemness marker in sheep (Sanna <i>et al.</i> , 2009)
Buffaloes: First reports on establishing stem cell-like cells from IVF-derived buffalo embryos (Verma <i>et al.</i> , 2007; Huang <i>et al.</i> , 2008), development of multilineage ES cells from parthenogenetically developed embryos (Sritnaudomchai <i>et al.</i> , 2007), isolation and characterization of embryonic germ cell-like cells (Huang <i>et al.</i> , 2008), <i>in vitro</i> culturing of spermatogonial stem cells (Xie <i>et al.</i> , 2008), isolation and prolonged culturing of amniotic fluid cells (Yadav <i>et al.</i> , 2008a, b; Dev <i>et al.</i> , 2011), expression of pluripotency-related antigens in stem cell-like cells (Anand <i>et al.</i> , 2011), expression of pluripotency-related genes in amniotic fluid stem cells (Yadav <i>et al.</i> , 2011).

However, in contrast to mouse and human ES cells lines, true ES cell lines have not been established from livestock. Expression of antigenic and molecular markers is reported to vary in stem cells or stem cell-like cells in different species. Yadav *et al.* (2005), for example, found that expression of Oct-4 was optimal in the primary cultures of bovine ICM cells, which reduced to undetectable levels during the later passages *in vitro*. It implies that the utility of Oct-4 as a

marker for stem cell-pluripotency is limited to a certain stages of cell cultures. Rapid loss of Oct-4 and pluripotency in cultured blastocysts and derivative cell lines are also reported in rodents as well as in bovine and porcine epiblasts.

Nevertheless, the key aspects of animals ES cells, specifically the identification of species-specific ES cell-markers need to be elucidated. It has been pointed out that currently used human or mES cell-specific molecular markers are not specific for bovine ES cells, hence, further research is warranted on identification of valid ES cell markers in bovines (Munoz *et al.*, 2008). It was also suggested that until validated pluripotent bovine stem cell markers are identified, it might be advisable to combine the use of epiblast- and trophoblast-specific markers to rule out the presence of trophectoderm cells in ES cell cultures.

Opportunities and challenges

For reasons that are unclear the production of ES cells from ICM of blastocysts and the epiblast of slightly older embryos has proven elusive in ungulates (Ezashi *et al.*, 2009). The attempts to establish ES cell lines of domestic animals have been unsuccessful in part due to the inability to develop suitable culture conditions for these species.

This is because most of the culture systems for animal stem cells are based on those reported for murine or human ES cells. It is likely that these culture conditions do not support the growth of ES cells in other mammalian species, which differ in factors and cellular signaling cascade indispensable for survival and maintenance of ES cell pluripotency. Moreover, lack of cellular as well as molecular markers for validation of pluripotency and species-specificity are yet to be identified. Therefore, if we want to succeed into isolation and establishment of ES cell lines from species other than human and mice, it is imperative that we perform careful cross-species comparison without overlooking species-specific differences.

Conclusion

ES cells provide an attractive platform for examining basic developmental processes and disease pathophysiology. A major advantage of embryonic stem cells is that they are capable of differentiating into all types of body cells, as opposed to adult stem cells that seem to be more limited in terms of "to which kind of somatic cells they can be developed." Recently reported improvements in ES cell-related tools have the potential to further accelerate the use of ES cells in this arena. Chief among these advances are the novel tools for genetic manipulation of ES cells and improvements in ES cell culture conditions. In view of the ethical problems in deriving ES cells embryonic stem cells, the researchers are interested in discovering ways of obtaining embryonic stem cells or pluripotent stem cells without compromising the embryo from which the cells are drawn. In domestic

ungulates, the embryonic stem cells, embryonic germ cells and adultstem cells are still at initial stages of development

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