Bovine stem cells: methodology and applications

Aleena A. Saidova[1,2]*

[1]*Biological Faculty, M.V. Lomonosov Moscow State University, Moscow, Russia, 119991*

[2]*Center of Experimental Embryology and Reproductive Biotechnology, Moscow, Russia, 127422*

Abstract

Different types of stem cells have been extensively explored and described in human and rodents, but not in the domestic ungulates. Large animal models provide an essential opportunity to expand our knowledge in developmental biology and have significant advantages when modeling the long-term regenerative therapy, and disease-specific conditions. Moreover, transgenic animals with desired characteristics could be generated from modified pluripotent stem cells with novel reproduction approaches. Tissue and cell engineering of disease-resistant cattle could have an outstanding impact on the world economy. However, the validation and establishment of bovine stem cell lines have not received the deserved attention. This review provides systematic information on the special considerations for the establishment of the bovine stem cell lines and covers the different applications of bovine stem cells in regenerative therapy, genetic engineering, and translational medicine.

Keywords: Bovine Stem Cells; bESC; biPSC; Translational Medicine;

Introduction

Cows and cattle are of outstanding importance among all the ungulates for the livestock industry, being a source of meat and dairy products [1]. Full sequencing of cattle genome in 2009 opened new perspectives for the gene editing and transgenic modifications to produce a genetically superior cattle [2]. Examples of successful transgenesis include and expression of proteins with biomedically essential properties like human α-lactalbumin in milk and tissue engineering of mastitis-resistant cattle [3-5]. Technical limitations in the field of transgenic animals research include the limited lifespan of primary cell lines, as most of the cells (including fetal bovine fibroblasts) transfer to the senescent state in about 50 rounds of replication, that dramatically decreases the efficiency of the Somatic Cell Nuclear Transfer (SCNT), which was the first technology that was employed to obtain genetically modified animals [6]. Development of new methodological approaches led to the derivation of Embryonic Stem Cells (ESC) and Induced Pluripotent Stem Cells (IPSC) which not only provide the fundamental clues to the understanding of the pluripotency program in livestock species but also demonstrate a tremendous potential for producing cattle with the desired genetic properties through genomic selection and/or genome editing as well as for in vitro breeding schemes through genomic selection, germ cell differentiation, and in vitro fertilization.

To fit the criteria of stemness, stem cells have to pass the functional tests including the embryoid bodies formation in vitro and the ability to form teratomas in immunodeficient mice [7]. The molecular signature of stem cells for most mammalian species include a typical pattern of pluripotency markers Oct4, Sox2, Rex1 and Nanog [8]. Another set of stemness markers often used in fundamental studies is stage-specific embryonic antigens (SSEAs) that include SSEA-1, SSEA-3, and SSEA-4. Expression of SSEA molecules strongly depends on the source of stem cells and varies between species, as human ESC express SSEA-3 and SSEA-4, while mouse stem cells usually express SSEA-1 only [9,10]. The species-specific differences should also be taken into account for the development of culturing conditions and maintenance requirements. Despite that all stemness tests have been developed mainly for human and rodent cells and experimental models, the same molecular markers were applied to describe different types of stem cells from ungulates. Regardless of the significant and extensive efforts, the validation of stem cell lines from cattle has not been accomplished.

The current review focuses on the basic concepts relating to the fundamental biology of bovine stem cells and describes the state of art for derivation of ESC and iPSC in cattle, including successful and unsuccessful efforts to establish and validate viable bSC cell lines together with specific requirements and culturing conditions for the maintenance of these cells in undifferentiated state. Another critical issue of the review is the translation of bovine in vitro models to clinical in vivo assays.

Bovine Mesenchymal Stem Cells

Stem cells are defined as a specific group of cells with self–renewing capacities that remain undifferentiated in embryos, fetuses, and adults. The highest order of the developmental potential is totipotency which is an intrinsic property of a zygote (Figure 1). This means that zygote can generate all cell types inside the embryo and also contribute to the extraembryonic tissues [11]. The sequential step of development is the pluripotent cells that include embryonic germ cells (EGCs), cells from the inner mass of the blastocyst (ICMCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (IPSCs). Embryonic stem cells are the ancestors of the pluripotent cells from pre-implantation embryos, while induced pluripotent stem cells can be reprogrammed from the adult somatic cells. Both cell types are

pluripotent and possess the capacity to differentiate into most cells excluding the trophoblast and extra embryonic endoderm.

The more lineage-restricted cells are defined as adult stem cells. Among them, mesenchymal stem cells (MSC) (or mesenchymal stromal cells) require more special consideration. The most common and classical source for MSC is bone marrow [12]. However, MSCs can also be derived from the adipose tissue, peripheral blood, skin, and lungs [13-15].

Despite a heterogeneity and poor phenotypic description of MSC population, these cells are defined as pluripotent and differentiate not only to multiple types of connective tissue but also demonstrate a cross-over into non-mesodermal cell lineages [16]. In that respect, MSC can be considered as an ethically acceptable alternative to ESC for regenerative medicine, as reported for the regeneration of bone, cartilage and spinal cord injuries for different model organisms [17-23]. In the veterinary clinic, MSCs are introduced into protocols to treat lameness in dogs and horses, however, taken into account the facts above this technique should be practiced more carefully [24-26].

The primary source for isolation of the bovine MSC (bMSC) is umbilical cord and bone marrow27-29. Similar to other organisms, the characteristic phenotype of bMSC is CD29+/CD73+/CD90+/CD105+, while the negative phenotype markers have not been reported. bMSCs in vitro demonstrate a fibroblast-like morphology and can differentiate into three classic mesenchymal lineages: chondrogenic (with an expression of collagen II and aggrecan), adipogenic (with an expression of PRAR-γ and ALP) and osteogenic (with an expression of collagen I) [27, 30-32]. Most studies on bMSC were performed on hydrogels or in pellets and evaluated the characteristics of chondrogenic bMSC differentiation, as bMSC easily enter the chondrogenic differentiation in the absence of growth factors, which might be a natural characteristic of these cells [30]. However, cell pellet or monolayer is not sufficient for regenerative purposes, as bMSCs that can grow in a 3D assay require the presence of TGFβ1 and start to express collagen II only after medium optimization [29, 31]. Although the bMSC demonstrate the spontaneous chondrogenic differentiation, their use in regenerative therapy is limited due to the low quantities of extracellular matrix components that chondroblasts express after differentiation and the assay protocols require further optimization.

Bovine embryonic stem cells

The first ESC lines were generated from the mouse blastocysts [33-34]. Such cells formed a sphere-a like colonies, LIF and FBS were reported as first supplemental factors for the propagation of these cells [35]. Later it was reported that serum could be replaced with BMP4, whereas inhibitors of GSK3 or MAP/ERK signaling can be used as LIF surrogates [36,37]. These colonies can be trypsinized and clonally propagated; they can also colonize ICM in the embryos to form chimeras and be introduced as a part of embryonic germ cells [38].

The pig was the first large animal model used for ESC derivation due to the large size and immunological similarity to human tissues 39. Extensive efforts have been made to obtain porcine ESC lines from different sources, including outgrowths of the epiblast and embryonic ICM, parthenogenetic embryos and embryos obtained by SNT. Although all the lines demonstrated some pluripotency features, all of them with a few exceptions failed to survive at extended passages [40-42]. Most of them also failed to expand into teratomas in SCID/nude mice and could not contribute to chimeras, thus confirming the inability, to match the pluripotency criteria.

Porcine cell lines most capable of forming teratomas were derived from the parthenogenetic embryos, in this case, basal ESC medium with FBS contained three critical supplemental factors, namely LIF, FGF2, and KITLG [42]. These data demonstrated that the primary culture conditions suitable for human and mouse embryos could be insufficient for other ESC and that a unique combination of growth factors and supplements should be validated for each animal model. Some data showed a promising effect of LIF and FGF2 together, the addition of GSK3beta and MEK inhibitors (so-called 2i medium, initially modified for rat ESC) was also described as a putative strategy [40].

The first bovine ESC were isolated as primary cell lines from ICM cells and could retain the normal karyotype for a few passages [43]. The next step to create the ESC culture was the development of suspension system that allowed to culture cells in low concentrations [43]. ES-like cells produced from the cultured blastocysts were used as donors for NT, and this method allowed to generate calves at a comparable to SCNT frequencies [44]. An idea to isolate cells from morulae and blastocysts allowed to culture viable cells for more than 50 passages. Stice, at al. [45] employed these cells as donors for nuclear transfer and managed to obtain embryos ready for implantation, albeit all the embryos were unable after 60 days due to the placental defects. Iwasaki et al. 46 made an advance with the isolation of viable and stable bESC line from the ICM. They reported that ES-like cells from the ICM of in vitro embryos could be aggregated with tetraploid bovine embryos in a feeder-free system. 36% of these embryos passed the blastocyst stage, and 15% were implanted to recipient cows. Two of six calves were chimeric in the hair roots and liver, though all of them died shortly after birth. First, a long-term line of bovine ESC that was viable for about 258 days was obtained on a mixed feeder layer consisting of bovine and mouse fibroblasts selected and purified for 30 passages. Unfortunately, ES cell morphology, in this case, was confirmed only by alkaline phosphatase staining and embryoid bodies formation tests. In 2001 Mitalipova et al. described the Z2 line of bESC that was stable for more than 150 passages and proved that, as for murine ESC, bESC cells were positive for SSEA-1, SSEA-3, SSEA-4, c-kit and passed EB formation test but failed to form teratomas in nude/SCID mice [47]. Subsequent studies reported the SSEA-1, SSEA-4, Oct-4 and Nanog-positive bESC [48]. The next generation of methodology efforts focused on modification of culturing conditions and isolation of primary cells from the alternative sources. Verma et al. reported the efficient isolation of eight parthenogenetic cell lines (bpESC) from 15 in vitro parthenotes [49]. Five of these cell lines were viable for more than 140 days

and expressed for Oct4, Nanog, SSEA-1, and SSEA-4 (confirmed both on mRNA and protein level), bpESC could form embryoid bodies and differentiated into three germ layers. Epigenetic regulation also turned out to be an important tool in optimizing the bESC colonies derivation. According to the murine assays, epigenetic modification of ES-like cells with decitabine (5'-aza-2'-deoxycytidine) increased the number of ES-like colonies [50]. Using the pressed zona pellucida-free blastocysts and embryonic explants treated with five µM of 5'-azacytidine Lim et al. demonstrated the 18-fold increased efficiency of bESC isolation [51]. Importantly, these bESC-like cells passed all the pluripotency tests including teratoma formation in nude/SCID mice.

The best-suggested combination for the bovine ESC was FGF2, LIF and in FBS-containing medium (20) and 2i medium that had the same effect as for pigs [52]. It has also been stated that supplement of 2i medium to embryos between the morula and blastocyst stages led to the subsequent recovery of all cell lines from the blastocysts, although all of them demonstrated a limited proliferative capacity [53]. Gong et al. successfully applied a protocol of the enzymatic dissociation to maintain the ESC-like morphology during culturing, but these cells were viable for only ten passages [54]. The above summary suggests that well-established and genuinely pluripotent cell; lines do not exist for bovine ESC and that protocols suitable for human and rodents should be revised and expanded. Bogliotti et al. applied a custom TeSR1 base medium (growth factor-free) supplemented with FGF2 and IWR1 (CTFR), for the derivation of bESCs [55]. CTFR culture conditions allowed to obtain pluripotent ESC that could be established by three or four weeks after ICM plating and remained stable for more than 50 passages. These cells demonstrated stable karyotype and expressed the pluripotency markers SOX2 and POU5F1 (OCT4) and passed the teratoma assay test. Moreover, bESCs displayed transcriptional and epigenetic characteristics of primed pluripotency. bESC cultured in CTFR were also good donors for the somatic nuclear transfer that opens good perspectives for the genome editing studies. Another critical consideration for culturing the bESC is regulation of pluripotency-related genes in bovine inner cell mass (ICM) explants. Pant et al. showed that inhibition of the BMP4 pathway with noggin successfully up regulated the expression of Nanog in ICM explants [56]. Thus the evaluation of in vitro conditions to keep cells in an undifferentiated state for long passages should be the next step for bESC maintenance.

Bovine induced pluripotent stem cells

The IPSC technology in which embryonic or adult somatic cells gain the pluripotency features using gene reprogramming was first introduced for mouse [57]. The IPSC technology has been successfully applied to the full range of domestic animals [58-61].

Several efforts have been made for iPSC generation from cattle and cows. In most cases, transgene delivery methods included retroviral and lentiviral vectors. However, the use of viral transfection imposes the possibility of transgene expression after

the completion of reprogramming and can potentially escalate the possibility of mutation. To avoid the non-desirable effects, different groups applied the non-integrating plasmid vectors and transposons [62-66]. As for ESC, the primary trigger for colony derivation and cell reprogramming was the media composition and the vital supplemental factors. In the presence of LIF on its own or in combination with BMP4 or 2i medium, naïve cell phenotype was obtained [62-64, 67]. Reprogramming of the bovine fibroblasts with transposon delivery of 6 genes in a medium with FGF2 and LIF was also successful. However, the transgenes remained transcriptionally active after the reprogramming [65]. In 2016 Malaver-Ortega et al. showed that LIF alone or in combination with bFGF increased the mRNA expression of Oct4 and REX1 genes, while bFGF alone led to a dramatic decrease in expression of the same genes [68]. The results with GSK3/MEK inhibitors were also promising: 6-bromoindirubin-3-oxime and Sc1 inhibitors alone or in combination with 5-AzaC induced significantly higher levels of expression of endogenous REX1, OCT4, NANOG, and SOX2. Thus, the biPSC gene expression profile is malleable by modification of the cell culture conditions, and the culture conditions determined the differentiation capacities of iPSC. Transfection of biPSC with AID (activation-induced cytidine deaminase) also led to increased levels of OCT-3/4 and NANOG in biPSC due to the enhancement of DNA demethylation69. The novel approach to the derivation of biPSC was single cell cloning of bovine, iPSCs generated by the introduction of piggyBac transposons with cloning the efficiency at 40%; these cells were able to passage not less than 50 passages after the enzymatic dissociation and fit all the iPSC criteria.

The gold standard of IPSC generation method employs the lentiviral or retroviral transduction of the necessary transcription factors into the target cells. However, the new non-integrating techniques that transduce target cells with replication-competent RNAs of the reprogramming factors are widely used and extensively developed [70]. Another novel methodology focuses on optimization of mRNA-based procedures, like the synthesis of self-replicating DNA that eliminates the step of repeated transfections [71]. Furthermore, certain chemical compounds can in some cases substitute the endogenous reprogramming transcription factors, though that expression of OCT4 usually an essential prerequisite [72-73]. Hou et al. were the first who showed a successful reprogramming of mouse somatic cells with a cocktail of seven small molecules and called these cells ciPSC (chemically induced PSC) [74]. Unfortunately, to date, there are no reports for reprogramming biPSC with more high-end methods like non-integrating viral of mRNA based, that is also fair for other farm species.

Moreover, the germline contribution potential was not confirmed for the majority of the biPSCs, so most of the biPSC cells in the studies should be termed as IPSC-like cells. To date, all the extensive efforts failed to obtain the biPSCs that can produce viable and fertile offspring due to the possible presence of exogenous transcription factors [75]. The problem of exogenous expression must be solved for the field to move on, and the possible perspectives are the optimization of culture conditions

that could be favourable for the activation of the endogenous signalling pathways, autologous feeder layers (that in turn raise a point of assay reproducibility) and possible synthesis or derivation of supplemental growth factors that are more specific for the target species (like bovine LIF instead of human or mouse LIF).

Translational Medicine and Bovine Stem Cells

Bovine model is an underestimated model for studying human diseases. Cattle, like all large animal models, can provide significant advantages when establishing the long-therapy approaches, and modeling the disease-specific conditions. The most critical applications of bovine models include cartilage repair, mammary gland regeneration and hereditary genetic diseases of the blood and immune system.

Cartilage

Because of poor regenerative capacities of the articular cartilage, this tissue is one of the central targets of regenerative therapy. Experimental assays on bovine models addressed the problems of osteoarthritis, cartilage degeneration, induced chondrogenesis, and cartilage aging. The latter has been described on a 3D cell culture of native bovine cartilage chondrocytes from fetal, juvenile and adult animals. In this system, adult chondrocytes demonstrated diminished collagen formation capacities and altered biochemical properties [28]. Likewise, fetal and juvenile MSCs in hydrogels for the same animals had a greater matrix and mechanical properties, confirming that donor age is one of the significant determinants of regenerative capacities both for chondrocytes and MSC. Steinert et al. demonstrated that the differentiation of MSC into chondrocytes could be enhanced by transduction BMP-2, IGF1, and TGFb1 in the adenoviral system [76]. Simultaneous expression of three chondrogenic proteins in MSC resulted in larger cell aggregates and a higher level of cartilage-specific proteoglycans and collagen, indicating that gene-induced chondrogenesis may be a basis for stem –cell-based therapy for cartilage repair. Another approach to cell-based therapy is derivation and cloning of chondroprogenitor from native immature cartilage. Individual cells were isolated from bovine immature metacarpalphalangeal joints by differential adhesion to fibronectin. Cloned colonies of these cells had transcription profile similar to MSC and successfully differentiated into cartilage in 3D pellet cultures, so critical determinants of the progenitor phenotype could be maintained in in vitro system [77]. The next problem of chondrogenesis assays was that MSC constructs for cartilage repair could not achieve the functional properties of the native tissue due to possible absence of additional stimuli. It was natural to assume that mechanical stimulation could be the optimal strategy to overcome the functional limitations of in vitro assays. First studies on human and rabbit MSCs showed that dynamic compression enhanced the expression of collagen II and aggrecan and that presence of TGF-beta influenced this process [78-79]. Huang et al. applied long-term compressive loading to bMSC-seeded scaffolds and evaluated the mechanical properties of the constructs after six weeks [79]. Mechanical loading initiated shortly after induction of chondrogenesis and

matrix aggregation improved the mechanistic properties of differentiated chondrocytes and expression of aggrecan I and collagen type II in the presence of TGF-beta.

One more strategy to enhance the regenerative potential of MSC was to mimic the embryonic processes that occur during the development of healthy cartilage. Allon et al. designed the bilaminar cell pellet consisting of bMSC in a shell of nucleus pulposus cells [80]. In stress or inflammation conditions, this bilaminar cell pellet employed inductive mechanisms from development to trigger differentiation in the absence of growth factors, that seems to be a definite advantage of this model. Another co-culture system of neonatal chondrocytes with adipose-derived stem cells in 3D hydrogels demonstrated the synergy that correlated with the cell density increase and resulted in the formation of large cartilage nodules; this work raised the potential of stem cells to catalyze tissue formation via paracrine signaling81. The next step that pointed towards the clinical translation of cartilage repair on bovine models was to overcome the potential problems with integration of in vitro constructs into in vivo models of cartilage injury. Erickson et al. proposed to use bMSC in encapsulated methacrylated hyaluronic acid hydrogels that were precultured during four weeks before implantation, and, like in case of dynamic loading, showed that initial chondrogenesis before implantation allows avoiding the undesirable effects of environmental stress in vivo [28].

Mammary gland

A shared hierarcy of human and bovine mammary glands provides evidence of common mechanisms regulating the maintenance and differentiation of mammary stem cells from human and cattle [82]. Martignani et al. reported the in vitro colony formation assay of bovine mammary stem cells that lack aldehyde dehydrogenase activity and resembled the luminal epithelial stem cell through the differentiation [82]. The regenerated structures recapitulated the organization of bovine mammary tissue. Among the complex and heterogenic cell types in the mammary gland, epithelial and myoepithelial progenitors are of particular interest for the regeneration of the vascular network in this tissue.

In this direction, Rauner et al. tried to establish in-vivo expansion of bovine mammary stem cells using a xanthosine administration to induce the proliferation of mammary stem cells [83].

The outputs of this research will have a significant impact on several aspects of mastitis and will improve current treatment regiments, but to date, the experimental assays with iPSC and MSC differentiation in the mammary gland are still at the beginning.

Genetic diseases of immune and blood cells

Several human genetic diseases have counterparts in large animal models. In most cases, bovine models contain the mutation of the same gene or employ the similar pathogenesis, and thus can serve as valuable preclinical models for testing the cell transplantation or gene therapy approaches.

Bovine Model of Leukocyte Adhesion Deficiency Type I (BLAD) was first described in a Holstein's calf with marked neutrophilia and impaired neutrophil function. BLAD calves have pneumonia, enteritis, periodontitis, and altered wound healing. The primary defect in BLAD leukocytes, as in human, lies in a deficiency of β2 integrin subunit of CD18 due to the point mutation in a highly conserved region of the CD18 gene [84]. Gene therapy approach using SCNT using bone marrow cells from a healthy cow allowed to obtain 0.3-0.5% of donor neutrophils in the peripheral blood of the calf in a year after therapy [85]. The heifer also demonstrated a sustainable clinical improvement in 28 months with increased levels of CD18 positive donor leukocytes86.

Chediak-Higashi Syndrome (CHS) is an autosomal recessive disorder that affects nervous, and the immune system is considered to be a lysosomal disease [87]. An animal model for CHS include Hereford, and Japanese black cattle Japanese black cattle have abnormal giant granules in leukocytes, decrease the number of granules in platelets and bleeding from minor trauma [88-89]. CHS in Hereford cattle and Brangus cattle with chronic infections, and a marked susceptibility to infection, petechial hemorrhaging, pulmonary abscesses, and pneumonia demonstrated a more severe phenotype compared to Japanese black cattle. CHS in Brangus cattle had a similar phenotype to that of Hereford cattle [90]. The severe CHS phenotype contrasts with the disease in Japanese black cattle, which was moderate and rarely fatal. It is possible that the causative mutation in CHS-affected Brangus and Hereford cattle is different from that in Japanese black cattle. Gene therapy and stem cell-based approach have been not attempted on these models due to the large size of the affected gene, but a novel vector packaging and delivery models may overcome these difficulties.

Conclusions and future perspectives

Though the indisputable importance in agriculture and biotechnology, bovine stem cells and have not received the proper attention as a model for preclinical research. Cattle represent a valuable model for the development of cell-based treatments before introducing them to the clinical practice. In this review, we showed that bovine stem cells from different origins require a unique set of culturing and maintenance conditions and possess a phenotype that does not correlate with human or rodent stem cells. At the same time, applications of bovine stem cells in regenerative therapy, and gene editing therapy can be a useful translational preclinical model. It is necessary to develop the exact phenotype criteria to manipulate bovine stem cells in clinical practice. Bovine stem cell therapy offers new perspectives for exploitation and exploration in livestock industry research.

Acknowledgments

Author contributions

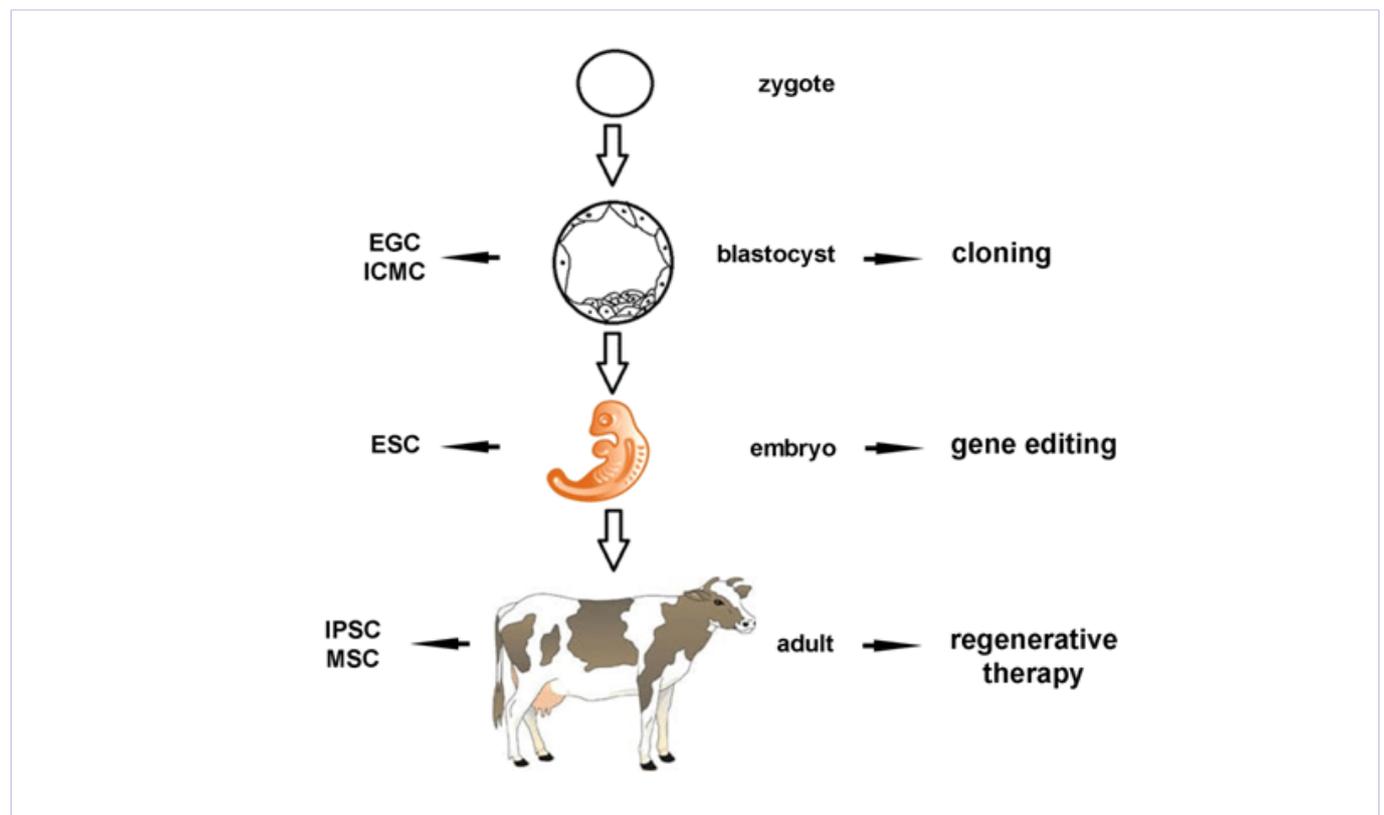Aleena A. Saidova designed, drafted and critically revised the manuscript

Figure 1: Bovine stem cells with decreasing potencies are derived from the animals at different developmental stages. The only exception is induced pluripotent stem cells (iPSCs) that are derived from adult animals.

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