Chapter 1

An Overview of Pluripotent Stem Cells

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1. Introduction

This book is entitled **Pluripotent Stem Cells** (PSCs) and various contributors have written on different aspects of the PSCs. But I will fail as an editor of this book if I do not bring to the reader's attention the all the sources of PSCs (Figure 1).

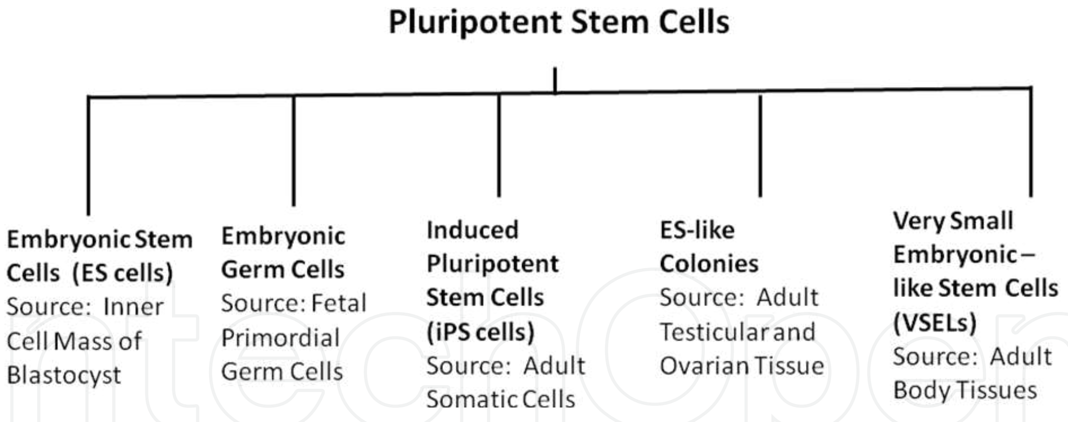

Figure 1. Potential sources for pluripotent stem cells

Professor Thomson and Prof Gearhart published landmark papers in 1998 wherein they published derivation of PSCs from inner cell mass of spare human blastocyst [1] and from early fetal germ cells [2] respectively. Recently Professor Yamanaka was awarded the Nobel prize for medicine for establishing protocols to reprogram somatic cells to embryonic state with the help of 4 factors [3, 4]. Besides this there are several papers which have reported derivation of ES-like colonies from adult testicular biopsies in both mice [5, 6] and men [7-10]. Similarly Gong et al [11] reported ES-like culture using ovarian tissue. There is a huge body of literature suggesting that mesenchymal stem cells (MSCs) have pluripotent characteristics and can transdifferentiate [12]. We have recently published that adult gonads [13, 14] umbilical cord blood/tissue, bone marrow [15] etc. harbor a sub-population of similar kind of pluripotent stem cells termed very small embryonic-like stem cells (VSELs). We also developed a case for VSELs which may be resulting in ES-like colonies rather than de-differentiation of spermatogonial stem cells into pluripotent state [16]. Moreover, the VSELs have confused the field of MSCs, since they are always present as a sub-population amongst MSCs but have remained unnoticed and the pluripotent properties were conferred incorrectly on to the MSCs. VSELs are not widely accepted at present, but have been shown to have promising application towards regenerative medicine.

Thus the aim of the present chapter is to update the readers with the recent advances with embryonic stem cells, induced pluripotent stem cells and VSELs which have been implicated with maximum potential for use in cell-based therapies.

2. Embryonic stem cells

Embryonic stem (ES) cells, as the name suggests, are derived from embryos, more specifically from the inner cell mass (ICM) of the blastocyst. ES cells are characterized by two hallmark properties viz., self-renewal - ability to proliferate indefinitely and pluripotency - capacity to give rise to cells of all the three embryonic germ lineages such as ectoderm, mesoderm and endoderm. They possess a high nucleo-cytoplasmic ratio and telomerase activity. ES cells display high activity of endogenous alkaline phosphatase and express several nuclear and cell-surface markers of pluripotency. They tend to cluster together when cultured in suspension on a non-adherent surface to form 3D aggregates known as embryoid bodies that may be simple or cystic. Moreover, they produce teratomas on injection in immune deficient (SCID) mice, are clonogenic and are capable of producing chimeras when injected into blastocysts in the mouse model.

3. Mouse ES cells

ES cells were first derived from ICM of mouse blastocyst stage embryos [17, 18]. Besides ICM of blastocyst mouse ES (mES) cells have also been derived from cleavage stage embryos and even from biopsied individual blastomeres of two- to eight-cell stage embryos [19- 21]. In general, mES cells can be cultured on a layer of mitotically inactive mouse embryonic fibroblasts (MEF) in the presence of serum and leukaemia inhibitory factor (LIF). The cytokine LIF sustains the self-renewing and pluripotency features of mES cells. LIF, a soluble glycoprotein of interleukin (IL)-6 family of cytokines acts via binding to heterodimers of the LIF-receptor and the signal transducer gp130 resulting in activation of STAT3 signaling [22-24]. In absence of serum, LIF is incapable of maintaining pluripotency of mES cells; however, in combination with bone morphogenetic protein-4 (BMP4) prevents differentiation of mES cells [25]. BMP4 induces expression of *Id* (Inhibitor of differentiation) genes via the Smad pathway. Overexpression of *Id* indeed allows proliferation of mES cells in the presence of LIF and without need of BMP4 or serum.

4. Human ES cells

A breakthrough occurred with the derivation of human ES (hES) cells in 1998 [1]. Since the first report on derivation of hES cell lines at least 1071 hES cell lines have been derived worldwide [26]. Besides spare human blastocysts, hES cell lines have also been derived from morula stage embryos [27], abnormally developing and arrested embryos [28], single blastomeres of 8-cell stage embryos [29] and 4-cell stage embryos [30, 31]. Mitotically inactivated feeder cells and serum containing medium along with basic fibroblast growth factor (bFGF) are generally used to maintain hES cells. LIF and its related cytokines fail to support hES cells in serum-containing media that supports mES cells despite the existence of a functional LIF/STAT3 signaling pathway in hES cells [1, 32, 33]. In contrast to mES cells, FGF and TGF/Activin/Nodal signaling are essential for the self-renewal of hES cells [34]. Although, elements of the BMP pathway exist in hES cells [35], but unlike mES cells, BMPs added to hES cells in conditions that would otherwise support self-renewal, cause rapid differentiation [36]. Recent studies have revealed multiple interactions between the FGF, TGFβ, and BMP pathways in hES cells. Activin induces bFGF expression [37], and bFGF induces Tgfβ1/TGFβ1 and Grem1/GREM1 (a BMP antagonist) expression and inhibits Bmp4/BMP4 expression in both fibroblast feeders and in hES cells [38].

Although similar in their characteristics such as expression of Oct-4, Nanog, alkaline phosphatase activity, formation of embryoid bodies, teratoma formation, some potential differences exist between mES cells and hES cells. In contrast to mES cells which show expression of SSEA-1, hES cells express SSEA-3/4, TRA-1-60/81. Further, the average population doubling time for hES cells is longer compared to mES cells (30-35 hr vs. 12-15 hr).

5. *In vitro* culture and differentiation of hES cells

Although hES cell lines were first derived on MEF feeder layers, continuous efforts towards developing xeno-free culture system has resulted in establishment of human feeders derived from fallopian tube epithelium [39], fetal foreskin, muscle [40, 41], or amniotic epithelium [42]. Attempts have been made to derive new hES cell lines in more defined conditions including serum-free or feeder-free conditions in the presence of extracellular matrices such as matrigel and fibronectin [43-45]. Crook et al [46] derived six clinical-grade hES cell lines using GMP-grade human feeder grown in a medium with GMP-quality FBS and propagated the cell lines using a GMP formulation of Knockout Serum Replacement (KO-SR). Although not xeno-free, the cell lines meet clinical quality. Sidhu et al [47] reported the derivation of hES cell line in culture using human-derived collagen coated plates and KO-SR to maintain human feeder fibroblasts. A fully defined xeno-free medium (RegES), capable of supporting the expansion of hES cell lines, induced pluripotent stem (iPS) cells and adipose stem cells has been described [48]. Recently, Wang et al [49] have developed a xeno-free and feeder-cell-free culture system for propagating hES cells and hiPS cells using human plasma and human placenta extracts.

Human ES cells have the ability to form 200 odd cell types in our body. Essentially, ES cells can be differentiated spontaneously by embryoid body formation or by directed differentiation using a cocktail of growth factors. Several growth factors have been shown to direct differentiation of ES cells namely activin-A and transforming growth factor (TGF-β1) mainly induce mesodermal cells; retinoic acid (RA), epidermal growth factor (EGF), BMP-4, and bFGF activate ectodermal and mesodermal cells; β nerve growth factor (NGF) and hepatocyte growth factor (HGF) differentiate all three embryonic germ layers [50-53]. Directed differentiation is a more controlled process involving stage specific sequential addition of growth inducers and inhibitors which are known to effect key pathways. For e.g. activin A and BMP4 are two such growth factors which have been used widely for cardiogenic differentiation. Various studies have shown that hES cells can be differentiated into neuronal [54], hematopoietic [55], endothelial [56], muscle [57], cardiac [58, 59] pancreatic [60, 61], hepatic [62] lineages. Although hES stem cell lines are similar with respect to self-renewal and expression of pluripotency markers, published literature however suggests that they exhibit differences in their differentiation ability under identical culture conditions [63, 64].

6. Potential use of ES cells

The remarkable features of hES cells has served as an important breakthrough for basic research and has great potential for regenerative medicine. ES cells may act as key research tools for understanding the complex events that occur during embryonic development which may explain the causes of birth defects. They are ideal candidates for studying apoptosis in early stage of embryo, mechanism of differentiation, mutagenesis, immune rejection and aging. Human ES cells and their derivatives may be used for testing therapeutic drug efficacy and toxicity. They also have wide applications in tissue engineering. Following their culture on polymer scaffold, it has been reported to coax stem cells to form tissues with characteristics of developing human cartilage, liver, neurons and blood vessels.

Despite being associated with the risk of inducing teratomas and immune rejection, the vital potential application of hES cells is the generation of cells and tissues that could be used for cell-based therapies. Human ES cells directed to differentiate into specific cell types offer the possibility of a renewable source of replacement cells and tissues to treat a myriad of diseases and disabilities including Parkinson's and Alzheimer's diseases, spinal cord injury, burns, heart failure and diabetes etc. The first FDA-approved phase-1 clinical trial for safety began with Geron's (Menlo Park, CA, USA) GRNOPC1 derived oligodendrocyte progenitor cells to treat complete thoracic-level spinal cord injury [65]. The trial was initially stalled for occurrence of microscopic cysts in animal transplants but was later approved [66, 67]. However, in November 2011 Geron dropped out of stem cell research for financial reasons and said that they would continue to monitor existing patients, and were attempting to find a partner that could continue their research. The recent success of a prospective clinical study of Advanced Cell Technology (CA and MA, USA) to establish the safety and tolerability of subretinal transplantation of hES cell-derived retinal pigment epithelium (RPE) in patients with Stargardt's Macular Dystrophy (SMD) and Dry age-related Macular Degeneration (Dry AMD) represents an important step towards therapeutic use of hES cells [68]. Although long-term follow up is essential and eye is an immune-privileged site; it is still encouraging to note that there are no associated signs of hyperproliferation, tumorigenicity, ectopic tissue formation, or immune- rejection after 4 months of transplantation.

7. Induced pluripotent stem cells

A major progress in the stem cell field was generation of induced pluripotent stem (iPS) cells by the reprogramming of somatic cells to an embryonic stem cell state using a cocktail of transcription factors. In 2006, Takahashi and Yamanaka reprogrammed mouse fibroblasts through retroviral transduction with 24 candidate genes [3]. The pool of genes was gradually reduced to four transcription factors, Oct4, Sox2, c-Myc, and Klf4. The results were rapidly confirmed by various researchers [69-71]. Soon the technology was successfully applied to generate iPS cells from human fibroblasts [4, 72, 73]. Concurrently, another group identified Oct4, Sox2, Nanog, and Lin28 to be sufficient to reprogram human cells, with Oct4 and Sox2 appearing essential and the other two factors either strongly (Nanog) or modestly (Lin28) influencing the efficiency of reprogramming [74].

The different ways for generation of mouse and human iPS cells using various reprogramming factors has been well summarized by Maherali and Hochedlinger [75] and Kiskinis and Eggan [76]. The choice of a gene delivery system is a key aspect for generation of iPS cells and has been very well reviewed by Oh et al [77]. Many researchers have reported use of integrating viral vectors such as retroviral [4, 73, 78] and lentiviral vectors [74, 79], non-integrating viral vectors such as adenoviral [80] and Sendaiviral vectors [81], nonviral methods such as plasmid DNA [82], piggyBac transposons [83, 84], recombinant proteins [85, 86], mRNAs [87] and small molecules such as valproic acid [88]. Moreover, derivation of iPS cells from patients suffering from the neurodegenerative disease amyotrophic lateral sclerosis (ALS) [89] as well as patients with other diseases, including juvenile onset type 1 diabetes mellitus, Parkinson disease (PD) [90], and spinal muscular atrophy (SMA) [91] has been reported.

8. Advantages and disadvantages of iPS cells

As a potential application in cell based therapy, one of the major advantages of iPS cells is the avoidance of immune rejection, since they are derived from a patient's own cells, as well as ethical issues associated with the use of human embryos. Furthermore, iPS cells are similar to ES cells in many aspects, including cell morphology, expression of pluripotency markers, long telomeres and capability to form embryoid bodies, teratoma, and viable chimeras [92, 93]. Apart from use in cell-based therapy, iPS cells derived from patients with disease can serve as an effective model to understand the mechanisms of diseases.

However, use of iPS cells have several drawbacks and are mostly related to current reprogramming methods. Viral vectors employed for gene delivery has led to the integration of multiple viruses into iPS cell genomes, resulting in tumorigenesis due to genetic abnormalities in the cells. Moreover, the efficiency of reprogramming of human iPS cells from fibroblasts is very low, approximately less than 0.02% [94]. The use of Myc gene as a reprogramming factor and/or the reactivation of a silenced Myc gene might cause iPS cells to become cancer cells [95].

Recently, three studies published in Nature showed that the reprogramming process and the subsequent culture of iPS cells *in vitro* can induce genetic and epigenetic abnormalities in these cells. Gore et al [96] found on an average of five point mutations in each of the iPS cell line analyzed, with the majority of the mutations being non-synonymous, nonsense or splice variants, and were enriched in genes mutated or having causative effects in cancers. Hussein and colleagues [97] showed that copy number variations (CNVs) occurred at a high rate during the process of reprogramming leading to genetic mosaicism in early-passage iPSCs. Analysis of the CG methylation patterns by Lister et al [98] identified numerous differentially methylated CG regions (CG-DMRs) between iPS cells and ES cells. The presence of a core set of CG-DMRs in every iPS cell line suggests hotspots of failed epigenomic reprogramming. These studies raise concerns over the implications of such aberrations for future applications of iPS cells. A much more in-depth research is necessary to understand about the reprogramming process and the biological consequences of these genomic and epigenomic changes needs to be investigated.

9. Very small embryonic like stem cells

The ethical and other technical issues concerning the use of ES cells in regenerative medicine have led to search for alternative stem cells with therapeutic potential. In this regard adult stem cells can potentially provide a therapeutic alternative to ES or iPS cells. Though adult stem cells are known to be tissue specific and can only differentiate into cells of their tissues of origin, nevertheless several studies have reported that adult stem cells can differentiate in to cells of completely different lineage. The process is termed as adult stem cell plasticity. Wagers and Weissman proposed few potential mechanisms and explanations for the observed adult stem cell plasticity [99]. The potential mechanisms include trans-differentiation or de-differentiation of stem cells, presence of multiple different stem cells in a tissue, presence of pluripotent stem cells in addition to adult stem cells and cell fusion of stem cell with cell of different lineage. However, several lines of evidence support existence of pluripotent stem cells in adult tissues that can differentiate into all three lineages explaining adult plasticity the best. Many investigators have reported presence of pluripotent stem cells in adult tissues and were defined either as mesenchymal stem cells (MSCs) [100], multipotent adult progenitor cells (MAPCs) [101], marrow isolated adult multilineage inducible cells (MIAMI) [102], multipotent adult stem cells (MASCs) [103], very small embryonic like stem cells (VSELs) [104]. Although these cells may represent an overlapping type of stem cells, the most characterized among these cells to the single cell level is VSELs and they have been isolated and identified in several adult body organs.

VSELs are defined as epiblast derived stem cells, which are deposited early during organogenesis and may serve as source of tissue commited stem cells. Pluripotent VSELs (Oct4^{+}, SSEA1^{+}, Sca1^{+}, Lin^{-}, CD45^{-}) were first reported by Ratajczak and group in various adult mice tissues [105]; highest numbers being in brain, kidneys, muscles, pancreas and bone marrow [106]. These are diploid cells with high telomerase activity, express other pluripotent (Rex-1, Nanog, SSEA and Klf-4) and germ cell (Mvh, Stella, Fragilis, Nobox and Hdac-6) markers and decrease in numbers with age [107]. An important evidence for pluripotency of VSELs is hypomethylated status of OCT-4 promoter and its association with transcription promoting histones [108] as well as presence of bivalent domains [109]. Like embryonic stem cells they do not express MHC class I and HLA-DR antigens and are also negative for mesenchymal stem cell markers like CD90, CD105, CD29. They are very small in size (3-5 um in mice), have a large nucleo-cytoplasmic ratio, and open chromatin structure for OCT-4 and Nanog promoter [107]. OCT-4 expression at mRNA and protein level in VSELs has been confirmed using sequence specific primers. VSELs have the ability to differentiate into three germ layers *in vitro*, however unlike ES cells, VSELs neither complement during blastocyst development nor form teratomas in immuno-deficient mice [110]. Attempts have been made to propagate them on feeder layers, but they do not self-renew as easily as the established embryonic stem cell lines possibly because of altered methylation status of some developmentally crucial genes. Similar VSELs have also been isolated from human umbilical cord blood, mobilized peripheral blood, and adult bone marrow by flow cytometry as CD133^{+}, lin^{-}, CD45^{-} [104] and also by differential centrifugation method [15, 111].

VSELs are descendants of epiblast stage pluripotent stem cells. They get deposited in various body organs including the gonads in early stages of development, as a quiescent stem cell population which possibly serves as a back up to the tissue committed stem cells (TCSCs) [112]. These two populations of stem cells (VSELs and TCSCs) together are responsible in bringing about tissue renewal, homeostasis and regeneration after injury throughout life and decrease in number with age. The co-existence of two stem cell populations (the more primitive being quiescent and the progenitor being more rapidly dividing) has been recently proposed [113, 114]. VSELs are the DNA label-retaining (e.g. BrdU), quiescent stem cells with lower metabolic state whereas the tissue committed stem cells actively divide and do not retain DNA label over time. They are highly mobile, respond to the SDF-1 gradient and enter into circulation in case of any injury to bring about regeneration and homeostasis. They are also considered as a missing link to support the germ-line hypothesis of cancer development [115, 116].

VSELs in Umbilical Cord Blood (UCB): A population of human cells similar to murine bone marrow derived VSELs was first reported by Kucia et al in umbilical cord blood [117]. These UCB derived VSELs (Lin-/CD45-/CD133+) ranged between 6-8 um in size, possess large nuclei and express nuclear embryonic transcription factors OCT-4, Nanog and cell surface SSEA-4. The strategy of isolation of VSELs from cord blood is hampered by their small size as they get discarded along with debris. Recently our studies reported that VSELs settle along with RBCs and are not enriched in interphase layer of MNCs obtained after ficoll separation of cord blood [15].These VSELs expressed pluripotent markers OCT-4,primitive marker CD133 along with primordial germ cell marker stella and fragilis indicating their epiblast origin. Our studies have also shown the presence of VSELs in the discarded fractions of bone marrow and cord blood obtained after processing [15].

VSELs in adult mammalian gonads: Initial studies by Ratajczak group have shown that mouse testis harbor VSELs [106]. Our group has identified presence of VSELs in testis of human and mice as well as in ovaries of human, sheep, monkey, rabbit and mice [13]. These VSELs are localized in the basal layer of cells adjacent to the basement membrane in seminiferous tubules [13] and were found interspersed with the ovarian surface epithelial cells [14]. The main approach in identifying the VSELs in adult mammalian gonads involves studying differential expression of a pluripotent marker OCT-4. OCT-4 is an octamer binding transcription factor required for maintaining pluripotency of cell. Published literature on OCT-4 in somatic stem cells has confused stem cell researchers [118-120] because of the presence of several pseudogenes and alternatively spliced transcripts [118, 121]. Thus a careful designing of primers for RT-PCR analysis and proper selection of antibodies becomes essential to detect specific transcripts. Also a careful selection of OCT-4 antibodies is essential to detect pluripotent stem cells [119]. We used a polyclonal OCT-4 antibody that enabled the simultaneous identification of VSELs with nuclear OCT-4 and tissue committed stem cells with cytoplasmic OCT-4. In addition, careful selection of primers for OCT-4A and total OCT-4 for Q-PCR studies has helped us generate interesting results [13-15, 122].

VSELs in Testis: We have documented that adult human testis harbors a population of pluripotent VSELs (with nuclear OCT-4A) which are more primitive to A_{dark} Spermatogonial Stem Cell (SSC) (with cytoplasmic OCT-4B). The VSELs possibly give rise to A_{dark} SSCs, which in turn undergo clonal expansion as evident by the presence of cytoplasmic bridges between the rapidly dividing cells [13]. OCT-4 is not immuno-localized in more differentiated male germ cells. Based on this study a new hierarchy of testicular cells was proposed with all testicular cells originating from VSELs and not from SSCs as generally believed. Similarly presence of VSELs distinct from SSCs was also identified in mouse testicular tissue.

VSELs in Ovaries: The long- held dogma in female biology is that women and other mammalian females are born with fixed and non-renewing pool of germ cells, which are enclosed in structures called follicles. Their number decrease with age due to ovulation or atresia and their exhaustion lead to menopause. However in last 8 years several investigators with access to modern molecular techniques have convincingly demonstrated that adult mammalian ovaries harbor stem cells and undergo postnatal oogeneisis and thus have challenged the central dogma. Presence of PSCs in adult ovary has been demonstrated by many groups [11, 14, 123, 124]. Our group has identified two distinct types of stem cells in ovarian surface epithelium (OSE) of human and other mammalian species [14, 122]. The two stem cells are VSELs that express OCT-4 in nucleus, which are pluripotent and slightly larger progenitor committed cells (termed Ovarian Germ Stem Cells-OGSCs) that express OCT-4 cytoplasmically. This is very similar to reported presence of VSELs and Spermatogonial stem cells in adult mammalian testis as mentioned earlier. We have recently reviewed various publications on ovarian stem cells and explained the results in the context of VSEL biology [122]. Readers are encouraged to read the review for more details.

Based on our studies in ovarian stem cells and other literature, we have proposed a model for oogenesis and follicular assembly in adult mammalian ovaries [122]. According to the model, VSELs with nuclear Oct-4 that are located in the OSE undergo asymmetric cell division and give rise to cells with cytoplasmic Oct-4 (OGSCs, which intensely stain with Haematoxylin). The OGSCs undergo further proliferation, meiosis and differentiation to assemble into primordial follicles in the OSE. The granulosa cells are formed by the epithelial cells through epithelial mesenchymal transition. As the follicles grow and further mature they shift into the ovarian medulla.

10. Clinical potential of VSELs

The clinical potential of VSELs, isolated from cord blood or bone marrow by flow cytometry, is just beginning to emerge. In various disease models like myocardial infarct [125, 126], stroke [127], skin burn injury [128], neural regeneration [129] etc. these cells get mobilized into circulation within 24 hours. For myocardial regeneration, the VSELs are very efficient to improve LV ejection fraction and attenuation of myocardial hypertrophy [126]. As they become scarce with age, regeneration becomes inefficient resulting in age-related disease manifestations.

The identification of VSELs in gonads has far reaching implications in reproductive health issues. Understanding the biology of VSELs in gonads may help explain the mechanisms of different pathologies of gonads and may pave for new treatments for infertility. However, application of VSELs to improve reproductive health needs to be researched and established. We have recently studied the differential effect of busulphan on the relatively quiescent VSELs versus rapidly dividing germ cells in adult mice gonads (unpublished results). The VSELs were found to be resistant to the treatment, however were unable to differentiate probably due to the altered niche of VSELs due to treatment. Ratajczak group recently reported that VSELs in mouse bone marrow are resistant to total body irradiation [130]. They observed that there was increase in proliferation of VSELs post treatment, although were unable to reconstitute the bone marrow. These studies open up newer and exciting avenues for fertility preservation in cancer survivors who are rendered infertile by various cancer treatments.

11. Advantages of VSELs over ES cells

VSELs can be derived easily from autologus source and do not form teratoma easily [131]. Thus both the major concerns associated with ES cells of immune-rejection and risk of teratoma formation is taken care of.

12. Advantages of VSELs over iPS cells

There is no need for reprogramming somatic cells (which may harbor mutations) to embryonic state when pluripotent ES-like stem cells can be harvested from adult tissues. They may also be superior to iPS cells since they are derived from a very quiescent stem cell population and are thus 'young' cells with long telomeres that could be isolated from an aged body, in contrast to iPS cells which are derived from terminally differentiated somatic skin fibroblasts (with shortened telomeres) that tend to accumulate DNA mutations over time. In addition, VSELs do not have epigenetic issues associated with iPS cells. Unlike iPS cells, there is no requirement of viral vectors and hence risk of transformation of VSELs into cancer cells is avoided.

13. Future perspectives

Embryonic stem cells are considered to be 'magic bullets' having a great potential for cell-based therapy, however future clinical use of ES cells are still plagued by ethical issues. Hence there is urgent need to expand research in derivation and culture of pluripotent stem cells from alternate sources. Induced pluripotent stem cells though believed to be ideal candidates need to be exploited further to realize their clinical potential. Considering the potential advantages of VSELs over ES and iPS cells, the need for research to harness potentials of VSELs is high. Currently the availability of large number of VSELs for effective use in clinical applications is limited. Research is progressing towards expansion of VSELs in culture and is still in nascent stages. Also, many key questions have to be answered before realizing the full potential of stem cells.

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