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Viewpoint

Stem cells, progenitors & regenerative medicine: A retrospection

It is well evident that the embryonic stem cells (ESCs) are pluripotent, can differentiate into all the three germ layers namely ectoderm, mesoderm and endoderm and into 200 odd cell types present in the body, are immortal, can expand in large numbers *in vitro*, and are genetically stable over long periods in

culture. Three groups in the country have successfully derived well characterized human ES (hES) cell lines including Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bengaluru[1], Reliance Life Sciences[2] and National Institute for Research in Reproductive Health (NIRRH)[3], Mumbai. However, hES cells have the associated issues of immune-rejection and risk of teratoma formation. On the other hand, the adult stem cells (ASCs) do not expand in culture but these can be isolated from the patients' own bone marrow and thus are considered safe. Though studies/ trials have been undertaken using autologus stem cells in India[4], at the global level this approach has not shown the desired results[5-7]. The National Guidelines for Stem Cells Research and Therapy became available in 2007, and has been recently revised (*http://icmr.nic.in/guidelines/NGSCR%202013.pdf*) and are named National Guidelines for Stem Cells Research indicating that stem cells are not yet ready for therapy and more research is required before these are put to translational use. Simultaneously, Central Drugs Standard Control Organization (CDSCO), New Delhi has put up a draft guidance document for regulatory approval of Stem Cell and Cell Based Products (SCCPs) (*http://www.cdsco.nic.in*). Several companies/clinics have started culturing and using mesenchymal stem cells (MSCs) for treating various conditions in India. The situation is alarming as it is not clear whether these therapies would benefit the public. Are mesenchymal cells true stem cells or just stromal cells? Similar situation exists with cord blood banking to treat multiple diseases and claims that cord blood may serve as a future health insurance for the baby. It has become evident that *(i)* besides blood related disorders, cord blood stem cells may not regenerate other tissues as these do not possess trans-differentiation potential, and *(ii)* autologus cryopreserved cord blood sample will never suffice when the baby grows up as an adult[8]. Use of fresh unrelated donor cord blood sample may be a viable alternative as emerging studies suggest that mismatched allogeneic cord blood stem cell transplant is easily tolerated with 1-2 antigen mismatch and is associated with lower anticipated risk of graft versus host disease (GVHD)[9,10]. Moreover, the present article suggests that pluripotent stem cells exist in adult body organs and the need to bank cord blood as a source of stem cells may be a futile exercise. The concerns raised in a recently published editorial[11] are timely. More research is required to understand how normal body stem cells interact with their niche, differentiate and get mobilized under disease conditions to bring about regeneration. Such information is crucial to exploit therapeutic potential of stem cells in the field of regenerative medicine. Here the author discusses the existing confusion as to what are the true stem cells in the body, how these are different from the tissue-specific progenitors and whether these may be implicated in initiating cancers. It also provides an interesting perspective to the stem cell field based on available literature and highlights the importance of microenvironment/niche in deciding the stem cell fate.

To begin with, we need to remind ourselves that the stem cells are basically cells that reside in a specialized somatic microenvironment (niche) and undergo asymmetric cell divisions, can self-renew and at the same time give rise to the tissue-specific progenitors. The progenitors (immediate descendants of stem cells) multiply in large numbers, differentiate into tissue-specific cells and thereby maintain homeostasis. The normal body stem cells are expected to get mobilized, bring about regeneration and also possibly transform into cancer stem cells under certain yet not well understood conditions (Fig. 1).

ES cells derived from the inner cell mass of the blastocyst, undergo symmetrical cell divisions 'self-renewal' in culture[12] and have been correctly labelled as a tissue culture artifact[13] as these do not reflect *in vivo* conditions. The inner cell mass from which the embryonic stem cells are derived is a transient phase during development and does not behave as stem cells *in situ*. The ES cells rather arise through selection and adaptation under *in vitro* conditions. These stem cells are pluripotent, produce teratoma on being injected in SCID (severe combined immunodeficiency) mice and integrate easily in a developing blastocyst to form a chimera. Differentiation of human ES cells into fully committed cell types *in vitro* is an inefficient process, thus attempts are made to transplant progenitors and allow *in situ* maturation. However, such efforts have resulted in short-term benefits[14]. These stem cells have the potential to differentiate into various cell types *in vitro* but whether these can sustain long-term regeneration *in situ* remains to be demonstrated. It is indeed fascinating that recently human ES cells were successfully differentiated into mini-brains[15]. Early success on safety and efficacy has been reported using human embryonic stem cells derived retinal epithelial cells but it is still a long way to go[16]. Tabar and Studer[17] have discussed the existing challenges in translating ES based cell therapies to the clinic.

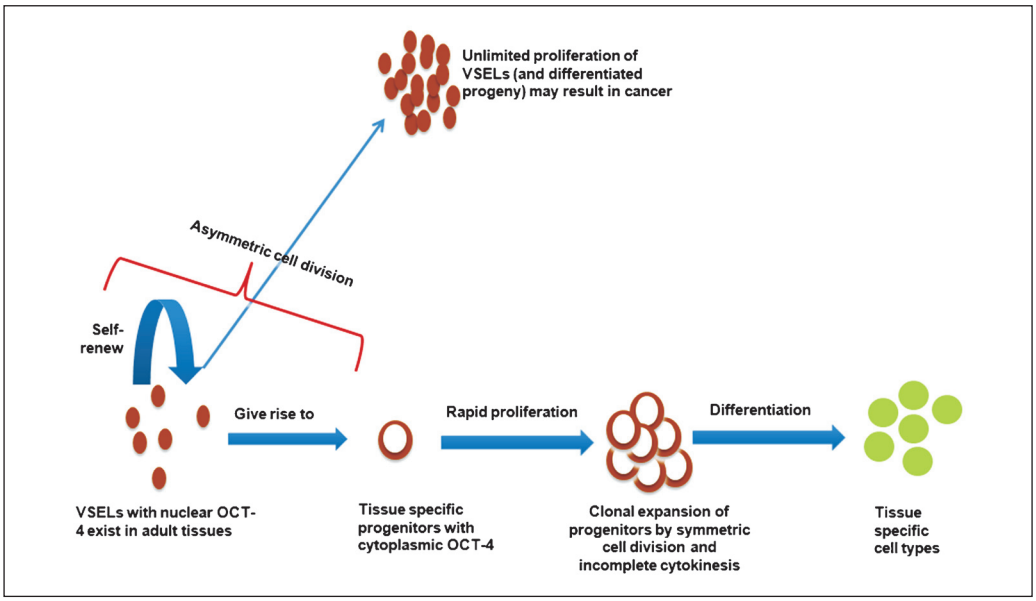

Fig. 1. Proposed basic concepts in stem cell biology. VSELs, very small embryonic-like stem cells; OCT-4, a stem cell marker, nuclear OCT-4A isoforms responsible for pluripotent state; ASCs, adult stem cells which are tissue specific, actually progenitors; CSCs, cancer stem cells.

Best examples of ASCs are the haematopoietic stem cells (HSCs) and MSCs which have been extensively studied in the bone marrow, cord blood, Wharton's jelly, *etc*. and spermatogonial stem cells (SSCs) in the testes. HSCs undergo symmetric cell divisions to maintain themselves but whether they undergo asymmetric cell divisions remains elusive[18]. Ting *et al*[19] have found that the endocytic protein Ap2a2 possibly regulates asymmetric cell renewal of HSCs since it enhances HSC function without any substantial increase in HSC numbers and that the protein was asymmetrically distributed during cell division. However, more work needs to be done to prove this conclusively[18]. Further, like HSCs, the testicular SSCs undergo symmetric cell divisions[20]. If both HSCs and SSCs undergo symmetric cell divisions, which are the stem cells in these tissues that undergo asymmetric cell division? Are we missing out on them? At the same time it has been reported that various adult body organs (gut epithelium, hair, bone marrow, skin, *etc*.) harbour two stem cell populations including a relatively quiescent and actively dividing population[21-23].

There is yet another rapidly expanding body of literature that needs to be acknowledged. This is the presence of very small embryonic-like stem cells (VSELs) in adult body organs and have been described by different names such as ELH (elutriation, lineage depletion and the ability to home to bone marrow) cells, spore cells, MAPCs (multipotent adult progenitor cells), MIAMI (marrow-isolated adult multineage-inducible cells), Muse cells (multilineage-differentiating stress-enduring stem), *etc*[24]. VSELs first reported by Ratajczak's group[25], are believed to be the primordial germ cells (or their precursors) that arise from the embryonic germ cells in the yolk sac and in addition to migrating along the dorsal mesentery and settling in the gonadal ridge to differentiate into germ cells these migrate and settle in various body organs and persist throughout life. VSELs are extremely quiescent in nature and studies done using mouse bone marrow VSELs have shown that IGF-1 mediated growth factor signaling pathways play a crucial role in their quiescence[26], these are mobilized under disease condition, give rise to three germ layers in both mice[27] as well as in humans[28], and have been proposed to be the embryonic remnants that could result in various cancers[29]. We have reported spontaneous differentiation of ovary surface epithelial cells into oocyte-like structures[30] and also that testicular cell suspension from a busulphan treated mouse testes spontaneously differentiating into sperm (unpublished data). Both had VSELs in the initial culture whereas similar success of differentiating hES cells into gametes *in vitro* has not yet been achieved. Reasons for this remarkable potential of VSELs over hES/iPS cells have been recently reviewed[31]. We have also demonstrated that

VSELs regenerate the adult mouse pancreas after partial pancreatectomy[32]. However, the scientific community at large is not yet convinced by the existence of VSELs. This has resulted mainly because of their very small size and tendency to get discarded as debris during processing since these cells do not easily settle down on centrifugation[33]. The recent report[34] casted serious doubts on the very presence of VSELs. However, Ratajczak's group explained the technical reasons that could lead to mistaken results by others[23,35].

Initial work from our group resulted in the derivation of two hES cell lines KIND1 and KIND-2[3], we studied their propensity[36], adapted both the cell lines to feeder-free conditions and established directed differentiation protocols to make pancreatic[37] and tripotent cardiovascular progenitors[38]. At present, pre-clinical evaluation of safety, efficacy and feasibility of these progenitors is being studied in animal models. Working with ES cells taught us the meaning of pluripotency and importance of transcription factors OCT-4, NANOG and SOX2 as the 'Triumvirate of Pluripotency'. Of the three, OCT-4 appears to be crucial (especially the OCT-4 transcript which is expressed in the nucleus) as it belongs to Octamer class of transcription factors that recognize 8bp DNA site with the consensus ATGCAAAT. Along with Pit and Unc protein, OCT defines the POU class of transcription factors that interact with DNA. OCT-4 is crucial for pluripotency and self-renewal and silencing OCT-4 results in differentiation of ES cells[39,40]. Oct-4 is also crucial to re-establish pluripotency in somatic cells as one of the main 'Yamanaka factors'[41]. OCT-4 biology has confused stem cell biologists as they failed to discriminate between various transcripts of Oct-4 and this has led to a lot of mix-up[42,43].

Using a polyclonal OCT-4 antibody and specific primers for Oct-4A and for OCT-4 (comprising OCT-4A, Oct-4B/B1) we have demonstrated the presence of two distinct cell types expressing OCT-4 in adult human testis[44], ovary[39,45], pancreas[32], cord blood, cord tissue and bone marrow[33] including nuclear expression in VSELs and cytoplasmic OCT-4 in slightly bigger cells. The slightly bigger cells are the tissue specific progenitors that arise from the VSELs and cytoplasmic OCT-4 gradually disappears as the cells undergo further differentiation. Thus pluripotent VSELs that exist in various adult body organs are expected to be similar but the progenitors that arise are tissue-specific. The VSELs are invariably discarded along with the red blood cells during cord blood banking and processing bone marrow samples for autologus use[33].

Ratajczak's group has shown that total body irradiation completely destroys the HSCs in mice whereas the VSELs survive and have the ability to proliferate as evident from BrdU uptake[46]. Similarly, we have observed that chemotherapy destroys actively dividing germ cells in both ovary and testis; however, the VSELs persist in the gonads[47,48]. These results suggest that VSELs are relatively quiescent (dormant) stem cells in the body organs whereas the HSCs, OGSCs (ovarian germ stem cells) and SSCs are the actively dividing (restless) progenitors that arise from the VSELs. Then what are the adult stem cells? The existing terminology appears to be a misnomer! The adult body organs harbour nuclear OCT-4 positive, relatively quiescent VSELs that resist oncotherapy and actively dividing progenitors with cytoplasmic OCT-4. The progenitors are tissue specific and differ based on their location (somatic microenvironment or the niche), in testis these are the SSCs, in ovary these are the OGSCs, in bone marrow HSCs whereas in the Wharton's jelly these are the MSCs. Thus the understanding based on differential expression of nuclear OCT-4 in VSELs and cytoplasmic OCT-4 in the progenitors has led to better understanding of stem cells biology[49]. We have also observed that under normal conditions it is the cytoplasmic OCT-4 positive progenitors that expand in number to maintain tissue homeostasis whereas uncontrolled expansion of nuclear OCT-4 positive VSELs results in testicular tumour[50]. To summarize, a confusion exists in the basic terminology of stem cells. Are adult stem cells indeed stem cells or just progenitors? As suggested earlier[33], we believe that the term ASC is a misnomer, these are progenitors that arise from VSELs which are the true stem cells existing in various adult body organs (Fig. 2).

MSCs derived from adult bone marrow are considered to be pluripotent and several studies show their ability to trans-differentiate into all three germ layers[51]. But pluripotent properties are possibly due to a sub-population of VSELs that exists amongst MSCs[49]. We believe that the ubiquitous nature of MSCs is because these are the niche forming cells in various adult body organs and these are indeed multipotent rather than pluripotent. Similarly, the claims that testis is the only organ that can undergo spontaneous reprogramming to ES-like status *in vitro*[52] are controversial and the pluripotent ES-like colonies reported *in vitro* could be

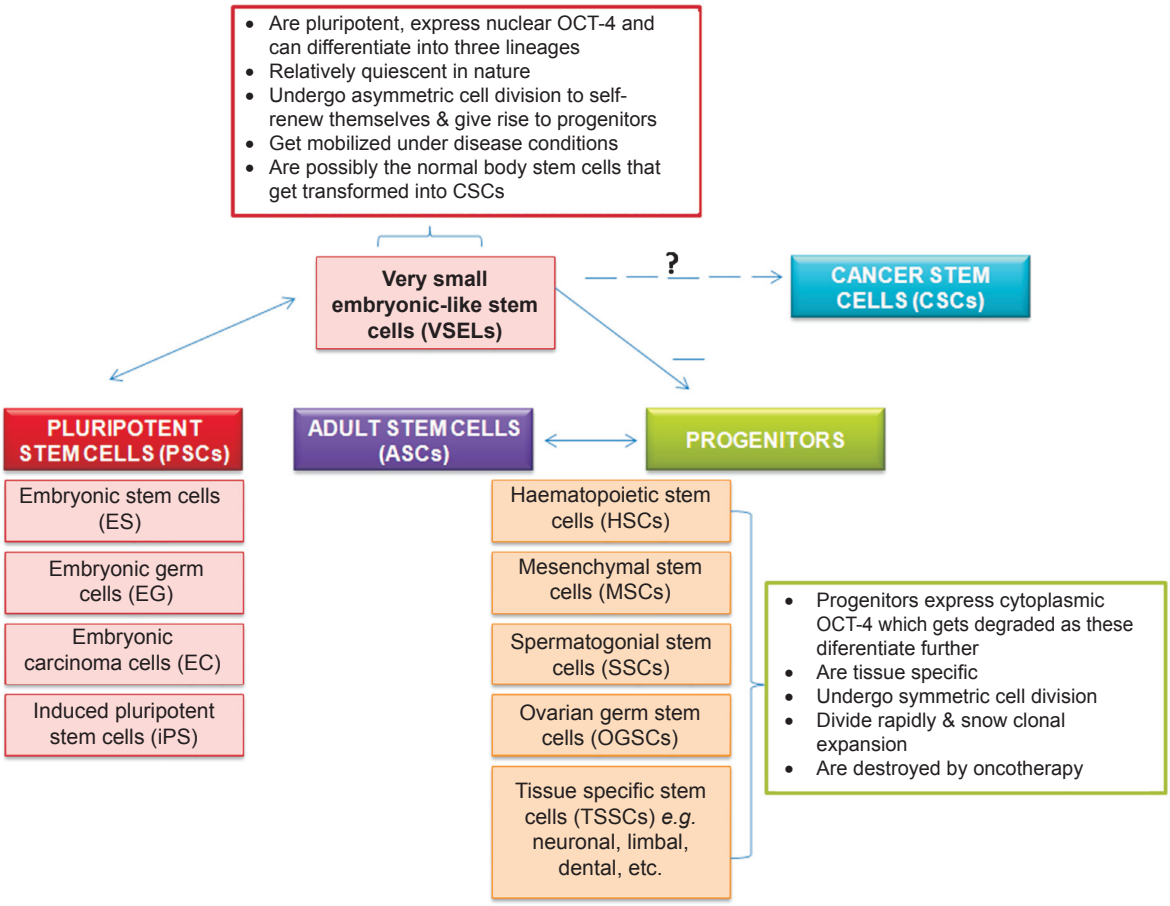

Fig. 2. Basic understanding on stem cells and progenitors.

because of the presence of VSELs as a sub-population[53]. Why is the efficiency of derivation of iPS cells so low? Why not all skin fibroblasts get reprogrammed to embryonic state? It is likely that VSELs in primary skin fibroblasts culture overcome quiescence and start growing when exposed to reprogramming factors. Although bone marrow transplant is a standard method of care for blood disorders, but it has failed to regenerate other organs during autologus bone marrow stem cell therapy[5-7]. The injected cells in various trials perhaps largely comprised progenitors which did not have the ability to trans-differentiate. This might be the reason why various multi-centre trials world-wide resulted in negative findings[5-7]. During these trials, regenerative potential of VSELs was never evaluated as these were invariably lost during processing[33]. Total nucleated cells are injected during autologous adult stem cell therapy and limbal epithelial cells are injected during limbal stem cell therapy[54] as we still do not know the true identity and how to purify the stem cells in these tissues.

Against this backdrop, presence of pluripotent VSELs in various adult body organs needs to be accepted as *(i)* these have the ability to self-renew and differentiate into various cell types, *(ii)* their presence can explain asymmetric cell division and *(iii)* these could be the elusive cancer initiating cells as nuclear OCT-4 has now been reported in several cancers (Fig. 1; Table). VSELs with nuclear OCT-4 *per se* are not carcinogenic but the changes in their microenvironment alter their behaviour from remaining quiescent to uncontrolled proliferation. Similar views have been published earlier[27].

Our country needs to invest more into VSELs research. India has not yet heavily invested in any particular stem cell type, rather supported research on all of them but has a chance to now lead in the field of VSELs biology. Brain storming is required to facilitate both basic research as well as pilot trials targeting VSELs biology. Also VSELs biology in the field of cancer initiation needs to be deciphered as these stem cells could be the root cause of recurrence (because of

Table. Expression of pluripotent markers including Oct-4 in various cancers

Reference	Tissue/organ
Luo *et al*[55]	Nasopharyngeal carcinoma
Li *et al*[56]	Lung cancer
Samardzija *et al*[57]	Ovary
Yang *et al*[58]	Human neuroblastoma
Rijlaarsdam *et al*[59]	Germ cell tumours
Guo *et al*[60]	Human gliomas
Ge *et al*[61]	Hypo-pharyngeal squamous cell carcinoma
Monsef *et al*[62]	Prostate cancer
Schoehals *et al*[63]	Cancers
Atlasi *et al*[64]	Bladder cancer
Gidekel *et al*[65]	Oct-3/4 as a dose-dependent oncogenic fate determinant
Monk & Holding[66]	Human embryonic genes re-expressed in cancer cells

their quiescent nature and cancer drugs generally target actively dividing cells) during remission period.

We need to think carefully before allowing mass culture of MSCs in the country. MSCs therapy should not suffer from similar outcome like autologus stem cells therapy[5-7]. Most perplexing is the observation in our laboratory that VSELs are mobilized and increased in numbers (more than 5-folds based on flow cytometry studies) in a streptozotocin treated mouse pancreas (unpublished observation). Then why these stem cells do not regenerate the diabetic pancreas? Do we really need to inject more stem cells in a diabetic pancreas during stem cell therapy or do we need to target the niche or both that will allow restoration of normal biology of endogenous stem cells (VSELs)? It is not easy to decipher well kept secrets of Mother Nature.

To conclude, there is a huge scope for basic research in the field of stem cells biology before translating from the bench to bedside.

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