



Viewpoint

Shifting gears from embryonic to very small embryonic-like stem cells for regenerative medicine

Autologous bone marrow mononuclear cells [presumed to include haematopoietic stem cells (HSCs)] are a standard of care for blood-related diseases but have failed globally to regenerate other organs. It is increasingly becoming evident that human pluripotent stem cells (PSCs) including embryonic stem cells (hES) and induced pluripotent stem cells (iPS) tend to differentiate into their foetal counterparts and have resulted in the formation of organoids which are perfect to study early embryonic development, but their ability to regenerate adult organs remains questionable. In contrast, very small embryonic-like stem cells (VSELs) are PSCs that exist in adult organs, express pluripotent markers, differentiate into cells of all three germ layers *in vitro*, are mobilized to various organs under conditions of stress/disease, and give rise to tissue-committed progenitors that maintain lifelong homeostasis¹. Thus, the research focus of our group has shifted from hES cells to VSELs to develop strategies wherein one can manipulate endogenous VSELs and their somatic microenvironment to achieve regeneration, to understand disease pathology and also their role in cancer initiation.

We initiated our research on stem cells with a focus on hES cells and derived two well-characterized hES cell lines KIND1 and KIND2² on human feeder layers and have now been adapted to expand under feeder-free conditions. Both the cell lines showed good propensity³ to differentiate into endoderm and mesoderm, and our efforts were focused on their differentiation into pancreatic⁴ and tripotent cardiac⁵ progenitors. A pre-clinical study using KIND1-derived pancreatic progenitors in streptozotocin-treated diabetic mice showed that mice transplanted with a microcapsule containing hES-derived pancreatic progenitors were capable of regulating blood sugar levels for up to 90 days compared to control streptozotocin-treated

diabetic mice that did not survive beyond 30 days (unpublished observations).

While working on hES cells, the importance of transcription factors, especially nuclear OCT-4 was realized which is crucial for maintaining pluripotent state of a stem cell. Nuclear OCT-4 positive VSELs were reported by our group in adult human testes and ovaries, and were recently reviewed¹. Both hES cells and VSELs are PSCs wherein hES cells are derived from inner cell mass (ICM) of blastocyst-stage embryo and VSELs are postulated to be an overlapping population of the migrating primordial germ cells (PGCs)⁶ that settle down in various adult organs during early development. Ratajczak *et al*⁷ recently reported reversal of quiescent state of VSELs by treating with histone deacetylase inhibitor valproic acid and nicotinamide *in vitro*. They could expand VSELs *ex vivo* by about 10³ folds in chemically defined medium after culture for 1-2 months in the presence of growth factors and hormones including follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

Cells with pluripotent properties have been described in adult organs by multiple groups (Fig. 1) and also named differently such as spore-like cells, multipotent adult progenitor cells (MAPC), amniotic fluid stem cells (AFSC), marrow-isolated adult multilineage-inducible cells (MIAMI), VSELs, or multi-lineage differentiation stress-enduring stem cells (MUSE)⁷. However, these comprise a single stem cell population in a 'dark room' being examined through different key holes by various investigators. It has been suggested that the controversy surrounding isolation protocols for VSELs needs to be sorted out by organizing workshops⁸. These stem cells express pluripotent transcription factors and also exhibit the ability to differentiate into three germ layers. Transcription factor OCT-4 has alternately spliced

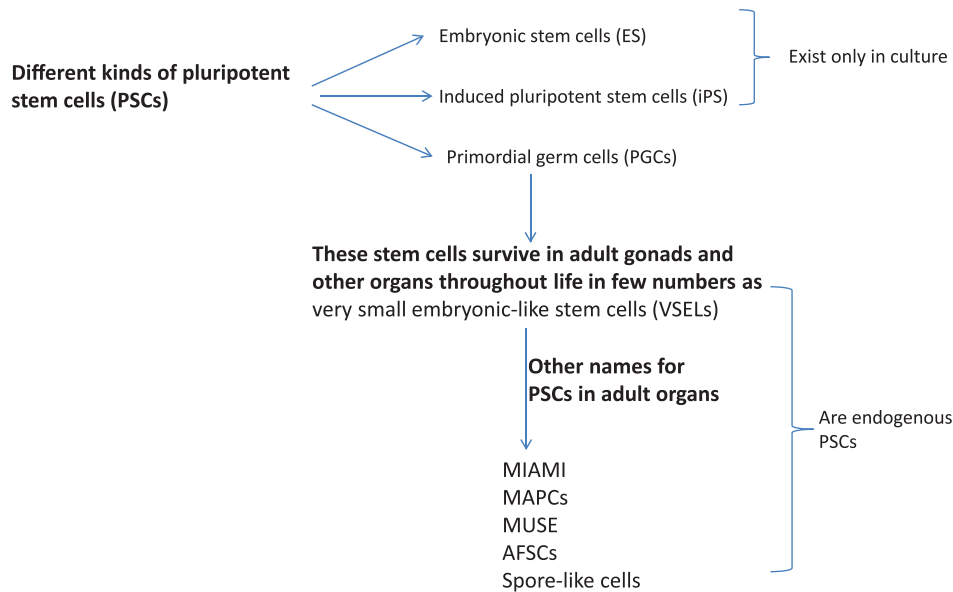


Fig. 1. Different types of pluripotent stem cells. MIAMI, Marrow-isolated adult multilineage inducible cells; MAPCs, Multipotent adult progenitor cells; MUSE, Multilineage-differentiating stress-enduring (Muse) cells; AFSCs, Amniotic fluid-derived stem cells.

isoforms and it is OCT-4A which is responsible for pluripotent state and not cytoplasmic OCT-4B. Our group has demonstrated that VSELs express nuclear OCT-4A whereas the progenitors express cytoplasmic OCT-4B that eventually degrades as cells differentiate further¹.

Human ES and iPS cells exist only in culture and have been described as tissue culture artefacts in literature⁹. Human ES cells are understood to exist in 'primed' (flattened monolayer colony morphologies, FGF and Activin/nodal signalling pathways, expressions of SSEA4, TRA 1-60 and TRA-1-81, X chromosome inactivation in females) and 'naïve' (small, round or dome-shaped colony morphologies, LIF and BMP4 signalling pathways, SSEA-1 expression and two active X chromosomes in female) states⁷. Attempts are being made to convert 'primed' hES cells into 'naïve' state as it enhances their ability to differentiate⁶. Thus, the scientific community is possibly going through a phase of denial regarding the differentiation potential of ES/iPS cells into desired cell types (which remains highly inefficient) and has diverted research efforts on understanding 'naïve' and 'primed' states of hES cells and also lot of research efforts are focused on organoids developed *in vitro* from ES/iPS cells¹⁰. Rather than moving in these basic research directions, we have diverted our research efforts on endogenous, pluripotent VSELs in adult organs to exploit their regenerative/translational potential. Differentiation

potential of hES cells and VSELs are compared below using two examples (*i*) regenerating a diabetic pancreas, and (*ii*) making 'synthetic' gametes. VSELs are also compared to adult stem cells.

Regenerating a diabetic pancreas

Work done in this field using different kinds of stem cells has been recently reviewed¹¹. A US-Food and Drug Administration-approved clinical trial is ongoing by ViaCyte, USA to treat type 1 diabetes mellitus (DM) using pancreatic progenitors derived from hES cells¹². No outcome results have come so far. It has been realized over time that hES as well as iPS cells tend to give rise to their foetal counterparts^{10,13} and thus may not be efficient enough to behave like or regenerate adult organs. In contrast, VSELs actively regenerate adult mouse pancreas (and differentiate into all cell types) after >80 per cent is removed surgically¹⁴. We have also shown that VSELs exist in adult pancreas and that their dysfunction with age may lead to both type 2 DM and pancreatic cancer¹⁵. Flow cytometry studies show that LIN-/CD45-/SCA-1+ VSELs increase in numbers in a diabetic pancreas and can regenerate a diabetic pancreas after partial pancreatectomy (unpublished data from our laboratory). It is known that if >80 per cent of diabetic pancreas is surgically removed, it regenerates back to normal state and we have demonstrated that VSELs bring about this regeneration¹⁴. Thus, compared to >90 day survival of diabetic mice which were transplanted hES cells-derived pancreatic progenitors

Table. VSELs compared to hES, iPS, HSCs and MSCs for regenerative medicine

<p>VSELs compared to hES & iPS cells</p> <p>Unlike hES cells, endogenous VSELs will not face immunological issues. Induced pluripotent stem cells harbour mutations in both genomic and mitochondrial DNA and thus cannot be an ideal starting material to make gametes. Also, due to safety concerns of using autologous iPS cells in the first clinical trial, allogeneic use of iPS cells is being considered for clinical applications - that will have associated immunological issues.</p> <p>Compared to ES & iPS cells which give rise to foetal counterparts and form organoids <i>in vitro</i>, VSELs easily regenerate adult organs.</p> <p>Unlike hES & iPS cells, use of VSELs for regenerative medicine has no associated risk of tumour formation.</p>
<p>VSELs compared to HSCs & MSCs (Adult stem cells)</p> <p>In the haematopoietic system, VSELs are pluripotent stem cells with regenerative potential and not HSCs/MSCs.</p> <p>VSELs express nuclear OCT-4 whereas HSCs/MSCs express cytoplasmic OCT-4. Thus, VSELs are the true pluripotent stem cells whereas HSCs/MSCs are committed progenitors.</p> <p>VSELs, very small embryonic-like stem cells; hES, human embryonic stem; iPS, induced pluripotent stem; HSCs, haematopoietic stem cells; MSCs, mesenchymal stem cells</p>

(experiment had to be terminated as the mice became very sick), diabetic mice after partial pancreatectomy and endogenous regeneration from VSELs survived for almost five months and maintained normal glucose levels and body weight (unpublished data). These results prompted us to work further on VSELs and develop strategies to achieve endogenous regeneration of various pancreatic cell types rather than depending on ES/iPS cells grown in Petri dish as a source of β cells alone.

It is an expensive affair to make hES cells clinical grade and then produce progenitor cells in cGMP facility for clinical use¹⁶. The first clinical trial by Geron using hES cells for spinal cord injury (<http://www.nytimes.com/2011/11/15/business/geron-is-shutting-down-its-stem-cell-clinical-trial.html?r=0>) was stopped due to financial constraints. The first clinical trial using iPS cells is on hold at present for genetic reasons¹⁷, and both genetic instability and presence of mutations in mitochondrial DNA in iPS cells have put a big question mark on their potential for regenerative medicine^{18,19}. VSELs are endogenous PSCs which have no associated immunological issues nor impose any risk of teratoma formation (Table). Besides regenerating adult pancreas, VSELs are also involved in regeneration of liver, lung and neurons in various animal models.²⁰

Making gametes from hES and iPS cells versus VSELs

Making gametes from stem cells to help infertile couples is considered to be the highest goal of regenerative medicine, but obtaining human gametes from ES/iPS cells is still a thing of future²¹. The major hurdle being to convert hES/iPS cells into PGCs-like

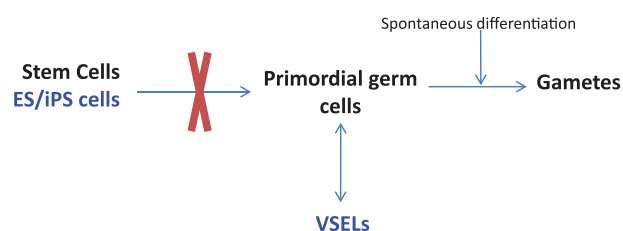


Fig. 2. Making human gametes from pluripotent stem cells. It is technically difficult to differentiate ES/iPS cells into primordial germ cells (PGCs) as it involved lot of epigenetic changes which may be difficult to replicate *in vitro* VSELs are developmentally linked to PGCs which are natural precursors to the gametes. Thus VSELs spontaneously differentiate into gametes.

cells (PGCLCs). PGCs are precursors and spontaneously differentiate into gametes (Fig. 2). Success achieved and gametes have been differentiated from mouse ES/iPS cells but the process remains inefficient and fraught with safety issues. Being similar to PGCs, VSELs spontaneously differentiate into oocyte-like structures and sperm *in vitro*. We have recently reported that even mouse bone marrow VSELs can differentiate into germ cells *in vitro*²². Choosing between ES/iPS/VSELs to make gametes has been debated^{23,24}.

VSELs compared to adult stem cells

VSELs also score better compared to adult stem cells (Table) including HSCs because HSCs are committed progenitors that arise from the VSELs. Very small, CD45 negative VSELs have been reported to differentiate into CD45 positive HSCs when cultured on OP9 feeder layer²². A review on VSELs discussing their developmental link to PGCs and that these are most primitive stem cells in the haematopoietic system capable of giving rise to HSCs, MSCs and EPCs has been recently published²⁵.

Trials using autologous bone marrow mononuclear cells (presumed to include HSCs) have failed globally because HSCs are committed progenitors and not pluripotent as reported earlier²⁶ and their ability to trans-differentiate remains questionable. As a result, HSCs will help recolonize bone marrow during blood-related diseases and have been greatly successful during bone marrow transplantation but do not possess the ability to regenerate other organs. Autologous adipose tissue derived stem cells have resulted in blindness in patients with age-related macular degeneration²⁷. Developing therapeutic approaches using stem cells and associated risks and benefits have been recently discussed by a group from US FDA²⁸. They stressed that rather than rushing to the clinics, our approach to exploit clinical potential of stem cells should be based on sound evidence. VSELs isolated from human cord blood²⁹ and bone marrow³⁰ can differentiate into cells of all three germ layers. Our group has also demonstrated that VSELs isolated from 5-fluorouracil (5-FU)-treated mouse bone marrow can differentiate into progenitors of three germ layers (neuronal, pancreatic and cardiac) and also give rise to HSCs and germ cells *in vitro*²². However, the clinical potential of VSELs remains to be tested since these have been unknowingly discarded during processing of samples for transplantation^{1,31}. VSELs in the haematopoietic system survive total body irradiation and treatment with 5-FU and increase in numbers as evidenced by BrdU uptake, thus suggesting an important role played in bone marrow regeneration³².

Ontogeny of embryonic stem (ES) cells and VSELs explains differences in their regenerative potential

The underlying reason for better potential of VSELs over ES cells is based on their ontogeny. PSCs are first established in the ICM of pre-implantation blastocyst, and after implantation, ICM transitions into post-implantation epiblast cells. During gastrulation, some of the post-implantation epiblast cells are specified as PGCs which retain pluripotent state. ES cells are derived from ICM of blastocyst-stage embryo whereas VSELs are overlapping with migrating PGCs which appear for the first time in epiblast-stage embryo. The ICM and post-implantation epiblast cells are distinct, based on the differences in their gene expression profiles, epigenetic status and differentiation capacity³³. PGCs migrate along the dorsal ridge to the gonadal ridge, and while the genetic material of parents is maintained, their epigenome undergoes extensive reprogramming including demethylation of DNA (at CpG islands, transcription start sites, gene

bodies and surrounding intergenic regions), histone modifications, expression of epigenetic modifiers and chromatin remodelling on a genome-wide scale. Being equivalent to PGCs, VSELs methylome is distinct compared to ICM from which ES cells are obtained *in vitro*. Kim *et al*³⁴ have reviewed molecular nature of VSELs in adult organs.

One can differentiate ES cells into pancreatic progenitors based on gene expression and protein profile, but for the cells to acquire similar epigenetic status as adult cells may be difficult to achieve *in vitro* and this could be the possible bottleneck. Epigenetic status of pancreatic progenitors obtained from hES cells is distinct from adult pancreas^{4,14}, and for the same reason, it is difficult to convert ES/iPS cells into PGCLCs. VSELs are developmentally as well as epigenetically more mature compared to ES cells. Thus, VSELs spontaneously differentiate into gametes and also regenerate adult, diabetic pancreas. Compared to hES cells which are envisaged to provide an alternative replacement for islets alone, VSELs regenerate all pancreatic cell types after partial pancreatectomy.

Do stem cells initiate cancer?

Gross and Emanuel³⁵ discussed that since the launch of 'War on Cancer' in 1972, the USA government alone has spent over \$100 billion on cancer research, resulting in fundamental discoveries and millions of publications. However, the actual clinical progress has remained modest with cancer mortality decreasing from about 200 to 166 deaths per 100,000 as of 2012. This almost 17 per cent reduction has largely been attributed to 50 per cent decreased smoking over the past 50 yr. It has been postulated that cancers occur due to altered behaviour of VSELs³⁶. Why nuclear OCT-4 and other markers CD133, FSH receptors (FSHR) and human chorionic gonadotropin show ubiquitous expression in various kinds of cancers, has been discussed^{1,37}. Whether somatic cells de-differentiation/reprogramming to pluripotent state leads to cancer initiation and explains expression of pluripotent markers in various tumours or pluripotent VSELs existing in various adult organs in small numbers undergo uncontrolled proliferation and get transformed into cancer stem cells has also been discussed³⁸.

Thus, it becomes essential that we focus on VSELs to understand how their biology gets affected leading to various diseases, to achieve endogenous regeneration and also to understand cancer initiation. Carefully planned clinical trials need to be undertaken targeting endogenous VSELs for regenerative medicine. Studies

done on VSELs will result in several paradigm shifts in due course of time.

Highlights of some of the results achieved using VSELs are listed here: (i) VSELs possibly undergo asymmetric cell divisions to self-renew and give rise to the tissue-specific progenitors (HSCs/SSCs/OSCs) which further undergo symmetric cell divisions and clonal expansion followed by differentiation into tissue-specific cell types^{1,39}; (ii) Rather than HSCs, VSELs have the true potential to differentiate into three germ layers. HSCs can only differentiate into blood cells. VSELs are mobilized under various kinds of stress to restore homeostasis in affected organs^{1,7,14}; (iii) There is a need to spin cells at 1000×g to obtain VSELs rather than widely used speed of 250-270 g^{1,31}. Also after Ficoll-Hypaque density gradient centrifugation, VSELs settle down with RBCs³¹ and need to be enriched (not to be discarded) and used for regenerative medicine; (iv) VSELs exist in larger numbers during foetal development⁴⁰ and are possibly the yet elusive 'foetal stem cells'. The use of foetal stem cells for regenerative medicine is not justified (<http://www.jsonline.com/news/opinion/use-of-fetal-tissue-is-unethical-and-unnecessary-b99572742z1-326513781.html>) as VSELs exist in adult organs and there is a need to manipulate them to achieve regeneration; (v) VSELs regenerate pancreas after partial pancreatectomy^{14,15}; (vi) Being relatively quiescent, VSELs survive toxic insults including radiotherapy and chemotherapy¹. These can be targeted to restore gametogenesis⁴¹. There may be no need to bank gonadal tissue prior to oncotherapy⁴² and associated legal, ethical and safety issues could be avoided⁴³; (vii) VSELs are pluripotent and most primitive stem cells that co-exist along with spermatogonial stem cells in the testis; undergo asymmetric cell divisions and can restore spermatogenesis both *in vitro* and *in vivo*³⁹; (viii) VSELs and OSCs co-exist in ovary surface epithelium and possibly result in post-natal neo-oogenesis throughout life⁴⁴. Age-dependent compromised function of niche supporting the stem cells results in menopause and can be manipulated to regenerate the non-functional ovary from VSELs³⁹. VSELs have also been reported in large numbers in human ovarian tumour tissue⁴⁵ and mice studies⁴⁶ suggest that these may be responsible for formation of leiomyoma that arise from uterine myometrium. (ix) FSHR are expressed on VSELs/HSCs/SSCs/OSCs in addition to Sertoli cells in testis and granulosa cells in the ovary. Thus, FSH acts directly on the stem cells (in ovary, testis and bone marrow)^{1,25,32,37,39,44,47,48}.

In view of the reasons discussed above, the focus of research efforts of our group has shifted to VSELs for their application in regenerative medicine.

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