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VERY SMALL EMBRYONIC-LIKE STEM CELLS: A REVIEW OF BASIC SCIENCE, APPLICATIONS, AND POTENTIAL USE IN ORTHOPEDICS

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Abstract

Continuous and growing research studies regarding the clinical applications of the pluripotent or multipotent stem cells with their potential to differentiate into three germ layers are very well conducted in regenerative medicine (RM). In this review, we report the recent clinical applications and potential use of very small embryonic-like stem cells (VSELs) in orthopedics. VSELs are nonhematopoietic (CD45 - / Lin -), rare, and very small cells; they were reported as "dormant" cells in the bone marrow (BM), but are also found in cord blood, peripheral blood (PB), and in adult organs. Based on their capability to express markers of pluripotency (such as Oct-4 +/Nanog +/SSEA-1/4+/CXCR4+), it has been hypothesized that these cells could be early deposited during the embryonic development as descendants of epiblast-derived stem cells and perhaps from some primordial germ cells. VSELs can be released or mobilized from the BM to the PB during tissue injury and stress, facilitating the regeneration of damaged tissues. As well as mesenchymal stem cells, nowadays VSELs can be expanded *ex vivo*. Their pluripotency could be suitable for applications in RM, solving several problems regarding the use of both controversial embryonic stem cells and induced pluripotent stem cells. VSELs studies will hopefully open new frontiers to better understand their potential that would be relevant for future applications in RM and translational research.

Keywords: *Orthopedics*; *Pluripotent stem cells*; *Regenerative medicine*; *Very small embryonic-like stem cells*; *VSELS*

INTRODUCTION

Mesenchymal Stem Cells: A Valuable Stem Cell Type in Orthopedic Field

The most widely used cell type studied and applied in cell therapy and orthopedics is the mesenchymal stem cells (MSC). They were discovered in 1955,[1,2] and isolated from the rat's BM by Friedenstein in 1970 as a nonhematopoietic, multipotent, plastic adherent, and fibroblastic-like stem cells.[3] These cells were isolated in small numbers in culture and could differentiate *in vitro* into bone, cartilage, adipose tissue, tendon, muscle, and fibrous tissue. Since their discovery, numerous names have been suggested and Caplan in 1991[4] proposed to call them "Mesenchymal Stem Cells," being the most common name in the international nomenclature.[5] After 25 years of studies and debates, Caplan in 2017 suggested a new definition: "Medicinal

Signaling Cells."[6,7] Another definition recently proposed is "Maintenance Stromal Stem Cells."[8] Both these definitions are more accurate in describing the characteristics of these cells because they have a homing activity toward injured or degenerated tissues. MSCs are able to activate the site-specific and tissue-specific residential stem cells by secreting bioactive factors (growth factors, cytokines, extracellular vesicles). Given that, MSCs should be considered for their paracrine or signalling or immunomodulatory effect instead of a real stemness function with regenerative potential. The extensive use of MSCs in regenerative medicine (RM) is essentially related to their easy harvesting from fat tissue, bone marrow (BM), cord blood (CB), and other sources, and their capability to be expanded and amplified in laboratory cultures. However, MSCs should be considered as a "cell-drug" delivered *in situ* able to drive and regulate and give maintenance to the tissue healing process through the activation of residential progenitor stem cells, which are probably the ones able to differentiate and regenerate the tissue. MSCs represent a kind of "medicine" but do not represent a real backup pool of cells able to regenerate tissue. Accordingly, studies reported that only a small percentage of implanted MSC survive and undergo limited self-renewal and proliferation, but the rest undergo apoptosis after releasing the bioactive factors.[9,10] Most of them get trapped in the capillary network and disappear from the injection site in a short amount of time.[11,12] Due to their low viability, treatment protocols in RM suggest multiple administrations of MSCs (2–4 weeks apart) and recently, few studies attempted to optimize their survival and engraftment to increase their performance.[13]

Different Types of Stem Cells and their Potential Application in Regenerative Medicine

Researchers' aim is to find "perfect" stem cells able to differentiate and regenerate all kinds of tissues once requested. The use of embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) as a therapeutic tool in RM is related to ethical issues and controversies. Regarding the human ESCs (hESC), previous studies have demonstrated their inability to differentiate into mature and specific cell types, risk of teratoma formation, immunologic issues, genomic instability, arrhythmias, and the fact that differentiated cells retain a fixed major histocompatibility complex phenotype.

Regarding the iPSCs, they have inefficient derivation, tendency to differentiate into fetal counterparts, safety issues, risk of teratoma formation, risk of harboring mitochondrial mutations, genomic instability, the fact that they keep the epigenetic state of somatic cells, donor-patient match/HLA compatibility, immunological issues, and finally the concern that the use of autologous iPSCs is expensive and time-consuming.[14]

Hematopoietic stem cells (HSCs) represent another potential source of stem cells in RM applications. In physiological conditions, HSCs harboring multipotent capacity are the pioneer in constituting the blood system by their differentiation into progenitors of blood cells. There are several sources of HSC, such as BM, CB, peripheral blood (PB) and mobilized PB.[15] It has been shown that HSCs can trans-differentiate into different cell types not belonging to the hematopoietic system. Indeed, this concept of plasticity or trans-differentiation of HSCs has been reported by many authors showing that HSCs from BM can give rise to hepatic cells,[16,17] skeletal muscle,[18,19] cartilage,[20] brain microglia as well as macroglia,[21,22] endothelial precursors,[23] and cardiac muscle cells.[24,25] Several basic and clinical researches focus on the application of HSCs as a regenerative therapy in various nonhematological conditions such as neurological disorders (Parkinson's disease), ischemic conditions (stroke, myocardial ischemia), and orthopedic conditions such as cartilage degeneration or defects. However, nowadays, more studies focus on the thorough characterization of the trans-differentiation potential of HSCs.

Almost 20 years ago, a population of cells called very small embryonic-like stem cells (VSELs) expressing pluripotency markers and possessing the ability to differentiate into the three germ layers was discovered by Ratajczak.[26] Despite their multipotency, VSELs are still struggling to get widely acknowledged by the scientific community, and more studies need to refine and optimize their

isolation, characterization, as well as identify their biological potential.[27] The scope of this review is to introduce VSELs, as a population of pluripotent stem cells, their application in RM and more specifically in orthopedics. The purpose of this review is to make these small but very powerful cells better known, and to inspire future studies and their potential clinical applications (Table 1).

METHODS

Literature research of online databases (PubMed, EMBASE, and GOOGLE SCHOLAR) was performed in February 2020. Searches included the following term from each of the following three groups. Group 1 consisted of "Very Small Embryonic-like Stem Cells" or "VSEL" or "VSELs" or "pluripotent stem cell," or "pluripotency." Group 2 contained "stem cells," "regeneration," "Tissue regeneration," "musculoskeletal osteoporosis," "bone defect," "bone," "osteoarthritis," "chondral lesion," "degenerative arthritis," "Tendon" "ligament," "muscle," "fracture," "orthopedic." Abstracts and any articles not written in English were excluded.

Very Small Embryonic-like Stem Cells: A Hidden Gem

Bone marrow is a well-known source of stem cells containing both HSC and NHSC populations, which are the key players in tissue regeneration and repair. Since the last two decades, HSC population have displayed plasticity features and the ability to regenerate nonhematopoietic organs.[28] Even though the HSC plasticity concept still remains questionable, several studies reported that in pathological conditions, HSCs can trans-differentiate in nonhematopoietic lineage with the ability to regenerate damaged tissues.[16,19,22,27,15–22] A study by Kucia et al. indicates the presence of a heterogeneous population of non-HSC (NHSCs),[30] in addition to HSCs in BM. It has been suggested that the presence of both populations of stem cells is related to their migration during the developmental process attracted by secretions of the resident stromal cells and osteoblasts (SDF-1; HGF).[31–33] A subpopulation of these NHSC has been identified and was called VSELs in 2006.[26] These rare cells can be characterized by their very small size (5–7 μm) in humans and (2–4 μm) in mice, and their expression of some surface markers (such as Lin-, CD45 -, CD34+, CD133+, CXCR4+, and Cmet, LIF-R).[34,35] VSELs express markers of pluripotent stem cells (PSC) such as SSEA-1 and 4, Oct-4, NANOG, KLF4, Rex-1, and several other primordial germ cell markers.[34,36] They can be found in a dormant quiescent state in the BM as well as in CB, PB, and adult organs. Based on their pluripotency, it has been hypothesized that VSELs are deposited in the early stage of the embryonic development in the BM and are direct descendants of epiblast-derived stem cells (EPSC) and most probably some primordial germ cells (PGC).[37,38] See Figure 1.

They have large nuclei surrounded by a thin rim of cytoplasm and contain euchromatin (open-type chromatin) that is characteristic of ESC. VSELs have a capacity of self-renewal and could be considered as a backup pool of stem cells that actively contribute to the turnover of other tissue-specific mono-potent stem cells that are in peripheral niches; once activated they can contribute to tissue or organ regeneration. Indeed, VSELs are mobilized to various organs under stressful conditions, injury, or disease such as myocardial infarction,[39] stroke,[40,41] leg ischemia,[42] pulmonary diseases,[43] or cytotoxic treatments.[41] It has been demonstrated that they can give rise to tissue-committed progenitors that maintain lifelong homeostasis.[37] Kucia et al.[28] postulated that because VSELs show tropism to stromal cells and undergo emperipolesis in cocultures with BM-derived fibroblasts, they could be co-isolated with the adherent fractions of BM cells including MSC,[44] multipotent adult progenitor cells (MAPC),[45] marrow-isolated adult multilineage inducible (MIAMI) cells,[46] and multilineage differentiating stress enduring cell (MUSE).[47,48] However, because of these overlapping pluripotent populations of cells, there is still a lack of consensus on the phenotypic markers used in the isolation protocols of pure VSELs, requiring further studies in this field.

VSELs mostly remain in the G0 state of the cell cycle and undergo rare asymmetrical cell divisions to self-renew and give rise to progenitors that divide rapidly by undergoing symmetrical cell divisions and clonal expansion followed by differentiation into

Table 1. Comparison of Stem Cells: Human Embryonic, induced Pluripotent, Hematopoietic, Mesenchymal, and Very Small Embryonic-like Stem Cells

Cells	Potency	Markers	Source	Immunological Issue	Origin	Differentiation/ Plasticity	Expansion	Stemness	Tumor formation (Teratomas)
hEC	Pluripotent	OCT4, SOX2, SSEA4, TRA-1-60, TRA-1-81	Embryo/ Placenta	yes	Embryo/ Placenta	3 layers	+++	++++	++
iPS	Pluripotent	OCT4, SOX2, SSEA4, TRA-1-60 TRA-1-81	Skin/Blood (reprogrammed)	yes	Somatic Cells	3 layers	+++	++++	+++
HSC	Multipotent	CD34+, CD59+, CD90/Thy1+ CD38low- c-Kit-/low, Lin-	BM PB UC	No	Red BM	MESODERM Hematopoitic Plasticity +		+++ Committeed Prognitors	-
MSC	Multipotent	CD73+, CD90+, CD105+ CD34-, CD45- CD11b, CD14-, CD19-, CD79a, HLADR	BM Adipose Tissue Dental Pulp Derma Myocardium	No	Pericyte/ stroma	MESODERM bone, cartilage, adipose tissue, tendon, muscle, and fibrous tissue.	+++	+ Committed Prognitors	-
VSELs	Pluripotent	CD90+ CD133+ CD44+ Sca1+ Oct-4+ NANOG+ SSEA 1/4+ Rex1+ CXCR4	BM PB UC Adipose Tissue Ovarian Testis Myocardial	No	Epiblast/ Germ Cells	3 layers	+	++++	-

OCT4: octamer binding transcription factor 4; SOX2: sex determining region Y-box 2; SSEA4: stage-specific embryonic antigen-4; TRA-1-60/ TRA-1-81: surface antigen podocalyxin

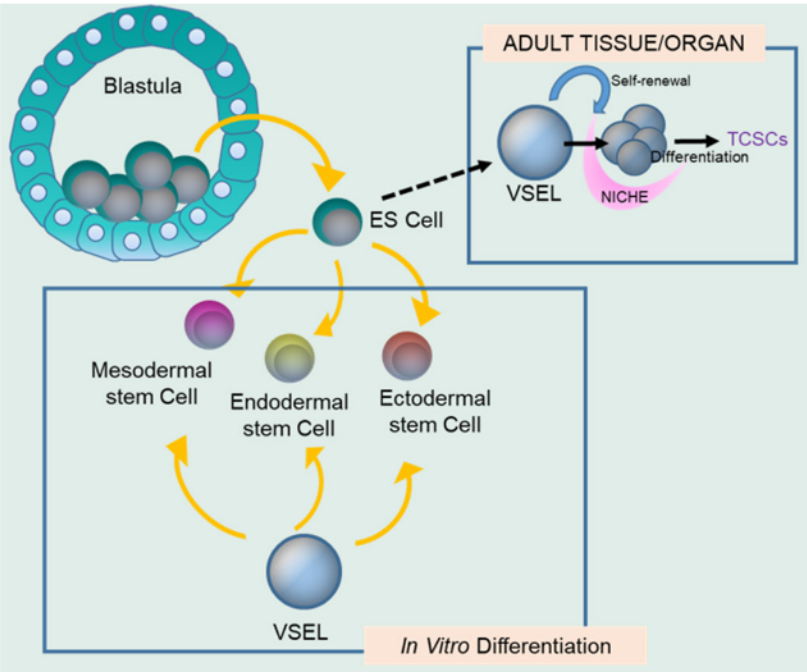

Figure 1. Proposed Scheme Illustrating the Potential *in vitro* and *in vivo* Differentiation Capacities of VSELs. VSELs are most probably originated from cells related to the germline and are deposited as reservoirs of adult stem cells in developing organs during embryogenesis. *In Vivo*, VSELs reside as a population of pluripotent stem cells (PSCs) able to self-renew and most probably differentiate by interacting with their niche to monopotent tissue committed stem cells (TCSCs). In vitro, VSELs demonstrate their multilineage cellular differentiation ability mimicking the embryonic stem cells (ES) properties.

tissue-specific cell types based on their location.[49] Unlike ESC, HSCs, or ES or iPSC, VSELs can spontaneously differentiate into adult cell types regenerating *in vivo* tissues and organs, such as the pancreas, and even gametes *in vitro*.[37] For these reasons, it seems that VSELs can be among the best endogenous stem cells, able to make regeneration of adult tissues.

The main problem to use VSELs in RM was addressed because of their scarce number. Will it be sufficient to achieve regeneration? Nowadays, even after sorting and other technical difficulties, VSELs can be expanded *in vitro* maintaining their pluripotency and ability to differentiate without manipulation.[50] In that sense, several methods have been tested to improve VSELs culture conditions (by using cytokine combinations, feeder cell cocultures, besides recombinant proteins, and small molecules).[51,52] Lahlil et al. have demonstrated that VSELs expand in pyrimidoindole derivative (UM171) medium and remain mononuclear by maintaining undifferentiated morphological features even after 12 days of culture.[53] To date, the knowledge of how and for how long native VSELs control their pluripotency and their differentiation potential remains unknown. Kucia et al. demonstrated in murines that highly purified BM-derived VSELs express a low level of mitotic genes and similar but not identical transcriptome to ESCs, which proliferate and differentiate normally.[54] It was reported that during VSELs differentiation by coculture with a C2C12 supportive cell-line, a unique pattern in imprinted gene methylation is reverted, which may explain in part VSELs quiescent status.[55] Unluckily their limited number, quiescence, and their poor ability to expand[56] *in vitro* are considered big challenges that limit the use of these cells as a candidate in RM.

Notwithstanding the *in vitro* challenges, VSELs are very powerful cells able to differentiate *in vitro* into all three germ-layer lineages

(ectoderm-mesoderm-endoderm) without forming teratomas.[57] VSELs do not form teratomas, and this ability can be explained by modified methylation of specific genes residing in their differentially methylated regions (DMRs); a deletion of paternally imprinted genes within the Igf2-H19 and Rasgrf1 loci and a hypermethylation of the Igf2 receptor (Igf2R), Kcnq1-p57KIP2, and Peg1 loci were reported. Because paternally expressed imprinted genes (Igf2 and Rasgrf1) enhance embryonic growth and maternally expressed genes (H19, p57KIP2, and Igf2R) inhibit cell proliferation, the unique genomic imprinting pattern observed in VSELs demonstrates the growth-repressive influence of imprinted genes on these cells.[54] As a result, there is no uncontrolled proliferation such as late migratory PGCs, and it may also explain the quiescent state of VSELs in adult tissues.[58,59]

Potential of Very Small Embryonic-like Stem Cells

Nowadays, VSELs can represent a valid, alternative candidate of stem cells in clinical application because their proliferation and differentiation *in vivo* are well and strictly monitored and controlled. Better understanding is still required for their biology and molecular mechanism governing their quiescence, activation, proliferation, targeting, and differentiation. Most probably there are few subpopulations of VSELs with different gene expressions profile or overexpressions. Lahlil et al. have demonstrated that VSELs Lin-CD34+CD45 expressing CD133 or NANOG have the same expansion and differentiation capacities toward the mesodermal and endodermal pathways, and that VSELs, which express the CXCR4 marker, have less ability to proliferate and differentiate.[50] This means that VSELs differentiation *in vivo* can follow different directions depending on their phenotype and pathophysiological needs. A deep phenotyping should provide a precise stratification of VSELs into subsets and therefore a better understanding of their biological mechanisms accordingly.

Additionally, more studies could be addressed to the transcriptome's investigation to find exactly which genes are governing VSELs quiescence.[60] A 10–15 fold expansion is now possible with some molecules, media, or growth factors. It is also necessary to prove that after differentiation VSELs can reconstruct damaged tissue in animal models and then in humans.

Potential Application of VSELs in Orthopedic

The VSELs have the ability to differentiate and become committed to the regeneration of tissues or organs. Research on VSELs will hopefully open new frontiers to better understand their potential that would be relevant for future application in RM and translational research.

The first study that hypothesized VSELs could play a pivotal role in the normal rejuvenation of adult tissues as well as involvement in the regeneration of damaged organs was published in 2008 soon after the discovery of VSELs by Ratajczak et al. and Kucia et al.[61] Authors envisioned that the potential of these cells for tissue and organ regeneration could also be applied toward the deceleration of the aging processes. Since then, several studies and researches have been conducted to better understanding the possible clinical application of these cells in several medical fields.

In 2013, Havens et al. published an interesting article demonstrating the capability of human VSELs (hVSELs) to generate skeletal structure *in vivo*. VSELs isolated from blood by apheresis following granulocyte colony–stimulating factor mobilization was fractionated and enriched by elutriation and fluorescence activated cell sorting. Sponge scaffolds made with collagen containing 2000–30,000 hVSELs were implanted into cranial defects in severe combined immune-deficient mice. A microcomputed tomography analysis showed that a cell population, including VSEL, produced mineralized tissue within the cranial defects compared with controls at 3 months. Histological studies showed significant bone formation and cellular organization within the defects compared with cellular or scaffold controls alone. When hVSELs cells were implanted into a cranial wound defect, woven human bone was generated with marrow cavities populated of osteoblasts, chondrocytes, and human neural adipocytes.[62] Authors observed that both hVSELs and murine VSELs cells can be induced to

express markers that are consistent with the acquisition of osteoblastic (Runx2, osteocalcin), adipocytic (PPAR-g), and endothelial phenotype (CD31, Factor VIII) cells that are mesenchymal derivatives.

In a study published in 2019 by Leppik et al. made large bone defects in the femurs of 38 Sprague Dawley female rats and treated them with β-TCP scaffold granules seeded with male VSELs. Authors have demonstrated that VSELs isolated from rat BM-derived mononuclear cells (BM-MNC) contribute to bone healing. BM-MNC, VSEL-depleted BM-MNC or scaffold alone, and bone healing were evaluated after 8 weeks post-surgery. Bone healing was remarkably increased in defects treated with VSELs and BM-MNC compared to defects treated with VSELs-depleted BM-MNC. Donor cells were detected in new bone tissue in all the defects treated with BM-MNC, whereas in defects treated with VSEL-depleted BM-MNC engraftment was detected only in fibrous tissue.[63]

As we know, osteogenesis and bone remodeling are complex processes that involve not only the osteoblastic and osteoclastic cell lineages but also several mechanisms and interactions between different cell populations.[64] A synergy occurs among bone progenitor stem cells, hematopoietic cells, and immune cells, thanks to the secretion of local cytokines, growth factors[65,66] and activation of transcription factors.[67,68] Osteoporosis is a metabolic disorder, related not only to the osteoblast and osteoclast imbalance but also to other functions, such as bone-vessel coupling and bone-adipocyte coupling.[69] VSELs are also promising for use in these kinds of skeletal disorders and to decelerate the aging processes in bone metabolism that leads to osteoporosis.[70] In 2015, Jung Younghun at the Michigan School of Dentistry injected tibial bone with hVSELs with and without estrogen in a mice model that induced osteoporotic by ovariectomy. DEXA and μCT scan have demonstrated a significant increase of trabecular number (11.24 ± 0.67 vs 6.78 ± 0.35, $P < 0.001$), bone volume (0.301 ± 0.05 vs 0.25 ± 0.01, $P = 0.025$), and bone mineral density (1108.56 ± 115.6 vs 913.02 ± 17.11, $P = 0.006$) in those animals treated with hVSELs and estrogen compared to those which did not receive estrogen. The authors declared that this study in SCID mice was the first to demonstrate that hVSELs are capable of inducing bone regeneration in osteoporotic animals.[71]

CONCLUSION

Based on the results, it can be concluded that VSELs could be a good source of pluripotent regenerative cells for orthopedics and sports medicine applications, both for acute and degenerative processes. Considering their propensity to form bones, they could be considered for the treatment of diseases such as osteogenesis imperfecta, bone defects, pseudarthrosis, and healing delay as well as avascular necrosis and osteoporosis. In terms of their pluripotency, VSELs represent a valid option to treat several tissue chronic degenerative conditions such as cartilage erosion and osteochondral defects, joint arthritis, ligaments or tendon injury and degeneration. However, further studies at the preclinical and clinical levels are necessary to confirm the efficacy of these pluripotency of musculoskeletal disorders.

CONFLICT OF INTEREST

The authors declare that there are no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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