Five generations of cell preparation: a translational framework for categorizing regenerative stem cell therapies

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Abstract

A description of a proposed categorization scheme of regenerative stem cell therapies illustrated by review of basic science and clinical studies involving the clinical application of mesenchymal stem cells (MSCs) is proposed. The aim of this review is to provide a novel framework for categorizing MSCs according to preparation strategy. Selected basic science studies and clinical trials were used to illustrate the applications in each category of stem cells. A review of the literature regarding stem cell sources and preparation makes apparent that there are five generations of stem cells in various states of study and clinical application, ranging from therapies currently being used in office-based practice to stem cell generations that hold considerable promise but with persistent concerns regarding safety and feasibility. In the last decade, stem cell research has spread to many different branches of regenerative medicine. Basic science and clinical studies examining the use of stem cell transplantation in the treatment of a wide range of human diseases have exponentially increased. The *Five Generations Model* may be a helpful way to describe stem cells in research and in clinical application. Describing stem cells in terms of cell preparation strategy, rather than source, may facilitate a greater understanding of this therapy by physicians and patients, and provides an opportunity for researchers to incorporate this helpful framework into a description of their background and findings.

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Key words: mesenchymal stem cells; stromal cells; regenerative medicine; bone marrow derived stem cells; adipose derived stem cells.

Acknowledgements: Mayo Clinic, Mayo Graduate School, and the Mayo Clinic Medical Scientist Training Program 5T32GM065841 are acknowledged.

Contributions: CLH and YFH provided original drafts of distinct portions of the paper, as well as final editing; all other authors also contributed to the concept of the paper and design of the conceptual framework, and as well as provided direct editing to the manuscript content; WQ, senior author, provided integral guidance, detailed original manuscript writing and editing, and provided the foundation and guidance for each section of the paper, as well as final approval of the version to be published.

Conflict of interest: the authors declared no potential conflict of interest.

Conference presentation: the concept of "5 Generations" was presented at a conference, the Regenerative Rehabilitation Symposium in Atlanta, GA, USA in October 2016.

Received for publication: 23 May 2017.
Accepted for publication: 6 June 2017.

Journal of the American Academy of Regenerative Medicine 2017; 1:7239
doi:10.4081/jaarm.2017.7239

Introduction

Interest regarding stem cell transplantation for the treatment of disease has attracted intense interest from the scientific and patient community, and research surrounding its potential clinical applications continues to elucidate its potential in many fields of medicine. Stem cells (SCs) have variable potency, differentiation potential, and capacity for self-renewal.[1] SCs have been shown to have both immunomodulatory and trophic effects in the environment into which they are transplanted.[2] Sources of SCs include: embryonic SCs (ESCs), induced pluripotent stem cells (iPSCs),[3,4] hematopoietic stem cells (HSCs), and mesenchymal stromal cells (MSCs). ESCs were first isolated

from humans as pluripotent cells in 1981.[5] However, due to tumorigenic risk and ethical concerns,[6] SC therapy has been focused on multipotent MSCs. Hematopoietic stem cells (HSCs) possess the capacity for self-renewal and multi-lineage differentiation, and are used clinically for treatment of hematologic disease.[7] Harvested for clinical use from bone marrow, peripheral blood, or cord blood, HSCs can develop into any type of hematopoietic cell. Clinically, HSC transplantation is used for cancer treatment, treatment of genetic or immunologic diseases that affect hematopoietic cell production or activity, tolerance induction, and graft-*versus*-tumor disease.[8] MSCs have the potential to differentiate into non-hematopoietic cells. They are derived from mesodermal tissues and organs including bone marrow,[9] adipose tissue,[10] umbilical cord,[11] peripheral blood,[12] amniotic fluid,[13] urine,[14] dental pulp,[15] breast milk,[16] periodontal ligament,[17] hair follicle,[9] synovial membrane,[18] endometrium,[19] and placenta.[20] The goals of SC therapy are to repair, replace, or regenerate tissue or organ function lost due to congenital defects, damage, disease, or age.[21] Since the discovery of MSCs, there has been a dramatic increase in their use in both preclinical research and clinical trials for treatment of a wide range of conditions.

Tremendous effort has been made in SC isolation, expansion, and phenotype modification for improving the efficacy of SC therapy. The methodology is complex and it has been challenging for physicians and patients to clearly understand the differences among SC products selected for specific indications. A heuristic model of SCs based on their preparation strategy, rather than source, may facilitate a better understanding of patients and clinicians going forward as we witness the prevalence of SC-based therapies offered for a wide variety of clinical uses increase. In this review, we provide a novel classification system of SCs based on preparation strategy. Selected basic science studies and clinical trials are used to illustrate the applications in each class. The literature reviewed is not exhaustive of all studies among all five generations; such a task is beyond the scope of the current discussion.

We have included key studies in our discussion to illustrate the concept of this classification framework based on the *5 Generations of SCs* model. This model spans the generations of SCs from preparations available for office -based clinical use today, through generations in experimental use only in humans, to SCs that are currently studied *in vitro*.

Classification of stem cells based on preparation strategy

A classification system of five generations of clinically applicable SCs based on SC preparation strategy is proposed (Figure 1). The first generation (G1) is defined as SCs prepared according to the Food and Drug Administration (FDA) current guidelines for minimal manipulation of human cells, tissues, and cellular and tissue-based products (HCT/P). The FDA has provided two definitions of minimal manipulation. For structural tissue, minimal manipulation means that the processing of the HCT/P does not alter the original relevant characteristics of the tissue relating to the tissue's utility for reconstruction, repair, or replacement [21 CFR 1271.3(f)(1)]. For cells or nonstructural tissues, minimal manipulation means that the processing of the HCT/P does not alter the relevant biological characteristics of cells or tissues [21 CFR 1271.3(f)(2)].[22] By this definition of nonstructural tissues or cells, MSCs obtained from bone marrow aspirate are an example of a G1 HCT/P. Such G1 cell therapies derive their therapeutic benefit from the immunomodulatory and trophic effects of MSCs.[23] Bone marrow aspirate concentrate (BMAC) is normally isolated mechanically and transplanted immediately in the office-based practice. The MSCs prepared by mechanical isolation are mixed with other cells of similar weight and limited in number per unit volume. The second generation (G2) includes MSCs that have undergone cell selection and culture expansion to substantially increase the purity and number of SCs available for transplantation. MSCs are selected based on the standard definition criteria established by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy including adherence to plastic in culture and a distinct cell surface marker profile.[24]

Culture expansion is required to be performed in Good Manufacturing Practice (GMP) facilities.[25] Once the MSCs could be expanded to tens of millions per milliliter, researchers started to investigate if lineage induction[26-30] or preconditioning[31-19] would improve the therapeutic potential, and this constitutes the third generation (G3) of SCs. The fourth generation (G4) is comprised of MSCs genetically modified by either vector transduction or gene editing technology.[40-42] If G3 modifies the phenotype of the MSCs, G4 permanently modifies the genes within MSCs. All G1 through G4 cells are based on MSCs, which can be traced *in vivo* for only limited time.[43,44] Cells prepared with those

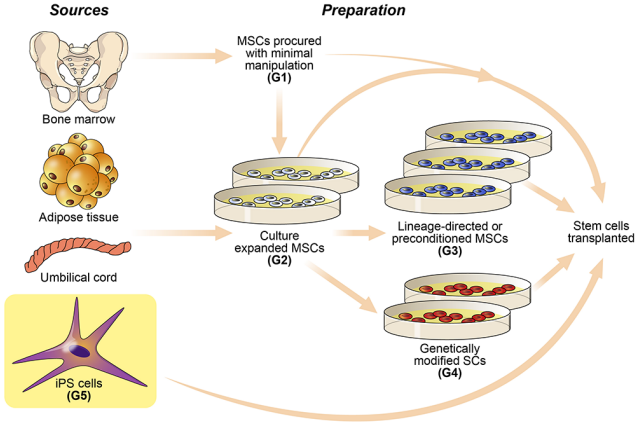

Figure 1. The *five generations of clinically applicable stem cells* model, based on preparation strategy.

strategies can be used for the hypothesized benefit of immunomodulation and trophic effect well established *in vitro*.[45] The fifth generation (G5) includes human induced pluripotent SCs (hiPSCs)[3,4] that are reprogrammed human somatic cells, which are pluripotent like ESCs, but without the ethical concerns of ESCs. Currently, hiPSCs are not studied or used in human subjects due to concerns of tumorigenicity.

Basic science and clinical applications of each generation of mesenchymal stromal cells

First generation: mesenchymal stromal cells prepared with minimal manipulation

BMAC and microfragmented adipose tissue are the most commonly used sources for G1 SCs. BMAC is the most commonly used source of MSCs in the office-based practice.[46] G1 has been extensively studied in both animal and human models of chondral defects. A systematic review of thirty-four basic science peer-reviewed studies concluded that G1 increased bone formation and improved bone healing based on histological, radiographic, and biomechanical analysis in animal models.[47] In human subjects, autologous G1 transplantation helped reduce pain and increased activity level in patients with chondral lesions in a dose dependent manner.[48-51] Published studies utilizing G1 sourced from bone marrow in the form of BMAC and from peripheral blood have studied injection targets including the intervertebral disc,[52] knee,[53-56] hip,[57-59] and patellar tendon.[60] Adverse events were generally mild and transient and included localized pain and/or swelling that were self-limited or responded to conservative management. Improvements in cartilage thickness, pain, and function were reported in studies examining the knee. Symptoms of chronic patellar tendinopathy continued to improve over the course of two years, and this improvement was maintained at five-year follow-up. Improvement in pain and rehydration of the intervertebral disc were both improved, as was Pfirrmann score in several subjects. Improvements in pain and articular cartilage repair were observed with post-operative or intra-operative injection of MSCs for the treatment of osteonecrosis of the femoral head. Autologous BMAC transplantation has been demonstrated to be safe in patients, with no increased cancer risk associated with treatment.[61] Limitations of G1 SC use include the limited number of MSCs procured during the procedure, and the fact that MSCs cannot be further purified based on surface markers in a timely fashion adequate for an in-office bedside procedure.

MSC transplantation must be autologous in order to be regulated solely under section 361 of the PHS Act and 21 CFR Part 1271.[62] By current FDA guidelines, G1 therapies can only be prepared from a patient's own tissues (such as microfragmented adipose or aspirated bone marrow) in order to be offered in the office-based clinical setting.

Second generation: culture-expanded mesenchymal stromal cells

To increase the number of cells available for therapeutic delivery, MSCs are further identified using surface markers and culture-expanded to yield a large number of cells for transplantation. Culturally expanded MSCs have been studied for safety and efficacy in the treatment of a variety of conditions, including neurological, cardiac, and musculoskeletal diseases.[63-65] In animal models, autologous G2 regenerated bone and healed chondral defects.[66] Allogeneic G2 was observed to be effective in treating calvarial defects in rabbits[67] and has been shown to regenerate cartilage[68,69] and repair the Achilles tendon.[70-72] In human clinical trials, patients with knee osteoarthritis treated with autologous G2 showed articular cartilage repair[73] and improved clinical outcomes in terms of pain and walking ability compared to controls.[74] The improvements in these patients persisted even after five years of follow-up.[75,76] Likewise, patients with chronic knee osteoarthritis treated with allogeneic G2 had better outcomes compared to controls at one year follow-up.[77]

G2 cells are being studied in a variety of conditions including hematologic conditions,[78-80] graft-*versus*-host disease,[81] cardiac disease,[82-84] traumatic brain injury,[85] stroke,[86] and amyotrophic lateral sclerosis.[87] A systematic review of MSC therapies for discogenic pain identified five published studies reviewing intradiscally injected G2 cells for discogenic pain. High-quality studies involving large numbers of subjects are lacking, but overall there is a low level of evidence to suggest that intradiscal injection with G2 cells are effective in alleviating discogenic pain, and that such injections are safe, with none of the studies reporting serious adverse events.[88] G2 cells have been reported to be associated with minimal risk of adverse events. A systematic review of intra-articular transplantation of culture-expanded bone marrow-derived MSCs yielded analysis of eight studies including 844 procedures (all of which involved autologous transplantation) with four reported serious adverse events (SAEs).[65] Only two of the reported SAEs were reported as probably or possibly related to the treatment and included infection resolving with antibiotics and one case of pulmonary embolism. The two SAEs that were reported as being unrelated to treatment both involved tumor formation at sites distant from injection, and were not thought to have been related to MSC transplantation. There were seven reported MSC-related adverse events (AEs), all of which were transient and included increased pain and swelling. G2 cells have not been demonstrated to cause tumor growth. Subcutaneous injection of culturally expanded adipose-derived MSCs showed no evidence for toxicity or malignancy in treated mice, and indeed transplanted cells were shown to have undergone complete removal by the host cell system at one year.[89] Intravenous injection of culturally-expanded adipose-derived MSCs were shown to have no evidence of toxicity or tumor formation in transplanted mice after thirteen weeks, nor in human subjects with chronic spinal cord injury after three months.[90]

Considerable study has been devoted to optimizing SC culture conditions. Cultured MSCs do not grow indefinitely, and the cellular senescence resulting from culture expansion may negatively impact the function and efficacy of MSCs, specifically by decreasing differentiation potential, altering immunoregulation, and impairing migratory ability. Approaches to trouble-shooting the problem of *in vitro* human MSC senescence include culturing in hypoxic conditions, adding growth factors to culture, and inhibiting metabolic pathways that contribute to DNA damage during culture expansion.[91] Platelet derivatives such as platelet lysate have largely replaced fetal bovine serum (FBS) in cell culture, due to concerns for safety in humans as well as animal welfare.[92] Culture in xenogenic serum is thought to lead to suboptimal cellular trafficking, possibly affecting the *homing* ability of MSCs to be recruited to sites of damage and/or inflammation. Among human sources for serum, including allogeneic and autologous serum, platelet lysate is thought to offer the best medium for cell culture to preserve the immunomodulatory, differentiation, MSC phenotype stability, and growth properties and potential of MSCs.[93] Finally, expanding MSCs on a three-dimensional scaffold such as hydroxyapatite enables the differentiation of MSCs toward a desired phenotype through the application of mechanical stimuli, as a way to overcome the loss of the complex environment of the bone marrow niche that helps preserve progenitor potency.[94] By optimizing the conditions under which MSCs are expanded in culture, the desired properties of this generation of SCs may be better preserved and enhance their therapeutic efficacy currently being studied in clinical trials.

Third generation: preconditioned or lineage-directed mesenchymal stromal cells

Preconditioning medium to optimize desired SC function represents the third generation of regenerative cellular therapies. Preconditioning strategies to enhance MSCs' therapeutic function *in vivo* include exposure to hypoxia, growth factors/cytokines, or conditioned medium.[2] It has been shown that culturing MSCs in hypoxic conditions enhances proliferation, angiogenesis, and neurogenesis in animal models.[34,35] MSCs exposed to IGF-1 in culture show increased viability.[36] MSCs treated with TNF-α, IL-1β, and nitric oxide demonstrated improved secretion of factors important for regeneration, immunomodulation, and cell trafficking in an animal model.[37] In terms of conditioned medium, MSCs cultured in myogenic medium demonstrated increased ability to repair heart defects by reducing scar formation thereby increasing survival, proliferation, and angiogenesis.[39]

Lineage-directed MSCs demonstrate enhanced therapeutic potential *in vivo*. One example is the derivation of cardiac progenitor cells from bone marrow-derived human MSCs (BMSCs).[26] In this study, BMSCs were induced to express cardiac transcription factors by a cocktail of stimulators to become cardiac progenitor cells. These cells were injected into an infarcted murine myocardium model. After one year, the lineage-directed cardiac progenitor BMSCs showed superior functional and structural benefit compared to mice injected with undirected BMSCs.[26] These findings led to one of the first human clinical trials investigating lineage-directed cell based therapy, the Cardiopoietic stem Cell therapy in heart failure (C-CURE) study. This trial reported that patients treated with lineage-directed MSCs realized significant improvement in left ventricular ejection fraction, physical performance, quality of life, and event-free survival at 2-year follow-up compared to standard care alone.[95] The study reported no evidence of systemic or increased cardiac toxicity. Based on the results of C-CURE, a phase III clinical trial, Congestive Heart Failure Cardiopoietic Regenerative Therapy (CHART-1), is in progress to evaluate the benefits of the lineage-directed MSCs in patients with chronic heart failure secondary to ischemic heart disease.[96]

Fourth generation: genetically modified mesenchymal stromal cells

The efficacy of MSCs *in vivo* is limited by their ability to engraft and proliferate in the area of their intended target. One compelling method to improve the survival and performance of MSCs is engineering via genetic modification. MSCs can be genetically modified using a non-viral or viral gene delivery method, or using gene editing technology. In the non-viral gene delivery method, lipid-based nanoparticles containing MSC-targeting and nuclear localization signaling peptides are used to chemically transfect MSCs.[42,97] Physical methods of MSC transduction include sonoporation via mechanical vibration[98] as well as nucleofection[99] and electroporation,[100] both of which use an electrical pulse to transiently open cell pores to facilitate the transfection of nucleic acid. In the viral-based method, retrovirus, lentivirus, or adeno-associated virus is used to transduce transgenes into MSCs.[40] The viral-based method is superior in terms of high transduction efficiency and stable expression of the gene of interest[41] as well as viability of the modified cells. Long-term safety concerns and regulatory issues have historically precluded its use in many translational studies.[101] However, vectors such as the recombinant adeno-associated viral vectors (rAAV) have been shown to be a safe and stable gene delivery system capable of enhancing the proliferative and chondrogenic differentiation potential of MSCs in a safe and stable manner with persistent transgene expression without the concerns for toxicity or immunogenicity of adenoviral, retroviral or lentiviral vectors.[102] The non-viral gene modification technique does not share the safety concerns of the viral technique but has an inferior profile in terms of transfection efficiency and cell viability.[101]

Given the superior performance of the viral-based method, only animal studies using virally transduced MSCs are discussed. Genetically modified MSCs have been used in cardiac repair, bone regeneration, and cancer treatment.

Injection of MSCs with overexpression of Akt1, a promoter of cell survival and growth, into infarcted swine myocardium reduced inflammation, regenerated myocardiocytes, and improved cardiac function.[103,104] MSCs transduced with vascular endothelial growth factor (VEGF) transplanted into a murine model of bone defect showed enhanced bone formation and increased vascularity compared to the control group that received only unmodified MSCs.[105] In terms of cancer research, MSCs producing interleukin-12 or interleukin-18 have been shown to inhibit tumor growth and prolong survival in animal model glioma.[106,107]

Fifth generation: human induced pluripotent stem cells

Because all G1 through G4 SCs have a limited life span *in vivo*, they are not able to directly differentiate into target tissue, proliferate and repair tissue. An immortal cell line, iPSCs could be a good candidate to function in tissue repair and are classified as G5. iPSCs can be generated from adult somatic cells by retroviral transduction of four key transcription factors: Oct3/4, Sox2, Klf4, and c-Myc.[3,4] Since iPSCs can be generated from any tissue, these cells avoid the ethical issues of using human embryos.[108] The unlimited lineage potential combined with lack of ethical concerns from using embryonic tissue make G5 an attractive therapeutic source for research in MSC-based tissue repair.

One field that has made major advances in both basic science and clinical studies using G5 is the study of retinal diseases. G5 can be differentiated into retinal photoreceptors[109-112] and used as model system to identify future therapies.[113-116] Allowed to proliferate on a collagen scaffold to perform sheets of cells, iPSCs have been differentiated into retinal pigment epithelium and then transplanted into the eye of retinally degenerated animal models.[117] The cell sheets were shown to have the same morphologies, gene expression patterns, and *in vitro* and *in vivo* function as that of authentic retinal pigment epithelium. iPSCs have also been used in applications including modeling of congenital cardiac and liver disease.[118,119]

Studies of G5 transplantation in human subjects have raised safety concerns. The main concerns with G5 are tumorigenicity, immunogenicity, and the lengthy preparation period.[120-122] A clinical trial using autologous G5 to treat age-related macular degeneration was stopped due to genetic mutations in the derived autologous cells.[121] A proposed idea to overcome these obstacles is to create a clinical grade stock of allogeneic G5 with minimal tumorigenic and immunogenic risks.[121,123] This would significantly shorten the preparation process and address concerns of tumorigenicity and immunogenicity. Enhancing efficiency of iPSC induction in the absence of exogenous c-Myc has been proposed as a specific method to reduce concerns around tumorigenicity,[124] as well as inducing overexpression of p27 to suppress tumorigenicity while preserving pluripotency.[125] Genetic modification of iPSCs holds promise to reduce tumorigenicity and improve precision in therapeutic applications.[126] More recently, *in vivo* studies have utilized cellular reprogramming using synthetic mRNA to develop retinal cell types from iPSCs without the risk of genomic integration.[127] Technological advances in preparation and culture of iPSCs are expected improve the demonstrated safety profile of this generation of SCs,[128] and clinical studies regarding G5 move forward.[129] iPSCs remain a compelling area of study in the hopes of wider application in translation studies in the future.

Conclusions

In this review we have proposed the first classification of SCs based on their preparation strategy. Categorizing SCs in terms of preparation strategy as opposed to source (adipose, bone marrow, or umbilical cord) is a key step in terms of describing the potential clinical application of SCs from a historic perspective, demonstrating the past, present and future development of translational SC therapy research, as the field of regenerative medicine moves beyond basic science to translational research. The classification scheme may improve the understanding of patients and clinicians regarding choice of type of cells for use in therapies, and provide a tool for assessing the merit of each type for further study. Conceiving of SCs in terms of preparation strategy may also be of benefit to clinical researchers in fields related to regenerative medicine in describing their study background and rationale.

Each of the five generations has been widely studied in basic science. However, in terms of clinical applications, only G1, G2, and G3 have been used to treat human diseases with report of very few serious adverse events. G1 is currently the only generation approved for office-based cell therapy and is being routinely offered in specialty practices for degenerative musculoskeletal conditions. G2, G3 and G4 remain areas of intense research interest, with on-going clinical trials with FDA approval as Investigational New Drug (IND) applications, holding considerable promise for a variety of neurologic, cardiac, oncologic, and musculoskeletal conditions. The engineering opportunities available in G4 SCs are very attractive for enhancing efficacy, targeting, and cell viability, and clinical trials rightfully continue examining their safety and practicality of use. G5 still has significant safety concerns to overcome before these cells can be used in translational studies in humans, but continue to attract significant interest from basic science researchers and clinical investigators interested in this more highly developed generation of MSCs. G5 is unique among the generations of SCs due to its ability for unlimited self-renewal and therefore tissue repair. The concern of tumorigenesis and significant time and cost necessary for its preparation limits its potential for application as an autologous cell-based therapy, but technological advancements to improve the efficacy and safety of G5 SCs preserve continued promise for its use as an allogeneic cell-based therapy.

The superiority of one generation over another in various clinical applications is not clearly established. The stud-

ies used to illustrate the *Five Generations model* of SC therapies collectively portray a field that is evolving as basic science research moves beyond the bench to studies in animals and humans, and as human clinical trials progress toward high-quality, randomized controlled trials beyond studies assessing basic safety and feasibility. As the field of regenerative medicine and biologics moves forward, a framework for understanding the basic principles of preparation methods of SCs is fundamental to understanding what is known, what questions are currently being asked, and future directions for high-quality research.

Future studies of cell-based regenerative therapy should consider whether a framework incorporating the *Five Generations model* may be of use in terms of improving understanding of SC therapy among clinicians as well as patients with a comprehensive view of all choices available, simplifying communication between clinicians and patients, and providing guidance for future research directions.

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