

Discovery Research and Scalable Production of Umbilical Cord Mesenchymal Stem Cells (Post-print)

Authors: Wang Dianliang

Date: 2018-07-15T00:00:00+00:00

Abstract

Umbilical cord mesenchymal stem cells, derived from human umbilical cord tissue, possess self-renewal capacity and multidirectional differentiation potential, representing a stem cell type with numerous advantages. They can be utilized to treat various diseases that are refractory to conventional therapeutic approaches, including senile degeneration, Parkinson's disease, vascular ischemia, pediatric autism, systemic lupus erythematosus, ankylosing spondylitis, multiple sclerosis, diabetes, diverse traumas, graft-versus-host disease, among others, with demonstrated therapeutic efficacy. This article reviews the research and discovery trajectory, clinical application value, isolation and preparation protocols, detection and identification methodologies, biological characteristics, and large-scale production technologies of umbilical cord mesenchymal stem cells. It should be emphasized that these cells exhibit distinct advantages over certain other stem cell types (such as umbilical cord hematopoietic stem cells, bone marrow hematopoietic stem cells, bone marrow mesenchymal stem cells, etc.), including abundant source material, robust proliferative capacity, and freedom from ethical controversies. The production and application of umbilical cord mesenchymal stem cells must adhere to relevant laws, regulations, and standard specifications promulgated by governmental or institutional authorities.

Full Text

Preamble

The Discovery, Research, and Standardization of Umbilical Cord Mesenchymal Stem Cells: Part 1 of "Umbilical Cord Mesenchymal Stem Cells"

Wang Dian-liang

Tissue Engineering and Regenerative Medicine Laboratory, Department of Pharmacy, General Hospital of the PLA Rocket Force, Beijing 100088, China

Abstract

Umbilical cord mesenchymal stem cells (UCMSCs) derived from human umbilical cord tissue possess self-renewal capacity and multi-lineage differentiation potential, representing a highly advantageous stem cell type. They can be used to treat numerous refractory diseases that are difficult to cure with conventional therapies, including senile degeneration, Parkinson's disease, vascular ischemia, childhood autism, systemic lupus erythematosus, ankylosing spondylitis, multiple sclerosis, diabetes, various traumas, and graft-versus-host disease, with demonstrated therapeutic efficacy. This article reviews the research and discovery history, clinical application value, isolation and preparation methods, detection and identification techniques, biological characteristics, and large-scale production technologies of UCMSCs. It should be emphasized that UCMSCs offer distinct advantages over other stem cell types such as umbilical cord blood hematopoietic stem cells, bone marrow hematopoietic stem cells, and bone marrow mesenchymal stem cells, including abundant material sources, strong proliferative capacity, and absence of ethical concerns. The production and application of UCMSCs must comply with relevant laws, regulations, and standards issued by governmental and institutional authorities.

Keywords

Stem cells; Mesenchymal stem cells; Umbilical cord mesenchymal stem cells; Isolation and identification; Biological characteristics; Large-scale preparation

Corresponding author, email: wangdianliang@sina.com

Stem cells are a population of primitive cells with self-renewal capacity and multi-lineage differentiation potential that can continuously replicate and differentiate to form other cells, tissues, organs, and even entire individuals [1,2]. Stem cells constitute a large family with numerous types, among which umbilical cord mesenchymal stem cells (UCMSCs) represent one important category [3]. Based on their origin, stem cells can be classified into embryonic stem cells and adult stem cells. Adult stem cells exist in various tissues and organs of the body, including umbilical cord, placenta, umbilical cord blood, peripheral blood, amniotic fluid, bone marrow, adipose tissue, nerve, muscle (cardiac muscle), skin, liver, pancreas, kidney, lung, and other tissues and organs. Therefore, UCMSCs belong to the adult stem cell category. Adult stem cells in tissues and organs generally remain quiescent under normal conditions but can exhibit varying degrees of regenerative and renewal capacity under pathological conditions or external induction. Precisely because different types of stem cells possess the ability to develop and differentiate into other cells, tissues, and organs, they are medically termed "universal cells" and are used to treat various intractable diseases, repair or reconstruct tissues and organs, and for anti-aging and cosmetic

applications.

1.1 Discovery and Research of Mesenchymal Stem Cells

In 1867, German experimental pathologist Julius Friedrich Cohnheim discovered stem cells while studying wound inflammation [4]. In his experiments, he intravenously injected animals with an insoluble aniline dye and subsequently found dye-containing cells, including inflammatory cells and fibroblasts associated with fiber synthesis, at sites distal to the injury. From this observation, he inferred the existence of non-hematopoietic stem cells in bone marrow, thus first proposing the concept of bone marrow stem cells. In 1974, Alexander Friedenstein and colleagues first isolated these stem cells from bone marrow, demonstrating that unlike most bone marrow-derived hematopoietic cells, they could rapidly adhere to in vitro culture vessels, generate fibroblast-like colonies, exhibit swirling growth patterns in culture, and possess self-renewal capacity. Friedenstein and his team also confirmed that when bone marrow cell suspensions were seeded, each stem cell could form distinct colonies, with a linear relationship between stem cell proliferation numbers and colony counts. Each stem cell represented a colony-forming unit fibroblast (CFU-F), which they studied using chromosome markers, 3H-thymidine labeling, time-lapse photography, and Poisson distribution statistics. Friedenstein encouraged fellow scientists and physicians to explore stem cell transplantation for treating major diseases. In 1991, Arnold Caplan officially named these bone marrow cells “mesenchymal stem cells” (MSCs), proposing that bone marrow-derived MSCs possessed the potential to differentiate into bone, cartilage, muscle, bone marrow stroma, tendon/ligament, adipose tissue, and other connective tissues. In 2005, the International Society for Cellular Therapy announced that the acronym “MSC” would represent multipotent mesenchymal stromal cells. Bone marrow-derived mesenchymal stem cells (BMMSCs) are sometimes also referred to as “bone marrow stromal cells” (BMSCs). Consequently, BMMSCs represent the earliest discovered and most extensively studied mesenchymal stem cells.

[Figure 1: see original paper]

1.3 Development of Stem Cell Therapy

Stem cell research truly began in the 1960s. In 1963, Canadian scientists McCulloch and Till first demonstrated the existence of stem cells in blood and discovered that hematopoietic stem cells could differentiate into hundreds of different types of human tissue cells. In 1967, Donal Thomas at the University of Washington reported that transplanting normal human bone marrow into patients could treat hematopoietic dysfunction, thereby initiating clinical stem cell research, as bone marrow contains both hematopoietic stem cells and mesenchymal stem cells. In 1981, Kaufman and Martin isolated embryonic stem cells from the inner cell mass of mouse blastocysts and established suitable in vitro culture conditions for embryonic stem cells, successfully developing stem

cell lines. In 1995, American scholar Broxmeyer discovered that umbilical cord blood is rich in hematopoietic stem cells and performed the first clinical cord blood transplantation in 1997. Modern clinical research has shown that in addition to hematopoietic stem cells, umbilical cord blood also contains mesenchymal stem cells, which can enhance the therapeutic efficacy of hematopoietic stem cells. In 1999, the New York City government funded the establishment of the world's first cord blood stem cell bank at the local blood center. Entering the 21st century, stem cell research and clinical applications have received significant attention from governments worldwide. In 2001, a British company announced the launch of newborn umbilical cord blood stem cell storage services, allowing parents to collect their infant's cord blood, isolate stem cells, and preserve them in liquid nitrogen for at least 20 years at a cost of £600. In 2008, China's first stem cell hospital was established in Tianjin, integrating with the Tianjin Cord Blood Hematopoietic Stem Cell Bank and the Tianjin Cord Blood Mesenchymal Stem Cell Bank to form a relatively complete stem cell engineering system encompassing product development, storage, and application. Concurrently, several stem cell drugs were approved for clinical use in countries including the United States, South Korea, Japan, Canada, Australia, and Italy, heralding a new era of cell therapy.

2 Sources and Biological Characteristics of Umbilical Cord Mesenchymal Stem Cells

Mesenchymal stem cells originate from the mesoderm and ectoderm during early embryonic development and represent the most abundant stem cell type in the body. Compared with bone marrow mesenchymal stem cells, which were discovered and studied earlier, umbilical cord mesenchymal stem cells (UCMSCs) possess distinct characteristics in terms of source and biological properties.

2.1 Sources

The umbilical cord appears grayish-white and serves as a cord-like structure connecting mother and fetus during fetal development, with one end attached to the fetal abdominal wall at the umbilicus and the other end connected to the fetal side of the placenta. The cord contains two umbilical arteries and one umbilical vein. The umbilical cord measures 30-70 cm in length (average 50 cm), 1.5-2 cm in diameter, and weighs 60-140 grams. The cord initially forms as the amnion envelops the stalk-like elongated portions of the allantois and yolk sac. As the yolk sac gradually degenerates, umbilical vessels develop, surrounded by loose gelatinous mesenchyme. The umbilical cord functions as a conduit for gas exchange, nutrient supply, and metabolic waste removal between mother and fetus. The umbilical arteries have thick walls and small lumens, while the umbilical vein has thin walls and a large lumen. The umbilical cord comprises three layers: the outermost layer is covered by amniotic epithelium, the umbilical vessels are centrally located, and between them lies the mucous connective tissue first described in 1656 by British anatomist Thomas Wharton,

who named it Wharton' s Jelly (WJ). Wharton' s Jelly provides support and protection for the umbilical vessels.

UCMSCs are derived from various umbilical cord tissues including neonatal umbilical cord blood, umbilical vein endothelium, umbilical artery endothelium, and Wharton' s Jelly. Typically, the umbilical cord is discarded as medical waste after delivery; thus, isolating and extracting mesenchymal stem cells from this source represents a process of transforming waste into valuable material. This approach offers low cost, environmental benefits, and causes no pain to patients. In contrast, bone marrow mesenchymal stem cells are derived from bone marrow, requiring bone marrow mobilization and aspiration procedures that cause patient discomfort and immune damage. Moreover, umbilical cord-derived mesenchymal stem cells offer advantages over their bone marrow-derived counterparts, including higher content, stronger proliferative capacity, and lower immunogenicity. Naturally, both types of mesenchymal stem cells share similarities in culture morphology, adhesiveness, surface markers, and differentiation potential.

2.2 Biological Characteristics

The primary biological characteristics of UCMSCs include robust proliferative capacity, strong plasticity, immunomodulatory effects, low immunogenicity, paracrine functions, hematopoietic support, promotion of stem cell engraftment, and suitability for industrialized production. Under in vitro culture conditions, UCMSCs exhibit adherent growth with vigorous proliferative ability, facilitating easy scale-up expansion and mass production. Since stem cell sources are limited and clinically effective treatment requires transplantation of sufficient cell numbers, large-scale preparation is particularly important. UCMSCs possess excellent plasticity with self-renewal and multi-lineage differentiation potential, capable of differentiating in vivo and in vitro into various functional cell types including bone, cartilage, tendon, ligament, adipose, neural, muscle, endothelial, liver, kidney, and pancreatic cells. This makes them clinically applicable for treating various diseases involving tissue damage and degeneration, especially those refractory to conventional therapies such as senile degeneration, Parkinson' s disease, vascular ischemia, childhood autism, systemic lupus erythematosus, ankylosing spondylitis, multiple sclerosis, diabetes, various traumas, and graft-versus-host disease.

UCMSCs exert immunomodulatory functions by secreting soluble cytokines and regulating the immunosuppressive effects of regulatory T cells, demonstrating clear therapeutic efficacy for autoimmune and inflammatory diseases including systemic lupus erythematosus, rheumatoid arthritis, diabetes, and infectious or traumatic systemic inflammatory responses. They exhibit low immunogenicity, as allogeneic or even xenogeneic UCMSC infusion does not trigger immune rejection reactions, ensuring high safety and absence of toxic side effects in clinical transplantation. UCMSCs also possess paracrine functions, secreting exosomes and various cytokines that promote tissue and organ repair by stimulating in

situ cell proliferation, angiogenesis, and inhibiting inflammatory responses at injury sites. Following transplantation, they provide hematopoietic support by secreting multiple cytokines (GM-CSF, G-CSF, SDF-1, VEGF, etc.) that promote hematopoietic stem cell proliferation and differentiation, thereby enhancing hematopoietic function. Additionally, UCMSCs promote engraftment of exogenous stem cells by secreting various cytokines that facilitate their proliferation and differentiation into functional cells after implantation in patients.

A particularly valuable characteristic is their suitability for industrialized and standardized clinical therapy production. A single umbilical cord can yield sufficient UCMSCs in vitro to treat tens of thousands of clinical patients, enabling standardized, large-scale, factory-style production to provide clinical-grade “drug products.” Given their abundant material source, convenient collection, easy expansion, lack of donor harm, absence of age-related proliferative and differentiation capacity decline (unlike BMMSCs), freedom from ethical controversies, and excellent biological properties, UCMSCs hold considerable promise for clinical applications.

3 Isolation, Identification, and Large-Scale Preparation of Umbilical Cord Mesenchymal Stem Cells

To ensure quality, the isolation, identification, and large-scale preparation of UCMSCs should adhere to relevant regulations and standards for stem cell research, including the “Guidelines for Quality Control and Preclinical Research of Stem Cell Preparations (Trial)” issued by the National Health and Family Planning Commission, the “Self-Discipline Norms for Quality Management of Stem Cell Preparation” issued by the China Medicinal Biotech Association, the “Guidelines for Clinical Translation of Stem Cells” issued by the International Stem Cell Association, and the “Criteria for Defining Mesenchymal Stem Cells” proposed by the International Society for Cellular Therapy.

3.1 Isolation and Preparation

UCMSCs primarily reside in the Wharton’s Jelly of the umbilical cord. Three main methods exist for primary isolation and culture: the tissue explant method, enzymatic digestion method, and a combination approach called the enzymatic tissue method.

The tissue explant method involves removing the outer membrane, blood vessels, and blood cells from the collected umbilical cord, then cutting the tissue into micro-explants of approximately 1 mm^3 . These explants are seeded onto plastic culture flasks for adherent culture, allowing UCMSCs to migrate out from the tissue blocks and grow radially around them. After mechanical removal of the tissue blocks, cells exhibit swirling or flame-like growth patterns with spindle-shaped morphology similar to skin fibroblasts and relatively uniform appearance. Cells typically reach confluence within 2–3 weeks, and the tissue blocks can be repeatedly used for multiple rounds of explant culture. This method offers the

advantages of simple operation and low cost, but suffers from lengthy primary culture time and poor cell adhesion.

The enzymatic digestion method employs single-enzyme digestion (collagenase, type I collagenase, type IV collagenase), double-enzyme digestion (collagenase combined with trypsin), triple-enzyme digestion (collagenase combined with hyaluronidase), or quintuple-enzyme digestion (type I collagenase, type IV collagenase, hyaluronidase, trypsin, DNase) to digest the 1 mm³ micro-tissue blocks into single-cell suspensions for seeding in culture flasks. Multi-enzyme combinations can improve UCMSC yield, reduce digestion time, and minimize detrimental effects on cell viability. However, this approach is characterized by higher economic cost, complex procedures, extended processing time, and significant cell death.

The enzymatic tissue method combines initial enzymatic digestion with subsequent tissue block culture. Its advantage lies in the loosened tissue structure after partial enzymatic digestion, facilitating easier cell migration from explants while minimizing cell damage due to shorter digestion times, thereby maintaining good cell viability even after multiple passages.

Regardless of the method employed, cells should be passaged when they reach confluence or 80–90% confluency using trypsin digestion to collect cells for subculture. Passaged UCMSCs grow faster than primary cells, typically reaching 85–90% confluence within approximately 6 days. Continuous passaging within 8 generations does not significantly reduce stem cell activity. During passaging, “stemness” maintenance factors and growth-promoting factors should be added to preserve stem cell activity.

3.2 Identification

The identification and characterization of UCMSCs must meet the following criteria:

3.2.1 Adhesion Characteristics Under Standard Culture Conditions

UCMSCs must exhibit plastic adhesion properties under standard culture conditions. Human umbilical cord-derived mesenchymal stem cells (hUCMSCs) observed under an inverted microscope initially appear spindle-shaped, polygonal, or multi-angular upon adhesion, and display fibroblast-like morphology with relatively uniform appearance when confluent, though occasional multi-angular cells may be observed. After 10–12 days of primary culture, cells exhibit clonal growth patterns and reach confluence by 14–12 days. Following passaging, cells show directional and swirling growth patterns. The doubling time of hUCMSCs is 60–85 hours, allowing passaging every 3–5 days. These cells demonstrate strong proliferative capacity, capable of reaching 20 passages in vitro, though proliferation rate declines after passage 15. By passage 7, hUCMSC numbers can increase over 300-fold while maintaining stable morphology. Throughout passaging, cell morphology remains relatively uniform without significant changes,

and proliferative capacity shows no obvious alteration. Cell cycle analysis reveals that 80% of cells reside in the G0-G1 phase, with relatively few cells in active proliferation. Early cell growth demonstrates an increasing trend with a lag phase generally not exceeding 36 hours, followed by a logarithmic growth phase lasting until day 5-6, after which cell proliferation enters a plateau phase, exhibiting a typical S-shaped growth pattern. Transmission electron microscopy shows that hUCMSCs possess relatively large, irregularly round or oval nuclei with prominent nucleoli, abundant euchromatin, and scarce heterochromatin. Cellular organelles are predominantly rough endoplasmic reticulum and mitochondria, with numerous free ribosomes.

3.2.2 Surface Marker Expression UCMSCs must highly express positive markers such as CD13, CD29, CD44, CD90, and CD105, while lacking expression of CD11a, CD11b, CD14, CD31, CD34, CD45, HLA-DR, and other markers. hUCMSC surface proteins can serve as immunological markers, though no highly specific surface markers have been identified [6-8]. Commonly expressed markers include CD13, CD29, CD44, CD90, and CD105 molecules, with weak expression of CD106 and low expression of MHC class I molecules. These cells do not express monocyte/macrophage markers CD14 and CD11b, endothelial cell-specific antigen CD31, lymphocyte function-associated antigen-1 (CD11a), or hematopoietic stem cell markers CD34 and CD45. UCMSCs also lack expression of MHC class II molecules such as HLA-DR and co-stimulatory factors CD80 and CD86 involved in HLA antigen recognition, enabling them to evade recognition by allogeneic T lymphocytes and NK cells and survive long-term in allogeneic or even xenogeneic hosts. Phenotypic analysis of UCMSCs is primarily performed using immunocytochemical staining and flow cytometry, with flow cytometry (FCM) recommended due to its advantages in analysis speed, precision, and accuracy. RT-PCR detection demonstrates positive OCT-4 mRNA expression in hUCMSCs; OCT-4 is an embryonic stem cell-specific gene that plays a crucial role in maintaining the undifferentiated state of stem cells.

3.2.3 Multi-Lineage Differentiation Potential The capacity to differentiate into mature cells of multiple tissue types from different germ layers represents a key characteristic of mesenchymal stem cells, and the presence of multi-lineage differentiation potential constitutes the most important criterion for evaluating UCMSCs. Specific *in vitro* induction culture systems should be selected for directed differentiation, with the induction type determined by observing cell morphology, phenotype, and function. Differentiation direction selection should consider functional cell types from different germ layers and tissues. Laboratories testing mesenchymal stem cells should generally examine cells from all three germ layers: directed differentiation into ectoderm-derived neural cells, mesoderm-derived osteoblasts, chondrocytes, and adipocytes, and endoderm-derived insulin-secreting cells.

3.3 Large-Scale Production

Although UCMSCs are derived from abundant sources, variations exist between different materials, necessitating standardized, large-scale factory production for extensive clinical application.

For *in vitro* expansion and culture of UCMSCs, adherent subculture methods are typically employed, which can satisfy the technical requirement of “one cord, one factory.” In subculture expansion systems, stem cells proliferate faster than primary cells, generally reaching 85–90% confluence within approximately 6 days. Continuous passaging within 8 generations does not significantly reduce stem cell activity. During passaging, “stemness” maintenance factors and growth-promoting factors should be added to preserve stem cell activity.

Common devices for large-scale UCMSC production include multi-layer flasks and spinner flasks. Multi-layer flasks employ static culture, which involves substantial labor and contamination risk but offers low equipment cost. Spinner flasks utilize motor-driven mechanical rotation for rolling culture, which facilitates mixing and contact between cultured stem cells, culture medium, and air, promoting stem cell proliferation and yielding higher cell numbers. The spinner flask method, traditionally used for vaccine production, is suitable for industrial-scale manufacturing.

Modern stem cell production employs bioreactors that can simulate the *in vivo* growth and development environment for high-efficiency proliferation. Bioreactor production offers several advantages: automated and scalable manufacturing, real-time monitoring of culture parameters, easy scale-up, reduced contamination risk, high stem cell culture density, and minimal equipment footprint. However, bioreactors are expensive and require operational expertise. They are typically combined with microcarriers, microencapsulation, and perfusion technologies to enhance cell quantity and quality, representing the future trend for industrialized production of various stem cell types.

[Figure 2: see original paper] Mesenchymal Stem Cell Filtration Separator (invented by Wang Dian-liang et al.; Patent No. ZL201120139405.5). Bioreactors are most suitable for subculture, while initial seed cell acquisition still requires adherent subculture in flasks. However, utilizing equipment such as mesenchymal stem cell filtration separators can substantially improve the efficiency of primary cell isolation and culture. A 40-cm-long umbilical cord processed via the tissue explant method can yield $1.5\text{--}2 \times 10^8$ primary cells, which can be expanded to over 1×10^{10} cells through 3–5 passages in multi-layer flasks, sufficient for treating thousands of patients. Bioreactor-based subculture can produce even higher quality and quantity of stem cells to meet the needs of more patients. Evidently, UCMSCs possess strong “drug-like” properties, making them suitable for large-scale industrial production to satisfy clinical demands. However, UCMSC research, production, and application must strictly comply with relevant laws, regulations, and standards issued by governmental and institutional authorities.

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