Stem Cells and Cancer Stem Cells 7
Therapeutic Applications in Disease and Injury

M.A. Hayat
Editor

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Although touched by technology, surgical pathology always has been, and remains, an art. Surgical pathologists, like all artists, depict in their artwork (surgical pathology reports) their interactions with nature: emotions, observations, and knowledge are all integrated. The resulting artwork is a poor record of complex phenomena.

Richard J. Reed, MD

One Point of View

All small tumors do not always keep growing, especially small breast tumors, testicular tumors, and prostate tumors. Some small tumors may even disappear without a treatment. Indeed, because prostate tumor grows slowly, it is not unusual that a patient may die at an advanced age of some other causes, but prostate tumor is discovered in an autopsy study. In some cases of prostate tumors, the patient should be offered the option of active surveillance followed by PSA test or biopsies. Similarly, every small kidney tumor may not change or may even regress. Another example of cancer or precancer reversal is cervical cancer. Precancerous cervical cells found with Pap test may revert to normal cells. Tumor shrinkage, regression, reversal, or stabilization is not impossible.

Another known example of cancer regression is found in pediatric neuroblastoma patients. Neuroblastoma shows one of the highest rates of spontaneous regression among malignant tumors. In addition to the well-known spontaneous regression in stage 4S disease, the high incidence of neuroblastoma remnants found during autopsy of newborns suggest that localized lesions may undergo a similar regression (Guin et al. 1969). Later studies also indicate that spontaneous regression is regularly seen in infants with localized neuroblastoma and is not limited to the first year of life (Hero et al. 2008). These and other studies justify the "wait and see" strategy, avoiding chemotherapy and radiotherapy in infants with localized neuroblastoma, unless MYCN gene is amplified. Infants with nonamplified MYCN and hyperdiploidy can be effectively treated with less intensive therapy. Infants with disseminated disease without MYCN have excellent survival with minimal or no treatment. Another example of spontaneous shrinkage and loss of tumors without any treatment is an intradural lipoma (Endoh et al. 1998).

Overtreatment

An example of unnecessary surgery is the removal of all the armpit lymph nodes after a biopsy when a sentinel node shows early stage breast cancer; removal of only the sentinel node may be needed. Limiting the surgery to the sentinel node avoids painful surgery of the armpit lymph nodes, which can have complications such as swelling and infection (such limited surgery is already being practiced at the Memorial Sloan-Kettering Cancer Research

Center). Radiation-induced second cerebral tumors constitute a significant risk for persons undergoing radiotherapy for the management of cerebral neoplasms. High-grade gliomas are the most common radiation-induced tumors in children (Pettorini et al. 2008). The actual incidence of this complication is not known, although it is thought to be generally low.

Presently, although approximately 80% of the children with cancer are cured, the curative therapy could damage a child's developing organ system; for example, cognitive deficits following cranial radiotherapy are well known. Childhood survivors of malignant diseases are at an increased risk of primary thyroid cancer (Sigurdson et al. 2005). The risk of this cancer increases with radiation doses up to 20–29 Gy. In fact, exposure to radiation therapy is the most important risk factor for the development of a new CNS tumor in survivors of childhood cancer, including leukemia and brain tumors. The higher risk of subsequent glioma in children subjected to medical radiation at a very young age reflects greater susceptibility of the developing brain to radiation. The details of the dose-response relationships, the expression of excess risk over time, and the modifying effects of other host and treatment factors have not been well defined (Neglia et al. 2006).

Among children with cancer, the application of radiotherapy, therefore, should not be taken lightly, and it should be administered only when absolutely necessary to successfully treat the primary tumor. When radiotherapy is administered, use of the minimum effective dose tends to minimize the risk of second CNS neoplasms (late effect). Prolonged follow-up of childhood cancer survivors (particularly those treated with radiation) is necessary because of the long period between treatment and the development of malignancy. This practice should be a part of the effective therapy of the primary disease.

There were an estimated 217,730 new cases of prostate cancer in the United States in 2010 with 32,050 deaths, making it the second leading cause of cancer deaths in men. Currently, there are more than 2,000,000 men in the United States who have had radical or partial prostate surgery performed. Considering this huge number of prostate surgeries and the absence of a cumulative outcome data, it seems appropriate to carefully examine the benefits of radical surgery.

Prostate cancer treatment is one of the worst examples of overtreatment. Serum prostate-specific antigen (PSA) testing for the early detection of prostate cancer is in wide use. However, the benefit of this testing has become controversial. The normal cut-off for serum levels of PSA is 4 ng/ml, so any man presenting a PSA above this level is likely to require rectal biopsy, but only 25% of men with serum levels of PSA between 4 and 10 ng/ml have cancer (Masters 2007). The PSA threshold being used for biopsy ranges between 2.5 and 3.4 ng/ml. Up to 50% of men presenting with prostate cancer have PSA levels within the normal range. It is apparent that screening of prostate cancer using PSA has a low specificity, resulting in many unnecessary biopsies, particularly for gray zone values (4–10 ng/ml). According to one point of view, the risks of prostate cancer overdetection are substantial. In this context, overdetection means treating a cancer that otherwise would not progress to clinically significant disease during the lifetime of the individual.

Overdetection results in overtreatment. The advantages and limitations of PSA test in diagnosing prostate cancer were reviewed by Hayat (2005, 2008).

Recently, the FDA cleared the use of NADiA (nucleic acid detection immunoassay) ProsVue prognostic cancer test. This proprietary nucleic acid detection immunoassay technology identifies extremely low concentrations of proteins that have not been routinely used as a diagnostic or prognostic aid. It is an *in vitro* diagnostic assay for determining the rate of change of serum total prostate specific antigen (PSA) over a period of time. The assay can quantitate PSA at levels <1 g/ml. This technique can be used as a prognostic marker in conjunction with clinical evaluation as an aid in identifying the patients at reduced risk for recurrence of prostate cancer for years following prostatectomy. It targets the early detection of proteins associated with cancer and infectious diseases. This technique combines immunoassay and real-time PCR methodologies with the potential to detect proteins with femtogram/ml sensitivity (10–15 g/ml). Additional clinical information is needed regarding its usefulness in predicting the recurrence.

A significant decrease in the risk of prostate cancer-specific mortality is observed in men with few or no comorbidities. Indeed, active surveillance in lieu of immediate treatment (surgery or radiation, or both) is gaining acceptance. Most men with prostate cancer, even those with high-risk disease, ultimately die as a result of other causes (Lu-Yao et al. 2009). Debate on this controversy is welcome, but narrow opinions and facile guidelines will not lead to fact and new information; men worldwide deserve it (Carroll et al. 2011). Automatic linking positive diagnosis with treatment, unfortunately, is a common clinical practice. Unfortunately, even men who are excellent candidates for active surveillance in the United States often undergo some treatment. Deferment of treatment is advised in men with low-risk disease.

First whole genome sequences of prostate tumors were recently published online in *Nature* journal (vol. 470: 214–220, 2011). This study revealed that rather than single spelling errors, the tumor has long "paragraphs" of DNA that seem to have broken off and moved to another part of the genome (rearrangement of genes), where they are most active. These portions of DNA contain genes that help drive cancer progression. The mutated genes involved include PTEN, CADM2, MAG12, SPOP, and SPTA1. This information may lead to the development of more efficient, less invasive ways to diagnose and treat this cancer. Such information, in addition, should lead to personalized therapeutics according to sequencing results of different gene mutations or chromosomal rearrangements. The urgent need of such studies becomes apparent considering the huge number of new cases of prostate problem reported every year.

In contrast to prostate cancer, cardiovascular disorders take the heavier toll of life. In other words, the risk of death for men in the United States between the ages of 55 and 74 years due to cardiovascular disease surpasses that of prostate cancer. Cardiovascular disease is the most common of the chronic non-communicable diseases that impact global mortality. Approximately, 30% of all deaths worldwide and 10% of all healthy life lost to disease are accounted for by cardiovascular disease alone.

It is known that chemotherapy can cause very serious side-effects. One most recent example of such side-effects was reported by Rubsam et al.

(2011). Advanced hepatocellular carcinoma (HCC) induced by hepatitis C virus was treated with sorafenib. It is an oral multikinase inhibitor that interferes with the serine/threonine kinases RAF-1 and B-Raf and the receptor tyrosine kinases of the vascular endothelial growth factor receptors and the platelet-derived growth factor receptor-beta. Although sorafenib is effective in regressing HCC, it shows serious side-effects including increasingly pruritic and painful skin changes (cutaneous eruption).

It is well established that radiation doses are related to risk for subsequent malignant neoplasms in children with Hodgkin's disease. It has been reported that increasing radiation dose was associated with increasing standardized incidence ratio ($p = 0.0085$) in survivors of childhood Hodgkin's disease (Constine et al. 2008). Approximately, 75% of subsequent malignancies occurred within the radiation field. Although subsequent malignancies occur, for example, in breast cancer survivors in the absence of radiotherapy, the rise increases with radiation dose.

Unwanted side effects of some drug excipients (e.g., propylene glycol, menthol) may also pose safety concerns in some patients. Excipients are defined as the constituents of the pharmaceutical formulation used to guarantee stability and physicochemical, organoleptic, and biopharmaceutical properties. Excipients frequently make up the majority of the volume of oral and parenteral drugs. Not all excipients are inert from the biological point of view. Although adverse drug reactions caused by the excipients are a minority of all adverse effects of medicinal products, the lack of awareness of the possible risk from excipients should be a concern for regulatory agencies, physicians, and patients (Ursino et al. 2011). Knowledge of the potential side effects of excipients is important in clinical practice.

The pertinent question is: Is it always necessary to practice tumor surgery, radiotherapy, or chemotherapy? Although the conventional belief is that cancer represents an "arrow that advances unidirectionally," it is becoming clear that for cancer to progress, it requires cooperative microenvironment (niche), including immune system and hormone levels. However, it is emphasized that advanced (malignant) cancers do not show regression and require therapy. In the light of the inadequacy of standard treatments of malignancy, clinical applications of the stem cell technology need to be expedited.

Eric Hayat

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Preface

Stem cell is nature's indispensable gift to multicellular organisms.

This is volume 7 of the nine-volume series, Stem Cells and Cancer Stem Cells: Therapeutic Applications in Disease and Tissue Injury. A stem cell is defined as a cell that can self-renew and differentiate into one or more specialized cell types. A stem cell may be pluripotent, which is able to give rise to the endodermal, ectodermal, and mesodermal lineages; an example is embryonic stem cells. A stem cell may be multipotent, which is able to give rise to all cells in a particular lineage; examples are hematopoietic stem cells and neural stem cells. A stem cell may be unipotent, which is able to give rise to only one cell type; an example is keratinocytes.

A cancer stem cell is a cell type within a tumor that possesses the capacity of self-renewal and can give rise to the heterogeneous lineages of cancer cells that comprise the tumor. In other words, a cancer stem cell is a tumor-initiating cell. A unique feature of cancer stem cell is that although conventional chemotherapy will kill most cells in a tumor, cancer stem cells remain intact, resulting in the development of resistance of therapy. All of these types of stem cells are discussed in this volume. Vast therapeutic applications of the following specific stem cells in disease and tissue injury are discussed: human embryonic stem cells, human mesenchymal stem cells, germ cell-derived pluripotent stem cells, induced pluripotent stem cells, human umbilical cord blood-derived stem cells, breast tumor stem cells, and hematopoietic stem cells.

As stated above, given that human embryonic stem cells possess the potential to produce unlimited quantities of any human cell type, considerable focus has been placed on their therapeutic potential. Because of the pluripotency of embryonic stem cells, they have been used in various applications such as tissue engineering, regenerative medicine, pharmacological and toxicological studies, and fundamental studies of cell differentiation. The formation of embryoid bodies, which are three-dimensional aggregates of embryonic stem cells, is the initial step in the differentiation of these cells. Such embryoid body culture has been widely used as a trigger for the *in vitro* differentiation of embryonic stem cells.

The basic capacity of self-renewal of human embryogenic stem cells is explained. The role of TGF-β in the propagation of human embryonic stem cells is discussed, so is the differentiation of these cells into neurons, hepatocytes, cardiomyocytes, and retinal cells. Molecular signaling pathways that modulate mesenchymal stem self-renewal are discussed. The regenerative

potential of stem cells and their mesenchymal progeny is explained. Clinical applications of mesenchymal stem cells are reviewed, and their use for treating cancer patients, diabetes, and neurodegenerative pathologies is detailed.

Donor policies for hematopoietic stem cell transplantation are explained. The usefulness of allogeneic stem cell transplantation of patients with chronic lymphocytic leukemia is underscored. Clinical use of hematopoietic stem cells for patients with myeloma is explained. Response of hematopoietic stem/progenitor cells to chemotherapy is clarified. Therapeutic applications of stem cells and induced pluripotent stem cells in treating Parkinson's disease are presented.

By bringing together a large number of experts (oncologists, neurosurgeons, physicians, research scientists, and pathologists) in various aspects of this medical field, it is my hope that substantial progress will be made against terrible human disease and injury. It is difficult for a single author to discuss effectively the complexity of diagnosis and therapy, including tissue regeneration. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of cancer cure and tissue regeneration. I hope these goals will be fulfilled in this and other volumes of the series. This volume was written by 62 contributors representing 10 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the readers in these important areas of disease and injury. I respect and appreciate the hard work and exceptional insight into the nature of cancer and other disease provided by these contributors. The contents of the volume are divided into four subheadings: Regenerative Medicine, Tissue Engineering, Transplantation, and Neural Applications for the convenience of the readers.

It is my hope that subsequent volumes of the series will join this volume in assisting in the more complete understanding of the causes, diagnosis, and cell-based treatment of major human diseases and debilitating tissue/organ injuries. There exists a tremendous, urgent demand by the public and the scientific community to address to cancer diagnosis, treatment, cure, and hopefully prevention. In the light of existing cancer calamity, government funding must give priority to eradicating deadly malignancies over military superiority.

I am thankful to Jennifer Russo for her help in many ways in completing this project.

M.A. Hayat

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Part I

Regenerative Medicine

Mesenchymal Stem Cell Expansion for Therapeutic Application

1

Teng Ma

Contents

Abstract

Human mesenchymal stem cells have emerged as an important candidate in cell therapy owing to their unique therapeutic properties. The clinical application of mesenchymal stem cells will require a rapid cell expansion process with high reproducibility. The cell expansion process must also preserve the native cell properties during expansion and meet regulatory requirements. The microenvironmental factors that influence the phenotype of the mesenchymal stem cells are being identified and reproduced in bioreactor-based cell expansion processes, moving the cell production technology toward clinical application.

Introduction

Recently, human mesenchymal stem or stromal cells (MSC) isolated from bone marrow and other tissues have generated a wave of enthusiasm in both scientific and clinical communities because of the promising results in clinical trials and the therapeutic prospect for many devastating diseases, from cardiovascular diseases and stroke to traumatic bone and cartilage injuries (Prockop and Olson 2007). To realize the potential of MSC for clinical applications, a significant challenge is to obtain MSCs in sufficient quantities in order to reach the required therapeutic potency. Because of the low frequency of MSC in bone marrow,

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only culture-expanded MSCs are likely to meet clinical demands of. The quantities of MSCs required for the majority of clinical applications are estimated to be in the range of 10^8–10^9 cells, whereas only 10^4–10^5 cells are typically generated from routine cultures (Sharma et al. 2011). However, traditional cell-culture techniques using sequential passaging not only results in a gradual loss of their self renewal properties and multi-lineage potential, but also are associated with other changes such as decreases in MSC's responsiveness to stimuli at wound sites, reduced secretion of therapeutic factors, and an increase of cell size with reduced cell mobility (Parekkadan and Milwid 2010). In addition, standard culture requires lengthy expansion using open vessels, which are labor intensive and at risk of microbial contamination (Sharma et al. 2011). These challenges have motivated the development of novel MSC expansion methods with an emphasis on preserving innate MSC properties. On the other hand, safety remains a major concern in cell therapy and must be addressed as MSCs move to clinical application. Processes of MSC production need to be in compliance with Good Manufacturing Practice (GMP) and must meet the final regulatory requirements. This review will focus on the recent advances in MSC expansion methods and on the progress of clinical scale MSC expansion.

Human MSC in Clinical Applications

The existence in the bone marrow of a stromal precursor that supports the development and maintenance of hematopoiesis was postulated in the early twentieth century (Maximow 1924). In the 1960s, Friedenstein and coworkers were the first to demonstrate that the stromal cells could be isolated from whole bone marrow aspirates based on adhesion to culture plastics and that these cells were colony forming unit fibroblastic (CFU-F) cells with high replicative capacity (Friedenstein et al. 1968). These cells were later shown to differentiate into a number of mesenchymal cell types including osteoblasts, chondrocytes, and adipocytes. As a result, these cells were called mesenchymal stem cells in reference to their high self-renewal capacity and their maintenance of adult mesenchymal tissues. These cells were also known as stromal cells to indicate their supporting role in hematopoiesis. Because of their origin, the early clinical applications of MSCs focused on treating diseases of connective and hematopoietic diseases. Recently, however, appreciation of MSC's trophic functions, including their enhancement of endogenous repair and attenuation of immunological and inflammatory responses through the secretion of regulatory macromolecules, has generated a broader interest for the treatment of conditions as diverse as spinal cord injury and multiple sclerosis (Caplan and Dennis 2006).

Attractive properties of these reparative adult stem cells are that they can be readily isolated from a small tissue sample and expanded in culture, that they will home to injured tissue, and that they enhance tissue repair. In current clinical studies, standard plastic culture has been the dominant method for MSC expansion. Autologous MSCs isolated from bone marrow aspirate were expanded in a standard CO_2 incubator and intravenously transplanted to stroke patients (Lee et al. 2010). The standard culture-expanded MSCs were also transplanted into patients with acute myocardial infarction (Chen et al. 2004), and were used for the treatment of severe limb ischemia (Lasala et al. 2010). However, studies have begun to reveal the adverse impact of the conventional culture method on MSC properties. Sequential passage of MSC using standard methods has been shown to be associated with a decrease in the expression of adhesion molecules, the loss of chemokine receptors, enlargement of cell size, and the lack of a chemotactic response to chemokines, thus compromising their therapeutic potency. The culture-expanded MSCs were entrapped at the pre-capillary level because of their large size after intra-arterially delivery, leading to micro-ischemia and significant loss of cell numbers (Furlani et al. 2009). Freshly isolated murine MSCs have high efficiency for homing to bone marrow following infusion but lost their homing ability after culture expansion (Rombouts and Ploemacher 2003). In stroke

treatment, culture expanded human MSCs at passage 2 (P2) have significantly higher trophic factor secretion (*e.g.*, VEGF, EPGF, BDNF, bFGF) compared to those of P6 MSCs, although both have similar morphologic features, viability, and tri-lineage differentiation capacity (Li et al. 2008). In addition, homing receptors such as CXCR4, a chemotactic receptor for SDF-1, is usually absent on the surface of culture-expanded MSCs (Karp and Teo 2009). As a result, limited targeting capability of culture-expanded human MSC and a very low graft survival rate require the delivery of a large number of cells to achieve the desired therapeutic effects. Since MSC's therapeutic value depends not only on the multi-lineage potency but also on the homing and engrafting abilities, strategies are being developed to expand the cell population while preserving the innate properties for clinical applications.

In Vivo Microenvironment of Human MSC

The immediate environment, or niche, of a stem cell provides important information about their interactions within the anatomical location, and is important in understanding their native functions. As such, mimicking or recapitulating the chief microenvironmental factors in the *in vitro* systems to "instruct" multipotent MSC fate has emerged as an important strategy in MSC expansion. The lack of unique MSC markers has made it difficult to locate or define the human MSC microenvironment *in vivo*, and the precise origin and identity of the archetypal MSCs from various tissue sources are debated continually. Increasing evidence suggests that MSCs reside in perivascular locations in almost all adult tissues and respond to systemic influences related to tissue injury or diseases. However, MSCs are not ubiquitously distributed with multi-potential and equal potency, but rather are a defined population of tissue specific progenitors that share some common characteristics of mural cells found within the microvascular walls (Bianco 2011).

Among the MSCs isolated from various tissue sources, the bone marrow-derived MSCs are the earliest and best characterized because of their close association with the hematopoietic microenvironment and their hematopoietic regulatory function. The anatomically relevant and structurally important microenvironmental characteristics of the bone marrow include the low oxygen tension and the abundance of the stromal elements including ECM proteins and regulatory macromolecules. Bone marrow has a hierarchical structure, and direct measurement has revealed that bone marrow in general is hypoxic, where some regions as low as 1–2% O_2 (Ma et al. 2009). In this hypoxic environment, MSCs are a significant source of many immunoregulatory and trophic factors and maintain the microenvironment by secreting and remodeling their ECM network. As such, the extracellular signals generated by the surrounding microenvironment, such as cell–cell interactions, secreted and ECM-bond growth factors, molecules of ECM, and local physiological environments, play important roles in influencing MSC identity and fate. On the other hand, the secretory profiles of MSCs are influenced significantly by the local physiological state, such as oxygen tension. Furthermore, MSCs are not static entities and they actively respond and modify their microenvironment by building or degrading the ECM network and by modifying their secretory profiles. Understanding these multifaceted niche factors and their dynamic and reciprocal interplay are important in revealing the mechanisms underlying MSC cellular properties and guiding the optimization of MSC expansion strategy ex vivo.

Hypoxic Expansion of Human MSC

Based on *in vivo* measurement and modeling the oxygen tension in bone marrow is considered to fall between 2% and 8% (Ma et al. 2009). Thus, low oxygen tension, traditionally termed "hypoxia", in fact represents an "in situ" normoxia of the bone marrow environment (Ivanovic 2009). In addition to occurring in the *in vivo* condition, hypoxia is a hallmark of ischemic injury, which is

also known to activate MSCs. Oxygen tension in the ischemic tissue is less than 1% and is believed to be an important factor in the elicitation of specific MSC responses such as migration and the secretion of angiogenic factors. Consequently, the utilization of hypoxia conditions in MSC culture is made to mimic either (a) the *in vivo* bone marrow environment or (b) ischemic tissue. The oxygen tension has been controlled typically in the range of 1–5% for long-term culture a less than 1% in short term pre-conditioning. In a comparative study, bone marrow-derived multi-lineage cells have highest growth under 3% O_2, followed by 5% and 1% O_2 (2% O_2 was not tested) (D'Ippolito et al. 2006). In addition, human MSCs maintain their proliferation ability for an additional 8–20 population doublings (PD) under 1% O_2 compared to normoxia but have comparable PDs during the initial 15 PDs, suggesting a longer lag phase at 1% O_2 (Jin et al. 2010). Together, these results suggest that oxygen tension in the range of 1–3% is most effective in supporting human MSC expansion.

It is important to note that MSC's hypoxia responses also depend on cell source, culture media, and duration of treatment. Holzwarth et al. (2010) showed that bone marrow MSC from children with hematopoietic malignancies have reduced viability and differentiation potential when cultured under 1% O_2 in the presence of human plasma instead of fetal bovine serum as commonly used in other studies. Furthermore, cells may also exhibit different cellular responses during short term exposure as they adapt to the new oxygen environment. In the conventional nomenclature of literature, oxygen tension refers to the gas composition in the culture ware, which varies from the peri-cellular oxygen tension depending on cell density, plastic culture ware, and duration of culture. Thus, oxygen tension as a parameter during cell expansion needs to be clearly defined in the context of other culture parameters in order to interpret the results. Moreover, confusion exists regarding the impact of hypoxic expansion on MSC's "stemness" and multi-lineage potential. While studies have shown that hypoxic MSCs have higher CFU-F and expression of such stem cell genes as Oct-4, MSC trilineage differentiations have different metabolic and regulatory requirements and may occur under their respective "optimal" oxygen environment. Thus, the hypoxia inhibition of a particular differentiation pathway is not necessarily in conflict with the role of hypoxia in preserving MSC's "stemness" during expansion. Finally, further studies are needed to characterize the impact of protracted hypoxia expansion on human MSC genomic stability and their *in vivo* properties.

Extracellular Matrix for Human MSC Expansion

Supplementation with growth factors such as FGF-2 and components of ECM proteins such as fibronectin have been shown to promote MSC expansion. However, the addition of a specific growth factor may favor the expansion of a particular lineage at the expense of multipotentiality, secretion of therapeutic factors, and long-term self-renewal capacity (Sotiropoulou et al. 2006). Given the intimate relations and important roles of ECM matrices *in vivo*, cell-derived ECM matrices have been used to provide an optimal niche to expand MSC and to direct their phenotype. The unique properties of the ECM are their abilities to sequester growth factors, modulate the activities of the growth factors through proteolytic actions, and affect the cell receptor activities. These interactions constitute a dynamic and reciprocal relationship between the cell and the ECM and cannot be readily reproduced using a single ECM component or growth factor. Compared to standard culture, MSCs expanded on decellularized ECM matrices had a lower level of reactive oxygen species, substantially increased response to BMP-2, and enhanced *in vivo* osteogenic differentiation (Lai et al. 2010). The impact of ECM matrices on MSC fate is supported further by the demonstration that the specific properties of cell-derived ECM matrices cause differential regulation of the MSC phenotype and influence stem cell fate. For example, matrices derived from endothelial cells rather than soluble EC-secreted factors induced functional changes in MSCs indicative of the

development of a vascular phenotype. The ECM matrices that represent the stepwise stages of MSC maturation down osteogenic and adipogenic lineages have been shown to regulate MSC lineage specification differentially (Hoshiba et al. 2010). These results suggest that the intricate structure of the cell-derived ECM cannot be fully reproduced with synthetic or purified components, supporting the concept of the "tissue specific ECM" and its pivotal role in directing stem cell fate.

The derivation of ECM matrices requires lengthy laboratory preparation and has a high batch-to-batch variation, impeding their adaption in GMP-compliant processes. These considerations have led to a novel MSC expansion strategy using thermo-responsive polymers such as poly(N-isopropylacrylamide) (PNIPAM) (Liao et al. 2010) to achieve sequential cell passaging with intact ECM proteins. The reversible temperature-dependent phase transition of the PNIPAM copolymers triggers alternation of cell adhesion/detachment and causes release of the adherent cells without enzymatic cleavage. As a result, cells can be harvested with intact ECM as single cells or cell sheets. Using this method, non-enzymatic sequential passaging of human MSC with intact cell-ECM and cell–cell interactions has been achieved. Human MSCs harvested from the thermo-responsive polymer surfaces have a larger number of CFU-F and have preserved their multilineage potential. Further analysis of the cell-ECM clusters revealed elevated ECM proteins including fibronectin and laminin, confirming the role of the ECM network in preserving the MSC stemness during expansion. The thermo-responsive polymer has been adapted in standard cell expansion devices, and the mechanisms of cell release are well understood. However, important differences between the cell-derived ECM and thermo-responsive culture exist. Thermo-responsive culture depends on the de novo synthesis of ECM matrices as opposed to the pre-formed, exogenous ECM network in the ECM culture. The functional role of these matrices and their impact on MSC fate remain to be elucidated. The extent to which the delayed cell-ECM interactions in the thermo-responsive polymer culture influences subsequent cellular events and cell fate requires further investigation.

Three Dimensional MSC Culture

Human MSCs have been cultured traditionally at low density on tissue culture plastics, because it was believed that the high cell density may reduce their stemness and result in differentiation. However, low-density cell expansion is a major hurdle for large scale expansion and may reduce the secretion of anti-inflammatory proteins critical for positive therapeutic outcome (Lee et al. 2009). In addition, the bare bone surfaces of the culture plastics are not conducive to the formation of an ECM network because of the lack of ECM anchorage sites and dilution of the endogenous regulatory macromolecules due to diffusion. As a result, cells on the 2D surface often depend on the exogenous nutrients and growth factors at un-physiologically high concentrations. These observations have led to the recent development of 3D aggregate culture of MSCs, which include porous scaffolds, spheroid culture, and cell aggregates (Grayson et al. 2004; Bartosh et al. 2010; Frith et al. 2010). Porous scaffolds provide a high specific surface area for cell adhesion, and thus sustain prolonged cell expansion at high density. Analysis of integrin profiles has shown that MSCs grown in 3D matrices possess 3D adhesion characteristics distinct from classical 2D adhesion complexes (Grayson et al. 2004). The distribution of ECM protein and growth factors in the 3D scaffold can be actively modulated by the flow configuration in the bioreactor, providing an effective means to influence the microenvironments with subsequent impacts on cell fate (Kim and Ma 2011).

The observation that cell aggregation in the lung as microemboli activated the secretion of TNF stimulated protein 6 has led to MSC culture in 3D spheroids to enhance their anti-inflammatory properties (Bartosh et al. 2010). It is believed that the enhanced cell–cell and cell–matrix interactions in the spheroids preserve MSC anti-inflammatory properties, cause increased