

Mesenchymal Stem Cell-Based HLA-Independent Cell Therapy for Tissue Engineering of Bone and Cartilage

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Abstract: Mesenchymal stem cells (MSC) can be obtained from human bone marrow aspirates and, thanks to their differentiation potential and excellent *in vitro* culture properties, represent an attractive cell line for the regeneration of mesenchymal tissue. Both *in vitro* and *in vivo*, they can differentiate into cartilage, bone, tendons and fat cells, and-in contrast to embryonic stem cells-they are not under ethical scrutiny. Cultured on three-dimensional scaffolds according to the tissue engineering concept, they have already been successfully employed for reconstruction of mesenchymal tissues in numerous studies involving both small and large animal models. Recently, immunological properties of MSC have been investigated by several groups. On the basis of the available literature, MSC have to be referred to as immune privileged, and they seem to be available for HLA-independent cell transplantation. While clinical MSC transplantation has also been successfully performed in pilot studies in humans, numerous points still remain to be clarified, underscoring the need for further intensive research before large-scale clinical application can be contemplated. Only then can it be shown whether the associated high expectations are justified.

Keywords: Mesenchymal Stem Cells, HLA-antigen, Tissue Engineering, bone regeneration, cartilage repair.

INTRODUCTION

Against the background of the ongoing controversial debate on the use of human embryonic stem cells for the regeneration of tissues and organs, research on ethically inoffensive adult stem cells is increasingly strong.

Adult haematopoietic progenitor cells with high regenerative potential derived from bone marrow or peripheral blood have been used successfully for several years to treat leukaemias and other malignant diseases. Transplantation of haematopoietic stem cells has become a standard routine and clearly shows the safety and feasibility of autologous and allogeneic adult stem cell therapies [1].

The discovery of the bone marrow as the major site of blood formation dates back to Neumann's and Goujon's experiments in the 19th century. Parallel to his haematopoiesis-related work, Goujon observed ectopic bone formation after transplantation of bone marrow under the renal capsule in rabbits. These experiments were successfully repeated by Maximov at the beginning of the 20th century [2]. Subsequent experiments by Alexander Friedenstein and co-workers shifted the focus of interest to the non-haematopoietic stem cell fraction [3].

MESENCHYMAL STEM CELLS

By analogy with the classic stem cell concept of haematopoiesis, Friedenstein, Owen and, later, Caplan

assumed that all mesenchymal cell populations found in bone marrow (bone, cartilage, fat and stromal cells) might be derived from a single stem cell. Arnold I. Caplan developed his model of mesengenes [4, 5], isolated cells with predicted mesenchymal differentiation potential and therefore introduced the term 'mesenchymal stem cell' or MSC [6]. To date, definite marker proteins to identify "the" genuine MSC are lacking. Several years ago, the cell surface protein STRO-1 was proposed as a promising candidate, but its position is still ambiguous [7]. Isolation of MSC is now mainly based on their adhesion affinity to cell culture plastic under characteristic media conditions [6, 8]. After expansion, cell populations isolated by this method can be examined for the MSC-characteristic expression pattern of cell surface antigens and for their differentiation potential *in vitro* and *in vivo*. In contrast to the standardised conditions used for isolation of haematopoietic stem cell, no general MSC expansion protocol has so far gained universal acceptance. One might speculate that different expansion conditions will lead to different stem cell populations. Therefore, these cells will behave differently in terms of proliferation and matrix synthesis when used in tissue engineering approaches [9].

Regarding their expression pattern of cell surface antigens, MSC are positive for (among others) CD13, CD29, CD44, CD73 (SH3 + 4), CD90; CD105 (SH2), CD166 and MHC class I. They are negative for haematopoietic markers such as CD34, CD38 and CD45 and for antigens involved in immunological signal transduction, such as HLA-DR (MHC class II), CD80, CD86, CD40 and CD40L (CD154) [10-12]. These immunological aspects of MSC will be illustrated below.

Although MSC are abundant in several tissues and harvesting from different sources such as umbilical cord blood [13], skeletal muscle, skin [14] and adipose tissue (so

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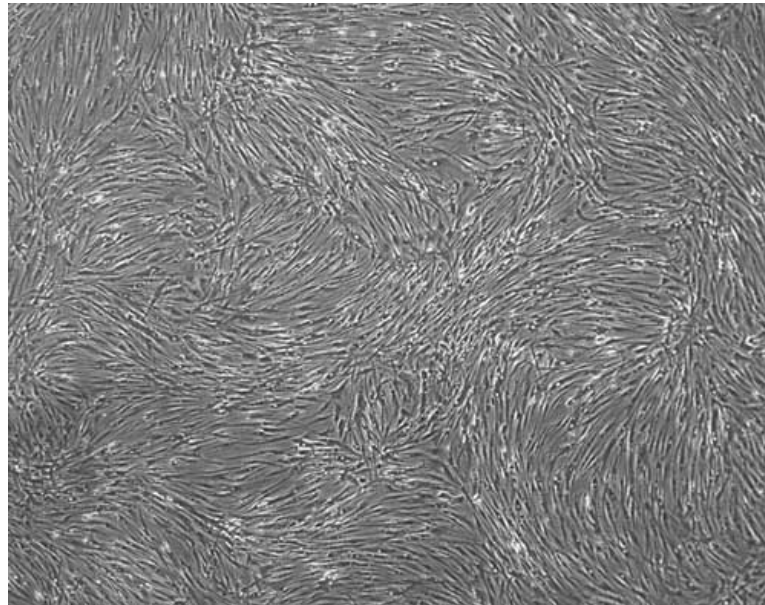


Fig. (1). Mesenchymal Stem Cells show characteristic fibroblast-like morphology after monolayer expansion *in vitro*.

called “ASC” – adipose-derived stem cells) [15] has been described, MSC are routinely isolated from bone marrow aspirates. The iliac crest is readily accessible, and sufficient cells can be found in a small volume. The frequency of MSC in human bone marrow ranges from 2 to 40 cells per 1×10^6 mononucleated cells; it varies with the patients’ age and general condition [8]. In contrast to chondrocytes, MSC have a high expansion potential and can easily be propagated to give many times their original number.

All these points are of great importance for the possible clinical regeneration of bone and cartilage in the context of tissue engineering.

TISSUE ENGINEERING

Tissue engineering is a multidisciplinary field in which the principles of biology and engineering are applied to develop tissue substitutes to be used in restoring, maintaining, or improving damaged human tissues. According to this concept, isolation and *in vitro* expansion of MSC followed by three-dimensional (3D) cultivation on an appropriate scaffold is one of the most promising approaches to the regeneration of damaged tissue. The cell/matrix construct that has developed and is formed *in vitro* is subsequently introduced into the tissue defect that is to be made good, if appropriate under the influence of tissue-specific growth factors. The combination of biological materials and cells thus forms the basis for obtaining dynamic transplants for tissue replacement from patients’ own cells [16, 17].

BONE REGENERATION

Substitution of bone is one of the major challenges in current orthopaedic and traumatological research. It is needed for repair or replacement of damaged tissues in cases of trauma, congenital and degenerative diseases, and cancer.

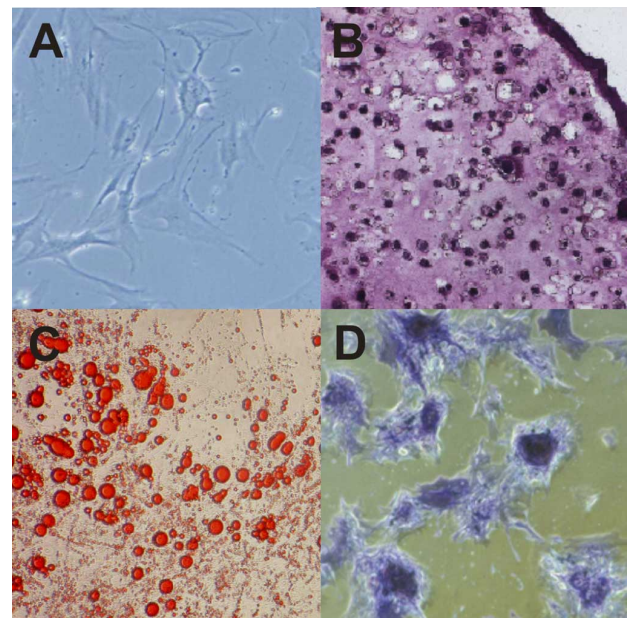


Fig. (2). Undifferentiated MSC (A) were cultured in control medium, chondrogenic medium, osteogenic medium and adipogenic medium for 14 days according to standard protocols. Glycosaminoglycan production as a marker of chondrogenesis (B), fat droplets as a marker of adipogenesis (C) and alkaline phosphatase as a marker of osteogenesis (D) and were histologically detected (LM, 40x).

In the United States of America, bone substitution is required in approximately 500,000 surgical procedures per year [18]. Although various attempts have been made to remedy the situation in the past, there is still a lack of optimal bone grafts for reconstruction of skeletal defects. A variety of techniques are available for management of this problem; each has its own advantages and disadvantages.

Autologous cancellous bone grafts, which are most commonly used to replace bone, are associated with significant donor-site morbidity [19]. In addition, the amount of autologous cancellous bone available for transplantation is limited.

Allogeneic bone grafts may be recognised by the host's immune system [20] or even transmit infectious diseases, such as HIV infection or hepatitis C [21]. Procedures to remove viral contamination (i.e. sterilisation) usually lead to denaturation of proteins and therefore to attenuation of their osteoinductive properties [22].

Nevertheless, a construct with osteoconductive, osteoinductive *and* osteogenic properties can only be achieved by combining artificial biomaterials with osteoconductive properties and viable osteogenic cells, such as osteocytes or mesenchymal progenitors. For these reasons, a substantial research effort has been invested in the development of artificial bone grafts. Over recent decades, a variety of synthetic bone substitutes have been developed for grafting, with the aim of minimising the complications mentioned above. The benefits of synthetic graft materials include availability, sterility and lower morbidity. Various materials have been tested, most of them containing calcium and phosphorus. Nevertheless, these artificial bone grafts usually have only osteoconductive, and no osteoinductive, properties [23]. Large defects cannot be repaired unless the grafting material used has osteoinductive or even osteogenic properties as well as osteoconductive properties.

Owing to their bone-like properties, scaffolds containing calcium phosphate and hydroxyapatite seem to be the most promising biomaterials for the use in tissue engineering of bone. Our group was able to demonstrate efficient three-dimensional cultivation and osteogenic differentiation of MSC on calcium-deficient hydroxyapatite (CDHA), demineralised bone matrix (DBM), tri-calcium phosphate (TCP) and mineralised collagen. According to these results, CDHA, DBM and mineralised collagen are the main materials to be characterised by excellent properties

concerning cell adhesion and interaction [24, 25].

In vitro, osteogenic differentiation of MSC takes place under the influence of β -glycerol phosphate, dexamethasone, and ascorbic acid [6, 8]. Following cultivation in the osteogenic medium, a calcium- and phosphate-containing extracellular matrix is secreted and important osteogenic marker genes, such as those for alkaline phosphatase, BMP-2, osteocalcin and bone sialoprotein, are expressed. This effect is further reinforced by cultivation in a biogenic matrix, which leads to a three-dimensional configuration of the cells [25].

The use of MSC for bone defect filling has already been tested in animal models of the "critical size defect" by several work groups, the critical size defect being the smallest bone defect that does not heal as a result of the body's own potential for regeneration. So far, the studies that seem most promising and are closest to clinical application in human subjects have shown a more favourable effect of scaffolds colonised with MSC than of acellular scaffolds in the long tubular bone defect model in dogs and sheep [26-29]. In these models healing of the bone defects was unequivocally and demonstrably better in the MSC group. Although these studies are very close to the clinical indications in humans, even in the size of the defects treated, there are still no reliable reports of experience with stem-cell-based bone replacement in humans.

Opinions vary on the origin of new bone formation started by transplanted MSC. While some groups suggest that MSC differentiate directly into precursor stem cells and then into mature osteocytes that induce new bone formation [6], Bianco suggests the following theory: in response to local and systemic stimuli (e.g. following fractures) MSC develop into osteoprogenitors that form extracellular matrix (ECM). According to Bianco, however, this matrix is only the foundation for an osteogenic environmental condition and leading structure into which the local osteoblasts originating in the bone then migrate and where they replace the deficient tissue [30, 31]. It is not yet possible, against the

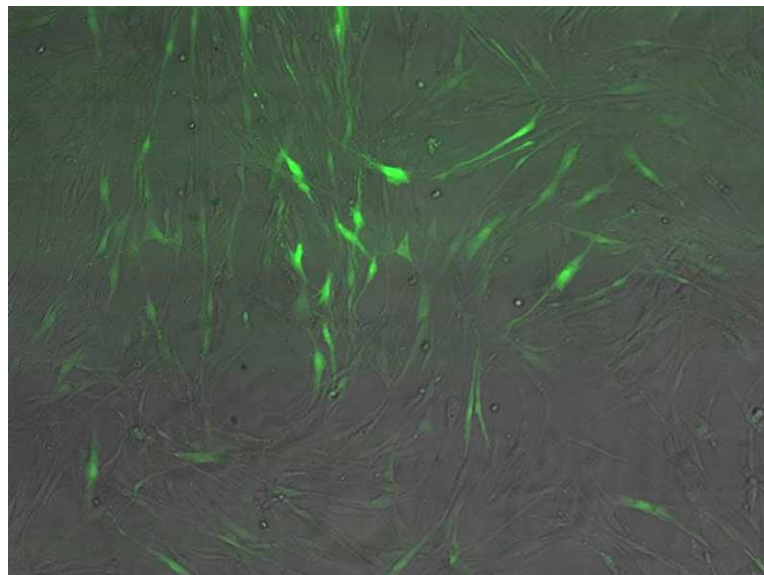


Fig. (3). For *in vivo* experimentes, cell labeling is possible in order to detect cell in histology: stable eGFP-expression can be detected in human bone marrow MSC lentivirally transduced with CMV-eGFP constructs after 3 months in culture.

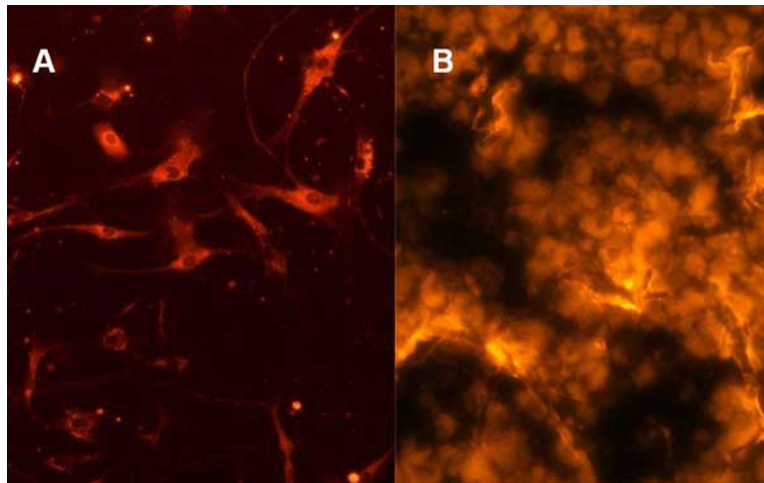


Fig. (4). Fluorescence labeled Mesenchymal Stem Cells *in vitro*: (A) Monolayer image reveals typical morphology; (B) three dimensional culture on mineralized collagen sponges for tissue engineering of bone.

backdrop of the data currently available, to discern which of the suggested theories truly underlies MSC-induced osteogenesis. There are hints supporting both. Cell labeling is commonly used in *in vivo* experiments to be conducive to answer such questions (Figs. 3 and 4). According to our own results, at least, the existence of vital transplanted MSC was demonstrable in the bone defect 4 weeks after transplantation [32], indicating the probability that MSC are at least partly involved in bone regeneration. Finally, the decisive motivating factor in the use of MSC for bone regeneration, regardless of the precise biological mechanism, is the demonstration of better bone healing in the presence of MSC *in vivo*.

Regardless of the cell type used, experts assume that bone is one of the first tissue types that could be replaced clinically by constructs preformed *in vitro* by tissue engineering techniques [18]. They justify their vision by saying that bone, relative to other, specialised organs and tissues, is less complex biologically in terms of the arrangement of extracellular matrix and cells.

CARTILAGE REPAIR

Compared with bone, cartilage has a limited capacity for regeneration, which may be due to its avascularity and acellularity. Thus, defects of the articular cartilage can lead to osteoarthritis and, ultimately, to loss of joint function.

Autologous chondrocyte transplantation (ACT) has become well established in orthopaedic medicine and is used for the treatment of superficial defects of the articular cartilage. Autologous chondrocytes are transplanted into cartilage defects and lead to the formation of cartilage repair tissue [33]. Articular cartilage is harvested from a non-load-bearing area of the injured joint in a first operation. It has been suggested that this harvesting procedure leads to cartilage injury, which predisposes the joint to osteoarthritis [34]. While expanding *in vitro*, harvested chondrocytes change their phenotype to that of a fibroblastic lineage [35] and are not able to generate an appropriate matrix in the same way as is possible in normal articular cartilage [36]. Therefore, attempts have been made to circumvent this

problem by using mesenchymal stem cells (MSC), which can easily be harvested from a bone-marrow biopsy and successfully expanded in monolayer culture *in vitro*. To engineer cartilage tissue *in vitro*, MSC are removed from monolayers and seeded into three-dimensional scaffolds or biogels. In the presence of growth factors of the transforming growth factor (TGF) superfamily, such as TGF- 1, TGF- 3, bone morphogenic protein (BMP)-2, BMP-6 and BMP-9, MSC turn into chondrocyte-like cells that produce chondrospecific extracellular matrix [24, 38]. The intracellular signalling responsible for chondrogenic differentiation has not yet been completely elucidated in MSC. It has been suggested that the chondrogenic effects observed are mediated by Wnt proteins [37, 38] and the MAP kinases p38, JNK (c-jun n-terminal kinase) and ERK (extracellular related kinase) [39, 40]. Another key regulator of chondrogenesis is the transcription factor SOX-9 (SRY-related high-mobility group box 9) [41], which has a specific binding domain of the Col2a1 promoter of chondrospecific collagen type II [42].

When MSC are used for cartilage regeneration *in vivo* it is not known whether MSC need to be differentiated *in vitro* prior to transplantation to cartilage defects or whether the local environment of the transplantation site alone can induce a chondrogenic phenotype in undifferentiated MSC. Cartilage defects of a canine femoral condyle were successfully treated with collagen gels seeded with undifferentiated MSC. Histological examination of 2-week grafts showed the synthesis of fibrocartilage containing chondrospecific collagen type II. The repair tissue of defects treated with cell-seeded grafts showed moderate biomechanical properties, but resembled native cartilage more closely than the empty controls [43]. Recently, autologous MSC have been successfully transplanted into cartilage defects in humans: 12 patients, each with an isolated cartilage defect of the medial femoral condyle, underwent a high-tibial osteotomy; MSC were harvested from the osteotomy site and were then implanted in the cartilage defect in a second operation. The patients achieved good clinical results in a 42-week follow-up, and arthroscopic and histological grading scores of the grafts showed better functional cartilage tissue than in the cell-free

control group [44]. Despite these promising results, many biological and technical problems have been observed in using MSC for cartilage regeneration *in vivo* and remain to be solved. It has been shown that MSC turn into hypertrophic chondrocytes, transdifferentiate to osteoblasts or are overgrown by osteoblasts from the subchondral bone [45]. Quintavalla implanted grafts seeded with fluorescence-labelled MSC in osteochondral defects to study whether transplanted cells might be detectable at the transplantation site 2 weeks after transplantation. It was shown that many MSC either migrated into the subchondral bone or underwent apoptosis, indicating fragmentation, dislodgement, and passive migration [46].

The use of MSC for cartilage regeneration seems to be a promising alternative to the transplantation of autologous, primary chondrocytes. Further progress is needed to develop methods and functionalised biomaterials that can control chondrogenic differentiation in superficial graft layers and the incorporation of the deeper layers into the underlying bone.

HLA-INDEPENDENT TRANSPLANTATION APPROACHES

MSC are attractive cells for use in defect repair, not only because of their differentiation potential, but also because of their immune privilege. As mentioned above, undifferentiated MSC do not express immunologically relevant cell surface markers including MHC-II, CD40, CD 40 ligand and T-cell co-stimulating molecules B7-1 and B7-2. Therefore they seem available for HLA-independent therapeutic strategies. *In vitro* studies suggest that HLA-mismatched MSC do not provoke an immune response in the host and are even able to suppress allogeneic lymphocyte proliferation. T-cell suppression is dose dependent and has no immunologic restriction because both autologous and third-party MSC equally reduce lymphocyte proliferation [47]. This phenomenon seems to be mediated by the production of cytokines, in particular interleukin (IL-) 2 and IL-10 [48], hepatocyte growth factor (HGF) and transforming growth factor β -1 (TGF- β -1) and not by induction of apoptosis [49]. Although the mechanism underlying these observations has not been described in detail, they suggest that human MSC have an immune privilege and can even modulate the immune system [11]. In the meantime, these *in vitro* data have been affirmed by several *in vivo* studies, which demonstrated an engraftment of MSC after allogeneic transplantation. The immunosuppressive capacities of MSC already led to their clinical use in treating severe graft-versus-host disease [50]. For MSC, an engraftment has been demonstrated after allogeneic as well as even after xenogenic transplantation of mouse MSC into infarcted rat myocardium. Nevertheless, a recent study observed lymphocyte proliferation after xenogenic transplantation of human MSC as an indication for a possible immune response, although on an *in vitro* level there has not been found a significant immune response [51]. This discrepancy on the one hand side clarifies that MSC derived from different species need to be evaluated separately for their immunological behaviour, on the other hand it reveals the need for further scientific work in this

field. The possibility of xenogenic availability of MSC for transplantation purposes remains to be unclear.

For tissue engineering purposes, it seems necessary to evaluate, if these immunosuppressive capacities of MSC remain even after *in vitro* differentiation, since there might be a benefit of *in vitro* differentiation prior to cell transplantation for tissue regeneration. As demonstrated by our group and others, the immune privilege of MSC persists in the face of *in vitro* osteogenic differentiation [12, 52]. Owing to these properties, it seems that MSC could be used to perform HLA-mismatched transplantation for tissue engineering purposes. This would be extremely beneficial, and not only for economic reasons; it would also lead to instant availability of these cells for tissue engineering purposes. In addition, since donor age and disease state of the patient may significantly influence stem cell number and quality, with an allogeneic approach, it would be possible to use approved MSC of high quality for transplantation purposes.

Before MSC can be used for clinical applications, the biomechanical properties of bone that has regenerated following transplantation of allogeneic MSC remain to be determined. In the case of MSC derived from canine bone marrow it has been shown that HLA-mismatched MSC can induce bone healing in a critical size defect model and in an alveolar saddle defect model of a comparable quality to that obtained with autologous MSC [32, 53]. In both papers no significant immune response versus transplanted allogeneic cells was found on an histological level and an engraftment of transplanted cells could be demonstrated. Because of the different characteristics of human and animal MSC, this observation needs to be investigated separately in human MSC and a biomechanical testing which produces evidence that allogeneic MSC lead to an equal stability and stiffness compared to autologous cells is still lacking.

In the context of possible allogeneic transplantation of MSC, possible side effects of the immunomodulating properties of MSC need to be discussed. In 2003, the group of Djouad demonstrated the local growth of malignant melanoma cells in the presence of allogeneic MSC [54], due to an induction of tolerance caused by the immunosuppressive properties of transplanted allogeneic MSC. Since these experiments have been published critical votes sound a note of caution concerning the use of allogeneic MSC. Anyhow, the possible usefulness of various therapeutic applications remains of great importance, but critical votes need to be taken seriously and possible side effects of immunomodulation by allogeneic MSC needs to be investigated in further clinical studies.

CONCLUSIONS

The first clinical application of MSC in tissue engineering is imminent. Following their *in vitro* studies, working groups throughout the world have combined to make it possible to investigate the replacement of mesenchymal tissue by means of MSC in a systematic manner, first in small and later also in large animal models. It is still only 15 years since the term 'mesenchymal stem cell' was first coined by Pittenger. For the future, high hopes are invested in cell-based therapy with MSC, in the

orthopaedics and traumatology sector for the treatment of cartilage and bone defects, but also in cardiology, for the treatment of myocardial infarct. The results of studies on tissue replacement for bone, cartilage and tendon tissue thus far have been very promising, but it remains to be seen whether the hope engendered by them will be proved justified when the new techniques are tried out in routine clinical practice. The possibility of transplantation without the necessity for HLA matching offers medical chances and options that make MSC particularly attractive relative to other cell types that are available for tissue engineering with mesenchymal tissues. Further studies will have to show whether transplantation of HLA-incompatible cells is really free of problems.

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