

TrinityTM Multipotential Cellular Bone Matrix Technical Monograph

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Abstract

Trinity™ Multipotential Cellular Bone Matrix is a viable bone matrix product containing adult stem cells, which can be used in place of autograft in essentially every surgical indication where autograft would normally be selected as a bone void fill or bone growth stimulator. Like autograft, Trinity™ Matrix provides all three essential bone growth properties: osteogenic cells, osteoinductive signals, and an osteoconductive scaffold. Unlike autograft, and other alternatives, Trinity Matrix contains consistently high concentrations of the adult mesenchymal stem cells (MSCs) that give rise to bone-forming osteoblasts. These adult stem cells are the ultimate source of the osteoregenerative machinery responsible for bone remodeling and repair. MSCs are especially amenable to therapeutic applications because they have been shown to be hypoimmunogenic and anti-inflammatory. The number and concentration of local MSCs determines the capacity for bone formation at the operative site. Therefore, direct delivery of MSCs may result in more rapid, reliable, and uniform bone formation. Through decades of intensive research, scientists and clinicians have generated an increasingly comprehensive understanding of the cellular and molecular events underlying bone formation. Drawing from this expansive knowledge base, Osiris® Therapeutics, Inc. has developed path-breaking technology related to the isolation, manufacture, and therapeutic use of mesenchymal stem cells. In partnership with Osiris Therapeutics, Blackstone Medical presents a unique and safe autograft substitute: Trinity Matrix.

Introduction

Bone has a natural capacity for self-repair. However, a number of variables—including operative location as well as patient age, sex, and health—can limit the potential for autoregeneration of human bone.¹⁻⁷ In such cases, a graft can be used to stimulate bone formation at the operative site. The ideal bone graft combines osteogenic cells (either endogenous or introduced) and associated bioactive osteoinductive factors with an osteoconductive structural matrix.⁸⁻¹⁰ Although the properties and interactions of these three components determine the characteristics and bone-forming capacity of a graft, osteogenesis is ultimately a cellular process¹¹ that begins with a unique type of multipotential adult stem cell called a mesenchymal stem cell (MSC), which gives rise to osteoblasts.¹²⁻¹⁵

More than 900,000 bone graft procedures are performed in the United States each year¹⁶ including approximately 250,000 spinal fusions. Although a wide variety of products and techniques are used in bone graft surgery, all are intended to stimulate bone formation and promote bony unions at the operative site. The seminal event for all bone formation is the mesenchymal stem cell's differentiation into an osteoblast. Despite the plethora of new spinal fusion products on the market, only Trinity™ Matrix combines osteoinductive and osteoconductive properties with consistently high levels of the osteogenic mesenchymal stem cells that are essential for bone formation. Trinity Matrix and autograft introduce MSCs into the operative site while alternatives such as bone morphogenetic protein and demineralized bone matrix rely solely upon the unpredictable availability of host MSCs. **(Figure 1)**

Autograft: Benefits, Challenges, and Alternatives

Autograft cancellous bone is the historic gold standard for bone grafting^{17,18} and spinal fusion.¹⁹ Autograft bone, traditionally harvested from the patient's iliac crest, is an effective bone graft because it contains the essential mesenchymal stem cells resident in autograft marrow as well as two other important components—the bioactive osteoinductive factors that stimulate cellular osteogenesis and an osteoconductive scaffold, which provides structural support for the formation of new bone.^{19,20} Bone marrow by itself has also been used as an autologous bone fusion aid for more than 100 years.²¹ The clinical application of bone marrow aspirate has been shown to facilitate spine fusion;²² marrow can be introduced via autologous transplant or by removing the cortical bone layer near the site to reveal the marrow-rich cancellous bone within.

However, autograft use is challenged by a range of problems including limited supply, donor-site morbidity,²³⁻³⁰ and inconsistent or low concentrations of endogenous MSCs.³¹ Autograft implants may also be less effective in patients from whom previous graft has been taken and in those who are experiencing a decline in their mesenchymal stem cell repository as a result of age, systemic illness, alcohol abuse, or osteoporosis.^{3,5,7,32,33,34} Moreover, due to the high degree of inter-individual variation in the MSC concentration of bone marrow,³¹ the osteogenic capacity of autograft may vary.

	Osteoconductive scaffold	Osteoinductive growth factors	Osteogenic living cells
Synthetic Ceramics	■	□	□
Banked Cancellous Bone	■	□	□
Banked Demineralized Bone (e.g. Osteofil®, Grafton®, DBX®)	■	■	□
rhBMP (e.g. INFUSE®, OP-1®)	□	■	□
Autograft	■	■	■
Trinity Matrix	■	■	■

Figure 1: Comparison between Trinity Matrix & other materials.

The clinical limitations of autograft have prompted vigorous investigation into suitable substitutes. Most autograft alternatives or extenders do not contain MSCs; they are simply intended to stimulate the activity of endogenous bone-forming cells through their osteoinductive and/or osteoconductive properties. Osteoinductive demineralized bone matrix (DBM), for example, stimulates bone fusion through the properties of naturally occurring bone morphogenetic proteins (BMP),³⁵ which broadcast chemical signals that induce osteogenic activity among endogenous cells.^{36,37} However, the osteoinductive activity of various DBM preparations is inconsistent due to variation in the amount and types of BMPs contained in the source bone.⁸ Alternatively, recombinant human BMPs provide a more consistent source of osteoinductive signals, but at two- to three-times the cost of DBM.¹⁶ Osteoconductive scaffolds made from synthetics such as ceramic hydroxyapatite/tricalcium phosphate (HA/TCP) have a porous structure that facilitates bony ingrowth.^{8,19,38} Although osteoinductive growth factors and osteoconductive matrices have been shown to independently stimulate arthrodesis,^{18,35,39} better results are achieved in combination with a source of osteoprogenitor cells.^{33,40} Direct delivery of MSCs results in more rapid, reliable, and uniform bone formation.^{33,41} In the absence of introduced osteoprogenitors, the success of autograft alternatives hinges on the presence of endogenous cells, which may not be abundant.

Stem Cells Basic Science

Stem cells, by definition, have the capacity for self-renewal. They can replicate numerous times without losing the ability to differentiate into multiple cell types. They are the primal undifferentiated cells that underlie the development, repair, and regeneration response of bodily tissues. In short, stem cells, found in a variety of tissues, are a reservoir of undifferentiated cells that replenish other specialized cells throughout life.

Adult stem cells play a daily role in bodily maintenance. Many adult tissues contain stem cell populations, which have the capacity for renewal after injury, disease, or as a result of aging.¹⁵ Varieties of adult stem cells may be multipotent, oligopotent, bipotent, or unipotent depending on their ability to differentiate into many, few, two, or one other cell types respectively.⁴² For example, multipotential hematopoietic (blood-forming) stem cells can produce several types of blood cells (e.g., red blood cells, white blood cells, and platelets).

In contrast, embryonic stem cells that result from the fusion of sperm and egg are totipotent; they have the capacity to grow into any cell type without exception. If implanted into the uterine wall, human embryonic stem cells will develop into a fetus. In the developing human, embryonic stem cells give rise to a range of adult stem cell types with significantly more limited differentiation potential. In the process of development from embryonic to adult stem cells, there is a loss of potential and an increase in specialization.⁴³ The increasingly specialized cellular lineages that an adult stem cell may follow are determined by both intrinsic factors and extrinsic microenvironmental cues, including signals from surrounding cells.^{13,33,42}

Adult Mesenchymal Stem Cells

Mesenchymal stem cells are a type of adult stem cell that serve as a repository for bone-forming cells. They have no capacity to develop into a fetus. As a component of a developing fetus, MSCs give rise to all the cells of the mesoderm, one of the three primary “germ layers” formed during embryogenesis from which many tissues are formed, including bone, cartilage and muscle as well as components of the circulatory, excretory, reproductive, and urinary systems. MSCs are developmentally intermediate to embryonic stem cells and terminally-differentiated adult cells.^{14,44} It is as a result of their relatively “young” biological age that MSCs can serve as an in situ source of osteoprogenitor cells throughout a lifetime.¹⁴ Whether derived from newborns or the elderly, MSCs maintain the same differentiation potential.³³

Bone marrow contains two types of stem cells, mesenchymal stem cells and hematopoietic stem cells. Mesenchymal stem cells are a relatively small, but essential component of bone marrow. They represent a fraction (0.001 to 0.01%) of all nucleated bone marrow cells.¹⁵ The highest concentrations of MSCs are found in bones in the pelvic girdle and vertebral bodies.³¹ An aspiration of iliac crest bone marrow contains between 1 to 5 MSCs per 500,000 nucleated cells,^{11,20} but the MSC concentration may be significantly reduced if a large volume (>2cc) of marrow is aspirated from a single site due to dilution by venous blood.⁴⁵ Therefore, it is recommended that the tip of the aspiration needle be moved after each 2cc aliquot until the desired volume of bone marrow is obtained. Recently, vertebral body aspirations have been reported to contain consistently higher average concentrations of MSCs than iliac crest bone marrow.^{31,46} Normally, 1cc of healthy adult marrow will yield between 1000 to 1500 MSCs. However, this concentration may vary significantly depending on aspiration technique, gender, age, and health of the donor.

Properties of MSCs

MSCs are multipotential, immune-privileged adult stem cells. They are capable of producing multiple types of specialized cells including bone, cartilage and fat cells.^{12,13,47,48} (Figure 2) MSCs can be transplanted between unmatched donor and recipient without stimulating an immune response.^{49,50,51}

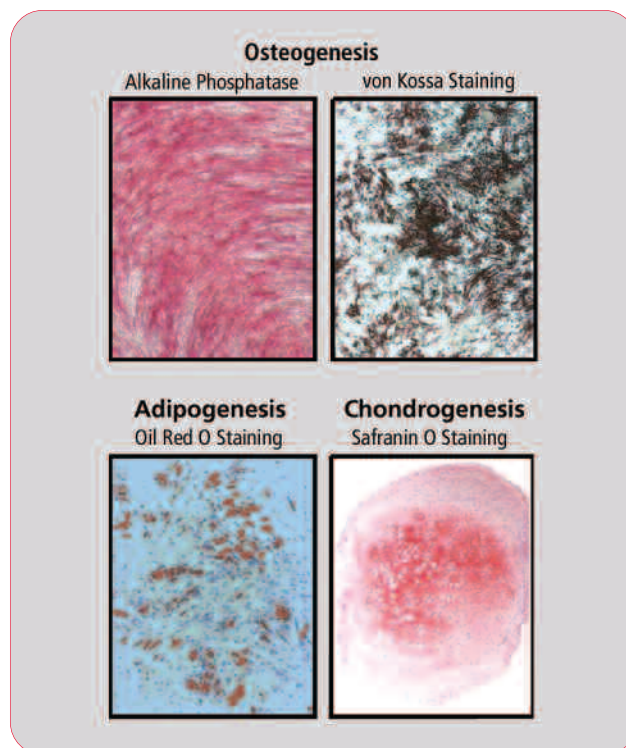


Figure 2: Trinity Matrix's mesenchymal stem cells are multipotential, as shown in this in-vitro study.

In the skeletal system, MSCs are the source of osteoregenerative cellular machinery required for bone remodeling and repair. The naturally occurring process of bone “turnover” follows an endlessly repeating sequence of definitive cellular and molecular transitions.⁵² Beginning with the repository of MSCs, these osteoprogenitors give rise to osteoblasts, which serve to make new bone for a defined period of time, then either die or terminally differentiate into osteocytes, to be followed by other newly differentiating osteoblasts.^{13,53} This exquisitely choreographed biological performance, staged so often and effortlessly in our bodies, can be exploited for therapeutic use in a clinical setting.⁵⁴⁻⁵⁶

As part of the normal healing process, the body's native MSCs concentrate around sites of tissue damage; they home to the site of injury and inflammation in response to endogenous signals.^{9,57} The extraordinary ability of MSCs to seek out the site of tissue damage has been observed in bone fracture⁵⁷ and damaged meniscus cartilage⁵⁸ as well as in non-skeletal injuries including myocardial infarction⁵⁹ and ischemic brain injury.⁶⁰

MSCs are especially amenable to therapeutic applications because they have been shown to be hypoimmunogenic and anti-inflammatory.^{61,62} A growing body of evidence suggests that, in contrast to other transplant procedures, implantation of allogeneic MSCs does not stimulate an immune-rejection response.^{49-51,56,61,63,64} In pre-clinical trials with large animal models, allogeneic MSC implants between genetically mismatched individuals have been shown to strongly enhance arthrodesis without the use of immunosuppressive therapy. In these trials, individuals were paired on the basis of a complete mismatch of the major histocompatibility complex (MHC) Class I and II antigens. Allogeneic MSCs do not express MHC Class II antigens, and therefore did not provoke an adverse host response; no histological evidence of an immune response was detected.^{50,51,64} Because of their hypoimmunogenic properties as demonstrated in large animal models including high-order primates (baboons),⁴⁹ MSCs have been referred to as "immune privileged."^{49,65} **(Figure 3)**

In addition to their immune privilege, allogeneic MSCs may also suppress proliferation of immune system T-cells when triggered by the foreign antigens of another allogeneic transplant regardless of donor source, including "third-party" MSCs.^{52,63,65,66} Whether undifferentiated or specialized, MSCs have immunosuppressive effects. In fact, differentiation, especially along the osteogenic lineage, appears to enhance the immunosuppressive effects of MSCs.⁶³ When administered following allogeneic skin grafts, MSCs, mismatched with host MHC antigens, improved graft survival compared with controls in pre-clinical trials using higher-order primates.⁴⁹ In a related clinical example, when infused with allogeneic MSCs, patients suffering from Hurler's syndrome or metachromatic leukodystrophy showed no evidence of Graft-Versus-Host Disease (GVHD).⁶⁷ This suggests that the immune suppressive properties of MSCs may have applications for the treatment of acute GVHD.⁶⁸

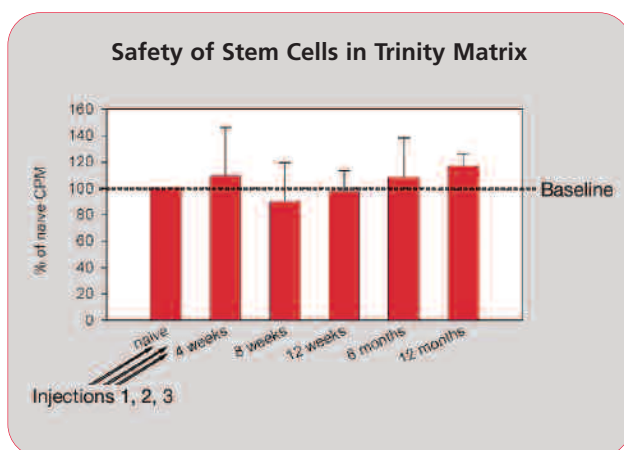


Figure 3: Allogeneic MSCs do not elicit an immune response in a caprine model system.⁶⁴

MSCs in Orthopedics

Since their properties were first discovered, it has been understood that MSCs have applications in spinal fusion.¹⁰ In vivo, these stem cells remain quiescent, replicating without differentiation, until they are stimulated to differentiate in response to endogenous signals.^{34,57,62} For many years, orthopedic surgeons have indirectly employed the osteoregenerative capacity of MSCs via autograft bone implants and bone marrow applications. More recently, direct application of concentrated autogenous MSCs has proven effective for treatment of spinal fusion in pre-clinical trials.⁶⁹

Among other factors, the number and concentration of local MSCs determines the capacity for bone formation at a particular site.⁷⁰ This is because the available number of bone-fabricating osteoblasts is directly proportional to the number of progenitor MSCs that enter and commit to the osteogenic pathway.³³ Therefore, an insufficient concentration of MSCs may not provide adequate osteogenic capacity, resulting in a clinical non-union.^{33,53} Bone marrow aspirates containing less than 1000 MSCs/cc have been shown to be ineffective for the treatment of tibial non-unions, suggesting that this is the minimum MSC concentration required for boney healing in that particular clinical setting.⁷⁰ Whether this can be extrapolated to spinal arthrodesis and successful fusion has not yet been confirmed in an animal model or clinical trial.

Identification of Osteogenic MSCs

Native, undifferentiated MSCs can be isolated ex vivo and cultured in vitro. They are adherent cells that will form colonies, adopting a spindle-shaped morphology in culture. They can be identified based on the presence or absence of certain cell surface antigens.^{12,15,32,47,73-75} Although no unique marker has been identified to date, mesenchymal stem cells do express a consistent pattern of positive and negative markers. Studies of MSCs have revealed that they typically display a profile positive for markers including SH2, SH3, CD29, CD44, CD71, CD90, CD105, CD106, CD120, CD124 and CD166. Amongst these are adhesion molecules, cytokines, cytokine receptors, and a variety of other types of molecules.^{76, 77} Positive markers for CD105 (endoglin, an accessory receptor for the TGF-, superfamily of ligands) and CD166 (ALCAM, an adhesion molecule) are consistently expressed at high levels by adult MSCs.

As a result of intensive study, the physical and developmental properties of osteogenic MSCs are well characterized and can be used to identify these cells. Osteogenic differentiation can be induced in vitro by treatment of cells with a chemical cocktail. Adoption of a cuboidal morphology is an early physical indication of MSCs that have undergone osteogenic differentiation.^{2,19,71,72} Other identifying characteristics of osteogenic MSCs include biochemical indicators—transient expression of alkaline phosphatase and osteocalcin—and production of a calcium rich, mineralized extracellular matrix.⁵⁷ **(Figure 2)**

The Future of MSC-Based Therapies in Orthopedics

Since the discovery of their properties, scientists and clinicians have worked to understand the regulation of MSC proliferation and differentiation with the aim of better controlling their activity in a clinical setting. Through such advanced understanding of the cellular and molecular events of bone formation, orthopedists will be empowered to apply stem cell-based therapies for treatment of the myriad pathological conditions resulting from insufficient osteoblastic activity or cell number.⁷⁸ In the words of MSC pioneer Arnold Caplan, “Just as power tools have changed orthopedists from carpenters to cabinet makers, so will biologics transform them into conductors of cellular symphonies.”⁵³

The Origin of Trinity Matrix

Trinity Matrix is a viable bone matrix product containing adult mesenchymal stem cells. It is designed to stimulate dynamic osteogenesis, which can lead to a robust and consistent bone formation at the implant site. The bone regeneration potential of Trinity Matrix relies on proprietary technology developed by Osiris® Therapeutics, Inc. of Baltimore, Maryland. The Company, founded in 1992, is based on the pioneering work of Arnold Caplan, Ph.D. and his colleagues at Case Western Reserve University. They showed that mesenchymal stem cells can engraft and selectively differentiate, based on the tissue environment, to such lineages as muscle, bone, cartilage, marrow stroma, tendon and fat. Due to their cellular origin and phenotype, these cells do not provoke an immune response, allowing for the development of products derived from unrelated human donors. The Osiris drug candidate Prochymal™ is now being evaluated in late stage clinical trials for treatment of GVHD and has received a Fast-Track designation from the FDA for this application (Jan. 31, 2005). Osiris is the first company to receive the FDA's Fast Track designation for a stem cell product. Prochymal is also in Phase II clinical trials for treatment of Crohn's Disease. Additionally, the company is conducting clinical trials to evaluate the use of MSCs for meniscus regeneration post meniscectomy and repair of damaged heart tissue following myocardial infarction.

Over the past 14 years, Osiris has established itself as an international leader in the science and application of MSC-based therapeutics with an intellectual property portfolio of 45 U.S. patents related to the isolation, manufacture, and therapeutic use of mesenchymal stem cells. Among the Company's intellectual property is a prominent patent that provides broad coverage for the isolation and use of multipotential stem cells from human tissue, a patent for the use of non-autologous stem cells (allogeneic MSCs), which provides the basis for the use of MSCs unmatched to the recipient, and 11 patents related to the use of MSCs in orthopedics. A new class of intellectual property has been developed for the unique processes used to create Trinity Matrix.

Trinity Matrix is a True Autograft Substitute

Trinity Matrix is a minimally-manipulated, human cellular and tissue-based allograft product that has undergone selective depletion of immunogenic cells, leaving a rich source of stem cells with demonstrated multipotentiality, but with negligible risk of inflammation or immune response. Trinity Matrix is extensively tested for consistently high concentrations of bone-forming mesenchymal stem cells. The result is a novel system for the regeneration of bone.

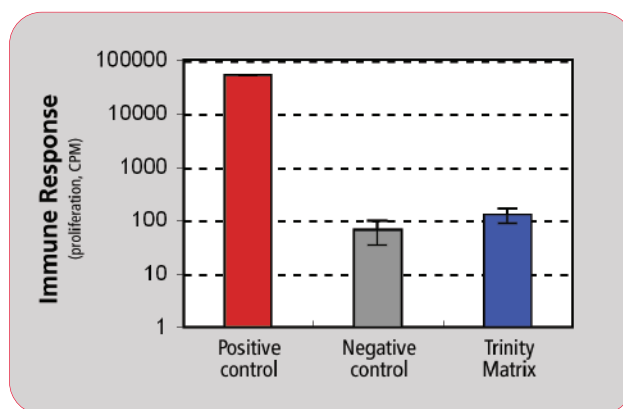


Figure 4: There is no immune response directed against Trinity Matrix, demonstrating its low immunogenicity. The positive control shows a typical immune response when immune cells from one individual (responder cells - PBMCs*) are cultured with (irradiated) immune cells from another individual (stimulator cells - unrelated PBMCs). This scenario simulates immune cell activation taking place in unmatched transplanted organ rejection. The negative control shows the baseline activation of responder cells cultured alone. The magnitude of the response when Trinity Matrix is incubated with responder cells is comparable to the negative control. The immune response is presented as PBMC proliferation in counts per minute (CPM) showing the uptake into DNA of 3H-thymidine by activated cells.

*Peripheral Blood Mononuclear Cells (PBMC's) e.g., lymphocyte and macrophage white blood cells of the immune system.

Trinity Matrix is similar to autograft not only because of its preserved biological activity, but also because it is the only product available that provides all three beneficial properties of autograft: osteogenic cells, osteoinductive signals, and an osteoconductive matrix. The product's cancellous bone matrix functions as an osteoconductive scaffold to support bone growth. Therefore, no additional carrier is required for Trinity Matrix to be clinically applied.

Trinity Matrix is an alternative to autograft that can be used for virtually all orthopedic bone repair, replacement or reconstruction applications. Trinity Matrix allows surgeons to provide their patients with robust bone growth conditions without the discomfort and potential complications of autograft harvesting, and eliminates the time spent on a secondary procedure. It can be used in place of autograft in essentially every surgical indication where autograft would normally be selected as a bone void fill or bone growth stimulator. Trinity Matrix is not intended to be an autograft extender; it is an autograft substitute.

The mesenchymal stem cells in Trinity Matrix are obtained from adult cadaveric tissue, avoiding the technical problems and controversy surrounding other stem cell technologies. MSCs are universal, meaning that they can be used in patients unrelated to the donor, without rejection, eliminating the need for donor matching and recipient immune suppression. (Figure 4)

Low Immunogenicity

Immunogenicity studies have shown that Trinity Matrix does not stimulate a cellular immune response, due to the immune privileged status of the cells and the manufacturing process. The product does not activate T-cell proliferation as shown in vitro from Mixed Lymphocyte Reaction (MLR) testing.

Trinity Matrix Contains Mesenchymal Stem Cells

The cells in Trinity Matrix were extracted and cultured for analysis to demonstrate their stem cell identity using fluorescence activated cell sorting (FACS). Flow cytometry was used to assess the cell surface expression of cells from Trinity Matrix for CD105-positive/CD166-positive markers utilizing either single color analysis or a simultaneous labeling with antibodies to perform a three-color analysis. The analysis was performed on a FACS Calibur System (Becton-Dickinson). The occurrence of CD105-positive/CD166-positive markers was recorded on a dot plot. Isotype controls were also run.

In general, the FACS data showed strong expression of CD105 and CD166. Representative results from two separate lots of Trinity Matrix (labeled A and B) are shown in Figures 5 and 6 demonstrating that >99% of the cells were positive for CD105 and CD166. The FACS analysis of the cell surface markers in Trinity Matrix confirmed that the cells have a mesenchymal stem cell profile because they express the characteristic markers CD105 and CD166.

Processing reduces components that could stimulate an immune response. The cellular identity has been characterized experimentally with fluorescent activated cell sorting (FACS).

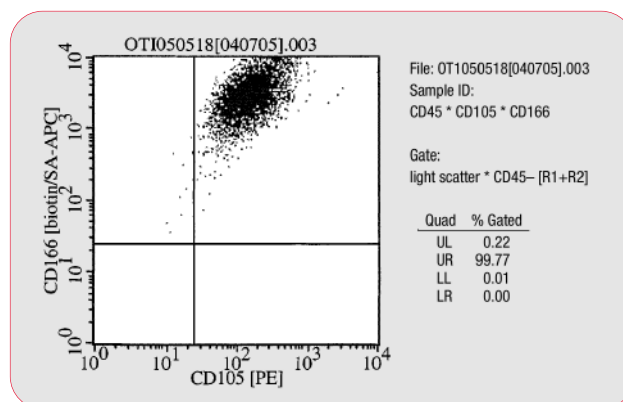


Figure 5: Dot plot showing positive expression of CD105 and CD166 from Trinity Matrix Lot A.

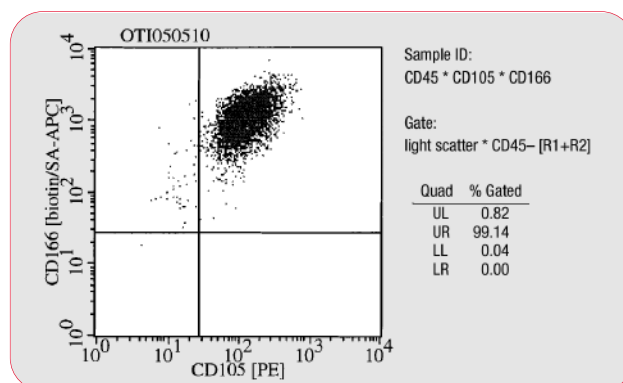


Figure 6: Dot plot showing positive expression of CD105 and CD166 from Trinity Matrix Lot B.

Trinity Matrix is an Allograft

The MSCs in Trinity Matrix are harvested with their native cancellous bone matrix; these adult stem cells are not expanded in culture. Therefore, Trinity meets FDA's criteria for regulation as a human cellular- and tissue-based product for transplant. Under the FDA regulations for human cells and tissue (21 CFR 1271), products do not undergo pre-market review provided they meet strictly defined criteria. Trinity Matrix meets these criteria and is labeled for use in any bone repair, replacement or reconstruction indication.

Making Trinity Matrix

Trinity Matrix is produced using Osiris's proprietary technique for preserving adult stem cells, which maintains their healthy, functioning state and multipotential characteristics that are the hallmarks of stem cells.

Rapid initial processing is followed by qualification testing, selective depletion to concentrate MSCs, cryopreservation, and extensive product testing. **(Figure 7)** Tissue processing at the Osiris facility begins within 72 hours of the donor's death. Trinity Matrix preparation takes place during an intensive two-day process. Using a proprietary aseptic processing method, Trinity Matrix is produced in a manner that preserves cell viability and osteogenic potential, while eliminating potential immunogenic cell components. **(Figures 7a and 7b)**

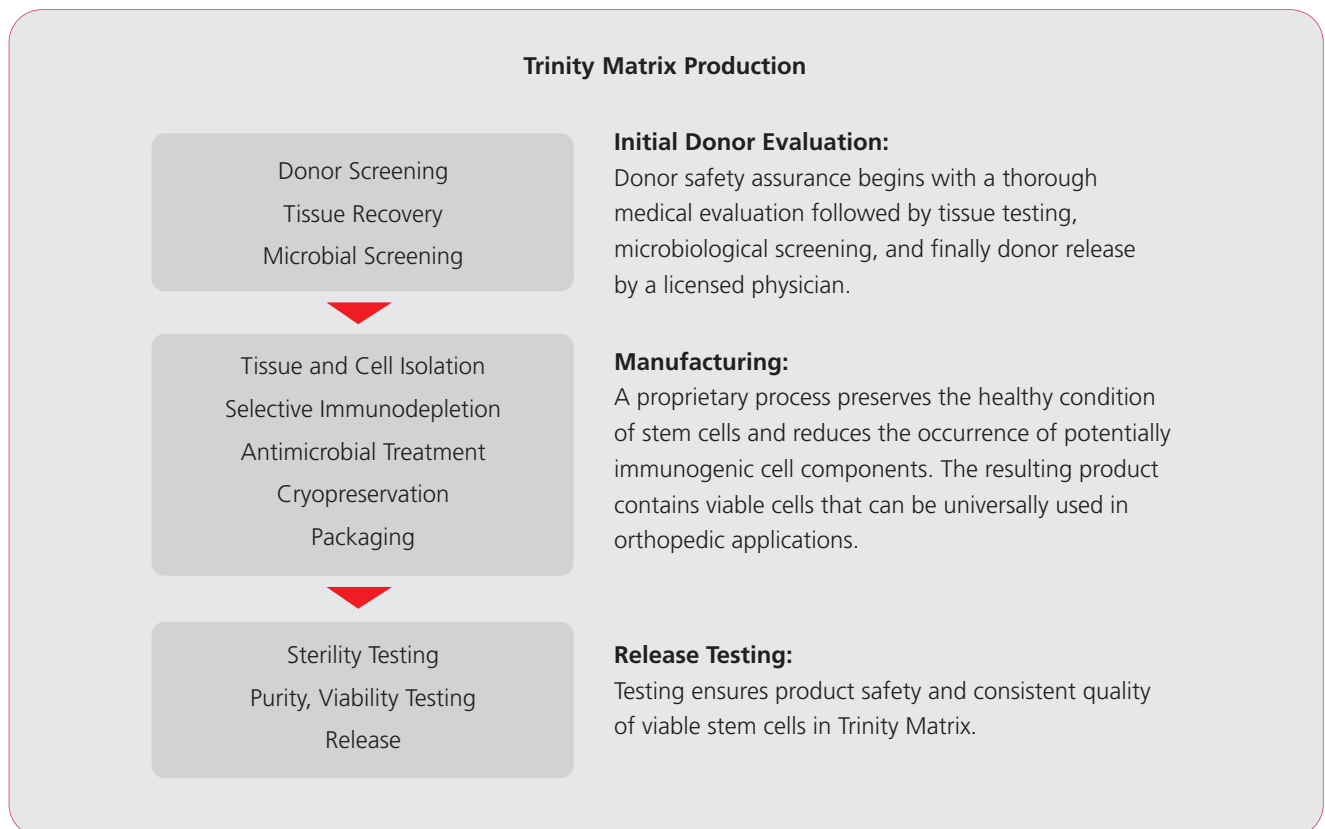


Figure 7: Trinity Matrix production, testing, and validation process.

Viable, Osteogenic Cells:

Every lot is tested to ensure that the cells are viable and osteogenic. The osteogenic potential arises from the stem cells in Trinity Matrix. Following processing of marrow-rich bone, testing demonstrates this potential.

Trinity Matrix Characterization: Each Lot Is Extensively Tested For Cell Viability, Concentration And Osteogenic Capacity:

Cell viability ≥ 70%

Cell number ≥ 1000 MSCs/cc

Verified in-vitro osteogenic capacity

Cryopreservation:

Immediately after processing, Trinity Matrix is frozen at -80°C, at which temperature it has a shelf life of 5 years.

Figure 7A

Safety Profile:

- *Aseptic tissue processing*
- *Microbiological disinfection of tissue*
- *Microbiological testing of every lot*
- *Strict determination of tissue eligibility for transplantation, exceeding FDA guidelines*
- *Quality Assurance review and release of every lot*

Donor Testing:

- *Hepatitis B Surface Antigen*
- *Hepatitis B Core Antibody*
- *Hepatitis C Virus Antibody*
- *Hepatitis C Virus Nucleic Acid Test*
- *HIV-1 and 2 Antibody*
- *HIV-1 PCR Nucleic Acid Test*
- *Human T-Lymphotropic Virus Antibody IIII*
- *Syphilis (Treponema pallidum)*

Donor Screening:

- *Medical and social history review*
- *Physical examination*
- *Medical record evaluation, including autopsy report (if performed)*
- *Licensed physician review of donor record*

Quality Assurance:

- *States of Maryland, New York, California, and Florida Licensure*
- *GTP compliant according to FDA regulations*
- *AATB accreditation in progress*

Figure 7B

Trinity Matrix Testing — Pre-Clinical Studies

Bone formation by MSCs has been shown to be safe and effective in pre-clinical studies using animal models. Although conclusive data from Trinity Matrix's clinical studies are not yet available, the results of four pre-clinical studies described in this section strongly suggest that the MSCs contained in Trinity Matrix are osteogenic, osteoinductive, and hypoimmunogenic.

The osteogenic and osteoinductive properties of human MSCs extracted from a thawed preparation of Trinity Matrix were demonstrated in pre-clinical Studies 1 and 2 using immune-compromised (athymic) murine models. Because these animals have no immune systems, human cells can be transplanted without the complications normally associated with xenograft. Athymic higher order animals are not available for pre-clinical studies. Therefore, Studies 3 and 4 evaluate the bone-forming capacity of MSCs in large animals using cells isolated from MHC mismatched individuals of the same species. The bone formation and hypoimmunogenic properties of allogeneic MSCs were demonstrated in canine models and high-order primates (baboons).

Study 1

Bone Formation by MSCs in an Ectopic Site

Methodology: The gold-standard assay for evaluating MSC-based osteogenic preparations is bone formation as a result of ectopic implantation of MSCs contained in a ceramic carrier.⁵³ In this pre-clinical trial using an athymic rat model, human MSCs were expanded in vitro, then implanted with a ceramic carrier to an ectopic site (back). Fluorescently labeled human donor MSCs appear green while host (rat) MSCs appear fluorescent blue. **(Figure A)**

Results: Assay confirms the presence and osteogenic activity of human MSCs. Osteoid seam formation seen adjacent to osteoblasts is derived from both the human donor MSCs (green) and the recipient rat MSCs (blue). This study demonstrates that the viable MSCs differentiate directly into osteoblasts to form bone (osteogenesis); no evidence of endochondral osteogenesis (bone formed from a cartilaginous precursor) was detected. Furthermore, human MSCs from Trinity Matrix also induced host cells to differentiate along an osteoblastic lineage to form bone (osteoinduction). It is hypothesized, the osteoinductive potential of Trinity Matrix results from MSC expression of growth factors such as BMP 2, BMP 6, BSP and others (e.g., VEGF).

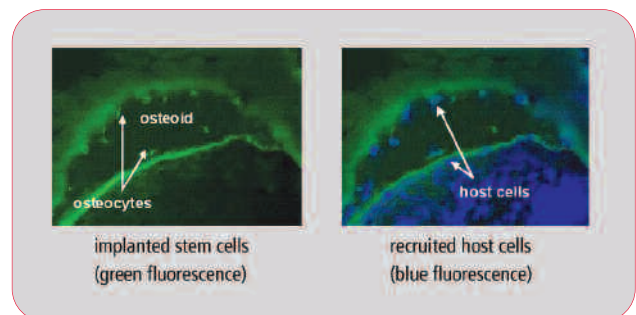


Figure A: Bone osteogenesis and osteoinduction by ectopically implanted MSCs.

Trinity Matrix Testing — Pre-Clinical Studies

Study 2

Spinal Fusion by MSCs in an Athymic Murine Model

Methodology: Male athymic rats underwent bilateral L4-L5 intertransverse fusion with Trinity Matrix using a posterolateral approach.⁸ Radiographs were taken before sacrifice at 8 weeks and postoperatively at 2 and 4 weeks. Fusion was evaluated biomechanically (by manual palpation) and histologically after sacrifice at eight weeks.

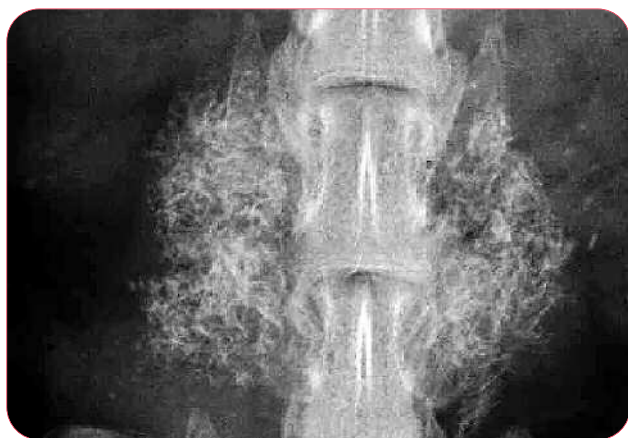


Figure B: Radiograph 8 weeks post L4-L5 fusion shows persistent mineral in Trinity Matrix making fusion determination difficult. However, manual palpation confirmed solid fusion at 8 weeks.

Results: Radiographs taken at eight weeks were obscured by persistent mineralization. **(Figure B)** However, solid fusion was confirmed by manual palpation at that time. Furthermore, results of histological analyses performed after sacrifice show osteoblasts lining areas of new woven bone formation adjacent to Trinity Matrix application area. **(Figure C)** No evidence of endochondral bone formation was detected.

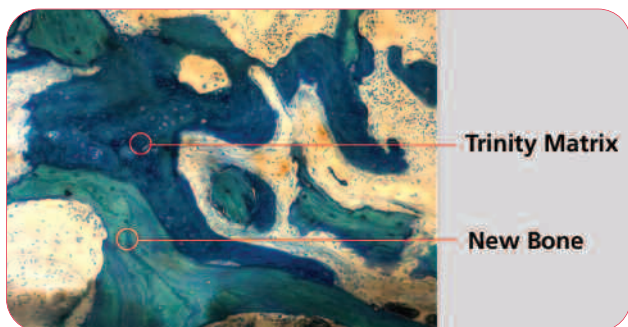


Figure C: Histological analysis after sacrifice at 8 weeks shows osteoblasts lining areas of new woven bone formation are directly attached to residual graft MSCs.

Study 3

Bone Formation by MSCs in Canine Segmental Defects

Methodology: Allogeneic donor MSCs were prepared from dogs by mismatching donor and recipient using one or more of the following three methods: dog lymphocyte antigen (DLA) testing; pedigree; and mixed lymphocyte reaction (MLR). An osteoperiosteal defect (21 mm) was created in the mid-femoral diaphysis of adult dogs and divided into 4 treatment groups:

1. Fixation only (control)
2. Ceramic carrier only
3. Ceramic carrier loaded with autologous MSCs
4. Ceramic carrier loaded with allogeneic MSCs

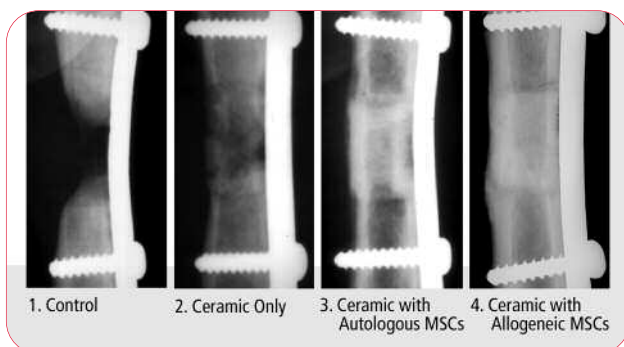


Figure D: Allogeneic MSC implants heal canine segmental defects as effectively as autologous MSCs.

Results: Radiographs taken at 16 weeks postoperatively **(Figure D)** showed that the treatment of allogeneic MSCs (4) resulted in segmental defect healing equivalent to the autologous MSC treatment (3). The untreated control (1) showed no spontaneous healing except for some remodeling of the bone ends. The ceramic only treatment (2) showed resorption and fracture due to brittleness. None of the animals demonstrated alloantibody (serum) or cellular immune responses to the allogeneic implants.⁵⁰

Trinity Matrix Testing — Pre-Clinical Studies

Study 4

Bone Formation by MSCs in Baboon Fibular Osteoperiosteal Defects

Methodology: As high-order primates, baboons provide an excellent study model because they are relatively large (adult males 20–26 kg, adult females 12–17 kg), long-lived (30–45 years), well defined, easy to use, and very closely related to humans. Allogeneic donor baboon MSCs were prepared from iliac crest bone marrow aspirates and mismatched to recipients using baboon lymphocyte antigen (BLA) testing. Fibular osteoperiosteal defects (2 cm) were surgically created then filled with the fluorescently labeled, BLA-mismatched MSCs mixed with demineralized baboon allograft bone.

Results: Radiographs (**Figure E**) of implants postoperatively (A), 12 weeks (B,C), 16 weeks (D,E), and 32 weeks (F,G). Variability was noted in the degree of mineralization within the implant at 12 weeks, but was more uniform by 16 weeks. Implants evaluated at 32 weeks were similar in density to the host bone and the host/implant junction is nearly obscured. Fluorescent micrographs (**Figure F**) show retention of dye-labeled cells within the implant. Labeled allogeneic MSCs (red) were found in the implant region within 12 (A), 16 (B) and 32 (C) week specimens. Reflected light provided tissue architecture (green). The 12-week healing response shows a woven bone appearance; labeled cells were found within areas of newly forming bone. Later times show dye-positive cells lining the areas of lamellar bone. Fluorescent cells were not found within the host marrow space or host cortical bone at the resected segment margins (C).

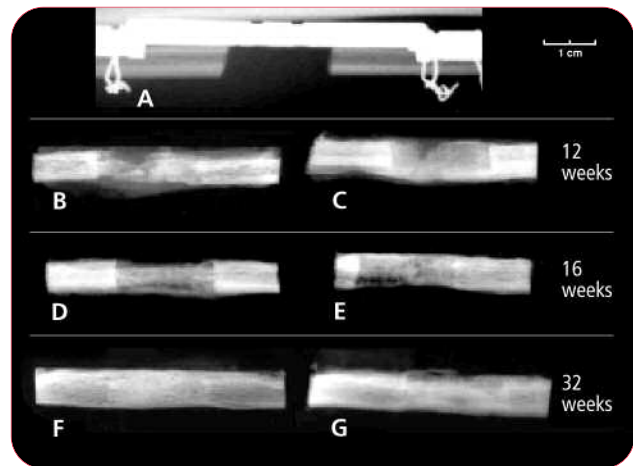


Figure E: Radiographs of post-operative implants show uniform bone mineralization within 16 weeks after MSC implantation.

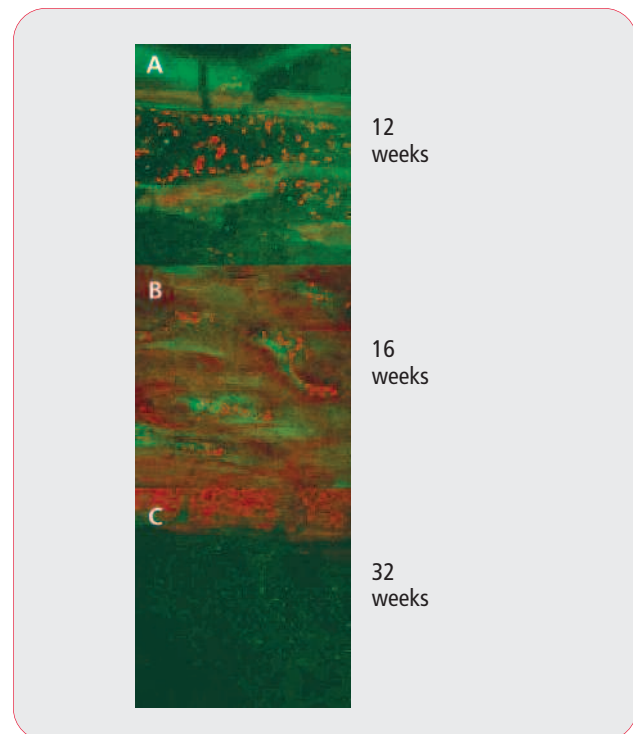


Figure F: Confocal microscopy shows retention of allogeneic MSCs (red) within the implant.

Closing Summary

Adult mesenchymal stem cells represent a powerful tool that is uniquely suited to bone regeneration. MSCs possess a tremendous capacity for self-renewal, they retain the ability to differentiate into bone-forming cells throughout their lifetime, and they are non-immunogenic, which enables these cells to be transplanted between genetically mismatched individuals without the risk of immune rejection. The proprietary Trinity Matrix manufacturing process developed by Osiris Therapeutics removes potential antigens from donated marrow-bearing bone, leaving only the osteogenic and osteoinductive “universal donor” MSCs and an osteoconductive bone matrix scaffold. Rigorous testing ensures that every lot of Trinity Matrix released (i) is aseptic, (ii) contains at least 1000 MSCs/cc, and (iii) has demonstrated osteogenic capacity in vitro.

Previously, graft materials containing allogeneic MSCs were only available for research purposes. Now, for the first time, MSC-based therapeutics can be widely used in a clinical setting. The pre-clinical animal trials and in-vitro studies described above provide compelling evidence that Trinity Matrix is a safe and effective autograft substitute with osteogenic, osteoinductive, and osteoconductive properties. In order to conclusively assess the safety and efficacy of Trinity Matrix, Blackstone Medical has initiated multiple clinical trials. The results of these clinical trials are the subject of a forthcoming monograph.

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