



ViviGen[®] Cellular Bone Matrix

The right cells. The right time. The right forms.



TECHNICAL MONOGRAPH

INTRODUCTION

DePuy Synthes, part of the Johnson & Johnson Family of Companies is the largest provider of orthopedic and neurological solutions in the world. Together the companies offer an unparalleled breadth of products, services, programs, and research and development capabilities that are designed to advance patient care and deliver clinical and economic value to health care systems throughout the world.

DePuy Synthes, part of the Johnson & Johnson Family of Companies include eight businesses that focus on joint reconstruction, trauma, neurological, crano-maxillofacial, spinal surgery, and sports medicine.

Since 1982, LifeNet Health® has helped to save lives, restore health, and give hope to thousands of patients each year. It is one of the world's most trusted providers of transplant solutions, from organ procurement to new innovations in bio-implant technologies and cellular therapies – a leader in the field of regenerative medicine, while always honoring the donors and healthcare professionals that enable the healing process.

Throughout a 17+ year collaboration, DePuy Synthes, part of the Johnson & Johnson Family of Companies and LifeNet Health have provided safe, high quality bio-implants to surgeons and patients. We continue to provide high quality products with the addition of ViviGen® Cellular Bone Matrix and ViviGen Formable™ Cellular Bone Matrix to our biomaterials portfolio. Both ViviGen and ViviGen Formable represent a paradigm shift in the field of bone and tissue repair. They are the first cellular allografts to be focused on recovering, processing, and protecting viable, lineage committed bone cells. ViviGen and ViviGen Formable contain all the properties required for bone formation and can be used as an alternative to an autograft.

CELL TYPES INVOLVED IN BONE HEALING

Bone is a highly dynamic tissue and undergoes continuous remodeling, which is a balance between the removal of old mineralized bone and the formation of new bone. As a result, bone is in a constant state of renewal. During the bone remodeling and repair processes, osteogenic cells must reach the sites of defect (aging or damaged bone) in order to directly participate in these processes.¹ Bone cells that are responsible for this remodeling process have very specific roles and functions (Figure 1):

Bone-lining cells are cells that line the surface of bone and the non-activated osteoblasts. They direct mineral uptake and release in the bone.

Osteoblasts are bone-forming cells. They secrete collagen matrix and synthesize proteins such as osteopontin and osteocalcin that are involved in the mineralization of the matrix.¹⁻⁴ Osteoblasts can differentiate into osteocytes.

Osteocytes are terminally differentiated osteoblasts that are entrapped in the mineralized matrix. They are able to send signals based on stress and strain present within the bone to begin the bone remodeling process.

Osteoclasts are bone-removing cells. They are responsible for the absorption and removal of old bone.

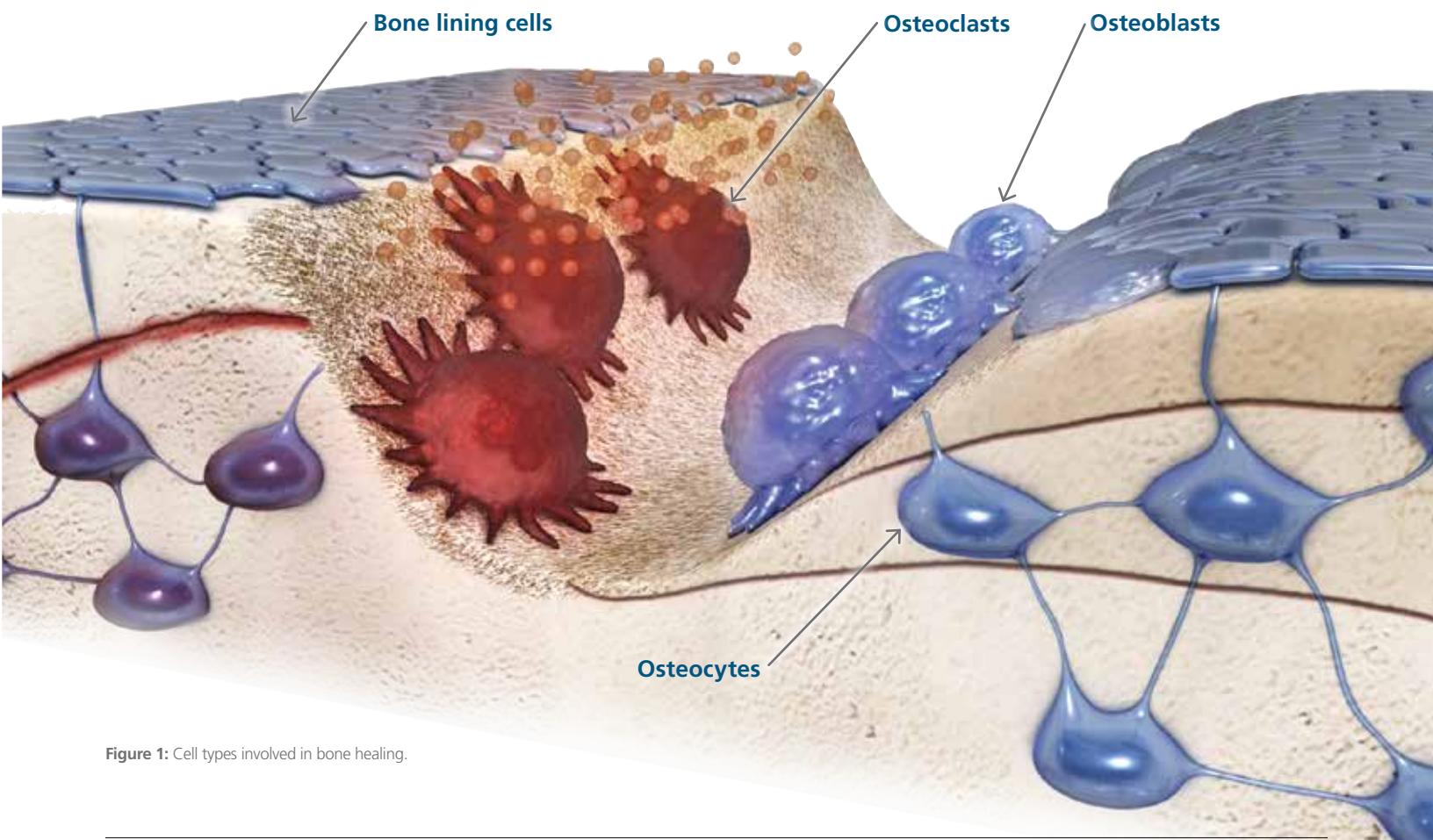


Figure 1: Cell types involved in bone healing.

BASICS OF BONE GRAFTING

The body does not build bone across large defects between bony fragments. In order for two bone fragments to fuse, they must be in very close proximity. Bone grafts are often required to facilitate the fusion of two bone segments that are not in close contact. There are several potential sources for bone grafts:

Autograft – Bone relocated from other parts of the patient's own body

Allograft – Bone from another member of the same species

Xenograft – Bone from a member of a different species

Synthetic – Substitutes created artificially to mimic bone in the body.

PROPERTIES OF BONE GRAFTS

Bone grafts differ in the characteristics they possess. Many bone graft substitutes have at least one of the following properties:

Osteoconductive – An osteoconductive graft has the ability to provide the framework for new bone growth. It satisfies the scaffolding requirements for a fusion.

Osteoinductive – An osteoinductive graft has the ability to induce bone growth. It satisfies the chemical signaling requirements for a fusion.

Osteogenic – An osteogenic graft has the ability to grow into bone. It contains live cells that are responsible for building bone and satisfies the biologic requirements for a fusion.

These properties often guide a surgeon's choice of implant based on the patient's condition and the desired clinical outcome. An ideal bone graft should possess all three properties.

Autograft has been considered the gold standard because of its inherent osteoinductive, osteoconductive, and osteogenic properties, as well as its long clinical history of use.

However, autograft is associated with several comorbidities including second incision site morbidity, risk of infection, longer operating room time, increased blood loss, and long term pain at the autograft harvest site.⁵ As a result, surgeons have sought alternatives.

THE RIGHT CELLS – LINEAGE COMMITTED BONE CELLS

In recent years, cellular allografts have emerged as an alternative to autograft. Until now, the focus of these cellular allografts has been centered on mesenchymal stem cells (MSCs). There has been a high level of interest in the surgical community surrounding MSCs in general, based on their multipotency. MSCs have the ability to differentiate into several types of cell lineages including osteoblasts, chondrocytes, fibroblasts, adipocytes, and myocytes (Figure 2).⁶ While MSCs have been shown in vitro to commit to different lineages, the molecular control of these processes is poorly understood.⁷ What is understood is that these MSC-based cellular allografts are being used in spine and orthopedic procedures with the goal of achieving fusion through new bone formation.

Osteoblasts are bone-forming cells that are responsible for the initiation of the deposition of mineral components, collagen matrix, and other non-collagenous proteins such as osteopontin, osteocalcin, osteonectin, osteoprotegerin, bone morphogenetic proteins (BMPs), and glycoproteins.^{1,2} They are derived from MSCs but are differentiated and committed to bone formation only.

MESENGENESIS

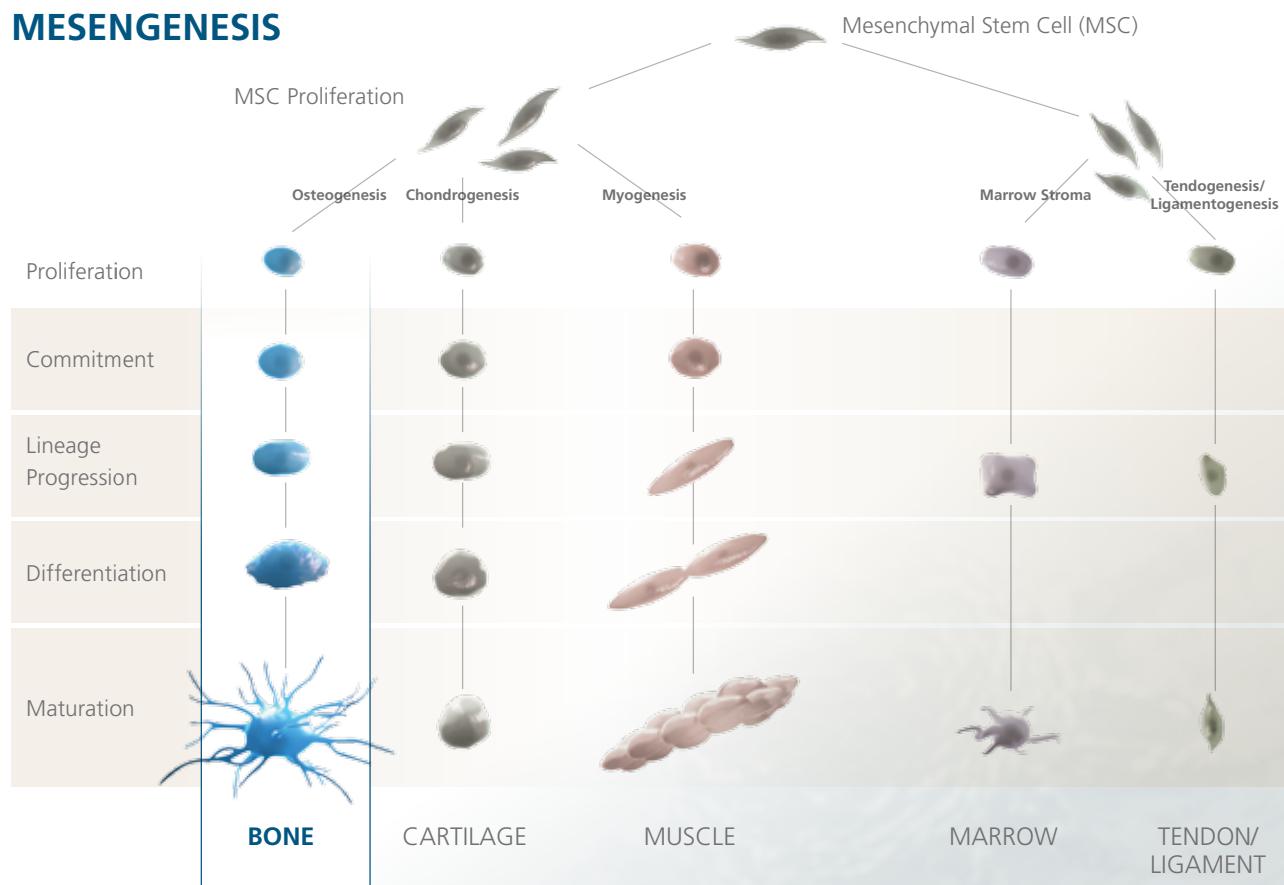


Figure 2: Differentiation of mesenchymal stem cells

There is a body of evidence supporting the fact that bone cells are the preferred cell type for bone formation. They are fully differentiated and are committed solely to laying down bone matrix. Multiple studies have shown that they remain at the defect site longer, directly participate in the bone formation process,⁸ deposit a higher quality of bone than MSCs,⁹ and secrete cytokines to stimulate angiogenesis and vascularization.¹⁰ Osteoblasts also secrete chemotactic factors such as IGF-1, which may play a major role in the recruitment of osteoblasts during bone formation.³

Additionally, osteoblasts and osteocytes can work in concert to facilitate further MSC differentiation.¹¹ Bone cells are also less influenced by age. MSCs significantly decrease in number and function as a person ages. In contrast, bone cells have been found to maintain viability, proliferation potential, and osteoblastic function later in life.^{12,13,14,15}

PRE-CLINICAL STUDIES SUPPORTING THE BENEFITS OF BONE CELLS

BONE CELLS REMAIN IN THE DEFECT SITE LONGER

In a study by Tortelli et al., ceramic scaffolds were seeded with either green fluorescent protein (GFP)-labeled murine MSCs or GFP-labeled murine osteoblasts and implanted into immunocompromised mice. The results showed that MSCs directed the formation of bone of host origin by activation of the endochondral ossification process. In contrast, osteoblasts directly participated in the formation of new bone through an intramembranous ossification process. Specifically, anti-GFP immunostaining showed that only osteoblast-seeded implants contained bone-depositing cells of donor origin (Figure 3). On the other hand, MSC-seeded implants showed no cells of donor origin after 30 days and led to the development of a tissue-engineered bone of host origin.⁸

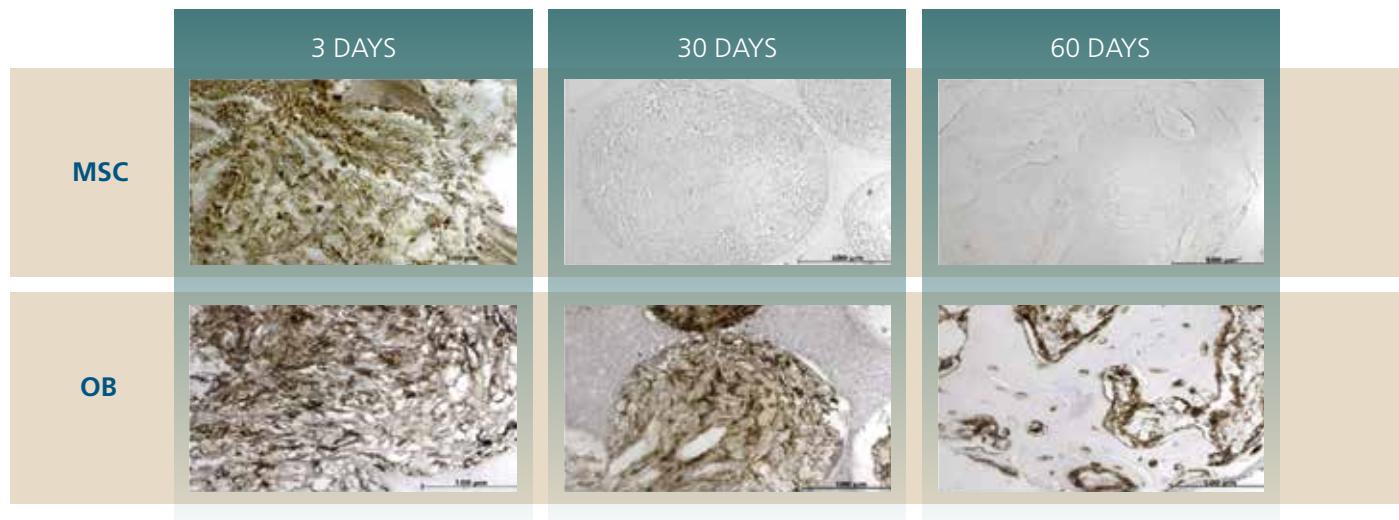


Figure 3: Fate of implanted exogenous MSCs and osteoblasts (OB)

The top panel shows GFP immunostaining of sections obtained from GFP+ MSC/Engipore and the bottom panel shows sections from GFP+ OB/Engipore implants. At Day 3 after implantation, exogenous GFP+ MSCs were observed within the pores of the scaffold. At Day 30, expression of GFP was negative in the pores of the GFP+ MSC/Engipore constructs. At Day 60, detectable bone tissue remained negative for GFP staining. For implanted exogenous GFP+ OB, GFP+ cells were detectable within the pores of the scaffolds from Day 3 up to the last time point; at Day 60 all osteocytes present in the newly deposited bone were GFP+. Scale bars are as indicated.

OSTEOBLASTS PRODUCE HIGH QUALITY BONE

A study by Reichert et al. showed that when ovine marrow-derived mesenchymal stem cells (bMSCs) or osteoblasts were seeded on a TCP scaffold and implanted subcutaneously in a severe combined immunodeficiency mouse, ectopic bone formation in terms of bone volume, maturation, and density was much more robust in the scaffold seeded with osteoblasts compared to bMSCs (Figure 4). The highest amount of new bone formation (NB) is shown for OB-seeded scaffolds supplemented with recombinant human BMP-7 (rhBMP-7). In particular, the scaffolds seeded with osteoblasts had higher mineralization than those seeded with MSCs and BMP-7 combined. The study suggested that mature bone is an adequate alternative cell source for bone tissue-engineering applications.⁹

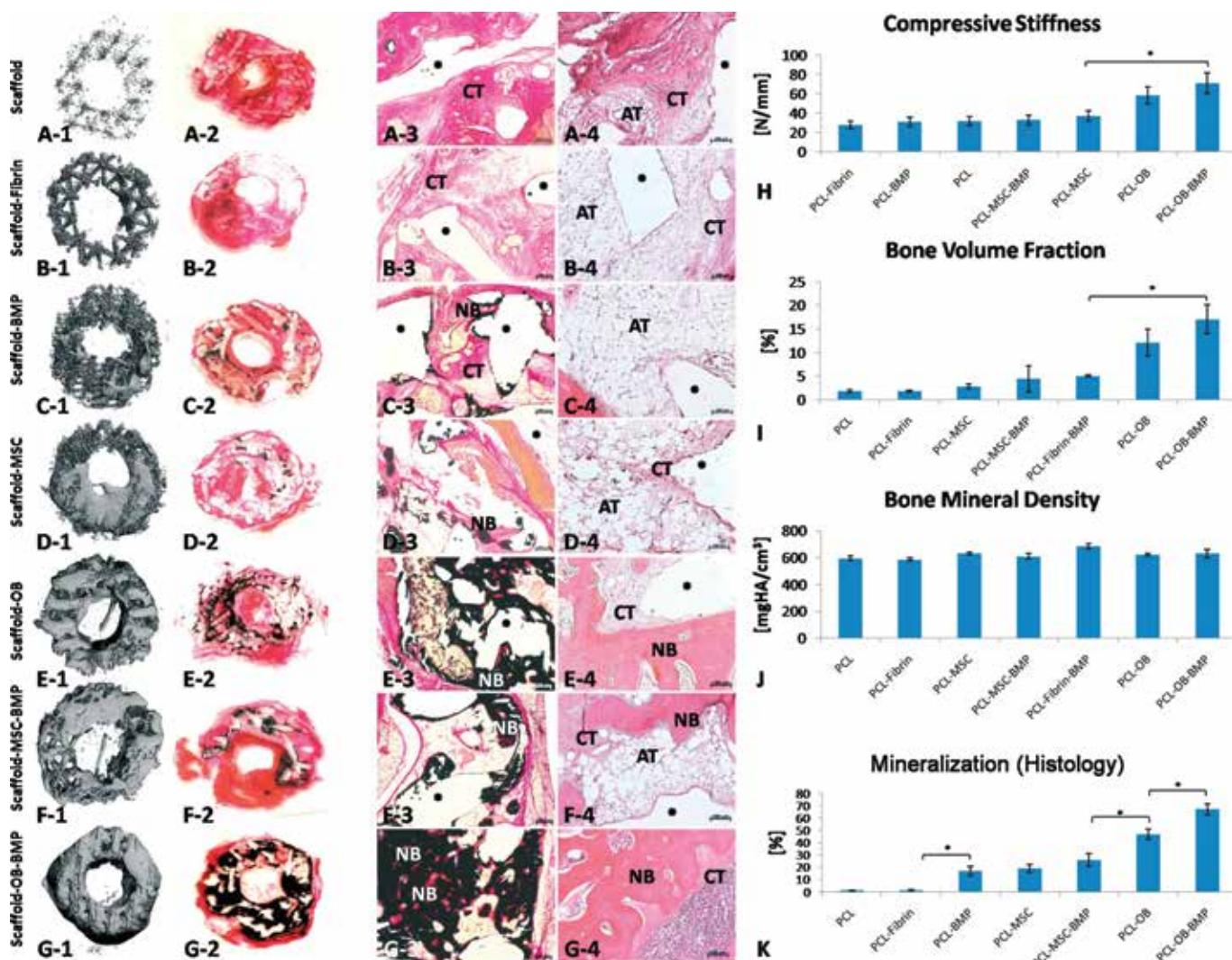


Figure 4: Histology, 2CT analysis, and biomechanical testing at 8 weeks

The highest amount of bone formation (NB) is shown for OB-seeded scaffolds supplemented with rhBMP-7 by histomorphometry on von Kossa-stained sections (A2–G2, A3–G3) and quantitative 2CT analysis (A1–G1, I). Results from compression testing (H) correlated well with these findings. There were no differences in bone mineral density between the groups (J). AT, adipose tissue; CT, connective tissue; *scaffold strut; scale bars = 100 µm.

BONE CELLS PROMOTE VASCULARIZATION

In order to determine the role of osteoblasts in vascularization, primary human osteoblasts (HOB) were pre-seeded on silk fibroin (SF) micronets in a study by Ghanaati et al.¹⁰ The results of this study demonstrated that osteoblasts were able to migrate throughout the entire scaffold and produce extensive bone matrix in vitro. After implantation, these osteoblasts induced a more rapid and significantly higher level of vascularization of the scaffold (Figure 5) with microvessels homogeneously distributed throughout the scaffold than SF alone with no pre-cultivation. This study concluded that osteoblasts can produce not only bone matrix but also soluble factors which can serve to instruct host endothelial cells to migrate, proliferate, and initiate the process of scaffold vascularization.¹⁰

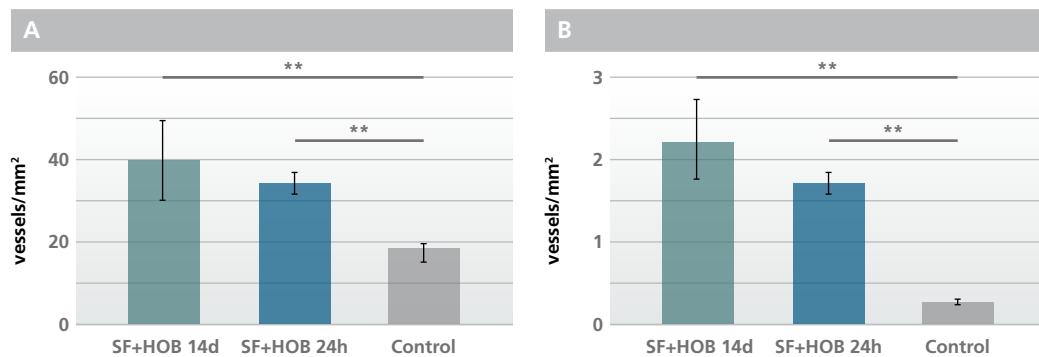


Figure 5: Histomorphometric analysis of host-derived blood vessels
Vessel density within the scaffold of the three study groups is shown in (A), and the percentage of scaffold that is vascularized is shown in (B). SF+HOB 14d, human osteoblasts seeded on SF and cultured for 14 days before implantation; SF+HOB 24hr, human osteoblasts seeded on SF for 24 hours before implantation; control, SF without cells.

OSTEOBLASTS SECRETE A POTENT CHEMOTACTIC FACTOR

Osteoblast recruitment to the site of future bone formation is essential for skeletal development, bone remodeling, and fracture healing. Nakasaki et al. showed that IGF-I secreted from osteoblasts induced directed cell migration of osteoblasts, promoted cell spreading, and regulated cell polarization in a monolayer model of wound healing. These results suggested a major role for IGF-I in osteoblast migration and contributed to the general understanding of osteoblast recruitment during bone formation and repair.³

OSTEOBLASTS AND OSTEOCYTES REGULATE OSTEOGENIC DIFFERENTIATION OF MSCS

The role of biochemical signaling from osteocytes and osteoblasts was investigated in an in vitro study by Birmingham et al. to understand how these cells direct osteogenic differentiation of MSCs.¹¹ Results from an intracellular alkaline phosphatase assay suggested that osteocytes are more influential than osteoblasts in stimulating osteogenesis in MSCs. Moreover, it was found that osteoblasts and osteocytes work in concert to secrete biochemical signals and synergistically stimulate the osteogenic differentiation of MSCs.¹¹

THE VIVIGEN DIFFERENCE

ViviGen is the first cellular allograft to focus on recovering, processing, and protecting viable, lineage committed bone cells. It is comprised of viable, cryopreserved corticocancellous bone matrix and demineralized bone.

THREE KEY COMPONENTS OF VIVIGEN

1

Viable Cells (Osteogenic)

The processing of ViviGen removes bone marrow components including Mesenchymal Stem Cells while retaining the desirable bone cells (osteoblasts, osteocytes and bone lining cells)¹⁷

2

Corticocancellous Chips (Osteoconductive)

Provide a natural scaffold for cell attachment, migration and proliferation

3

Demineralized Bone (Osteoinductive)

A patented demineralization process exposes natural growth factors within the bone matrix that recruit host cells and stimulate bone forming activity

These three components of ViviGen provide the properties necessary for bone repair that include osteoinductive signals, osteoconductive bone scaffolds, and osteogenic cells.



VIVIGEN AS A BONE GRAFT SUBSTITUTE

Because of its properties, ViviGen can be considered an alternative to autograft. Moreover, the need to harvest autograft intraoperatively may be eliminated, thereby avoiding the associated complications. ViviGen represents a paradigm shift in the field of bone repair and tissue engineering by focusing on lineage committed bone cells. At every step of ViviGen's processing, LifeNet Health has focused on maintaining the highest level of bone cell viability. This has been achieved by decreasing the time required for recovery and processing compared to that of its competitors¹⁶, developing proprietary processing reagents and cryopreservation media, packaging specifically designed to preserve bone cell viability, and to facilitate tissue preparation procedures prior to implantation in the operating room.¹⁷

REMOVAL OF MARROW COMPONENTS

Immunohistochemical analysis of ViviGen derived bone chips prior to washing and processing, as well as after cryopreservation and thawing, illustrate the absence of marrow components including MSCs. Prior to processing, large amounts of marrow components are present in the bone chips (Figure 6). Staining for CD45, a type I transmembrane protein present on all hematopoietic cells, confirmed the presence of hematopoietic cells in the bone matrix prior to processing. Post processing, cryopreservation, and thawing, marrow components as well as CD45-positive cells were absent, which confirmed that marrow components were absent. As a result, the processing reduces the number of potentially immunogenic cells from the bone marrow, also reducing the risk of eliciting an immune response.¹⁷

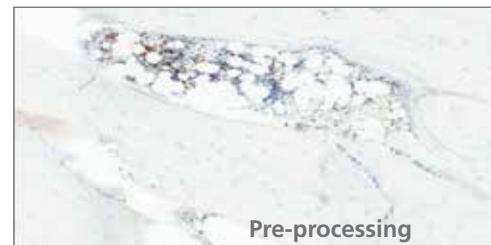


Figure 6: Immunohistochemical staining of CD45-positive cells (brown) pre-processing and post-cryopreservation and thawing.

The presence of MSCs was further evaluated by staining for CD166, an MSC cell surface marker. The initial staining of pre-processed tissue indicated that the marrow component contained a relatively small population of CD166-positive MSCs (Figure 7). When staining was repeated on ViviGen post-cryopreservation and thawing, MSCs were absent.

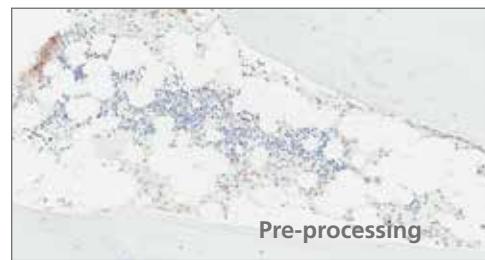


Figure 7: Immunohistochemical staining of CD166-positive cells (brown color) pre-processing and post-cryopreservation and thawing.

VIVIGEN COMPRISSES LINEAGE COMMITTED BONE CELLS

The gene expression profile of bone cells is very complex; however, there is extensive literature describing genes that are specifically expressed by osteoblasts and osteocytes. In order to illustrate that ViviGen derived cells are of osteoblast and osteocyte in origin, assays were performed to study the expression of genes and proteins most commonly expressed by bone cells.

Real-time quantitative reverse transcription PCR analysis was performed to examine the expression of the components of the secreted extra cellular matrix (ECM) and signaling molecules of osteoblasts and/or osteocytes (BMP-2, osteopontin, and osteocalcin).

Messenger RNA was isolated from cells derived from ViviGen bone chips. Cells isolated from six different donors (OB) were analyzed and compared to the commercially available normal human osteoblasts (NHOst; Lonza, Basel, Switzerland) and human MSC (hMSC; Lonza). ViviGen derived cells expressed higher levels of osteoblast characteristic genes when compared to NHOst (Figure 8). Further, when compared to the hMSCs, a large difference in expression of the osteoblast characteristic genes was observed. These results indicate that the primary cells derived from ViviGen bone chips express higher levels of osteoblast/osteocyte specific genes than NHOst or human MSCs, confirming they are mature bone cells.

Relative Fold Increase Compared to NHOst

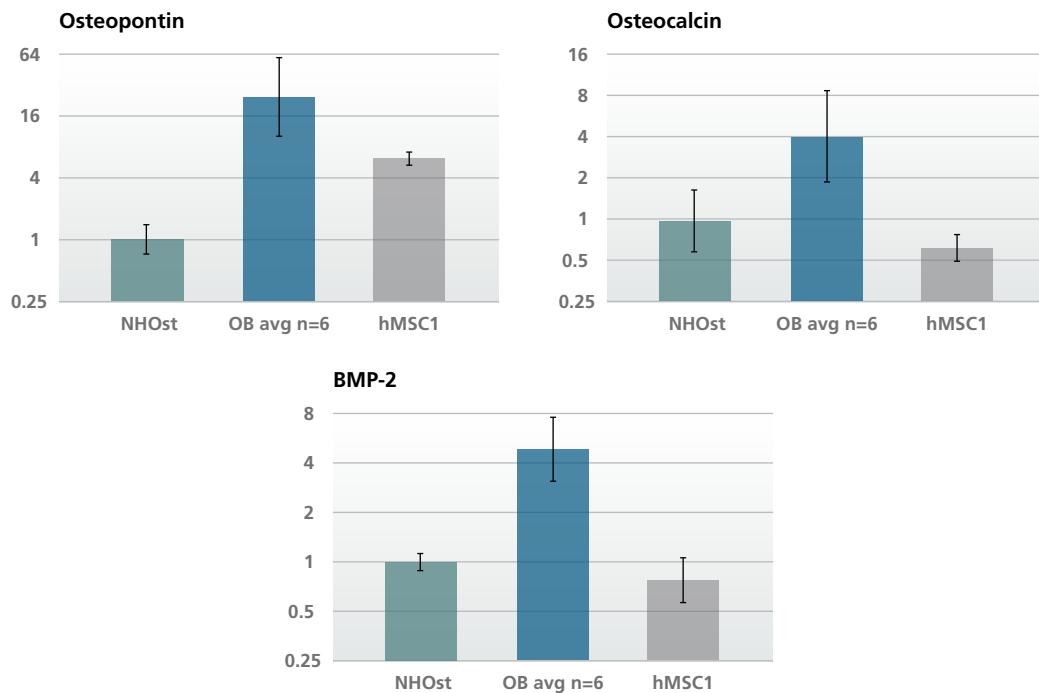


Figure 8: Average gene expression of ViviGen derived cells from six donors. Gene expression was measured using Taq-man primers for the specific genes of interest and standardized to the internal control (GAPDH). Expression levels are shown relative to the NHOst using the $\Delta\Delta Ct$ method.

ViviGen
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to MSCs.

Osteocalcin is one of the most abundant proteins expressed by both osteoblasts and osteocytes and is present intra- and extra-cellularly. In the pre-processed ViviGen samples, the expression of osteocalcin (brown, stained with anti-osteocalcin antibody) was observed in the bone cells and bone matrix, as well as in the bone marrow (Figure 9). In ViviGen samples that were cryopreserved and thawed (post-cryopreservation), osteocalcin was expressed in the bone cells and bone matrix. These results illustrate that the ViviGen derived cells are from a bone cell lineage.

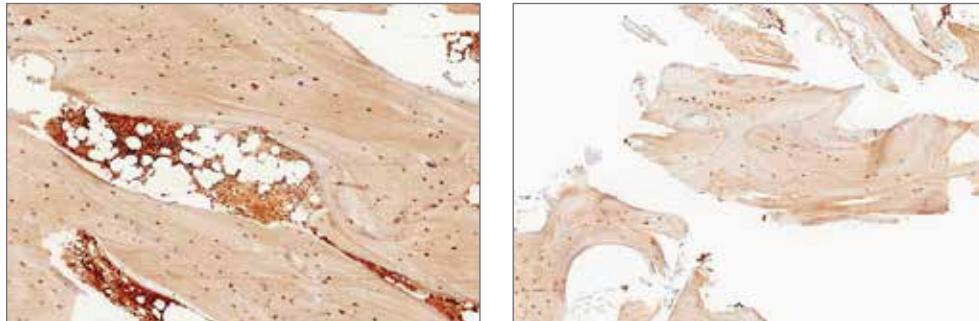


Figure 9: Immunohistochemical analysis of ViviGen bone chips pre-processing and post-cryopreservation and thawing. Both bone cells and bone matrix stained positively for osteocalcin (brown).

Bone cells that migrated out of the corticocancellous bone chips post cryopreservation and thaw were further analyzed using immunocytochemical analysis (Figure 10). It was shown that the cells that migrated from the bone also stained positive for osteocalcin (Figure 10). The results of these assays prove that the ViviGen derived cells post cryopreservation and thawing are indeed lineage committed bone cells.

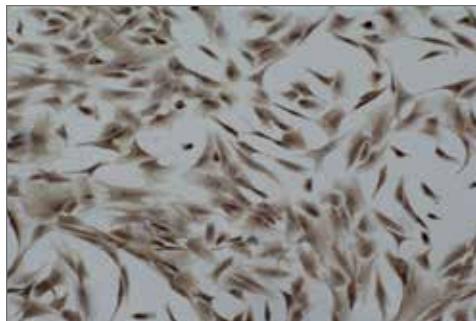


Figure 10: Immunocytochemical analysis of ViviGen derived cells. Cells were stained with anti-osteocalcin antibody (brown).

ViviGen
derived cells
are from
a bone cell
lineage.

VIVIGEN CONTAINS VIABLE CELLS

In order to determine the presence of viable cells, ViviGen bone matrices were thawed and plated in culture wells. The cells were able to migrate from the bone chips and attach to the culture plate, demonstrating that they survived the freezing and thawing process (Figure 11).¹⁷



Figure 11: Representative picture of cell migration, attachment, and growth of ViviGen derived cells.

The proliferation profile of the viable ViviGen bone chips was further evaluated using an Alamar blue assay. Assays that can demonstrate the proliferation potential of cells are the ultimate test of viability since they test cell function and not just the cell membrane integrity. Results show that ViviGen cells can not only survive cryopreservation and processing, but can also proliferate over time (Figure 12).

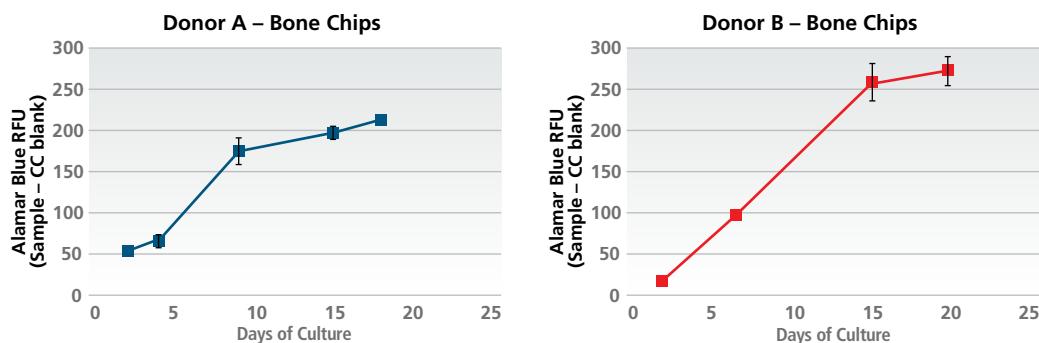


Figure 12: Representative ViviGen bone matrix from two different donors were thawed and tested for growth potential by the Alamar blue assay. Bone cells in ViviGen proliferate over time.

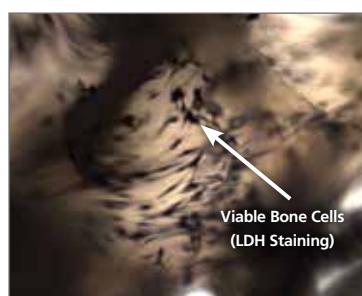


Figure 13: LDH staining of viable bone cells on the surface of bone chips after 21 days of culture.

After 21 days of culture, the bone chips were analyzed using a lactate dehydrogenase (LDH) stain (Figure 13). Only viable cells are able to maintain the LDH enzyme in their cytoplasm and thus stain positive (black). The black staining shown in Figure 13 represents viable cells that grew on the surface of the bone chips. Therefore, the bone cells not only grew on the culture plate surface but also on the surface of the bone chips. These results illustrate that ViviGen derived bone chips provide an osteoconductive scaffold for cellular attachment and growth.

VIVIGEN IS OSTEOGENIC

ViviGen derived cells were analyzed to determine if they had the ability to deposit calcium to initiate the bone formation process in vitro.¹⁷ Adipose-derived mesenchymal stem cells (ASC; LifeNet Health) and bone marrow-derived MSCs (bMSCs; Lonza, Basel, Switzerland) were used as controls. It was hypothesized that the ASC or bMSC population would have slower calcium deposition kinetics compared to osteoblasts due to their need to undergo differentiation from a stem cell to a mature osteoblast. In contrast, osteoblasts are expected to produce calcium deposits at a faster rate as they do not need to undergo this differentiation process to become committed bone forming cells in order to gain an ability to deposit calcium.

ViviGen derived cells, MSC, and ASC controls were plated into 24-well dishes and exposed to an osteogenic induction media and a growth media. After the cells were cultured in differentiation or growth media for 7, 14, and 21 days, they were stained for calcium deposition with Alizarin Red S (Sigma; St. Louis, MO). Upon examination, a substantial deposition of calcium was visible in the ViviGen derived cells as early as Day 7, but no calcium deposits were observed in the ASC and MSC populations at the same time point (Figure 14). Further, when allowed to differentiate to 14 days, extensive matrix deposits were evident

by their positive staining for calcium in the entire well. In contrast, the ASCs and MSCs were just beginning to deposit extracellular calcium at this time point, and it was necessary to culture the ASCs for 21 days before observing similar levels of deposits, while MSCs detached from the culture wells after 21 days and no additional calcium deposition was found compared to those on day 14. These observations indicate that ViviGen derived cells are in a primed state ready to produce calcium deposits faster than the undifferentiated ASCs or MSCs in vitro.

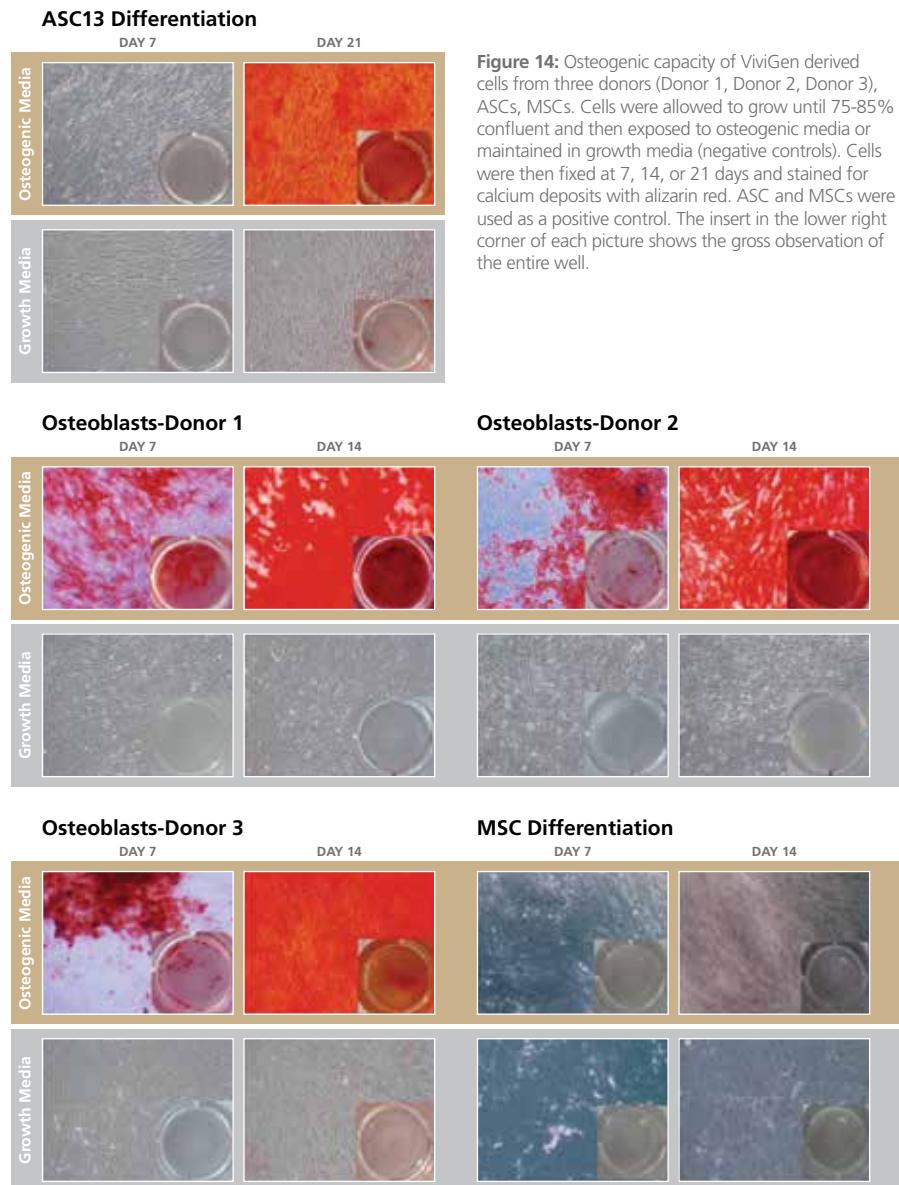


Figure 14: Osteogenic capacity of ViviGen derived cells from three donors (Donor 1, Donor 2, Donor 3), ASCs, MSCs. Cells were allowed to grow until 75-85% confluent and then exposed to osteogenic media or maintained in growth media (negative controls). Cells were then fixed at 7, 14, or 21 days and stained for calcium deposits with alizarin red. ASC and MSCs were used as a positive control. The insert in the lower right corner of each picture shows the gross observation of the entire well.

VIVIGEN FORMABLE

NEXT-GENERATION CELLULAR ALLOGRAFT

ViviGen Formable™ Cellular Bone Matrix is the second generation of ViviGen, providing surgeons with an alternative handling option. The demineralized particulate has been replaced with osteoinductive, precision-machined, demineralized fibers. These interconnected fibers provide putty like consistency, allowing the graft to be shaped and molded.

Many assays were conducted to assess whether these demineralized bone fibers possess the appropriate growth factors for osteoinductivity, demonstrate in vivo osteoinductivity, and support in vitro cell attachment, proliferation and viability.



VIVIGEN FORMABLE IN VITRO ASSAYS

Fiber Generation

Human cortical bones were recovered from six donors with research authorization through LifeNet Health's organ and tissue procurement service. Femurs were debrided and the marrow and trabecular bone were removed. The resulting bone segments were cut into fibers, processed, and demineralized utilizing proprietary procedures developed by LifeNet Health.¹⁷

Fiber Analysis

In vitro Growth Factor Analysis

Demineralized bone fiber lots (derived from 6 donors) were analyzed for the presence of BMP-2 and BMP-7 content in vitro by first digesting the tissue with a collagenase solution at 37°C to facilitate the extraction of proteins. The resulting solutions were analyzed in triplicate for the presence of BMP-2 and BMP-7 via ELISA (R&D Systems, Indianapolis, Indiana). Results are reported as ng protein/g of demineralized bone fibers.¹⁸

In vivo Osteoinductive Potential (OI)

Osteoinductive potential (OI) and the generation of new bone were assessed in vivo utilizing a 35 day athymic mouse muscle pouch model of OI. Four replicates (20-25g) of freeze dried demineralized bone fibers were weighed out from lots separate from those used in the growth factor analysis and cell attachment studies, rehydrated in saline, and loaded into a syringe for delivery. The rehydrated sample was used within 5 minutes of preparation and implanted inter-muscularly between the biceps femoris and gluteus superficialis muscles of athymic mice. The implants were recovered at 35 days post-implantation and processed for histological microscopic assessment of hematoxylin and eosin (H&E) stained sections. The osteoinductivity of the implanted bone fibers was determined by the presence of bone elements and bone matrix deposition.¹⁹

In vitro Cell Attachment and Sustained Cellular Proliferation

Demineralized bone fibers (6 donor lots previously analyzed for growth factors) were placed in cell culture and seeded with either previously frozen ViviGen derived bone cells, or thawed bone marrow-derived mesenchymal stem cells (BM-MSCs) recovered from human donor bone marrow. Prior to experimentation, the BM-MSCs were identified on the basis of cell surface marker expression via flow cytometry. The osteoblasts and BM-MSCs were seeded separately at 62,500 cells per 60.5 ± 1 mg fiber sample on day 0 and cultured over the course of 7 days in their respective growth media. These cultures were examined at 1 hour, 1 day, and 7 days post-seeding by scanning electron microscopy (SEM) to assess cell attachment and concomitantly by Alamar Blue staining to assess sustained cellular proliferation.²⁰

RESULTS

Presence of Growth Factors and Osteoinductivity

Using a proprietary demineralization process, bone fibers generated from 6 individual donors were processed and freeze dried. The resulting demineralized fibers were subsequently treated with a collagenase solution to digest the tissue and facilitate the extraction of proteins for analysis. Specifically, the BMP-2 and BMP-7 (Figure 15) content were measured in triplicate utilizing ELISA. Figure 15 depicts the average BMP-2 and BMP-7 levels from six donor samples.¹⁸ Other studies have reported a wide span of BMP-2 and BMP-7 levels in demineralized bone, with ranges from 6.5-110 ng and 44-125 ng per g demineralized bone, respectively.²¹⁻²³ The data presented here demonstrates that BMP-2 and BMP-7 were present, and fell within expected levels, in the demineralized bone fibers of ViviGen Formable after processing.¹⁷

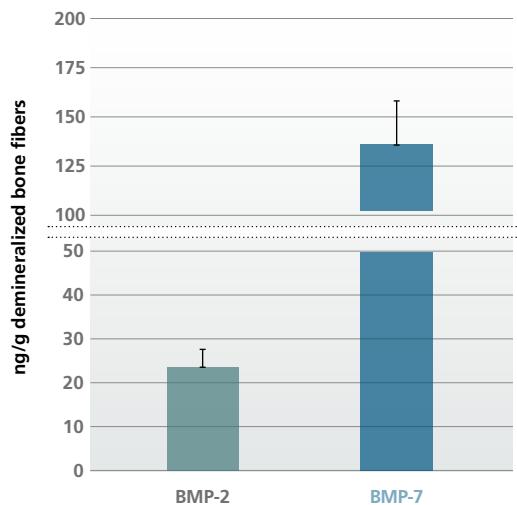


Figure 15: Average level of BMP-2 and BMP-7 content in ViviGen Formable demineralized bone fibers from six donor samples, each performed in triplicate, as measured by ELISA.

After demonstrating in vitro that new ViviGen Formable demineralized bone fibers contain BMP-2 and BMP-7, demineralized bone fibers generated in the same manner were intra-muscularly implanted into athymic nude mice to test their in vivo osteoinductivity (OI). After 35 days, the implanted material and surrounding tissue were removed and histologically prepared for hematoxylin and eosin (H&E) staining. Images from an H&E stained sample are presented in Figure 16 (A-C). Panel A shows a set of merged images that robustly illustrate more than 50% new bone elements present in the entire ectopic site (4x magnification). Panels B and C highlight at 4 and 10x magnification, respectively, the presence of new bone elements such as bone marrow (&), new vascularization (^), osteoblasts (□), chondrocytes (%), and new bone (*) dispersed amongst the remaining implanted demineralized fibers (#). These observations demonstrate the new ViviGen Formable demineralized bone fibers retain OI potential: They not only support the colonization of cells, but also the formation of new bone and bone marrow, as well as neo-vascularization, an important aspect of the healing process.¹⁹

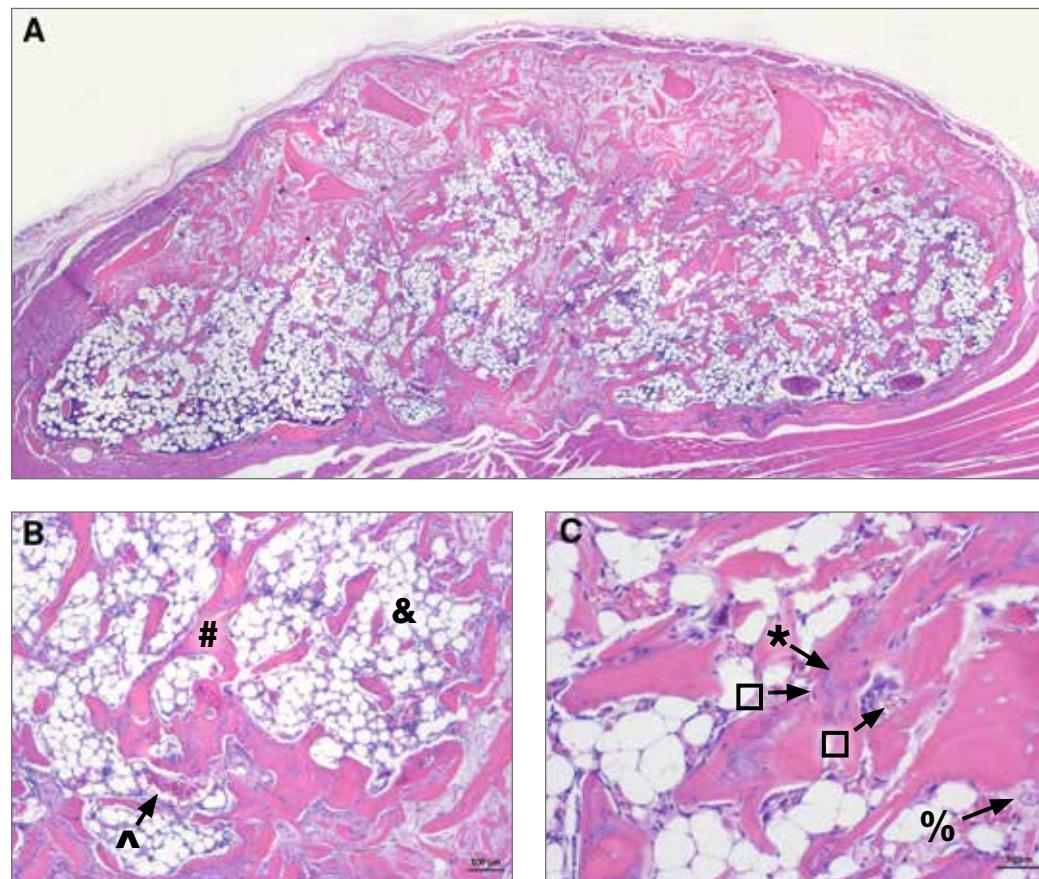


Figure 16: ViviGen Formable demineralized bone fibers demonstrated osteoinductivity in athymic nude mice. Panel A shows a set of merged H&E images that demonstrate more than 50% new bone elements present in the entire ectopic site at 35 days post-implantation (4x magnification). Panels B and C are also H&E images shown at 4 and 10x magnification, respectively, that depict the presence of new bone elements such as bone marrow (&), new vascularization (^), osteoblasts (□), chondrocytes (%), and new bone (*) dispersed amongst the remaining implanted demineralized fibers (#) at 35 days.

SUPPORT OF CELLULAR ATTACHMENT

The next set of experiments were designed to examine in vitro the colonization of the demineralized bone fibers by human bone marrow-derived mesenchymal stem cells (MSCs) and bone cells derived from human corticocancellous bone. The purpose of these experiments was to model bone cell migration from the corticocancellous chips to the demineralized bone fibers. Additionally, this may also represent how a patient's own bone forming cells might interact with the demineralized bone fibers in ViviGen Formable. Demineralized bone fibers generated from the original 6 donor lots were stored in a cryopreservative. The fibers were treated in this manner to model the ViviGen Formable product. Prior to their use, the demineralized bone fibers were thawed and rinsed per the same instructions for preparing ViviGen Formable for implantation. Previously isolated and frozen ViviGen derived bone cells were thawed, cultured, and seeded along with the demineralized bone fibers at a density of $62,500 \text{ cells}/60.5 \pm 1 \text{ mg}$ demineralized bone fibers in low-attachment cell culture wells.²⁰

Another cell population that supports new bone formation are mesenchymal stem cells (MSCs). To model how a patient's own MSCs might interact with the new demineralized bone fibers, human bone marrow-derived mesenchymal stem cells (BM-MSCs) were also separately cultured with ViviGen Formable demineralized bone fibers.²⁰

After 1 hour, 1 day, and 7 days, the samples were prepared for SEM analysis. Representative images at 3000x magnification are shown in Figure 17. Cells are highlighted in the figure by the asterisks (*). As seen in the images, both ViviGen derived bone cells and BM-MSCs attached to the ViviGen Formable demineralized bone fibers. As expected, both cell populations spread and formed processes over 7 days. The colonization of the fiber scaffolds by networks of cells confirm that they provide a biocompatible environment in supporting two cell types (i.e., ViviGen derived bone cells and BM-MSCs) that are involved in the process of generating new bone.²⁰

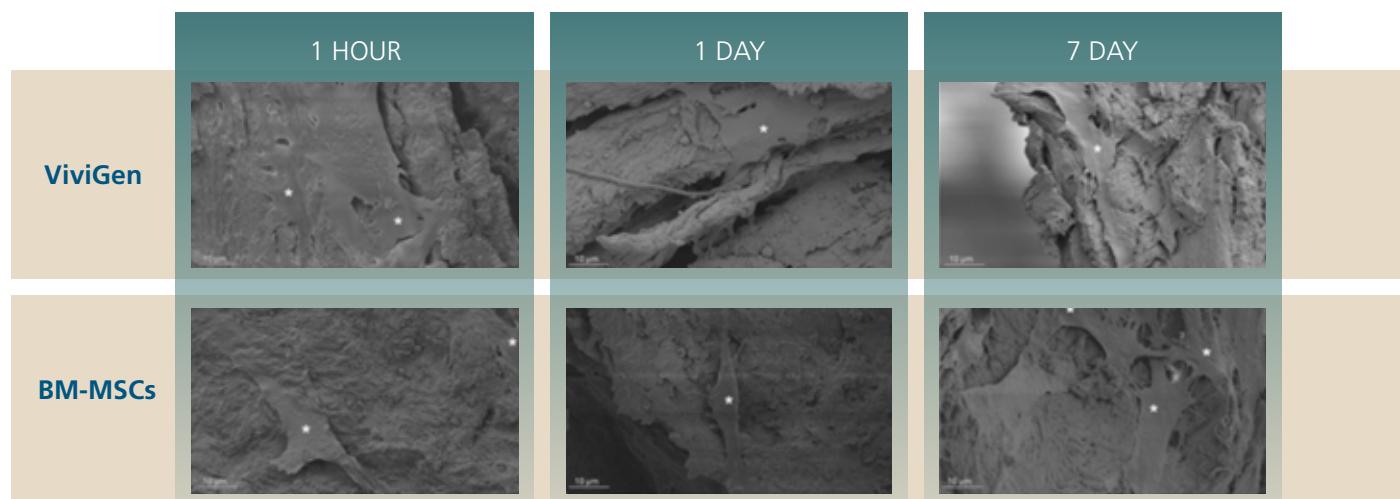


Figure 17: ViviGen Formable demineralized bone fibers support cellular attachment of both ViviGen derived bone cells and human BM-MSCs. Over the course of 7 days, cells (*) were observed spreading along the fibers, elongating processes, and interacting with other networks of cells.

SUPPORT OF SUSTAINED CELLULAR PROLIFERATION AND VIABILITY

To further confirm that the new demineralized bone fibers in ViviGen Formable provide a biocompatible environment for bone cells, an Alamar Blue assay was used to measure cellular proliferation of ViviGen derived bone cells seeded on demineralized bone fibers. The data illustrates that following seeding on day 0, the ViviGen derived bone cells demonstrated increasingly robust levels of cellular proliferation when cultured with ViviGen Formable demineralized bone fibers over the course of 7 days.²⁰

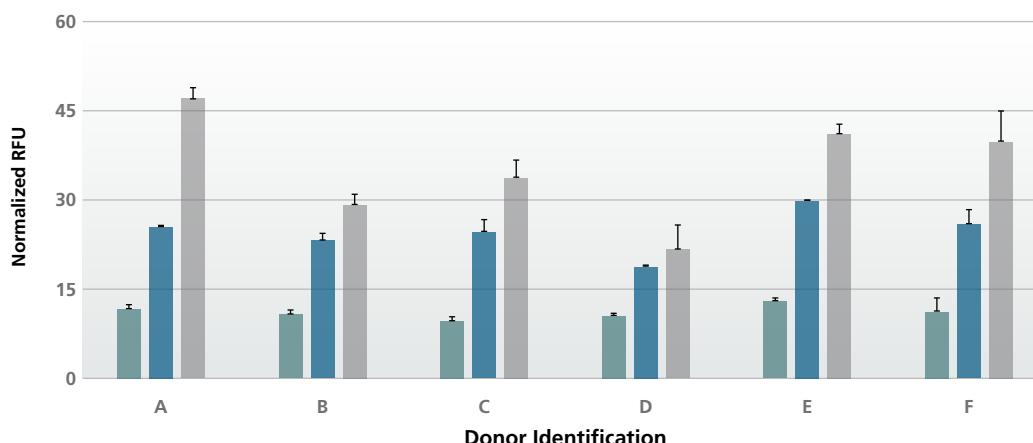
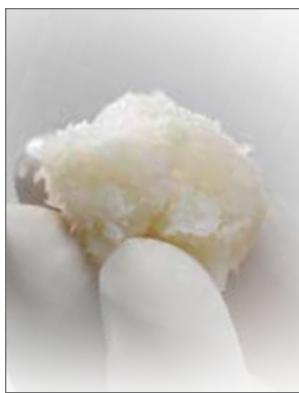


Figure 18: The results of an Alamar Blue assay showed that ViviGen Formable demineralized bone fibers supported cellular proliferation with ViviGen derived bone cells. The results show averages from 6 donor samples (A-F) conducted in triplicate. RFU = relative fluorescent units.

A COMPREHENSIVE SOLUTION TO MEET SURGEONS' CLINICAL NEEDS

ViviGen and ViviGen Formable provide the same advantages with alternative formulations to meet surgeons' clinical needs.

ViviGen



- Available for pathologies that require packing of a contained void or lumen, such as an allograft spacer
- Bone graft foundation in large revision or deformity cases where a large volume of graft is needed

ViviGen Formable



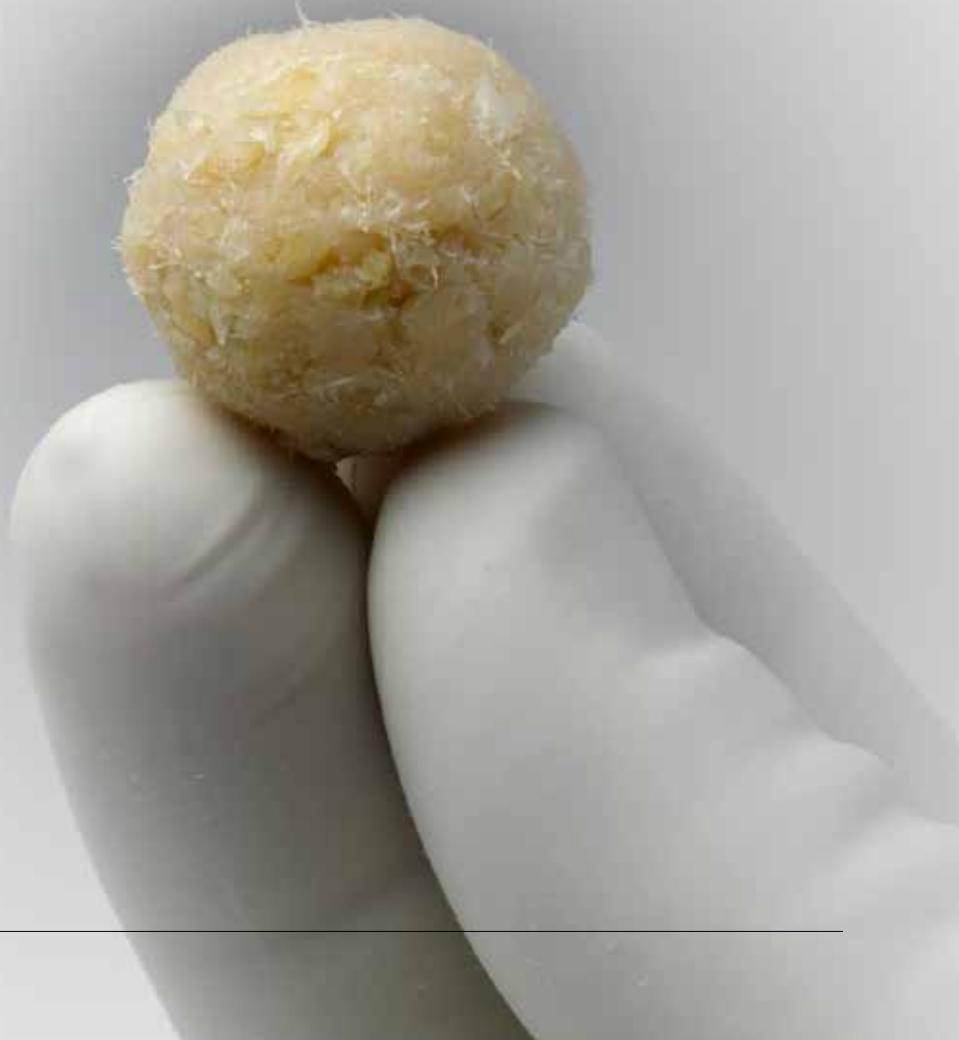
- Option for cases where migration can be a concern, with the ability to lay down as a defined shape
- Available for use in open voids as it resists migration such as in the posterolateral gutters

SAFETY

LifeNet Health prides themselves on their safety record over the last 30+ years. They hold the longest continuous accreditation from the American Association of Tissue Banks, and have a comprehensive range of measures in place to ensure the safety of their allograft bio-implants; this includes stringent donor screening methods and release criteria. To obtain suitable donors, LifeNet Health maintains an extensive network of recovery partners. Additionally, LifeNet Health is a leading, federally designated Organ Procurement Organization. LifeNet Health only accepts donors from federally designated Organ Procurement Organizations and qualified tissue recovery partners. These partners are regularly audited to guarantee that their recovery process meets current FDA regulations, AATB standards and LifeNet Health's own stringent guidelines.

SELECTING THE IDEAL DONOR

Every donor for ViviGen must meet LifeNet Health's strict medical and behavioral risks assessment in addition to microbial and serological testing.



VIVIGEN BONE CELLS IN VITRO DO NOT ELICIT AN IMMUNE RESPONSE¹⁷

While allograft tissue has a proven safety and biocompatibility history as a bone graft substitute, LifeNet Health wanted to ensure the risk of a host immune reaction when implanting ViviGen was minimized. As shown in Figures 6 and 7, the marrow components are removed during the proprietary cleaning process, which minimizes the potential risk of an immune response. In addition, LifeNet Health wanted to test the safety of implanting lineage committed bone cells. A mixed lymphocyte reaction (MLR) assay was conducted to illustrate that bone cells derived from ViviGen in vitro do not elicit an immune response.¹⁷

The MLR assay has been recommended by the FDA to measure a functional immune response mediated by T-cells against foreign antigens.²⁴ In the MLR immune response assay, cells from one donor act as a stimulatory cell population and are mixed with a responding HLA-mismatched lymphocyte population. If the stimulatory cells are immunogenic, the lymphocytes respond by proliferating.

Bone cells derived from three ViviGen donors were assessed in the MLR assay to test their capacity to stimulate an immune response from allogeneic immune cells. HLA-mismatched peripheral blood mononuclear cells (PBMC; HemaCare Corporation, San Fernando Valley, CA) were purchased and used as responding cells. Lymphocytes from the same ViviGen donors were used as positive controls and mixed with PBMC. PBMC alone was used as a negative control. As expected, the lymphocytes stimulated a statistically significant proliferative response from the non-matched PBMCs ($p<0.05$; Figure 19) showing that the lymphocytes were immunogenic. In contrast, when ViviGen derived cells recovered from the same donors were combined with PBMCs, there was no proliferation, illustrating the absence of an immune response arising from the bone cells. Therefore, unmatched immune cells did not recognize the bone cells as foreign. This functional immune response assay supports the safety of ViviGen.

A Mixed Lymphocyte Reaction (MLR) Assay was conducted to ensure the bone cells derived from ViviGen are non-immunogenic.

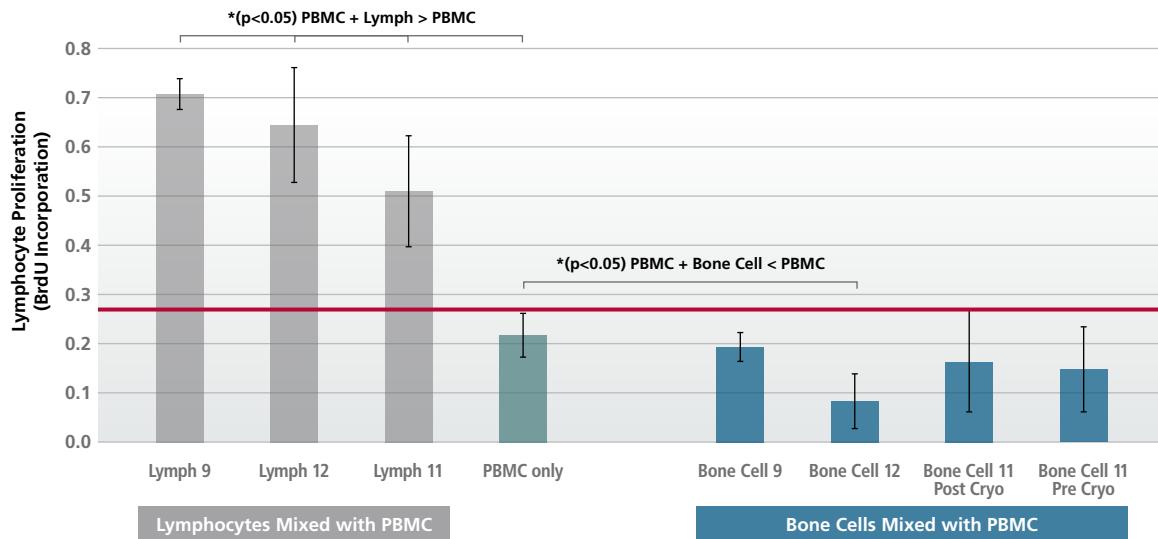


Figure 19: Mixed lymphocyte reaction. Lymphocytes from ViviGen donors were combined with PBMC to elicit an immune reaction (grey bars). ViviGen derived bone cells from the same donors were mixed with PBMC (blue bars). PBMC alone was used as a negative control (green bar).

VIVIGEN DERIVED BONE CELLS ARE NOT ANTIGEN PRESENTING CELLS.

To help explain the lack of immune cell proliferation response seen in the MLR assay, it is important to understand the surface antigen characteristics of bone cells. It is noted that all nucleated human cells possess major histocompatibility I (MHC I) class surface receptors which present intracellular proteins to immune cells and also identify the cells as "self". In addition, some cells also possess major histocompatibility class II (MHC II) surface receptors that present extracellular antigens to immune cells and are thus referred to as antigen presenting cells. However, some cells may either not express MHC II, express low levels of MHC II, or express altered conformations of MHC II receptors, and as a result may avoid detection by immune cells.^{25,26,27} In order to test this, ViviGen processed bone chips were stained for the presence of the MHC II antigen presentation receptors. As seen in Figure 20, immunohistochemistry for presence of MHC II receptors show that very few cells stain for MHC II receptors in the Haversian canals and no cells within the bone matrix stain positively for MHC II receptors in the final post-cryopreserved ViviGen product.¹⁷ This finding is consistent with the lack of immune cell proliferation response noted in the MLR assay.

Figure 20: Immunohistochemistry for MHC II in Post-Cryopreserved ViviGen.

20A) Few cells in the Haversian canal stained positively for MHC II (closed arrow). Cells inside the bone matrix and lining the Haversian canal were not stained positively for MHC II (open arrow).



20B) Cells within the bone matrix tissue do not stain positive for MHC II (open arrow).



PROCESSING

The processing of allograft tissue plays a major role in determining the properties of the graft that the surgeon receives and what is ultimately implanted into the patient. Processing is even more vital for a cellular allograft such as ViviGen due to the live cell or osteogenic component. LifeNet Health has scrutinized every step in the processing of ViviGen to achieve the highest level of cell viability and to identify factors that might reduce cell viability. This is done to provide a graft with the necessary properties for bone formation. There are many factors in the processing of ViviGen that could impact bone cell viability. These include:

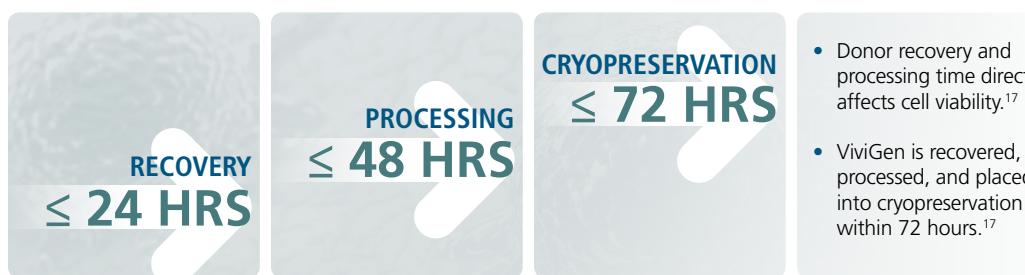
- Recovery and processing time
- Antibiotics used during processing
- Cryopreservation media
- Packaging
- Freezing and thawing protocol.

The processing of ViviGen is a proprietary method that confirms the live cells within the corticocancellous matrix are preserved and combined with demineralized bone from the same donor.

ASEPTIC PROCESSING

LifeNet Health utilizes aseptic techniques in ISO-certified clean rooms. Every lot of the final product is tested for sterility.

RECOVERY AND PROCESSING TIME



Processing time is an important factor in maintaining the highest level of cell viability once a donor becomes available. The duration between the time of donor death to cell recovery and the time of cell recovery to processing can have a negative effect on cell viability. The shorter the processing time, the higher the viability of bone cells.¹⁷

ViviGen is
cryopreserved
24 hours
faster than the
major cellular
allograft
competitors.¹⁶

PACKAGING

Packaging is an extension of a product. This is particularly important for a cryopreserved graft. The basic principle of successful cryopreservation while maintaining cell viability is a controlled slow freeze and a rapid thaw.²⁸ ViviGen's packaging design is unique. The thin walls of the ViviGen packaging allow for an efficient energy transfer, which facilitates a controlled freezing and a rapid thaw. The rapid heat transfer of the ViviGen packaging not only allows pouches of all sizes to thaw in less than 5 minutes, but is also vital for cell viability. The rapid thaw prevents ice crystals from forming intracellularly during thawing, ultimately maintaining viability. In addition, the port at the end of the ViviGen pouch allows for the quick and efficient removal of the cryopreservation media and rinsing solutions.



Rapid thawing is crucial for cell viability.

VIVIGEN PREPARATION

The viability of ViviGen bone cells is preserved until the final step of preparation for grafting in the operating room. LifeNet Health screened multiple post-thawing protocols using ViviGen derived bone cells as a model system. These bone cells were exposed to 100% cryopreservation media for 2 hours followed by rinsing with Lactated Ringer's supplemented with 5% Dextrose (D5LR). This mimics the process of retaining the tissue post-thawing in 100% cryopreservation media followed by rinsing with D5LR before implantation. ViviGen derived bone cells were 96% viable compared to cells in complete culture media (DMEM + 10% fetal bovine serum; Figure 21). In contrast, cells exposed to 30% v/v cryopreservation media in D5LR for 2 hours, which mimic rinsing the tissue with D5LR immediately after thawing and holding the tissue in D5LR for 2 hours, were only 62% viable compared to cells in complete culture media. Similarly, cells exposed to 30% v/v cryopreservation media in saline for 2 hours, which mimic rinsing the tissue with NaCl right after thawing and holding the tissue in NaCl for 2 hours, were only 52% viable compared to cells in complete culture media.

Based on these results, the thawing and post-thawing protocol for ViviGen was standardized as follows:

1. Quickly thaw ViviGen in the sealed pouch in $37 \pm 2^\circ\text{C}$ sterile saline.
2. ViviGen can be implanted immediately post-thawing or can remain in the ViviGen pouch containing 100% cryopreservation media for up to 2 hours.
3. Remove cryopreservation media.
4. Optional rinse with D5LR once immediately before implantation.

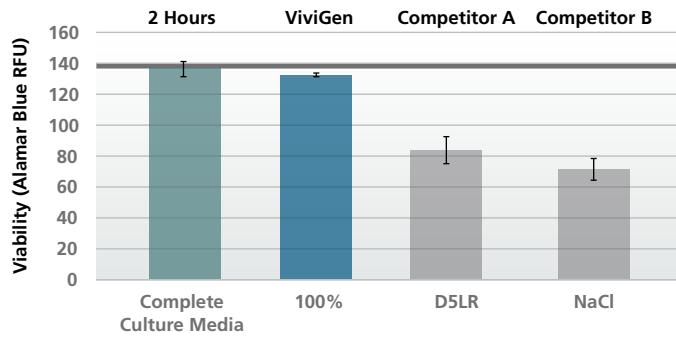


Figure 21: Viability for bone cells that were exposed to complete media (DMEM + 10% fetal bovine serum), 100% cryopreservation, 30% v/v cryopreservation media in D5LR, and 30% v/v cryopreservation media in saline for 2 hours. Viability was assessed using the Alamar blue assay with fluorescent excitation at 544 nm and emission at 592 nm. Mean \pm standard deviation (n=3) is presented.

CONCLUSION

The evidence presented here demonstrates the unique characteristics of ViviGen, which represent a paradigm shift in the field of bone repair. ViviGen comprises unique lineage committed bone cells shown to be the preferred cell type for bone formation by several studies. These bone cells in ViviGen have been shown to be viable, have the ability to proliferate, and are osteogenic. The processing time of ViviGen is 24 hours faster than that of competitor products, which ultimately contributes to an increase in cell viability. Other factors that maintain cell viability include ViviGen's unique processing reagents, proprietary cryopreservation media, optimal freezing and thawing characteristics, and preparation techniques in the operating room. Moreover, its novel packaging, specifically developed for cell-based products, allows all pouch sizes of ViviGen to thaw in 5 minutes or less, which is critical for maintaining cell viability.

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Findings from an in vitro assay are not always predictive of human clinical results.

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Editorial services were provided by Cactus Communications and funded by DePuy Synthes. The authors retained full control of the manuscript content.



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DSUS/SPN/1214/0607a 3/17 68-40-254.00