Application Note

OsteoMAX-XF™: A Novel Serum-free and Xeno-free Differentiation Medium for Accelerated Osteogenic Differentiation of Human Mesenchymal Stem Cells

Introduction

Stem cells are uniquely qualified for self-renewal and differentiation into mature somatic cells. They offer limitless potential for generating physiologically relevant, pathogen-free cells for multiple biological and clinical applications. Since the first haemopoietic stem cell (bone marrow) transplants over 40 years ago, cell replacement therapies using stem cells or their differentiated progeny have become more widespread, with applications in drug discovery, target identification, and high throughput screening in toxicology studies.

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can be isolated from various tissues and harbor the capacity to differentiate into bone, cartilage, and fat cells; however, under certain conditions they may also generate other lineages, such as neurons and muscle cells. MSCs, both autologous and allogeneic, have been used in multiple clinical trials for managing bone and cartilage defects. MSC-derived osteoblasts may also have applications in fracture repairs and generation of stem cell-derived bone grafts, and spinal fusion. The generation of osteoblasts from hMSCs has enormous value in developing and evaluating drugs for alleviation of orthopedic conditions. Similarly, the ability to generate MSCs from iPSc can unwrap the possibility of generating disease-specific MSCs, which may be useful in monitoring orthopedic disease progression and examining therapeutic efficacies of drugs.

Difficulties in directing MSCs and other stem cells to generate fully functional, specific cell types prevent the full exploitation of the enormous potential of these multipotent and pluripotent cells. Generating specific cell types in large scale, in a reproducible and cost effective manner, is even more challenging. One of the principal factors to consider here is the need for specific cell culture medium where variables and animal derived components are eliminated. This is particularly significant for osteoblast production since osteogenic differentiation protocols invariably include serum that contains essential components for generation of these cells.

To overcome these difficulties, EMD Millipore, in collaboration with Plasticell Ltd., has developed OsteoMAX-XF™ differentiation media, a novel, serum-free, xeno-free, differentiation medium for the generation of osteoblasts from human MSCs. This medium may be particularly suitable for drug discovery and clinical development. By screening roughly 3,500 combinations of serum and xeno-free media, we discovered a remarkably efficient, fully defined, reproducible medium to promote the differentiation of hMSCs into osteoblasts, offering an excellent, cost effective method to produce large amounts of human bone cells for multiple applications.

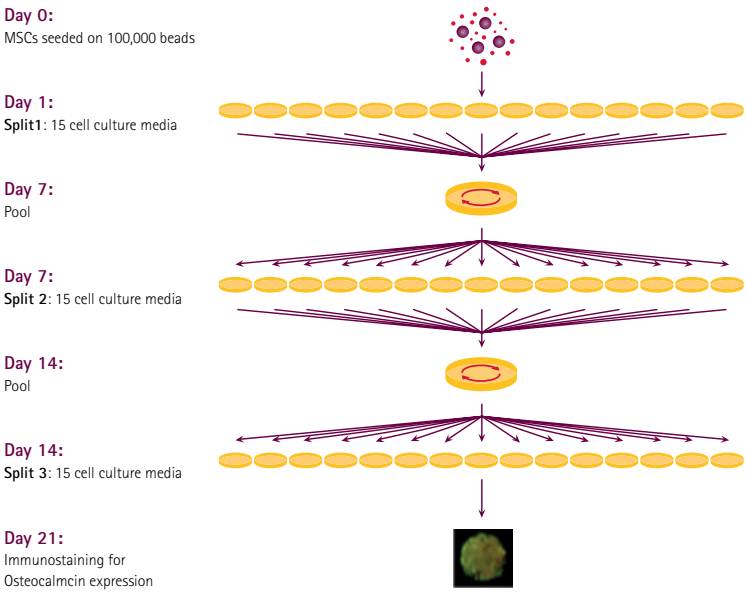

Figure 1.

Combicult® screen design. The iterative process of systematically splitting, culturing and pooling samples all possible combinations of media in a pre-determined matrix.

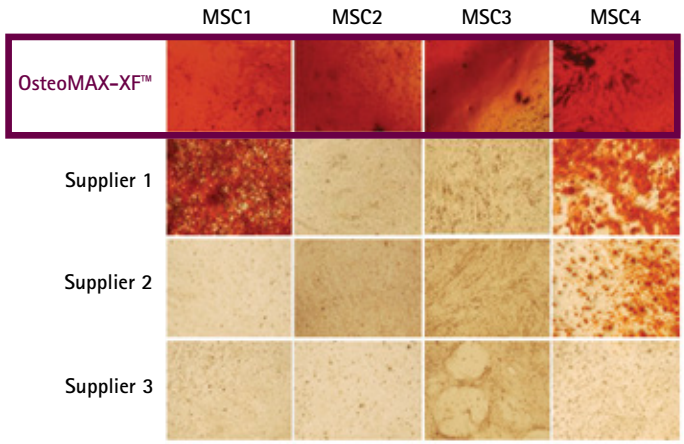

Figure 2.

Validation and refinement of serum-free differentiation protocols determined by CombiCult® technology resulted in identification of protocols that drive MSC differentiation to mineralizing osteocytes. The novel protocols are more effective than commercially available kits and give consistent results across multiple cell lines. Alizarin red staining of MSC cultures differentiated for 28 days; cell lines 1-3: bone marrow-derived MSC; cell line 4: adipose-derived MSC.

Materials and Methods

The discovery of OsteoMAX-XF™ differentiation medium was made possible by the use of Plasticell's Combicult® high-throughput combinatorial platform, which combines miniaturization of cell culture on microcarrier beads, a pooling/splitting protocol and a unique tagging system to allow multiplexing of thousands of experiments in one screen (Figure 1). In this system, stem cells grown on microcarrier beads are shuffled randomly, stepwise through multiple differentiation media using a split-pool method. The iterative process of systematically splitting, culturing and pooling samples all possible combinations of media in a pre-determined matrix. Each medium is spiked with a unique fluorescent tag that attaches to the beads. At the end of the differentiation process, beads bearing differentiated cells are identified by a screening assay, such as immunostaining or reporter gene expression. Individual positive beads are isolated using an automated large-particle sorter. The cell culture history of each positive bead is then deduced by analysis of the fluorescent tags attached to the bead. Typically 100 or more positive differentiation protocols are discovered in each screen. These are then analyzed using bespoke Ariadne® bioinformatics software, which uses criteria such as hierarchical clustering and probability analysis to select the optimal protocols for further validation.

We screened 15 different serum-free media on each of the 3 stages of differentiation, thereby testing 3,375 unique differentiation protocols. At the end of each experiment, beads were analyzed for cells expressing osteocalcin, a marker of osteoblasts. We found 97 unique protocols that were analyzed using bioinformatics software, which helped us identify those predicted to be the most efficient and optimal. Of these, 12 were selected for validation studies and were tested on microcarrier beads, as used in the screening experiment, in a monolayer culture system. From these validation experiments the most effective protocol, B372, was identified. In particular, this protocol gave very extensive mineralization of cultures compared to other selected protocols.

The next step was to optimize protocol B372 and the formulation for laboratory and larger-scale use. It was found that mixing together the media components from the 3 stages of differentiation into one formulation provided more efficient differentiation than the serial protocol. This 'one-application' formulation was termed B372+ (now known commercially as OsteoMAX-XF™, EMD Millipore Cat. No. SCM121) and was tested across several sources of MSCs and compared to other commercially available osteogenic differentiation kits (Figure 2). In contrast to the other kits that contain

serum, OsteoMAX-XF™ medium worked consistently well across all tested MSC cell lines.

Furthermore, by studying the time course of differentiation we found that OsteoMAX-XF™ rapidly generates mineralized osteoblasts, as early as day 7 consistently across several MSC cell lines (Figure 3).

Further experiments produced similar mineralization kinetics when human bone-marrow derived, adipose derived and ES cell derived MSCs were exposed to OsteoMAX-XF™ (Figure 3B). It was also determined that MSCs from bone marrow, adipose tissue and those derived from human embryonic stem cells (hESCs), whether cultured in xeno-free medium or serum-based medium, were able to efficiently differentiate into osteocytes when exposed to OsteoMAX-XF™ medium, as demonstrated by Alizarin Red and alkaline phosphatase staining (Figures 4 and 5). Since many MSC are isolated and cultured in serum-containing medium, it is important to show that they can be readily adapted to this novel serum-free differentiation medium. Interestingly, bone-marrow derived MSC (from EMD Millipore and Lonza) exhibited the most rapid and extensive mineralization as compared to adipose-derived MSCs and human ESC-derived MSCs (Figures 4 and 5). However, regardless of tissue origins, both adipose- and human ESC-derived MSC achieved maximal differentiation by day 22-24 (Figures 4 and 5).

For scale-up applications, it is desirable to have raw material components that are not only stable at 37 °C, but also have a long shelf life. To determine the stability of OsteoMAX-XF™ medium, the supplement was incubated at 37 °C for 7 days and subjected to two freeze-thaw cycles. There was no loss of activity as compared to the unstressed control. Using the Arrhenius model to extrapolate, the OsteoMAX-XF™ supplement was calculated to have a shelf-life of at least 2 years at -20 °C. An aliquot of the supplement that had been set aside for 1 year at -20 °C also yielded comparable activity. Activity was quantified by collecting conditioned medium from cells that had been exposed to OsteoMAX-XF™ for various lengths of time, ranging from 24 hours at room temperature (RT) to 7 days at 37 °C. Alkaline phosphatase activity was determined using the Quantitative Alkaline Phosphatase ES Characterization Kit (EMD Millipore Cat. No. SCR066). Figure 6 illustrates the dose-dependent activity of OsteoMAX-XF™ Medium. Even at accelerated temperatures, no reduction in activity was observed.

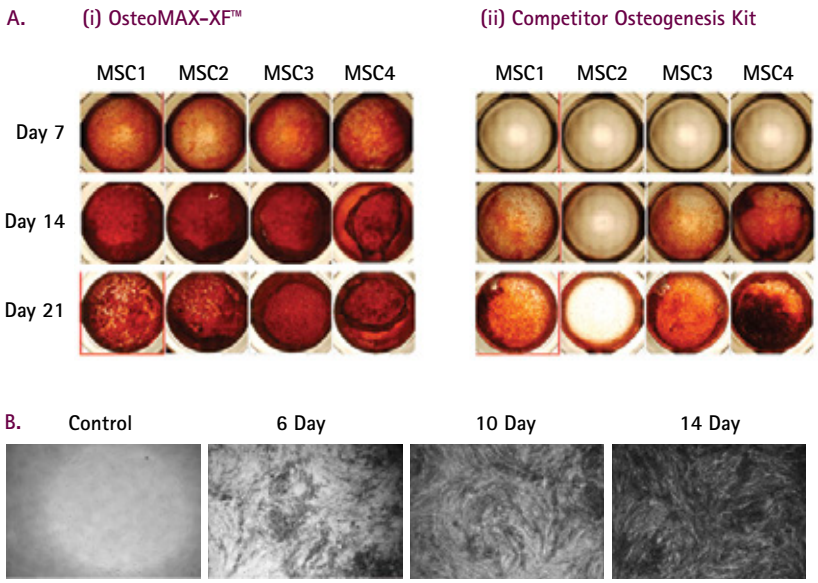

Figure 3.

Differentiation of multiple MSC cell lines using (A) OsteoMAX-XF™ Differentiation Medium (EMD Millipore Cat. No. SCM121); (ii) Supplier LT Osteogenic Differentiation Kit. Differentiation was induced over 21 days in 48-well plate cultures of four different human MSC cell lines (Promocell and Life Technologies). Alizarin Red staining of representative wells at day 7, 14 and 21 are shown. B) Mineralization kinetics of human bone-marrow derived MSC (Cat. No. SCC034) differentiated in OsteoMAX-XF™ Medium.

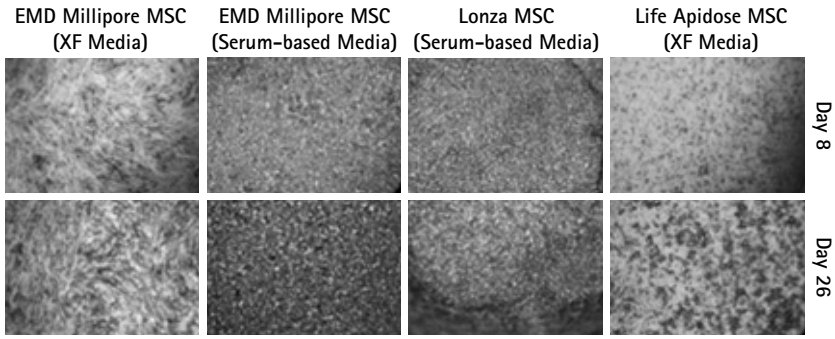

Figure 4.

Rapid mineralization of multiple MSC lines in OsteoMAX-XF™ Differentiation Medium. Cell lines were expanded in serum-based medium or xeno-free culture medium (Cat. No. SCM037) before exposing to OsteoMAX-XF™.

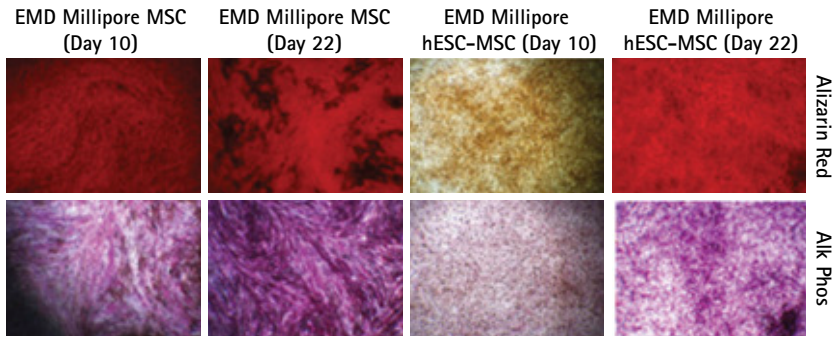

Figure 5.

Differentiation kinetics of human bone marrow (BM)-derived MSC (Cat. No. SCC034) and human ESC-derived MSC (Cat. No. SCC036) in OsteoMAX-XFTM medium.. Human ESC-derived MSC exhibit slower differentiation kinetics as compared to BM-derived MSC. However, by day 22-24, maximal differentiation is achieved in both cell types.

Finally, we have demonstrated that this formulation is suitable for the scale-up of osteoblast production as would be required for clinical applications or drug discovery use. In proof-of principle experiments, performing differentiation in roller bottles and cell factories, we were able to scale up differentiation from multi-well culture plates to volumes more compatible with these large-scale applications. For example, approximately 10^7 mineralizing osteoblasts could be obtained from a 600 cm^2 cell culture flask or 500 cm^2 roller bottle (data not shown). Osteoblast production could be further scaled by using more culture vessels, the limiting factor being the availability of the starting MSC material.

Conclusions

We describe the discovery and commercial development of a novel, serum-free and xeno-free MSC osteogenic differentiation medium. The formulation is extremely efficient, generating mineralized cultures in under 7 days, and works consistently across all sources of MSCs tested, including those derived from pluripotent stem cells. This is particularly important for generation of autologous therapies or disease and patient-specific cells for drug screening. Differentiation can be scaled to generate the number of cells required for large-scale applications. OsteoMAX-XF™ medium offers a reliable, simple, cost effective method to generate human osteoblasts for research, clinical and drug discovery applications.

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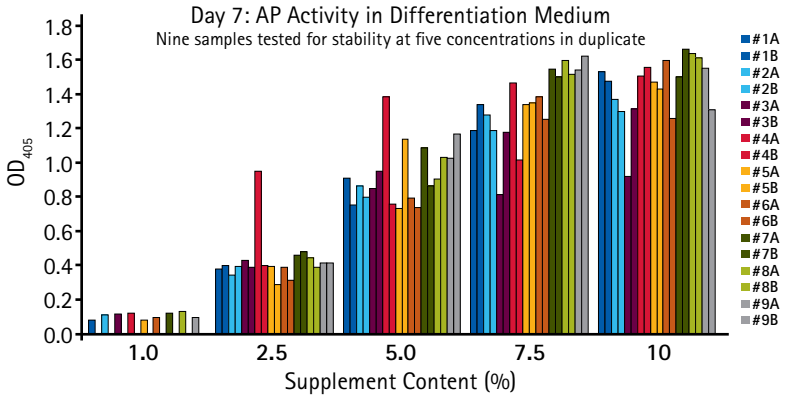

Figure 6.

Quantitative alkaline phosphatase determination of OsteoMAX-XF™ stability. OsteoMAX™ medium was subjected to stressed conditions ranging from 24 hours at RT to 7 days at 37 °C. Cells were exposed to varying concentrations of the stressed supplements for 7 days before the conditioned medium was collected for quantitative determination of AP activity. EMD Millipore's Quantitative Alkaline Phosphatase ES Characterization Kit (Cat. No. SCR066) was used. Stressed conditions were: #1: One freeze-thaw cycle (control); #2: Two freeze-thaw cycles; #3: Two freeze-thaw cycles, 24 hours at RT; #4: Two freeze- thaw cycles, 2 days at RT; #5: Two freeze-thaw cycles, 3 days at RT; #6: Two freeze-thaw cycles, 4 days at RT; #7: Two freeze-thaw cycles, 6 days at RT; #8: Two freeze-thaw cycles, 8 days at RT; #9: Two freeze-thaw cycles, 7 days at 37°C.

Description	Cat. No.
OsteoMAX-XF™ Medium	SCM121
FibroGRO™ Xeno-Free Human Fibroblast Expansion Medium	SCM037
Human Mesenchymal-LS Expansion Medium	SCM023
Human Adipose Mesenchymal Stem Cells	SCC038, SCR038
Human Mesenchymal Stem Cell Kit (Derived from Bone-Marrow)	SCC034, SCR108
Human Mesenchymal Stem Cells (derived from hES cells)	SCC036
Quantitative Alkaline Phosphatase ES Characterization Kit	SCR066
Alkaline Phosphatase Detection Kit	SCR004
Alizarin-Red Staining Solution	TMS-008-C
Human Mesenchymal Stem Cell Characterization Kit	SCR067
FlowCellect™ Human Mesenchymal Stem Cell Characterization Kit	FCSC100184

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