Applying mesenchymal stem cell technology to drug discovery and cell therapy

An important application of stem cells, in addition to cell therapy, is in the discovery of novel drugs, both those that act in a conventional manner and regenerative drugs that promote endogenous cells to repair lost or diseased tissue, overcoming the need for cell replacement therapies in some cases. This article describes how Plasticell has developed an efficient and reproducible cell culture medium to promote the differentiation of human mesenchymal stem cells to osteoblasts, enabling the production of human bone cells for screening of drugs for treatment of osteogenic diseases and promotion of bone generation and repair.

Stem cells are extraordinary cells, capable of self-renewal and differentiation to mature somatic cell types, both *in vivo* and *in vitro*. As such they offer the potential of limitless, consistent supplies of physiologically relevant cells from validated pathogen-free sources for use in multiple health care applications. Since the first haemopoietic stem cell (bone marrow) transplants more than 40 years ago, cell replacement therapies using stem cells and their differentiated progeny have become more widespread, particularly in the past few years. Stem cells also have application in drug discovery, giving many advantages over currently used methods at all stages of the process from target identification to high throughput screening through to toxicology studies.

Multipotent stem cells in drug discovery

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can be isolated from several tissues such as bone marrow, adipose tissue and umbilical cord blood and can be generated in vitro from embryonic and induced pluripotent stem cells. They typically have the capacity to differentiate into bone, cartilage and fat cells, although under certain conditions they have also been shown to generate other lineages including neurons and muscle. MSCs, both autologous and allogeneic, have already been the subject of more than 120 clinical trials for the treatment of a variety of diseases, including bone and cartilage defects. MSC-derived osteoblasts could have multiple clinical applications, including fracture repair, generation of stem cell derived bone grafts, and spinal fusion.

As an example, about 2.2 million bone graft procedures are performed worldwide every year. However, current treatments have several serious drawbacks that could be overcome through the application of stem cell therapies; such as, for autografts, pain and non-healing at the collection site and for allografts, immune-rejection and insufficient supply of material. Moreover, the generation of osteoblasts from hMSCs would be of great value in drug discovery for orthopaedic conditions; in target identification, *in vitro* modelling of disease and high throughput drug screening. The capability to generate MSCs from iPS cells also opens the possibility of generating disease-specific MSCs that could be used to study orthopaedic disease progression and phenotype in vitro as well as to study the effect of orthopaedic drugs across multiple genetic backgrounds.

Despite the enormous potential MSCs and other stem cells offer, full realisation of their application is currently hampered by the difficulty in routinely directing stem cell differentiation *in vitro* to generate fully functional, specific cell types of choice. Being able to do this at large scale in a reproducible and cost-effective manner, as required for cell therapy and drug discovery applications, is

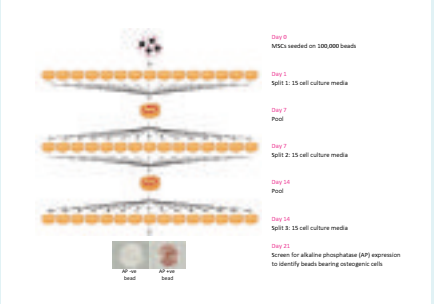
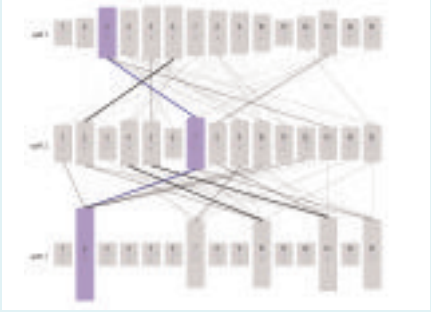


Fig. 1. Combicult® screen design: hMSCs were seeded onto beads and subjected to a 15x15x15 CombiCult® screen which multiplexed 3,375 protocols. Alkaline Phosphatase (AP) was used as a readout assay.



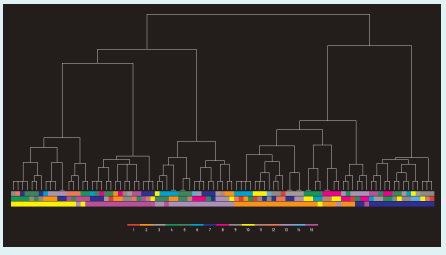


Fig. 2. Ariadne™ protocol deconvolution and analysis:
(left) Visual representation of the hit protocols overlayed on the screening matrix. Bar heights correspond to the number of positive beads that sampled each condition. Top-ranking protocol B372 is highlighted in purple.
(right) Dendrogram showing heirachical clustering of hit protocols.

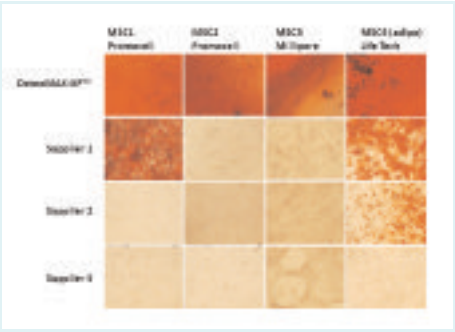


Fig 3: Validation and refinement of serum-free differentiation protocols determined by CombiCult® resulted in identification of protocols that drive MSC differentiation to mineralizing osteocytes. The novel protocols are more effective than commercially available kits and give consistent results across 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.

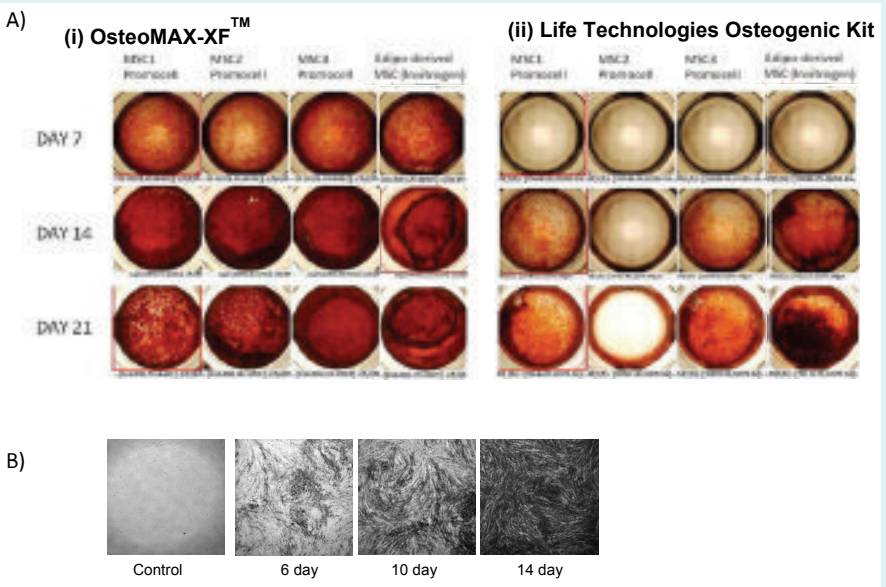


Fig 4. A) Differentiation of multiple MSC cell lines using (i) OsteoMAX-XF™ Differentiation Medium (Merck Millipore Cat. No. SCM121); (ii) LIFE STEMPRO® Osteogenesis Differentiation Kit (Cat. No. A10072-01). Differentiation was induced over 21 days in 48-well plate cultures of four different human MSC cell lines (Promocell and Life Technologies). Alizarin Red staining of representative wells at day 7, 14 and 21 are shown. B) Mineralisation kinetics of human bone-marrow derived MSC (Merck Millipore Cat. No. SCC034) differentiated in OsteoMAX-XF™.

even more of a challenge. One of the principal factors that needs to be considered is the cell culture medium and in particular the elimination of variable and animal-derived components such as serum. This is especially significant for osteoblast production, since osteogenic differentiation protocols invariably include serum, suggesting it contains essential components for generation of these cells.

Bearing these issues in mind, Plasticell aimed to develop novel, serum-free, xeno-free differentiation protocols for the generation of osteoblasts from hMSCs suitable for both clinical application and drug discovery use. By performing a screen of just under 3,500 combinations of serum- and xeno-free medium, the company discovered a remarkably efficient, fully defined, reproducible medium to promote the differentiation of hMSCs to osteoblasts. This offers an efficient and cost-effective method to produce large amounts of human bone cells for multiple applications such as cell therapy and drug discovery. Other applications, such as the use of these cells for *in vitro* modelling of osteogenic conditions and testing of novel scaffolds and medical devices for bone regeneration, are also envisaged.

Study design and results

In order to discover novel serum-free, xeno-free osteogenic MSC differentiation protocols, Plasticell utilised its high-throughput combinatorial platform Combicult®, which combines miniaturisation of cell culture on microcarriers, a pooling/splitting protocol and a unique tagging system to allow multiplexing of thousands of experiments in one screen (Fig 1). Stem cells grown on microcarrier beads are shuffled randomly, stepwise through multiple differentiation media using a split-pool method. The iterative process of splitting, culturing and pooling, systematically samples all possible combinations of media in a predetermined 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, eg immunostaining or reporter gene expression, and 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 analysed using bespoke bioinformatics software (Aridane™) which uses criteria such as hierarchical clustering and probability analysis to select the optimal protocols for further validation.

In this case, Plasticell screened 15 different serum-free media on each of three stages of differentiation, thereby testing 3,375 unique differentiation protocols (Fig.1). The researchers assayed beads at the end of the experiment for cells expressing alkaline phosphatase, a marker of osteoblasts. Ninety-seven unique protocols were discovered (Fig 2) and the bioinformatics software, Ariadne™, was used to analyse these and identify those predicted to be the most efficient and optimal. Twelve were selected for validation studies and were tested on microcarrier beads, as used in the screening experiment and in a monolayer culture system. From these validation experiments, the most effective protocol, B372, was identified. In particular, this protocol gave very extensive mineralisation of cultures compared to other selected protocols.

Having selected protocol B372 as the lead candidate, the researchers sought to optimise the formulation for laboratory and larger-scale use. In particular, it was found that mixing together the media components from the three stages of differentiation into one formulation gave as, if not more, efficient differentiation than the serial protocol. This 'one-application' formulation, termed B372+ (now known commercially as OsteoMAX-XF™, Merck Millipore Cat. No. SCM121), was tested across several sources of MSCs and compared to other osteogenic differentiation kits available on the market (Fig 3). In contrast to the other kits, all of which contain serum, OsteoMAX-XF™ worked consistently well across all tested MSC cell lines. Furthermore by studying the timecourse of differentiation it was found that OsteoMAX-XF™ very rapidly generated mineralising osteoblasts, as early as day 7, again consistently across several MSC cell lines (Fig 4).

Research kit development

Following discovery of B372+ medium at Plasticell's laboratories, the formulation was licensed to Merck Millipore for further

development into a kit available for sale to researchers (OsteoMAX-XF™ Differentiation Medium, Cat. No. SCM121). In experiments carried out by Merck Millipore, similar mineralisation kinetics were observed when their human bone-marrow derived MSCs were exposed to OsteoMAX-XF™ (Fig 4).

Furthermore, it was also determined that MSCs sourced from bone marrow, adipose tissue or derived from human embryonic stem cells (hESCs), whether cultured in xeno-free medium or serum-based medium, were all able to efficiently differentiate into osteocytes when exposed to OsteoMAX-XF™ as demonstrated by alizarin red and alkaline phosphatase staining (Figs 5 and 6). Since many MSC cells are isolated and cultured in serum-containing medium, it is important to show that they can be readily adapted to this serum-free differentiation medium. Mineralisation was readily observable with simple microscopic inspection. Interestingly, bone marrow derived MSC (from both Millipore and Lonza) exhibited the most rapid and extensive mineralisation as compared to adipose-derived MSC and human ESC-derived MSC. However, regardless of tissue origins, both adipose- and human ESC-derived MSC achieved maximal differentiation by day 22-24.

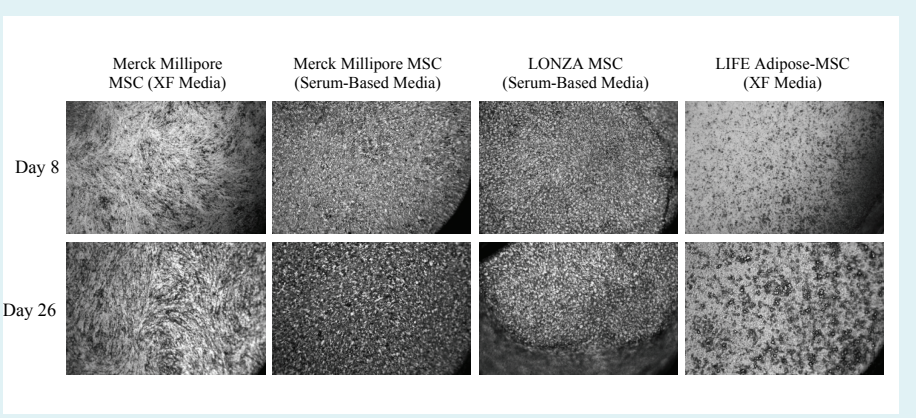


Fig 5. Rapid mineralisation of multiple MSC lines in OsteoMAX-XF™ Differentiation Medium: Cell lines were expanded in serum-based medium or XF culture medium (Merck Millipore Cat. No. SCM037) before being exposed to OsteoMAX-XF™.

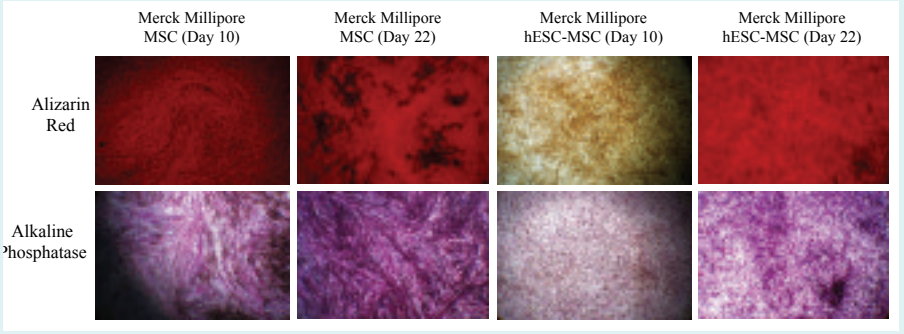


Fig. 6. Differentiation kinetics of human BM derived MSC (Merck Millipore Cat. No. SCC034) and human ESC-derived MSC (Merck Millipore Cat. No. SCC036) in OsteoMAX-XF™: Human ESC-derived MSC exhibit slower differentiation kinetics as compared to BM-derived MSC. However by day 22-24, maximal differentiation is achieved in both cell types.

Scale-up applications

For scale-up applications, it is desirable to have raw material components that are not only stable at 37°C, a temperature that is frequently used to grow MSC, but that also have long expiration dates to help reduce manufacturing costs. To determine the stability of OsteoMAX-XF™, the supplement was incubated at 37°C for seven days and underwent two freeze-thaws. Results from this accelerated stability study indicated no loss of activity as compared to the unstressed control. Using the Arrhenius Model to extrapolate, the OsteoMAX-XF™ supplement has an expiration date of greater than two years at -20°C. An aliquot of the supplement that had been set aside for one year at -20°C also yielded comparable activity. Activity was quantitatively assessed by collecting conditioned medium from cells that had been exposed to accelerated OsteoMAX-XF™ supplement ranging from 24 hours at room temperature to seven days at 37°C. Alkaline phosphatase activity was determined using the Quantitative Alkaline Phosphatase ES Characterization Kit (Merck Millipore Cat. No. SCR066). Even at accelerated temperatures, no reduction of activities was observed.

Finally, Plasticell has demonstrated that this formulation is suitable for the scale-up of osteoblast production as would be required for clinical or drug discovery use.

In proof-of principle experiments, performing differentiation in roller bottles and cell factories, the researchers were able to scale up differentiation from multi-well culture plates to volumes more compatible with these large-scale applications.

For example, about 10^7 mineralising osteoblasts can be obtained from a 600 cm^2 cell factory or 500 cm^2 roller bottle. Osteoblast production could be further scaled by utilising more culture vessels, the limiting factor being the availability of the starting MSC material.

In summary

To conclude, a novel, serum-free, xeno-free MSC osteogenic differentiation medium has been discovered and successfully commercially developed. The formulation is extremely efficient, generating mineralised cultures in under seven days and works consistently across all sources of MSC tested, including those derived from pluripotent stem cells. This is particularly important for the generation of autologous therapies or disease- and patient-specific cells for drug screening. Differentiation can be scaled to generate the numbers of cells required for large-scale applications. OsteoMAX-XF™ offers a reliable, simple, cost-effective method to generate human osteoblasts for research, clinical and drug discovery applications.

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