

A Micro Chamber-Based Instantaneous Synthesis of Humanised the Bone Marrow Micro-Tympanic and Stacks

Abstract

There is no high-throughput microtissue platform for generating microbone in bone marrow. Here, we describe a method for the assembly of microtissue arrays from bone marrow stromal cells (BMSCs) *in vitro* and their maturation into bone marrow ossicles *in vivo*. Discs with arrays of 50 microwells were used to assemble microtissues of 3×10⁵ BMSCs each on nylon mesh supports. Microtissues were cultured in chondrogenic induction medium and then in hypertrophic medium, which promotes endochondral ossification. They were then transplanted into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice and reconstituted into bone marrow ossicles. Mice were transplanted with 10⁵ CD34⁺ cells from human cord blood. Microbone contained more human CD45⁺ cells than mouse bone marrow, but almost no human CD34⁺ progenitor cells. Human hematopoietic progenitor cells cycle rapidly at an unphysiological rate in the mouse bone marrow and the decreased CD34⁺ cell content within the ossicles supports the hypothesis that the humanized niche better controls progenitor cell cycling. Microtissue assemblies within microwells connected by nylon membrane supports offer an elegant method for the fabrication and manipulation of microtissue assemblies with bone organ-like properties. More generally, this approach and platform will help bridge the gap between *in vitro* microtissue manipulation and *in vivo* microtissue transplantation.

Keywords: Microtissue • Micro-ossicle • Bone Marrow • Mesenchymal stem cell • Bone marrow stromal cell • Hematopoietic stem cell

Introduction

Microtissue cell culture models have shown that three-dimensional (3D) tissue appears to better mimic the organization of tissue compared to two-dimensional (2D) culture, and that recent technological advances have made microtissues more efficient. It is being used more and more as it has become available in tissue culture. How best to combine multiple micro-tissues on a large scale and to create larger tissues and organs remains an active area of research. An early study by Kelm et al. They used microtissues as building blocks, fusing smaller microtissues to form larger ones. Similarly, microtissues formed from different types of cells stack on top of each other to form more complex or physically larger macro-tissues. Recently, 3D printing has been used to imprint macrostructures onto microfabrics. The lack of blood vessels limits the number of microtissues that can assemble into larger tissues before nutrient diffusion limitations impair tissue function or viability. Because of this limitation, the elegance and usefulness of microtissues can be maximized by simply designing a system that maintains each microtissue as a separate entity and allows efficient processing or manipulation of multiple microtissue entities. There are many cases where it can be used to a limited extent. This paper focused on developing methods to manipulate microtissue array[1,2].

Aggregate into microtissues that can be stimulated with growth factors to form cartilage-like tissue *in vitro*. Although the tissue may look like cartilage, BMSCs tend to activate unique hypertrophic signaling pathways and these tissues develop to form bone and bone marrow when transplanted *in vivo*. Microtissues mineralize at the surface, followed by the

Arunodaya Bisht*

Department of Stem Cell and Research, India

*Author for correspondence:

bishtarun@gmail.com

Received: 01-June-2023, Manuscript No. srrm-23-101888; **Editor assigned:** 05-June-2023, Pre-QC No. srrm-23-101888 (PQ); **Reviewed:** 19-June-2023, QC No. srrm-23-101888; **Revised:** 24-June-2023, Manuscript No. srrm-23-101888 (R); **Published:** 30-June-2023, DOI: 10.37532/srrm.2023.6(3).69-72

gradual replacement of the cartilage-like nuclear template by myeloid structures, and finally microbone formation. Because these ossicles are formed from and contain human stromal cells, some researchers have used these tissues specifically to study human cell biology and to model disease. Studies have shown that these humanized bone marrow organs are capable of (1) superior engraftment of healthy human hematopoietic stem/progenitor cells (HSPCs) into mouse bone marrow, as well as engraftment of malignant human hematopoietic xenografts that do not develop in mice; It is shown that it can be compared and supported. Bone marrow proliferates readily and (3) these ossicles are more supportive of human prostate cancer cell populations than mouse bone marrow. Taken together, these data suggest that humanized ossicles are a better tool than mouse bone marrow for studying human cell biology and disease [3,4].

Serafini et al. formed microtissues from 3×10^5 BMSCs each cultured in chondrogenic induction medium supplemented with transforming growth factor- β (TGF- β 1 or TGF- β 3). The microtissues he cultured for three weeks and transplanted into mice. Over 16 individual microtissues were implanted in each animal, and by 8 weeks the microtissues had mineralized and contained nucleus pulposus structures, giving rise to numerous replicating microbones. In this case, the authors followed a commonly reported BMSC protocol for chondrogenic induction, but reported the formation of bone marrow ossicles. In another approach, Scotti et al. 5×10^5 BMSCs were seeded on Transwell membranes and cultured for 3 weeks in chondrogenic medium supplemented with TGF- β 1, followed by 2 weeks in hypertrophic medium (without TGF β 1 but with β -glycerophosphate and thyroxine). Did. Larger auditory ossicles formed. Instead of multiple repeated micro-knuckles as described. Therefore, the two main approaches described in the literature are the formation of multiple ossicles or the formation of 1-2 larger tissues.

The advantage of large ossicles is that there is more continuous volume to convert to bone marrow. Some studies have used this large number of cells to inject directly into these large tissues. Disadvantages of using a single large ossicle are the possible delayed

vascularization, the low number of replicates, and the possible need to increase the number of animals in the study. Although it is possible to generate bone marrow by an endochondral or an endomembrane differentiation program in mice, generating bone marrow by endochondral ossification best mimics most skeletal development. Since aggregation of cells during microtissue assembly mimics mesenchymal condensation, microtissues are thought to be a suitable substrate for this process compared to seeding cells at low density on large scaffolds. The number of cells within a single microtissue is usually adjusted to limit the maximum diameter and proper diffusion of metabolites within the tissue. For chondrogenic cultures, BMSCs are often pelleted into tissues of $2-5 \times 10^5$ cells each. By acting on this cell field, it would be possible to reliably generate a cartilage template that would be remodeled in the bone marrow upon transplantation into mice, theoretically allowing replication of endochondral ossification. Note that much work remains on this topic to fully elucidate the differentiation processes leading to ossicles. A disadvantage of using replicated microbones is that they are difficult to manipulate and are exacerbated by migration of the microtissues under the loose skin (face) of the mouse. This article addresses this microtissue/microbone management challenge [5,6].

Results

Experimental design

Microtissue array cassettes were prepared and used for microtissue array culture as indicated. After induced culture, the microtissue arrays were transplanted into mice and reconstituted into ossicles containing bone marrow nuclei. After 8 weeks of reconstitution, mice were irradiated and engrafted with human cord blood-derived CD34+ cells. We demonstrate different schedules for cell culture treatment, remodeling, and CD34+ cell transplantation.

In vitro growth, differentiation, and viability of microtissues

BMSCs seeded on the microwell array platform were characterized over a 5-week *in vitro* culture period. BMSCs formed discrete microtissues that increased in size and maintained viability as assessed by live/dead staining assays over a 5-week culture period.

The microtissue was smooth and shiny, resembling a typical BMSC pellet culture maintained in chondrogenic induction medium. Micro-CT analysis and alizarin red S staining revealed that microtissues maintained in thickened medium for the last two weeks of the 5-week culture period were mineralized, but maintained in chondrogenic medium throughout the 5-week culture period. It was found that the microtissues obtained were not calcified. Alcian blue staining showed high glycosaminoglycan (GAG) content in all microtissues cultured under both differentiation conditions. Approximately 50 microtissue arrays, each composed of 3×10^5 human BMSCs, were assembled on nylon mesh sheets using a microwell platform. An extracellular microtissue matrix forms around the fibers of the nylon mesh, firmly anchoring each microtissue to the mesh. Microtissue arrays were easily manipulated and were transferred to standard 6-well plates for most of the culture period and then transplanted into NSG mice. After 8 weeks of subcutaneous culture in mice, the microtissues transformed into microbones containing a mineralized shell of osteoid tissue and a bone marrow core. A mineralized, bony-like shell was evident on micro-CT images, and medullary structures were evident in histological sections of ossicles. A previous study that observed remodeling reported microtissue mineralization in the first 3–4 weeks after implantation in mice, followed by progressive replacement of the cartilage/bone-like core with medullary stroma. Similar to in the ossicles, similar to previously published studies by Bianco's team, we observed the development of mineralized tissue resembling trabeculae and cortical bone [7-10].

We conditioned mice bearing microbone arrays with sublethal irradiation and transplanted human cord blood-derived CD34+ cells into the animals. After 8 weeks, we compared levels of human hematopoietic cells in the bone marrow and ossicles of mice. The total content of hCD45+ cells was higher in humanized ossicles than in mouse bone marrow, whereas the content of human CD34+ cells was higher in mouse bone marrow than in humanized ossicles. Similar percentages of CD34+ cells in mouse bone marrow were observed in previous studies in which human hematopoietic

cells were transplanted intravenously (up to approximately 15% of total human bone marrow CD45 cells). The reduced number of human CD34+ progenitor cells in humanized ossicles compared to mouse bone marrow was consistent with previous studies reporting fewer circular human HSPCs in humanized ossicles than in corresponding mouse bone marrow. Human HSPCs are known to circulate in the mouse medulla instead of remaining quiescent, resulting in an increase in the number of human HSPCs in the mouse medulla. Fewer CD34+ HSPCs in humanized ossicles compared to corresponding mouse bone marrow may reflect reduced cycling of human progenitor cells in ossicles. In this sense, the behavior of human HSPCs in humanized ossicles better recapitulated their physiological behavior than the corresponding human HSPCs in mouse bone marrow. Therefore, as shown previously, humanized bone marrow is likely to provide a more physiological mimic than mouse bone marrow.

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