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Finding the winning combination

Combinatorial screening of three dimensional niches to guide stem cell osteogenesis

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The ability to predict and guide stem cell differentiation remains a major challenge in regenerative medicine. Numerous dynamic microenvironmental cues often provide synergistic or combinatorial signals that influence the fate of stem cells, and ultimately drive functional tissue formation. This interplay between microenvironmental cues within tissues is under intense investigation. Our goal was to better understand this interplay within the framework of a systematic 3D platform that would enable high-throughput screening (HTS) of factors that contribute to stem cell fate decisions. It is important that such platforms provide valid biomimetic microenvironments, which can be translated to macroscale constructs. Specifically, we reported on a technique for screening of combinatorial 3D niches to guide the osteogenic differentiation of human mesenchymal stem cells (hMSCs). This platform offers a rapid, cost-effective and multiplexed approach for a variety of tissue engineering applications.

Keywords: biomaterials, high-throughput screening, stem cell differentiation, biomimetics, tissue engineering

Abbreviations: HTS, high-throughput screening; hMSCs, human mesenchymal stem cells; ECM, extracellular matrix; 2D, two dimensional; 3D, three dimensional; FN, fibronectin; LN, laminin; OCN, osteocalcin; BMP, bone morphogenic proteins; ALP, alkaline phosphatase; GE, methacrylated gelatin.

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Commentary

The ability to generate cell-laden tissue mimetics for therapeutic purposes still remains an unmet goal of regenerative medicine.[1] The process of guiding stem cell differentiation into each specialized cell type is governed by a variety of factors.[2-4] To direct the differentiation of stem cells many studies have focused on dissecting biophysical properties of scaffold such as matrix stiffness or topography.[3,5,6] In addition, the role of biochemical signals (soluble factor or cell matrix cues and direct cell-cell contacts) have also been extensively studied.[7-9] However, most of these studies fail to recapitulate the full complexity of cellular microenvironments and only offer a glimpse into stem cell differentiation. In native tissues both biophysical and biochemical cues synergistically regulate stem cell fate to guide the formation of functional organs and tissues.[10-12] In the past, high-throughput screening (HTS) methods have shown success in large scale assessment of drugs and chemicals for their efficacy or toxicity.[13-15] HTS approaches represent versatile tools, which leverage high-content imaging, liquid handling and automation in screening large sample numbers to quickly determine bioactivity, that could be used to study stem cell differentiation.[16,17] Therefore, it is important to mature and adapt this technology for regenerative medicine applications.[18] HTS approaches could allow for the study of stem cell responses during the controlled presentation of desired individual or complex signal combinations within a three dimensional (3D) microenvironment, leading to better understanding of the stem cell niche.[10-12,19] Developing combinatorial 3D platforms would therefore offer a systematic analysis of how various biophysical and biochemical cues affect stem cell fate.

The initial strategy to screen combinatorial microenvironments was performed with multi-well based assays.[12,20] First of these techniques relied on encapsulating stem cells in hydrogels with various

extracellular matrix (ECM) compositions.[12] Other studies have focused on assessing the role of cell-material interactions, by generating libraries of 3D matrices, using similar multi-well type approaches.[20,21] However, multi-well based methods, especially those utilizing 96-well microtitter plates, are often plagued with limitations, including lower throughput and high cost requirements.[22] Other, HTS approaches have been based on two dimensional (2D) substrate micropatterning methods that utilize microarray robotics to lower cost, reagent volumes and increase throughput.[16-18,23] Such 2D systems also allow for the study of synergistic effects by integrating several varying parameters into the study.[16-18] However, 2D platforms do not fully recapitulate the native tissue architecture and microenvironment. These observations are supported by several studies that have shown cellular function significantly differs in 2D microenvironments relative to their 3D counterparts.[24-27] Therefore, developing 3D microarray platforms is important for studying stem cell differentiation in a high-throughput manner.[10,23,28] In one such study Dordick and colleagues[28] developed a 3D cellular microarray platform that could be used to track and quantify stem cell behavior. By encapsulating cells into an alginate based hydrogel they were not only able to examine mouse embryonic stem cell linage commitment in general, by also performed dual screens of tretinoin and fibroblast growth factor-4 effects on ES pluripotency.[28] Such miniaturized platforms could have broad applications such as in cytotoxicity and drug screening assays. Therefore, it is crucial to apply methods studying stem cell behavior in a combinatorial 3D microenvironmental setting. These combinatorial platforms could be exploited to present varying amounts and combinations of a variety of factors including both biophysical and biochemical cues.

Recently,[29] we have developed a combinatorial screening platform that merged microarray technology with 3D cell-laden hydrogels to analyze the effect of various soluble and ECM signaling cues on the differentiation of hMSCs to osteogenic fates. In this system, a rapid printing method was used to fabricate arrays of hundreds of microscale gels within a few minutes, by using several orders of magnitude less material and cells. Our approach encapsulated hMSCs within the photocrosslinkable gel, methacrylamide-modified gelatin (GE), containing different combinations of ECM proteins (**Fig. 1**). Within these microarrays the hMSCs were evenly distributed and maintained their viability. To determine the effects of various matrix factors on the osteogenic differentiation, we investigated the role of fibronectin (FN), laminin (LN) and osteocalcin (OCN) either individually or in combination. In addition, we analyzed the simultaneous role of growth factors, such as bone morphogenic proteins (BMP2 and BMP5), also known for their role in osteogenesis.

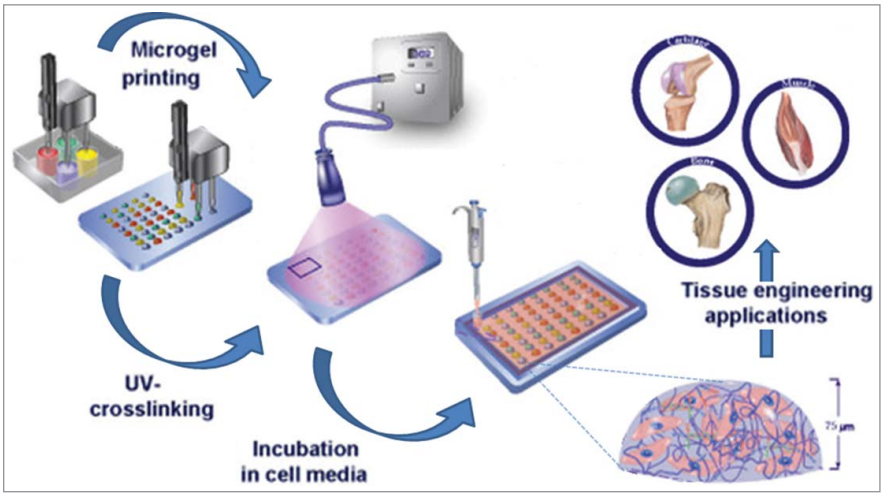

Figure 1. Fabrication of 3D combinatorial niches. A robotic microarray spotter was used to rapidly print droplets consisting of hMSCs, gelatin methacrylate, prepolymer solution and various ECM proteins on TMSPMA functionalized glass slide. The printing step was followed by a 15 sec UV light exposure to form the miniaturized cell-laden constructs. Following printing, cell-laden gel microarrays were placed inside sealed chambers.

To characterize the extent of osteogenic differentiation of hMSC, we analyzed the expression of alkaline phosphatase (ALP). It was observed that ECM combinations had a much more pronounced effect on ALP expression in comparison to single protein conditions, which did not significantly promote stem cell differentiation. In particular, we observed that GE-LN-FN-OCN hydrogel combination had the highest expression of ALP. At the tested concentrations, the supplementation with BMP 2 and BMP5 had less of an effect on ALP expression in comparison to combinatorial ECM components.

To validate the translational potential of our combinatorial 3D microarray platform we tested the predictions from the microarray response in larger (centimeter sized) constructs. Specifically, we tested the following conclusions from the cell-laden microarray studies for macroscale validation: A) ALP expression is similar in GE-LN and GE gels i.e., that contribution of a single ECM component is insufficient to drive differentiation; B) GE-LN-FN-OCN gels exhibit highest ALP expression; and C) supplementation of BMP2 has a minor effect on ALP expression in GE-LN-FN-OCN gels. We used optical imaging for ALP expression, measuring the production of osteopontin (bone-related extracellular protein) and a biomarker for osteogenesis and production of calcified matrix as hallmark of osteogenic differentiation and microarray result validation. At each stage we observed consistency between macroscale and microarray platform results. Furthermore, Raman spectroscopy and gel mechanical testing indicated hydroxyapatite presence, strengthening the potential to translate the 'hit' combinations into macroscale bone formation models. However, progress still needs to be made in identifying other combinatorial 3D stem cell niche platforms. One existing limitation with these

gel microarrays is that experiments are restricted to 7 d, mainly due to hydrogel degradation during the hMSC culture. In the future these issues can be resolved by incorporation or substitution with other natural polymers such as alginate or hyaluronic acid that have lower degradation rates.

However, other important challenges remain. Future platforms should also include a cell-culture system that is not static. As such, the platform would greatly benefit from a continuous exchange of media through a microfluidic based dynamic cell-culturing. Better temporal control of microenvironmental cues would then be possible, while also preventing unwanted cellular communication and crosstalk between neighboring gel spot microenvironments.[23] A recent report by Cosson adn Lutolf[30] attempts to solve some of these technical challenge with the use of hydrogel microfluidic chips. The system they propose has the potential for complex and long-term dynamic cell culture, is capable of perfusion and decouples cell culturing from microfluidic manipulation. The group is able to demonstrate spatiotemporal control of biomolecular deliver relevant to stem cell culture and differentiation. However, for regenerative medicine applications these in-vitro 3D combinatorial stem-cell microenvironments, need to be further validated and studied.

In conclusion, we demonstrated both the high-throughput combinatorial screening and translational potential of the cell-laden 3D microarray platform, showing that it is possible to analyze ECM and growth factor effects on stem cell differentiation in a combinatorial manner. We hope that future platforms will continue to be valuable tools in probing the complexity of multiple cellular microenvironments, well beyond osteogenesis. Combined with an improved design and flexibility we can envision much broader studies that will involve more intricate cell manipulation. We anticipate studies that could ultimately be a stepping stone toward incorporating and accounting for an individual's complexity, where the platforms would have the capacity to usher in the era of personalized medicine. As such, we imagine that this and similar platforms will have the potential to accelerate the discovery of novel biomaterials for an array of applications including those of regenerative medicine and drug discovery.

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