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Long term mesenchymal stem cell culture on a defined synthetic substrate with enzyme free passaging

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ABSTRACT

Mesenchymal stems cells (MSCs) are currently the focus of numerous therapeutic approaches in tissue engineering/repair because of their wide multi-lineage potential and their ability to modulate the immune system response following transplantation. Culturing these cells, while maintaining their multipotency *in vitro*, currently relies on biological substrates such as gelatin, collagen and fibronectin. In addition, harvesting cells from these substrates requires enzymatic or chemical treatment, a process that will remove a multitude of cellular surface proteins, clearly an undesirable process if cells are to be used therapeutically. Herein, we applied a high-throughput 'hydrogel microarray' screening approach to identify thermo-modulatable substrates which can support hES-MP and ADMSC growth, permit gentle reagent free passaging, whilst maintaining multi-lineage potential. In summary, the hydrogel substrate identified, poly(AEtMA-Cl-*co*-DEAA) cross-linked with MBA, permitted MSCs to be maintained over 10 passages (each time via thermo-modulation), with the cells retaining expression of MSC associated markers and lineage potency. This chemically defined system allowed the passaging and maintenance of cellular phenotype of this clinically important cell type, in the absence of harsh passaging and the need for biological substrates.

1. Introduction

Bone marrow transplants are the historical and dominant stem cell therapy, but since the initial isolation of colony forming units from bone marrow by Friedenstein in 1974 [1], the importance of specific mesenchymal stem cells (MSCs) has grown, with increasing numbers of accessible and more readily obtainable sources of these cells such as adipose tissue [2], umbilical cord blood [3], and Wharton's Jelly [4]. In addition, the range of cell types that MSCs can generate has expanded from the originally characterised adipocytes, chondrocytes and osteocytes [5] to smooth muscle cells [6] and neurons [7]. Furthermore, MSCs have also been shown to be capable of modulating the immune response, attenuating inflammation and graft *versus* host responses [8]. A large number of clinical trials are underway investigating MSCs ability to modulate the immune system in a number of diseases [9] as well as for tissue repair [10].

The increasing importance of MSCs in stem cell biology has made it crucial to develop fully defined culture systems to allow the expansion of these cells for research and therapeutic applications. The maintenance of MSCs in their 'stem cell' state in the absence of undefined components remains a challenge and represents a major limitation for their long-term clinical potential. Although standard tissue culture plastic is sufficient to support attachment and isolation of MSCs from a heterogeneous primary tissue isolate, it is insufficient to maintain the growth and properties of these cells for long term culture. Indeed, it has been shown that continued culture on tissue culture plastic results in loss of osteogenic [11] and adipogenic [12] differentiation potential. Culture on tissue culture plastic or on biological substrates such as gelatin [11] or fibronectin [13] also requires enzymatic or chemical treatments to release cells

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for passaging. This has the potential to damage cells by 'shaving' off vital cellular surface receptors [14], which must also lead to variation and uncertainty in apparent surface marker expression.

We have previously screened diverse cell types with polymer microarrays to identify polymers with high affinity to bind and support the growth of mouse ESCs [15], bind specific pathogens [16], activate platelets [17] and resist bacterial binding and growth [18]. The polymer microarray system has also allowed the identification of thermo-responsive hydrogels capable of maintaining hESCs in long term culture (>20 passages) in a serum free media while retaining their pluripotency [19].

In order to identify a thermally modulatable substrate for MSCs that could qualify as an alternative to currently used biological substrates and eliminate the need for harsh release treatments, a polymer microarray-based high-throughput screening platform was used (Fig. 1). The microarrays consisted of 609 polymers using monomers known to form hydrogels [20], with the aim of generating a substrate that could be modulated in response to a physical stimulus, *i.e.*, change in temperature, resulting in thermo-detachment of MSCs. The screening strategy targeted human embryonic stem cell derived mesenchymal progenitors (hES-MPs) as a standardisable and plentiful surrogate for adult tissue derived MSCs [21–23], which are susceptible to significant variation between donors [24]. After two iterative rounds of screening on polymer microarrays for hydrogels that support growth and retain MSC phenotype, 10 hydrogels were indentified and evaluated on a larger scale for their ability to trigger cellular release upon mild temperature reduction (Fig. 1C). Finally, 'lead' hydrogels were evaluated in long term culture with hES-MPs and adipose derived Mesenchymal Stem Cells (ADMSCs) using thermo-detachment, with analysis using a multitude of MSC markers. Differentiation was also evaluated to show multilineage capacity. This approach yielded a polyacrylate-based hydrogel (poly(AEtMA-Cl-*co*-DEAA) cross-linked with MBA) that showed good cellular binding, proliferation and MSC marker expression together with excellent thermo-detachment capability for both hES-MP and ADMSCs.

2. Materials and methods

2.1. Materials

Cell culture reagents were purchased from Invitrogen unless otherwise stated. Other chemicals were from Sigma–Aldrich accept tridecafluoro-1,1,2,2-tetrahydrooctyl-dimethylchlorosilane (FDS) which was obtained from ABCR GmbH Co. KG. Chemicals were used as received except *N*-isopropylacrylamide (NIPAA) which was recrystallised from hexane. *N*-acryloyl-*N*′-propylpiperazine (NANPPA) and 2,2′-(ethylenedioxy)bis(ethylamine) mono-acylamide (EOA) were synthesised as described previously [25] and [20]. The monomers and the cross-linker used in this study are listed in Supplementary Table 2.

2.2. Preparation of polymer hydrogel microarrays

The preparation of microarrays has been described in detail previously [20]. Briefly, arrays were fabricated by printing aqueous solutions of 18 acrylate/acrylamide monomers in drops on a masked glass slide using a MicroDrop scientific inkjet printer and polymerised *in situ* in the presence of a cross-linker

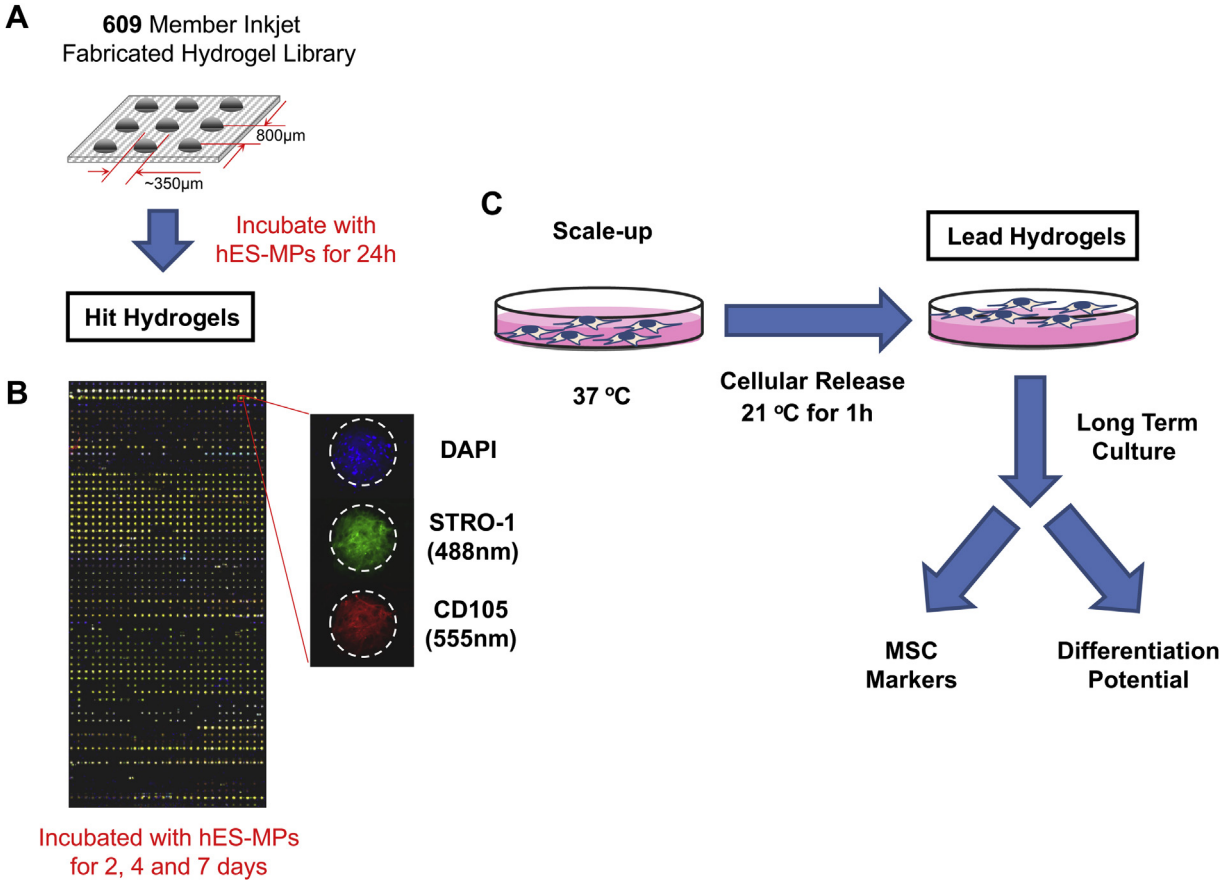

Fig. 1. Overview of the high-throughput polymer microarray system for the identification of thermo-modulatable hydrogels. A) An inkjet synthesised 609 member hydrogel library ($n = 4$) was fabricated. hES-MPs were incubated on the arrays for 24 h to assess binding. The top binding hydrogel 'hits' were identified. B) A 'hit array' was inkjet synthesised and incubated with hES-MPs cells for 2, 4 and 7 days and assessed for growth and the two MSC markers CD105 and STRO-1. C) The top 30 hits identified in step 2 were scaled-up and coated onto coverslips and incubated for 7 days with hES-MPs and assessed for growth with the markers CD105, STRO-1 and CD271. The top 10 hydrogels ('leads') were further assessed for their thermo-modulatable abilities. Candidates were examined for long-term cell growth and maintenance of phenotype using marker expression and differentiation capacity.