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Complete List of Authors:	Le, Ngoc Nhi; University of Wisconsin-Madison, Materials Science Program Liu, Tianran; University of Wisconsin-Madison, Biomedical Engineering Johnston, James; University of Wisconsin-Madison, Biomedical Engineering Krutty, John; University of Wisconsin-Madison, Biomedical Engineering Templeton, Kayla; Humboldt State University, Chemistry Harms, Victoria; University of Wisconsin-Madison, Biomedical Engineering Dias, Andrew; University of Wisconsin-Madison, Orthopedics and Rehabilitation Le, Hau; University of Wisconsin-Madison, Surgery, Orthopedics and Rehabilitation, School of Medicine and Public Health Gopalan, Padma; University of Wisconsin-Madison, Materials Science & Engineering Murphy, William; University of Wisconsin-Madison, Orthopedics and Rehabilitation; Biomedical Engineering, Materials Science and Engineering

ARTICLE

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Ngoc Nhi T. Le[a], Tianran Leona Liu[b], James Johnston[b], John D. Krutty[b], Kayla Marie Templeton[e], Victoria Harms[b], Andrew Dias[g], Hau Le[f,g,h], Padma Gopalan [a,c,d] and William L. Murphy[a,b,g]*

We describe a screening approach to identify customized substrates for serum-free human mesenchymal stromal cell (hMSC) culture. In particular, we combine a biomaterials screening approach with design of experiments (DOE) and multivariate analysis (MVA) to understand the effects of substrate stiffness, substrate adhesivity, and media composition on hMSC behavior in vitro. This approach enabled identification of poly(ethylene glycol)-based and integrin binding hydrogel substrate compositions that supported functional hMSC expansion in multiple serum-containing and serum-free media, as well as the expansion of MSCs from multiple, distinct sources. The identified substrates were compatible with standard thaw, seed, and harvest protocols. Finally, we used MVA on the screening data to reveal the importance of serum and substrate stiffness on hMSC expansion, highlighting the need for customized cell culture substrates in optimal hMSC biomanufacturing processes.

Introduction

Human mesenchymal stromal cells (hMSCs) are commonly used tools for research, and they have been used as putative cell therapies in hundreds of clinical trials. However, hMSC behavior in cell culture remains incompletely understood, and it has been challenging to predict the critical quality attributes that may correlate with in vivo efficacy of hMSCs. One contributor to this knowledge gap is reliance on ill-defined cell adhesion substrates and growth media during hMSC culture. hMSCs are routinely cultured on tissue culture polystyrene (TCPS) in growth factor-supplemented media, most commonly in the form of fetal bovine serum (FBS). TCPS is a complex substrate for hMSC expansion due to its supraphysiological stiffness and nonspecific protein adsorption. In particular, TCPS modulus is six orders of magnitude higher than that of bone marrow and five orders of magnitude higher than any human soft tissue. Long-term hMSC cultures on TCPS have resulted in increased cellular stress, decreased mechanosensitivity, and cytoskeletal coarsening and stiffening [1-4]. Additionally, TCPS allows nonspecific adsorption of hundreds of proteins, which makes it difficult to understand the mechanisms involved in cell-substrate signaling [3]. Further, the reliance on FBS as a component of cell culture medium leads to potential for xenogenic contamination, batch-to-batch variability, and volatility in the manufacturing process [5-9].

To address these issues, there has been significant interest in chemically defined, xeno-free (XF), serum-free (SF) cell culture substrates and media formulations [5, 6, 8-10]. Despite notable progress in development and commercialization of SF media formulations, significant challenges remain to be addressed. Serum removal can have unanticipated negative effects on cell adhesion and proliferation [5-7], and hMSC attachment and expansion in SF systems are often inferior when compared to serum containing media environments [5, 8, 9]. Prior studies evaluating performance of SF systems have revealed the need for concurrent optimization of both the media formulation and substrate in order to achieve hMSC growth comparable to levels achieved in serum containing culture systems [5, 11-14]. In addition, several commercially-available SF media formulations are proprietary, and developed to promote optimal growth only when used with proprietary culture substrates, leading to limited utility in research and clinical studies [5, 8, 9, 11-14].

Here, we used a combinatorial screening approach to identify chemically-defined substrates for hMSC culture in XF, SF media. Our screening method focused on variables that have been previously shown to influence hMSC behavior, including substrate stiffness, cell adhesivity, and growth medium [15-34].

[a]*Materials Science Program, University of Wisconsin-Madison, Madison, WI USA*
[b]*Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, WI USA*
[c]*Department of Material Science and Engineering, University of Wisconsin-Madison, Madison, WI USA*
[d]*Department of Chemistry, University of Wisconsin-Madison, Madison, WI USA*
[e]*Department of Chemistry, Humboldt State University, Arcata, CA USA*
[f]*Department of Surgery, University of Wisconsin-Madison, Madison, WI USA*
[g]*Department of Orthopedics and Rehabilitation, University of Wisconsin-Madison, Madison, WI USA*
[h]*School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI USA*
**Corresponding author can be contacted at*
Wisconsin Institute for Medical Research II, 1111 Highland Avenue Room 5405, Madison, WI USA 53705.
*Phone: (608)265-9978; Email: wlmurphy@wisc.edu*See DOI: 10.1039/x0xx00000x

From this screen, we identified three poly(ethylene glycol)-based, integrin binding and chemically-defined hydrogel substrates that supported functional hMSC expansion in multiple serum containing (SC) and SF media formulations. Finally, we used these substrates to study the influence of cell culture parameters on hMSC adhesion, expansion, and differentiation.

Results and Discussion

Identification of hydrogel substrates for functional SF hMSC expansion

The array-based screening method identified customized hydrogel substrates for functional hMSC expansion in SC and SF media. Slide-based hydrogel arrays enabled identification of integrin-binding peptides that could support hMSC adhesion to hydrogels in SC and SF media (Figure S2C, Figure S3, and Figure S4). hMSCs, manufactured by Lonza and sourced from multiple healthy bone marrow donors, were seeded at 1000 cell/cm^2, a density lower than recommended for XF SF culture, to prevent significant cell-cell interactions that could mask the effects of cell-substrate interaction. We first incorporated 2 mM of several integrin-binding peptides (containing laminin-derived IKVAV, fibronectin-derived RGD, and vitronectin-derived PHSRN sequences) into 8 kPa stiffness hydrogels and examined the ability for each substrate to support cell adhesion, cell spreading, and cell expansion (Figure S4). Substrates presenting 2 mM cyclic RGDf promoted the greatest combination of initial adhesion, cell spreading, and cell expansion in αMEM + 10% FBS culture. However, regardless of peptide concentration, cyclic RGDf alone was unable to support hMSC adhesion in TheraPEAK XF SF medium at levels comparable to those achieved on the TCPS control (Figure S5A).

Hydrogel spots that included cyclic RGDf combined with IKVAV supported robust hMSC adhesion to PEG hydrogels in SF medium. hMSCs were cultured on hydrogels with 8 kPa modulus, presenting either 2 or 4 mM cyclic RGDf, along with 0.5 mM IKVAV, in TheraPEAK XF SF medium (Figure S5). Regardless of cyclic RGDf concentration and media formulation, adding 0.5 mM IKVAV increased hMSC adhesion to levels equal to or greater than those achieved on TCPS controls. In αMEM + 10% FBS culture, increasing cyclic RGD concentration increased hMSC adhesion and the addition of IKVAV further increased cell adhesion to levels greater than what was achieved on TCPS. In TheraPEAK XF SF culture, increasing cyclic RGDf concentration alone also increased cell adhesion, but only the combination of cyclic RGDf and IKVAV promoted adhesion levels comparable to what was achieved on TCPS pre-treated with CellStart coating.

Multiple combinations of substrate stiffness and cell adhesivity supported hMSC expansion on PEG hydrogels in SF medium (Figure 2, Figure S6A). In particular, we varied hydrogel stiffness (1, 8, or 18 kPa), cyclic RGDf concentration (0 – 6 mM), and IKVAV concentration (0 – 2.5 mM) and examined cell adhesion, spreading, and expansion in serum containing (αMEM + 2% or 10% FBS) and SF (TheraPEAK XF SF) media. The stiffness range was chosen to mimic stiffness values previously used for hMSC culture on hydrogel substrates in SC media [1, 35-40]. Hydrogel compositions that concurrently supported adhesion, spreading, and expansion in both SC and SF media were deemed "hit" compositions. Of the 48 different substrate compositions examined (46 hydrogel substrates, TCPS, and TCPS + CellStart coating), there were 23 "hits" that promoted cell adhesion, spreading (> TCPS substrates), and expansion (> 1 fold change in cell number) in TheraPEAK XF SF medium (Figure 2 and Figure S6). The "hits" included 22 hydrogel substrates as well as TCPS + CellStart coating. hMSC expansion in TheraPEAK XF SF medium was significantly lower than hMSC expansion in αMEM + 2% or 10% FBS culture, regardless of cell culture substrate.

Three out of the twenty-two "hit" hydrogel substrates in TheraPEAK XF SF also supported hMSC adhesion, spreading, and expansion in other media (αMEM + 2% FBS, RoosterNourish XF, and StemPro XF SF media), and were thus termed "master hits" (Figure 3A). Notably, these 3 "master hit" hydrogel compositions not only supported bone marrow-derived hMSC (BM-hMSC) adhesion, spreading, and expansion, but also supported iPS-derived hMSC and mouse MSC adhesion, spreading, and expansion (Figure S6B). Additionally, the screening method identified customized hydrogel substrates for culture of other cell types in XF conditions, including human embryonic stem cells (hESCs) in Essential 8 (E8) medium and human umbilical vein endothelial cells (HUVECs) in Endothelial Growth Medium 2 (EGM2) (Figure S7). Importantly, these data indicate that the "master hit" hydrogel compositions have the proper combination of mechanical stiffness and cell adhesivity to support expansion of multiple cell types in multiple, disparate media conditions. These three "master hit" hydrogel formulations are: (1) 1 kPa/4 mM cyclic RGDf/0 mM IKVAV; (2) 8 kPa/ 2 mM cyclic RGDf/0.5 mM IKVAV; and (3) 8 kPa/4 mM cyclic RGDf/0.5 mM IKVAV.

"Master hit" hydrogel compositions identified in hydrogel arrays were readily applicable to more standard multiwell plate culture, and hMSCs could also be readily harvested for functional assays. hMSCs expanded on master hit hydrogels in SC and SF media retained their proliferative capacity, with expansion levels comparable to those seen on TCPS controls (Figure 3B). hMSCs also retained their multipotency and immunosuppressive functions after 8 days of expansion on "hit" hydrogels. Specifically, expanded hMSCs cultured on collagen I-coated TCPS in osteogenic induction medium for 21 days gave rise to mineralized cell cultures (Alizarin Red S+), and those cultured in adipogenic induction medium gave rise to cells with large lipid vacuoles (Oil Red O+) (Figure 4A, Figure S8, Figure S9A,B and Figure S10). Further, when co-cultured with activated T-cells, hMSCs expanded on hydrogels suppressed T-cell proliferation at levels comparable to those expanded on TCPS in αMEM + 10% FBS (Figure S9C).

"Master hit" hydrogels were also compatible with standard cell seeding and harvesting techniques (Figure S11). hMSCs passaged onto the hydrogels following TCPS expansion or directly from thaw both maintained cell adhesion and expansion capabilities similar to cells cultured on TCPS controls (Figure S11A,B). hMSCs dissociated from the surface using enzyme and enzyme-free reagents (trypsin, TrypLE, and

Versene) with high efficiency (> 80% of cells dissociated from the surface, Figure S11C) while maintaining high viability (> 80%, Figure S11C,D) and multipotency (Figure S8). Note that harvest from hydrogel substrates required longer incubation times with dissociation reagents in order to achieve the same dissociation efficiency (Figure S11C). For example, harvest with Trypsin required 10 minutes of incubation to dissociate > 91% of the cells from 8 kPa hydrogel substrates while harvest from TCPS required 5 minutes of incubation for > 95% dissociation efficiency. Despite the differences in incubation time, dissociation from hydrogel and TCPS surfaces both maintained ~83% cell viability. Finally, hMSCs could be dissociated off the hydrogels and reseeded onto a fresh hydrogel substrate using a procedure similar to the standard repassaging procedure on TCPS (Figure S11E).

The influence of cell culture parameters on hMSC adhesion, expansion, and differentiation

Linear, multivariate analysis (MVA) on the hydrogel screening data revealed independent and interactive influences of cell culture parameters on hMSC adhesion, expansion, and differentiation (Figure 4B). Independently, the media formulation, Cyclic RGDf concentration, and substrate stiffness all were positively correlated with hMSC adhesion and expansion. For interactive effects, stiffness and Cyclic RGDf had the greatest impact on hMSC expansion, and their synergistic effect increased cell expansion more than the effect of increasing Cyclic RGDf concentration alone or increasing stiffness alone. Interestingly, the interactive effects of stiffness x Cyclic RGDf x IKVAV caused a decrease in both hMSC adhesion and expansion. These results were confirmed via heat map of hMSC adhesion and expansion (Figure S12).

The influence of substrate stiffness on adipogenic differentiation of hMSCs following expansion (Figure 4A and Figure S10) served as a testbed to study substrate-dependent hMSC differentiation. hMSCs were expanded on a 1 kPa master hit hydrogel, a 8 kPa master hit hydrogel, or TCPS for 8 days, then harvested for induced adipogenic differentiation. Regardless of the XF SF media formulation used for expansion, hMSCs cultured on master hit hydrogel substrates of lower stiffness showed increased adipogenic differentiation in TheraPEAK XF SF and StemPro XF SF, as indicated by increasing Oil Red O+ lipid vacuole density (Figure 4A). There was significant difference in lipid vacuole density following induced adipogenic differentiation of hMSC expanded on softer (1 kPa) versus stiffer (8 kPa) substrates in TheraPEAK XF SF media: a 364% increase in lipid vacuole density for hMSCs expanded on 8 kPa hydrogels and a 591% increase for those expanded on 1 kPa hydrogels, relative to the TCPS condition. These results were consistent with differentiation of hMSCs expanded in StemPro XF SF (+241% on 8 kPa hydrogels relative to TCPS, and +285% on 1 kPa hydrogels relative to TCPS). There was no significant difference in hMSC adipogenic potential between hMSCs expanded in TheraPEAK XF SF versus StemPro XF SF on TCPS (Figure S9B). Interestingly, the influence of expansion substrate stiffness on subsequent adipogenic differentiation was only observed when expansion occurred in SF media, and was not observed when using hMSCs expanded in SC media (αMEM + 10% FBS).

Discussion

Here we have demonstrated the use of an enhanced screening approach to identify customized substrates for serum-free expansion of functional hMSCs. Of 46 different hydrogel substrate formulations examined, 3 formulations supported media agnostic, cell-source agnostic, functional MSC expansion. The customized substrates were integrated into routine cell culture workflows for thaw, seeding, and harvest, suggesting that they may provide a useful tool for serum-free expansion of therapeutic MSCs.

Consistent with previous studies, our screening results demonstrated the need to customize the substrate and media combination in order to support hMSC adhesion, survival, and expansion. Miwa et al. and Hartman et al. demonstrated that the Mesencult-XF medium supported cell adhesion when cultured using the manufacturer's proprietary attachment substrate, but did not support cell adhesion when Mesencult-XF was used in combination with fibronectin-coated TCPS [13, 14]. These two studies suggest that the performance of the medium could be improved by customizing the culture substrate [5].

Of the 48 different substrate formulations in our screen, 45 substrates supported hMSC expansion in 10% FBS, 36 substrates supported hMSC expansion in 2% FBS, and only 23 formulations ("hits") supported hMSC expansion in TheraPEAK XF SF media (Figure 3). Interestingly, 3 of the 23 "hits" supported hMSC expansion in 4 different serum-containing and serum-free media formulations, and thus represented media-agnostic "master hits" (Figure 4). The three "master hit" formulations also supported adhesion and expansion of multiple distinct cell types, including hMSCs from multiple sources (iPS-derived MSCs and primary, mouse-derived MSC), human embryonic stem cells (hESCs) and human umbilical vein endothelial cells (HUVECs).

Using multi-variate analysis (MVA), we assessed the effects of multiple cell culture parameters on hMSC expansion. Of the two parameters, substrate stiffness had a greater effect on the proliferation and spreading of hMSC. This aligns with observations from prior work showing that hMSC adopt a balled morphology and quiescent, non-proliferative state when cultured on soft surfaces [41, 42]. Notably, combinatorial control of substrate parameters (e.g. substrate adhesivity and media formulation) yielded an interactive increase in hMSC expansion to levels higher than what could be achieved with either parameter alone (Figure 4B). This result confirmed the general observations reported by Miwa et al. and Hartman et al. [13, 14], which demonstrated a substrate and media dependence for hMSC culture.

While we identified substrates that supported hMSC adhesion, expansion, and multipotency, we also observed decreased adhesion and expansion and altered cell morphology in SF, XF media. This observation was consistent with previous studies,

which reported reduced adhesion and expansion in SF, XF media, as well as on chemically-defined cell culture substrates [5-10]. For example, Jung et al. and Chase et al. demonstrated lower initial adhesion and expansion on in XF SF media during culture on TCPS that could be improved with the use of proprietary cell culture substrates developed specifically for each media formulation. While hMSC culture on the proprietary substrates improved adhesion and expansion, cell morphology was clearly different from that observed on TCPS or in SC media [5, 8-10]. For some media formulations (e.g. StemPro XF SF), hMSCs were smaller and cells tended to grow in clumps as opposed to the isolated, spread morphology typical of hMSC culture on TCPS in SC media [5, 8-10]. Our results demonstrated that some media formulations produced smaller, less-spread cells (StemPro XF SF) while other formulations (TheraPEAK XF SF) yielded polygonal, more well-spread cells, even on the same substrates (Figure 3A).

We also demonstrated hMSC expansion, and confirmed multipotency and immunomodulatory activity (Figure 4A and Figures S8-10). Our results and those from literature suggest that short-term hMSC expansion can produce cell populations with different functional behavior dependent on the media or substrate conditions. Here we did not specifically examine the effects of hMSC morphological differences on long-term cell behavior. However, based on previous studies that demonstrated direct correlations between morphology and cell behavior [1, 2], we can speculate that long-term hMSC culture in different media and substrate combinations could lead to functional differences. For example, here hMSCs expanded in TheraPEAK XF SF and StemPro XF SF underwent more efficient adipogenic differentiation than cells expanded in αMEM + 10% FBS (Figure 4A and Figure S10). Interestingly, we observed that adipogenic differentiation potential was dependent on the substrate stiffness used during hMSC expansion, but this "priming effect" during expansion was only seen in XF SF media, not in αMEM + 10% FBS. Other studies have also demonstrated different adipogenic and osteogenic differentiation efficiencies for hMSCs expanded with varying substrates and media, but our observation is the first to our knowledge to demonstrate serum-dependence in the ability to resolve stiffness-dependent hMSC lineage commitment after short-term culture [43].

Experimental

Hydrogel array formation and characterization

Two hydrogel array formats were utilized in this set of experiments: a slide-based hydrogel array and a multiwell-based hydrogel array (Figure 4.S1A). Both array formats utilized Norbornene-functionalized PEG (PEG-NB) hydrogels formed using thiolene chemistry and PEG-NB was synthesized and characterized as previously described [22, 44-46]. Briefly, 8-arm PEG-OH (20 kDa molecular weight, tripentaerythritol core, 8-arm with terminal OH, JenKem Technology) was dissolved in anhydrous dichloromethane (DCM, Sigma Aldrich) in one round bottom flask while N,N'-dicyclohexylcarbodiimide (DCC, Sigma Aldrich) and 5-Norbornene-2-carboxylic acid (Sigma Aldrich) were dissolved in a second round bottom flask. The PEG and norbornene solutions were combined and stirred overnight, protected from light, to covalently couple the 5-norbornene-2-carboxylic acid to the PEG-OH. The PEG-NB product was filtered through a medium fritted Buchner funnel (to remove urea salts byproduct) and the PEG filtrate was precipitated in 900 mL cold diethyl ether (Sigma Aldrich) and 100 mL hexane (Sigma Aldrich). The PEG solids were collected on qualitative grade filter paper and air dried at room temperature overnight, protected from light. The PEG-NB product was purified by dialysis (SNAKESKIN dialysis tubing, MWCO 3.5K, Sigma Aldrich) to remove residual norbornene acid. PEG-NB product was dialyzed against 4L of deionized H2O at 4°C for 72 hours, with water change every 8 hours, filtered through a 0.45 µm nylon filter to remove particulates and impurities, and lyophilized. Norbornene functionalization of >90% was confirmed with 1H nuclear magnetic resonance spectroscopy with free induction decay (FID) spectra obtained using spectroscopy services provided by the National Magnetic Resonance Facility at Madison on a Bruker Instruments Avance III 500i spectrometer at 400 MHz and 27°C. Samples were prepared at 6 mg/mL in deuterated chloroform (CDCl3, Sigma Aldrich) with tetramethylsilane (TMS) internal standard.

Silanized glass coverslips were prepared via liquid-phase silanization as previously described [22]. Briefly, clean glass coverslips were activated using oxygen plasma treatment at 40 standard cubic centimeters per minute (sccm) and 50 W for 5 minutes to increase the number of activated hydroxyl groups on the surface, immersed in 2.5 v./v.% 3-mercaptopropyl trimethoxysilane (3-MPTS, Sigma Aldrich) in toluene for 2 hours, rinsed with ethanol, dried, cured under nitrogen atmosphere at 100 °C for 1 hour, and immersed in 10 mM 1,4-Dithiothreitol (DTT, Sigma Aldrich) in PBS for 30 minutes at 37°C to increase free thiols available for thiolene reaction with PEG-NB.

Hydrogel precursor solutions were prepared by combining PEG-NB (4-20 wt/wt %), PEG-dithiol crosslinker (0.5-1 mole ratio of thiol-to-norbornene), peptides (1-6 mM), and 0.5 wt/wt% Irgacure 2959 photoinitiator (CIBA/BASF) and diluted to desired concentrations with phosphate buffered saline (PBS, pH 7.4) immediately prior to hydrogel array formation. Linear CRGDS, linear CRDGS, CREDV, CRGDSPG, cyclic (RGDfC), CPHSRN-(SG)5-RGD, CRGD-G13-PHSRN, and CIKVAV were purchased from GenScript USA. Note, per the manufacturer, the "f" notation denotes a D-amino acid. Linear PEG-dithiol (PEG-DT, 3.4 kDa) was purchased from Laysan Bio.

Slide-based hydrogel arrays were formed using a previously described differential wettability patterning method [22].

Multiwell-based hydrogel arrays were formed using a slight modification to the differential wettability patterned method and an array formation method. Hydrogels were immobilized to silanized glass coverslips to provide ease of handling. Briefly, gold-coated slides were cleaned via sonication in ethanol for 1 minute, dried with air, cleaned with oxygen plasma at 40 sccm and 50 W for 1 minute, and immersed in a 0.1 mM solution of HS–C11–(O–CH2–CH2)3–OH (PEG-terminated alkanethiols, ProChimia Surfaces) in ethanol solution for 2 hours to form a hydrophilic alkanethiol SAM layer. 12 mm round coverslips

were silanized using the procedure detailed above. Hydrogel precursor solutions were spotted onto the hydrophilic gold-coated slides, coverslips were used to sandwich the hydrogel precursor solution, hydrogel precursor solutions were crosslinked by UV-initiated photopolymerization through the silanized coverslips with 365 nm wavelength light for <6 seconds at 90 mW/cm^2, the resulting immobilized hydrogels were immersed in 70% ethanol for ≥ 72 hours, treated with UV-C in a biosafety cabinet for 3 hours to decontaminate, thoroughly washed 3X with PBS, immersed in cell growth medium at 37 °C for 72 hours with media changes every 12 hours, and stored in growth medium at 37 °C until use.

Hydrogel shear storage modulus and compressive modulus were determined using procedures previously published [22, 29, 44, 47-50].

Cell culture

Bone marrow-derived hMSCs from multiple donors (Lonza, Cat#PT-2501) were expanded in 10% fetal bovine serum (FBS, Invitrogen) in minimum essential medium alpha formulation (αMEM, MediaTech) supplemented with 1% penicillin/streptomycin (Invitrogen) on tissue culture polystyrene plates at 37 °C in a 5% CO_2 atmosphere until 70% confluence. hMSC (population doubling level 8-16) were harvested using trypsin (Invitrogen), resuspended in 10% FBS in αMEM, and seeded on sterilized hydrogel arrays or TCPS (for TCPS control). After 24 hours, unattached cells were removed by gently replacing the culture media. Cells on hydrogel arrays were maintained at 37 °C in a 5% CO_2 atmosphere with culture medium changed every 2 days. hMSCs were also cultured in xeno-free and serum-free media: TheraPEAK XF SF (Lonza) with TCPS control, StemPro XF SF (Gibco) with CellStart-coated TCPS control, and RoosterNourish XF (RoosterBio) with TCPS control. All hMSCs used were cryopreserved in αMEM + 10% FBS + 20% DMSO by placing 1mL cryovials in a -80°C freezer for 24 hours, before being transferred to liquid N_2 for storage. Cells were thawed into the respective medias of interest, and allowed to recover from cryopreservation for 72 hours prior to harvest and use for hydrogel experiments. hMSC were seeded onto TCPS and hydrogel substrates at 1000 cell/cm^2 (to prevent significant cell-cell interactions that could mask the effects of cell-substrate interaction) or 3000 cell/cm^2 (based on the media manufacturers' recommended seeding density for optimal growth). For xeno-free harvest, hMSCs were dissociated from the surface using TrypLE (XF trypsin, Thermo Fisher Scientific) and Versene (1X EDTA, Thermo Fisher Scientific).

hMSCs osteogenic and adipogenic differentiation utilized established protocols previously reported [51, 52]. Briefly, osteogenic medium (0.1 μM dexamethasone, 10 × 10 mM β glycerol phosphate, 50 nM ascorbic acid 2-phosphate) was prepared in αMEM + 10% FBS with penicillin (100 U/mL) and streptomycin (100 μg/mL). Adipogenic medium (1 μM dexamethasone, 500 μM isomethyl isobutyl xanthine, 10 μg/mL insulin) was prepared in 10% FBS in Dulbecco's modification of Eagle's medium high glucose with penicillin (100 U/mL) and streptomycin (100 μg/mL). hMSCs were expanded on TCPS or hydrogels for 8 days, dissociated, and seeded at 5000 cells/cm^2 on 48-well plates (Corning ™ BioCoat™ Collagen I, 48-well multiwell plates, Thermo Fischer Scientific) in αMEM + 10% FBS, allowed to grow until confluent, before media change into osteogenic or adipogenic media for differentiation or αMEM + 2% FBS for no differentiation control. Media was changed every 3 days for 21 days of differentiation.

Immunomodulatory function was assessed using hMSCs expanded on hydrogels or TCPS for 8 days and using instructions based on Miltenyi Biotec's human MSC Suppression Inspector with a ratio of 1:100 hMSCs to activated human peripheral blood CD4+ T cells (Lonza, Cat#2W-200) for 5 days. A CyQUANT Cell Proliferation Assay Kit (Thermo Fisher Scientific) was used to quantify DNA as an indicator of cell proliferation.

Induced pluripotent stem cell- (iPS) derived hMSCs were provided by Dr. Igor Slukvin's lab at the University of Wisconsin-Madison (Madison, WI) and cultured in complete Vasculife medium (Lifeline Cell Technology, Frederick, MD) supplemented with 10% FBS. Mouse bone marrow-derived MSCs were collected from bone marrow aspirate using procedures previously published and cultured in αMEM + 10% FBS [53]. Human umbilical vein endothelial cells (HUVECs, Lonza) were cultured in growth medium consisting of medium 199 (M199, Mediatech Inc) supplemented with EGM-2 Bulletkit (Lonza). H1 human embryonic stem cells (hESCs) were purchased from WiCell, expanded on Matrigel-coated TCPS, and cultured in Essential 8 (E8, Stem Cell Technologies) medium.

Data Acquisition and Analysis

hMSC viability after harvest was determined by Trypan Blue exclusion assessment. Hydrogel arrays and samples were placed in a heated environmental chamber and imaged on the Nikon Eclipse Ti microscope (Nikon) at each desired time point. Cell number was manually determined using NIS Elements software (Nikon) every 24 hours after seeding, cell area of single cells were determined using NIS Elements' threshold and automated measurement features at 72 hours after seeding, and cell expansion was quantified as previously reported by normalizing cell number at 72 hours relative to 24 hours, where fold change in cell number greater than 1 was used to indicate expansion and proliferation [19]. To avoid bias in image selection, an entire hydrogel was imaged for each data point, and the gels' positions on the slide were randomized before cells were counted and analyzed.

To assess osteogenic differentiation, cells were fixed with 10% Formalin, stained with Alizarin Red (40 mM, pH 4.1–4.3), washed three times with water, and assessed for mineral-stained red cells as positive indicators of osteogenic differentiation. To assess adipogenic differentiation, cells were fixed with 10% Formalin, incubated in Oil Red O working solution (3 parts of Oil Red O at 3 mg/mL in 99% isopropanol to 2 parts distilled water and filtered with 0.2 μm syringe filter to remove undissolved particulates) for 20 minutes, washed with water until clear, and assessed for lipid vacuoles stained red as positive indicators of adipogenic differentiation. Additionally, Oil Red O-stained cells were imaged using TxRed fluorescence

imaging on the Ti Eclipse microscope, and lipid droplet size and density was determined using automated thresholding on NIS Elements analysis software.

Design of experiment (DOE) and multivariate analysis (MVA) was performed using JMP statistical analysis software using a linear model. The multivariate value of each culture parameter was calculated as a ratio relative to the total summed effect value for all parameters ($\sum \text{All effects} = 1$). Statistical analysis for significance was performed using the GraphPad Prism software via Student's t-test (2-tailed, α=0.05) or ANOVA with post-hoc Tukey (HSD or Kramer depending on sample size variability) tests as indicated. Error bars denote standard deviation.

Screening method and platform technologies

An enhanced throughput screening method utilizing hydrogel array platform technologies identified substrates that supported functional hMSC expansion in SF media. The approach used design of experiments (DOE) and hydrogel array platforms, and was amenable to high-content, label-free characterization approaches (Figure 1A and Figure S1A,B). Both the slide-based and multiwell-based array platforms employed in this screening method comprised PEG-DT crosslinked PEG-NB hydrogels with networks formed via thiolene photopolymerization, a step-growth reaction mechanism with rapid gelation time that yields homogeneous polymer networks (Figure 1B and Figure S1C) [22, 23]. The composition of each hydrogel "spot" in each array was controlled such that substrate stiffness and cell adhesivity could be modified independently (Figure 1B,C and Figure S2A,B). Here, hydrogel stiffness could be regulated independent of adhesivity, regardless of total peptide concentration or identity (Figure 1C). In particular, we controlled the identity and concentration of cysteine-terminated moieties incorporated into the hydrogel network to regulate both stiffness and adhesivity. We controlled the stiffness of each hydrogel spot in the array by changing the concentration of PEG-NB polymer and the molar ratio of crosslinker-to-norbornene in the hydrogel precursor (referred to as "prepolymer") solution (Figure 1C and Figure S1B). We controlled the cell adhesivity through altering peptide identity and concentration in the hydrogel precursor solution. Several fibronectin-, vitronectin-, and laminin-mimetic, integrin-binding peptides were immobilized into the hydrogel network to support cell adhesion (Figure S2A,B).

Additionally, array-based screening examined the independent and combinatorial effects of substrate stiffness, cell adhesivity, and media formulation on hMSC behavior, and identified combinations of substrates and media that supported hMSC expansion (Figure 1A). Each spot in the array could be customized in terms of mechanical stiffness, cell adhesion peptide identity and concentration, GF sequestering peptide identity and concentration, cell type and density, and media formulation.

The screening workflow first used preliminary data from literature and prior experiments to design primary screen(s) on slide-based hydrogel arrays (Figure 1A and Figure S3). This initial screening set examined variables previously shown to influence hMSC phenotype: substrate stiffness and cell adhesivity. First, short-term hMSC culture was used to identify cytocompatible "hit" hydrogel compositions that supported hMSC adhesion and spread morphology. Second, the initial "hits' were scaled up into multiwell-based arrays for secondary screens to identify "master hit" hydrogel compositions that supported functional hMSC expansion.

Conclusions

The approach used in this study could provide a near term tool for hMSC expansion, and also a more general tool for cell culture optimization. We used an enhanced throughput screening approach to identify customized hydrogel substrates that support functional SC and SF expansion of MSCs from multiple sources (human bone marrow-, iPS-, and mouse-derived MSCs). The formulations themselves are promising for research and clinical studies, and the screening approach can be more broadly applied to develop customized materials for several different cell types and applications. Additionally, while we screened for "hits" using cell expansion as a criterion, this screening approach can also be used to identify materials for optimized production of lineage-specific cell populations from hMSCs, including adipogenic cells, immunomodulatory cells, and GF secreting cells.

Conflicts of interest

Disclosure Statement: William L. Murphy holds equity in Stem Pharm Inc. and Dianomi Therapeutics Inc.

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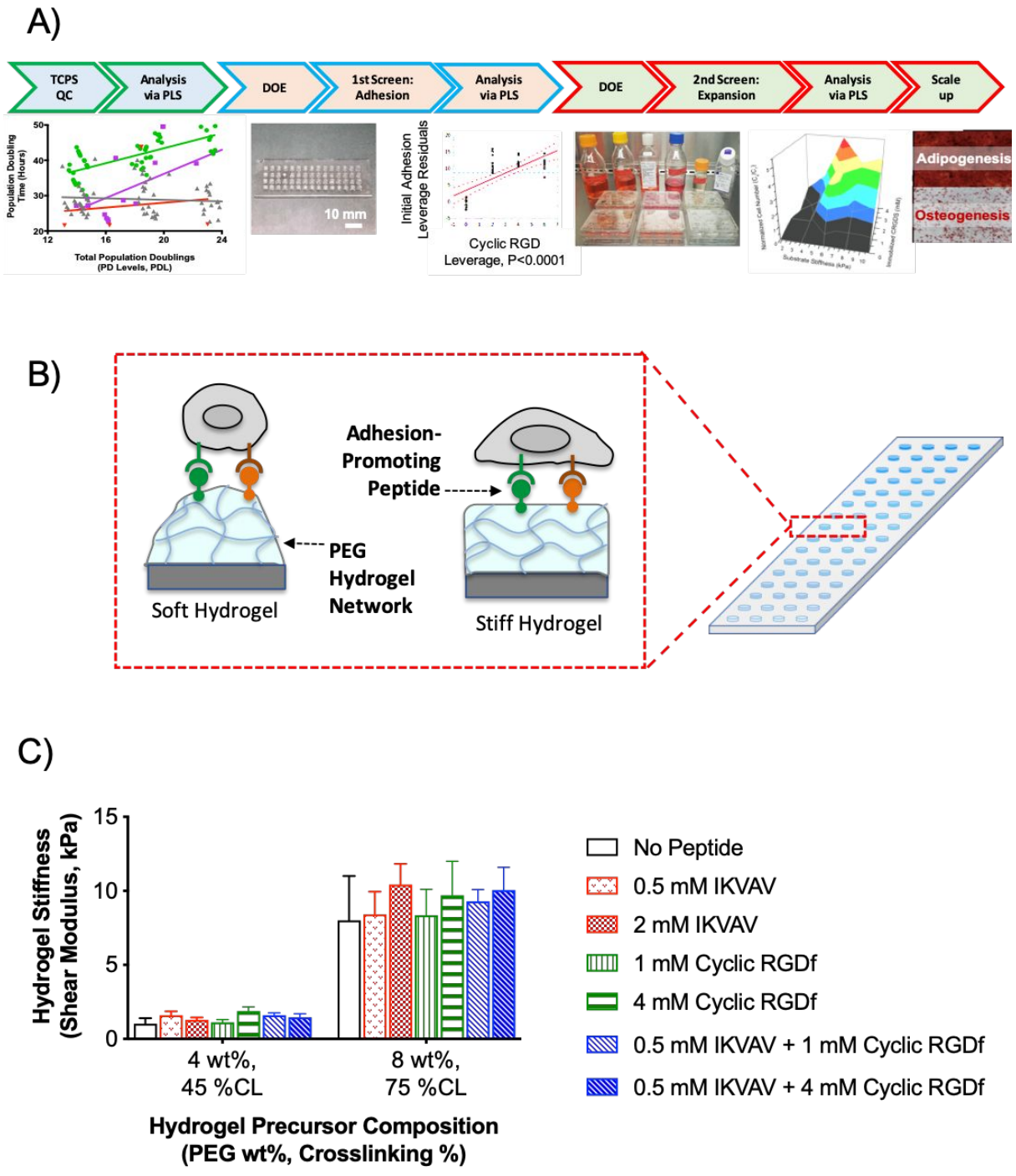


Figure 1. Hydrogel array screening. A) Workflow for enhanced throughput composition screening on hydrogel arrays formed on glass slides and scale up on bulk hydrogels formed in 6-well plates for hMSC expansion and long-term culture. B,C) Hydrogel stiffness and adhesivity interpedently tailored by controlling network density, crosslinking, and the identity and concentration of immobilized peptides.

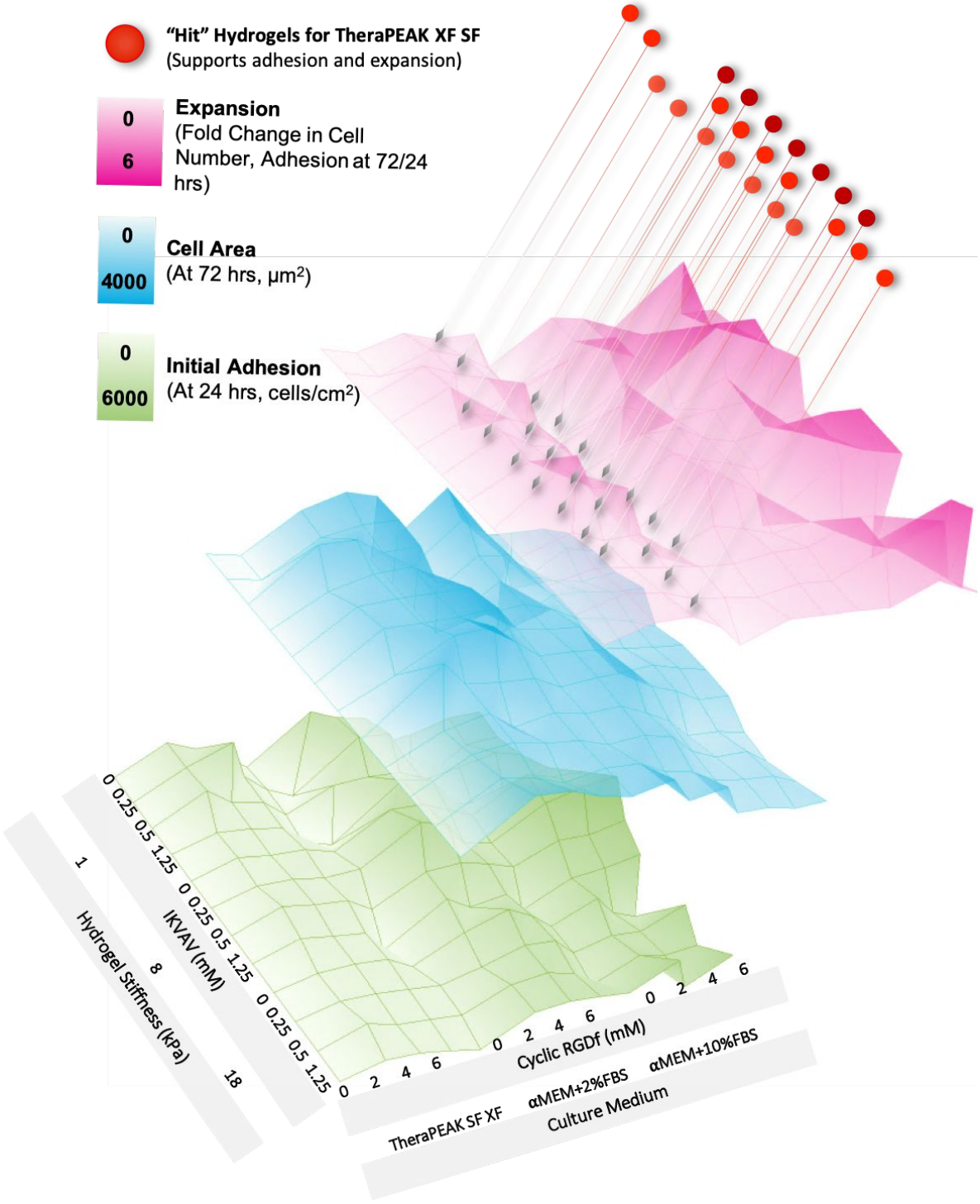


Figure 2. Identifying hydrogel compositions for promoting hMSC attachment, expansion, and spread morphology in TheraPEAK chemically-defined, serum-free, xeno-free medium. Expansion over 2 days (pink; C3/C1 = cell number at day 3 normalized to count at day 1), spreading at day 3 (blue), and hMSC attachment at day 1 (green). The Z axis is the response level for each parameter.

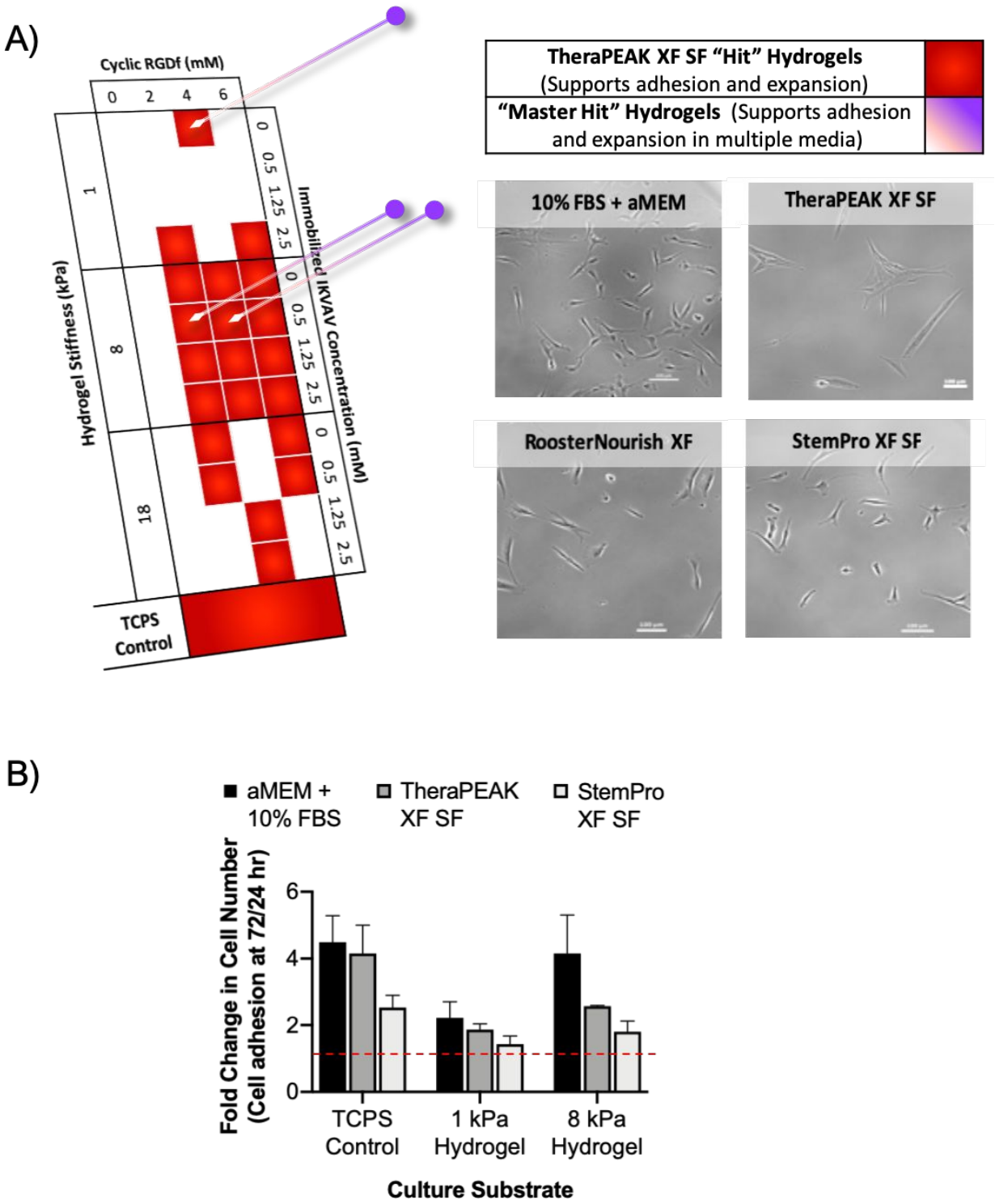
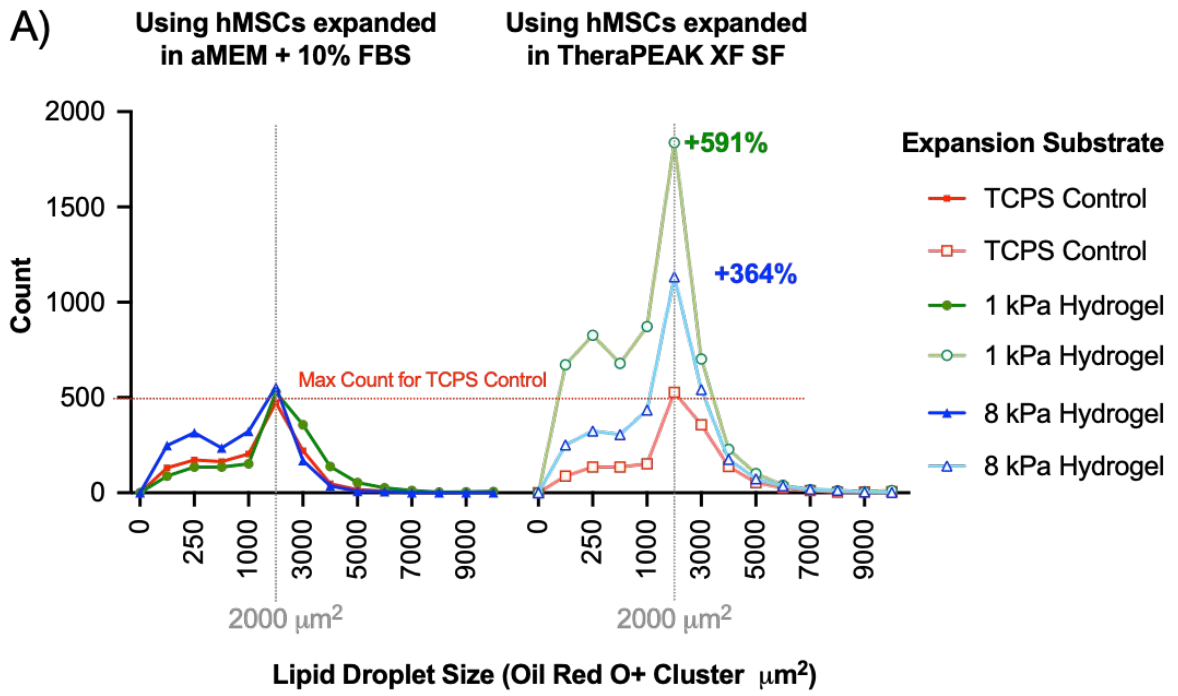


Figure 3. Customized "master hit" hydrogel substrates support A) hMSC adhesion in multiple media conditions; and B) hMSC expansion (dotted line indicates level of no increase in cell number, and thus no expansion).



B) Culture Parameters	Effect Magnitude	
	Adhesion (at 24 hrs)	Expansion (at 72/24 hrs)
Stiffness x Cyclic RGDf x IKVAV	-0.036	-0.239
Stiffness x Cyclic RGDf x IKVAV x Media Formulation	-0.0828	-0.0549
Cyclic RGDf x IKVAV x Media Formulation	-0.0598	-0.0397
Cyclic RGDf x IKVAV	-0.0205	-0.0136
IKVAV	-0.0057	-0.0038
IKVAV x Media Formulation	0.0012	0.0008
Stiffness x Cyclic RGDf x Media Formulation	0.0124	0.0082
Stiffness x Media Formulation	0.035	0.0232
Stiffness x IKVAV x Media Formulation	0.0531	0.0352
Stiffness x IKVAV	0.0673	0.0447
Stiffness	0.1328	0.0881
Cyclic RGDf x Media Formulation	0.1476	0.098
Cyclic RGDf	0.1803	0.1197
Serum in Media Formulation	0.4561	0.3027
Stiffness x Cyclic RGDf	0.0486	0.323

Figure 4. Culture parameters and their effects on hMSC adhesion, expansion, and differentiation. A) Directed adipogenic differentiation potential of hMSCs following 8-day expansion on hydrogel substrates in TheraPEAK XF SF or in αMEM + 10% FBS culture media. B) Multi-variate analysis (MVA) of the main and interactive effects of culture parameters (e.g. media formulation, substrate stiffness, and substrate adhesivity) on hMSC adhesion and expansion.

Supplementary Figures

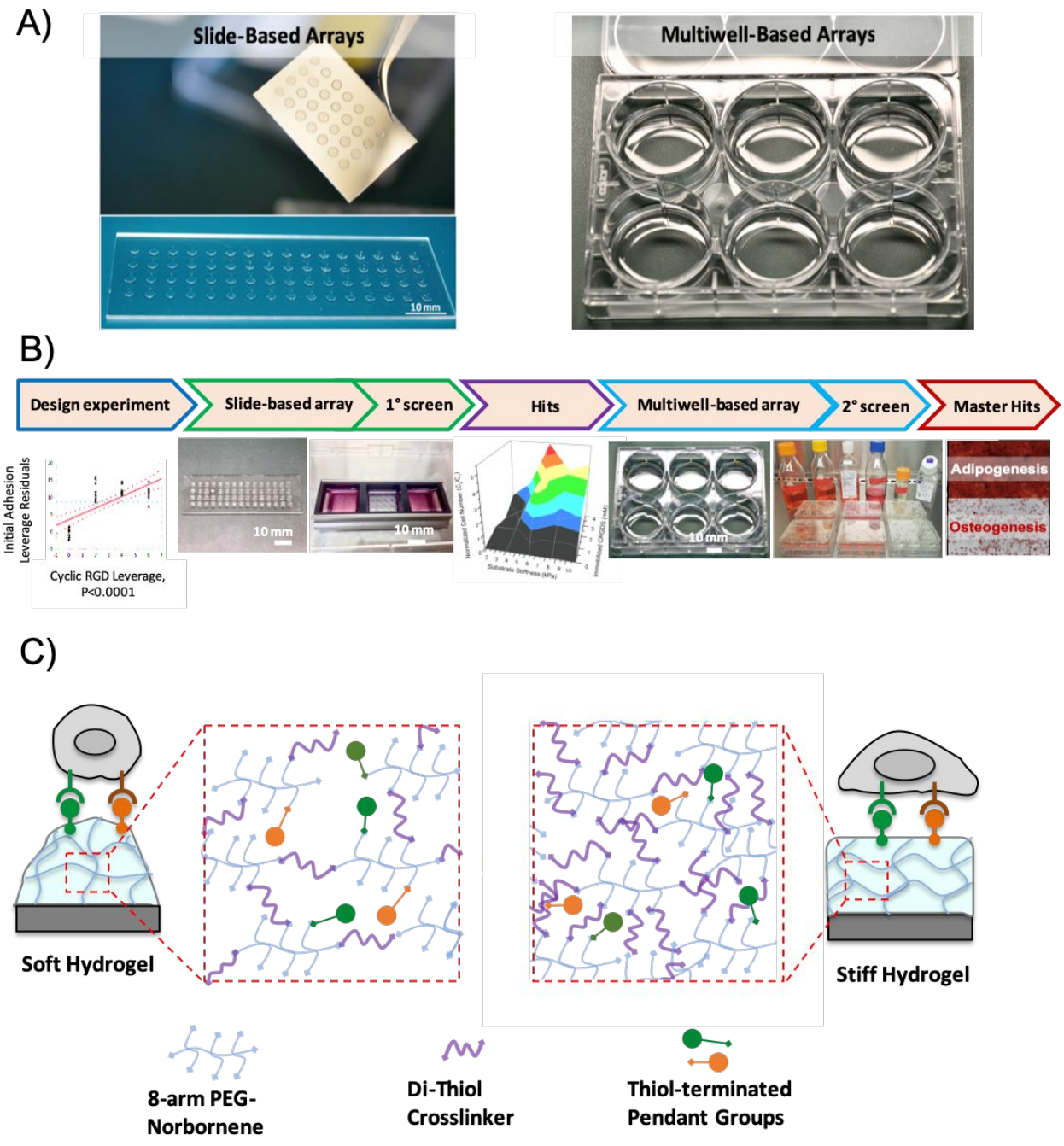
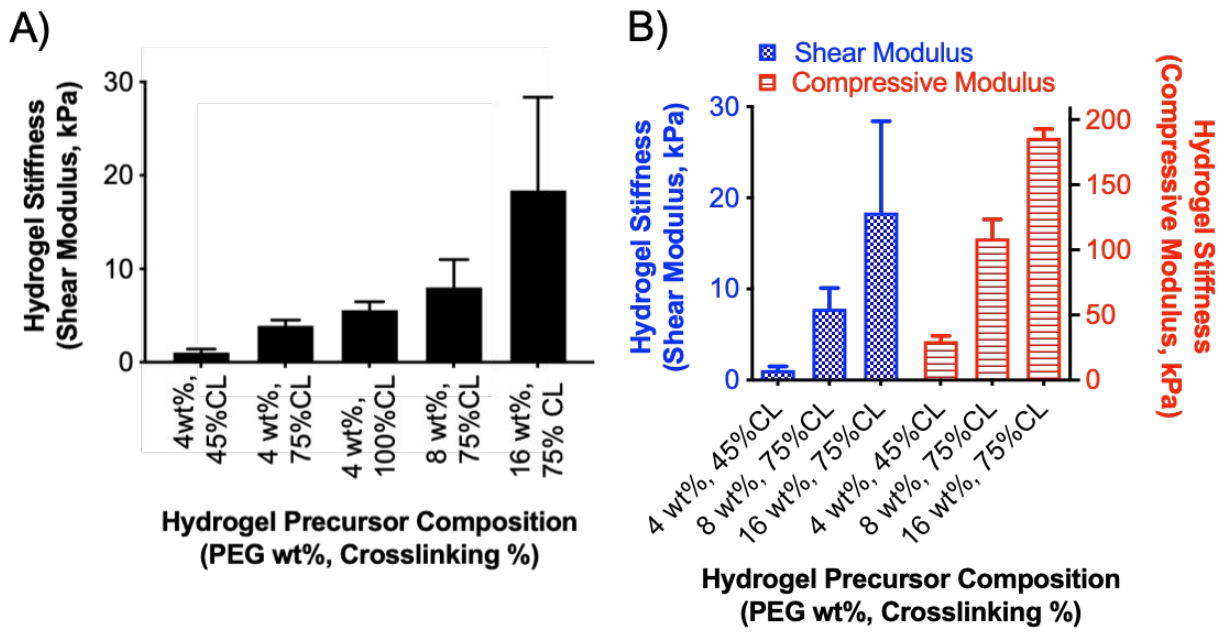


Figure S1. Screening workflow and platforms. A) Slide- and multiwell-based hydrogel arrays for screening. B) Workflow for enhanced throughput composition screening on hydrogel arrays formed on glass slide and scale up on bulk hydrogels formed in 6-well plate for hMSC expansion and long-term culture. C) Hydrogel networks formed using thiolene chemistry with an 8-arm PEG-norbornene polymer backbone, PEG-dithiol crosslinker, and thiol-terminated peptide pendant groups to promote adhesion. Stiffness is modulated using control of PEG-norbornene and PEG-dithiol crosslinker density in the unpolymerized hydrogel precursor solution.



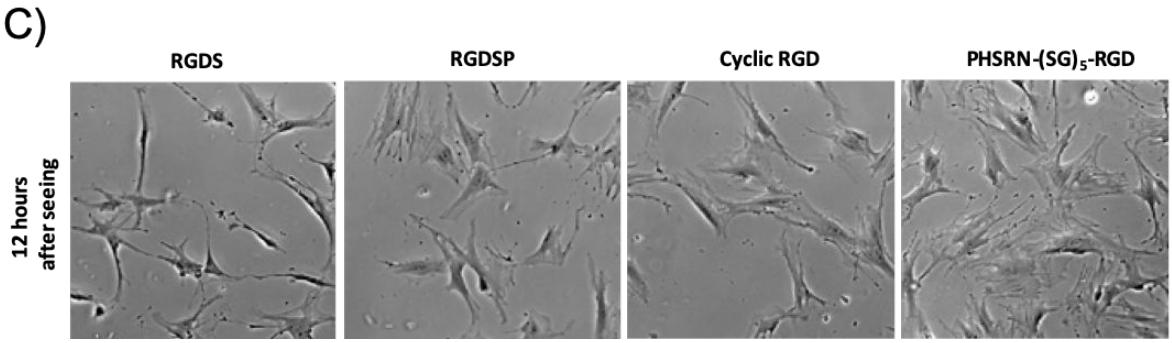


Figure S2. Controllable hydrogel substrate stiffness and adhesivity. A,B) Hydrogel stiffness is tunable by changing the polymer concentration (weight percentage) and crosslinking density (total percentage of norbornene arms crosslinked). C) Adhesivity is controlled by changing the identity and concentration of integrin-binding peptides.

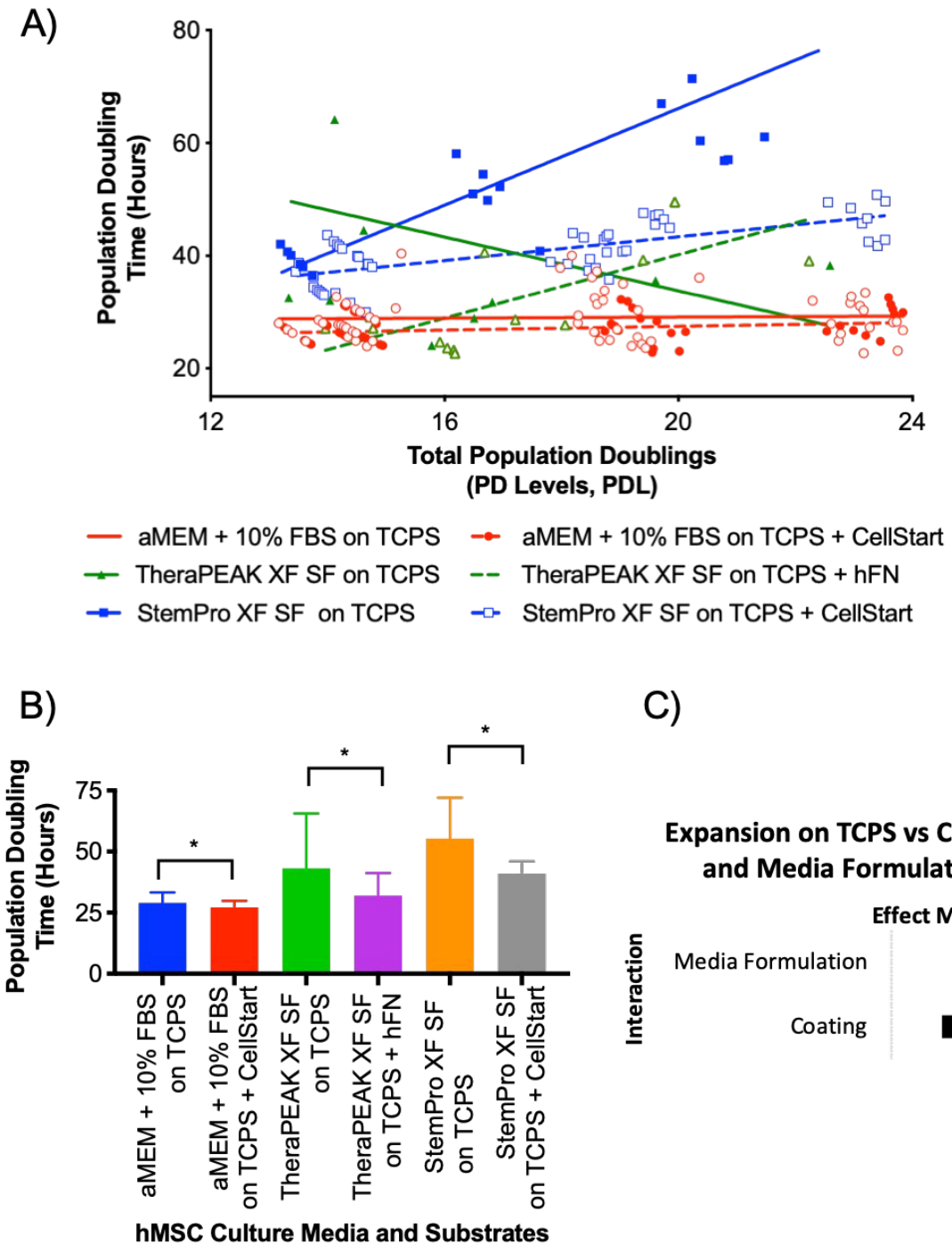


Figure S3. hMSC growth and maintenance in different xeno-free (XF) and serum-free (XF) media and protein-coated tissue-culture polystyrene (TCPS) substrates. A,B) Media and substrate properties independently and combinatorially affect age-dependent hMSC reduction in proliferative capacity. C) Multivariate analysis of previous quality control data reveals the importance of both media formulation and substrate adhesivity on hMSC adhesion.

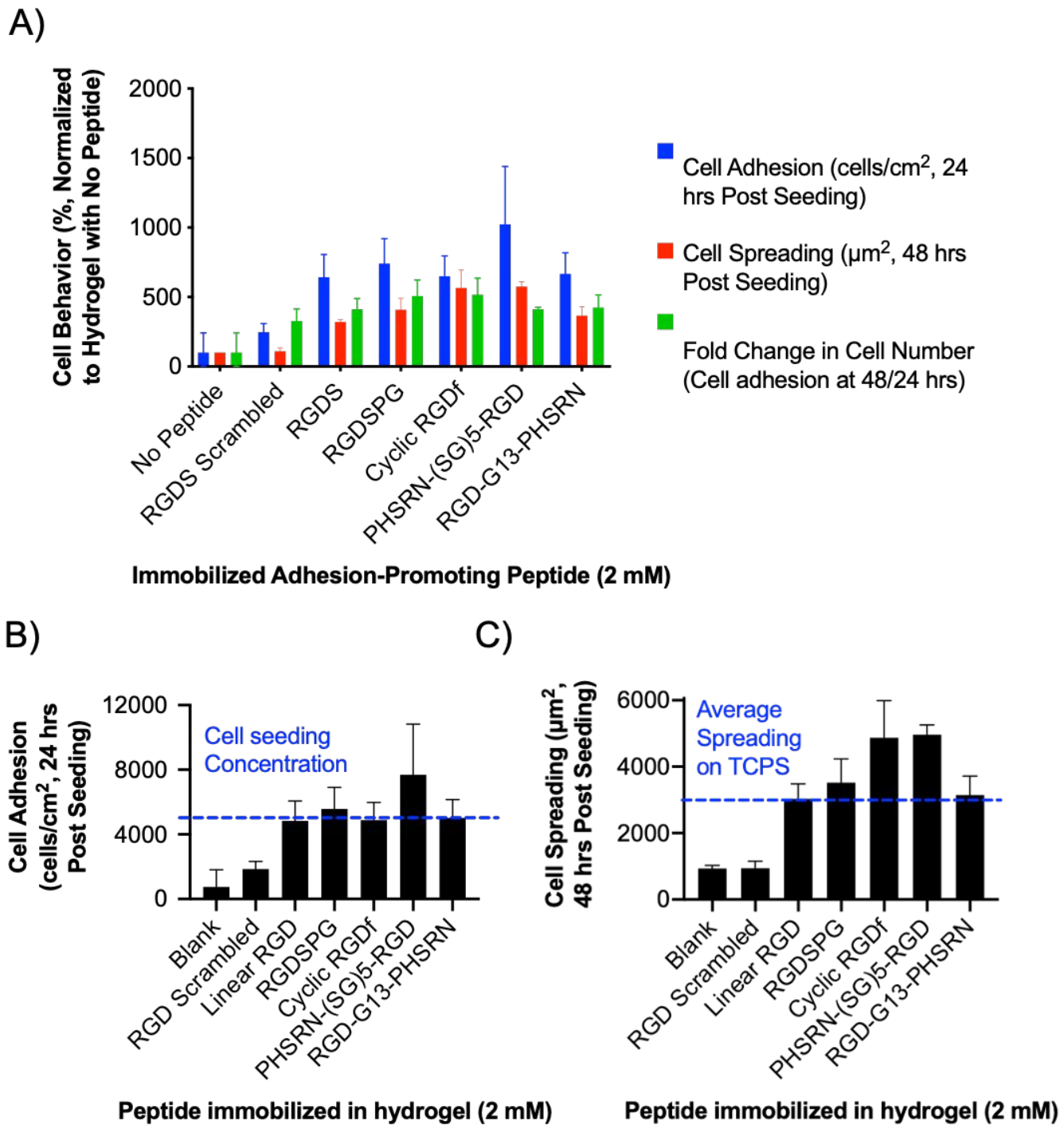


Figure S4. First screen of hydrogels containing various RGD-containing adhesion-promoting peptides and their effects on hMSC A,B) adhesion, A,C) spreading, and A) expansion.

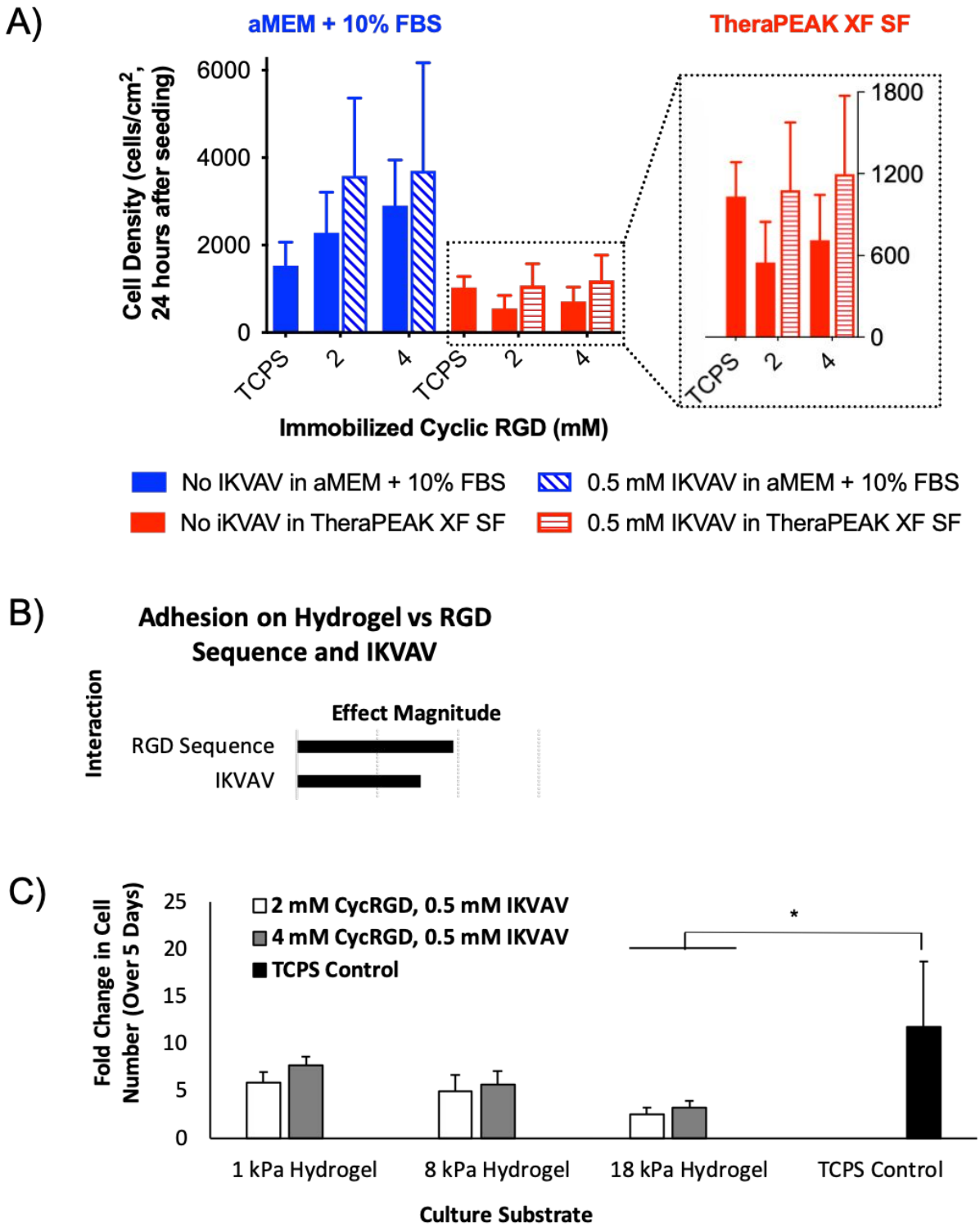


Figure S5. Second screen of the effects of hydrogels with immobilized A) Cyclic RGDf and IKVAV on hMSC adhesion in SC and SF media. B) MVA of RGD and IKVAV and their effects on hMSC adhesion. A,C) The combinatorial effects of adding IKVAV to Cyclic RGDf-containing hydrogels on stable hMSC adhesion and long-term expansion in SF culture.

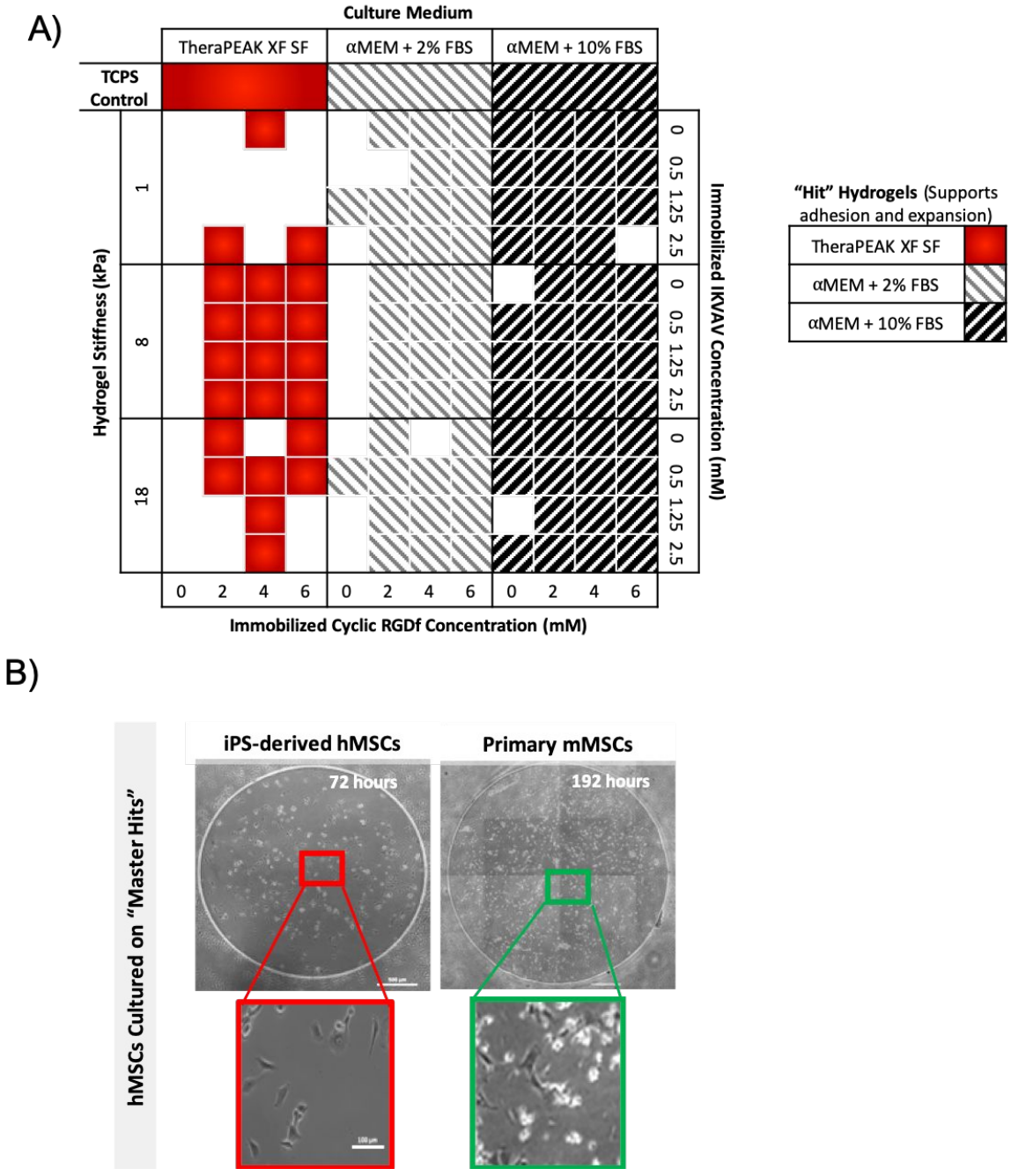


Figure S6. Screening for A) hydrogel substrates that support hMSC adhesion and expansion in TheraPEAK XF SF, αMEM + 2% FBS, and αMEM + 10% FBS media ("hits") and B) "master hits" that support media- and cell source-agnostic hMSC culture.

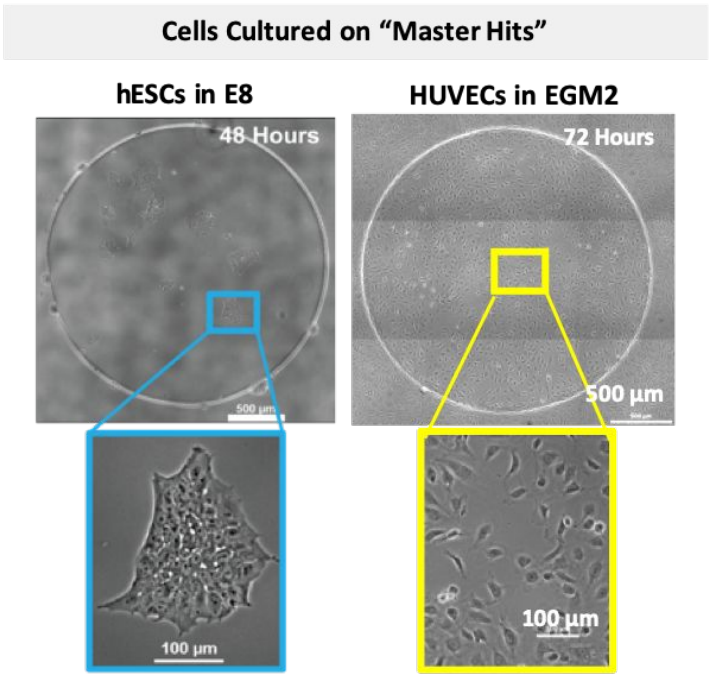


Figure S7. Screening workflow for use in identifying substrates for serum-free hESC and -HUVEC attachment and proliferation

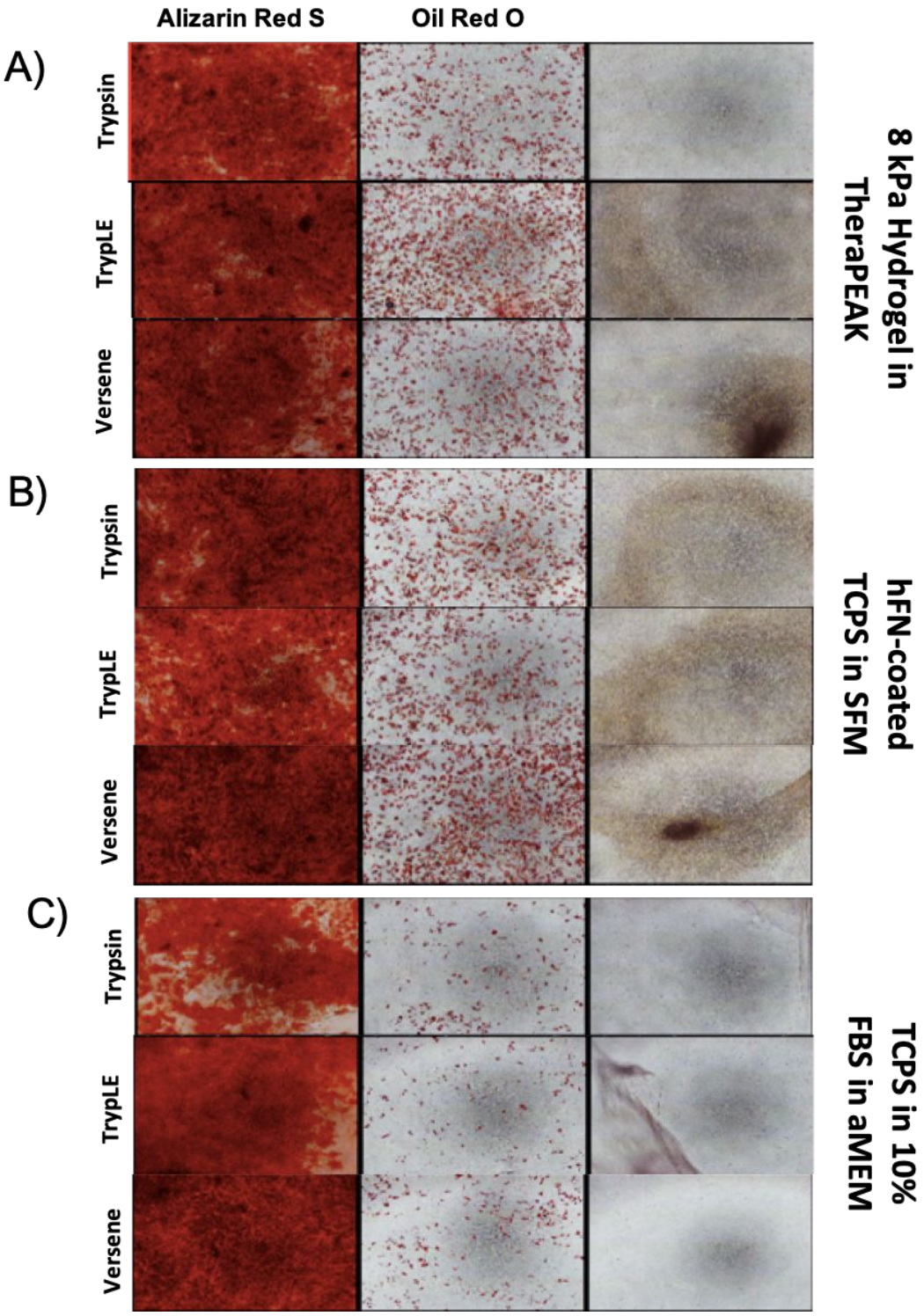


Figure S8. hMSC multipotency analysis after 8 days of culture on A) 8 kPa hydrogel in SFM, B) hFN-coated TCPS in SFM, or C) TCPS in 10% FBS in a αMEM and dissociated with trypsin, trypLE, or versene during harvest. All differentiation experiments conducted on collagen-coated TCPS. Osteogenic differentiation and no differentiation control (culture in αMEM + 2% FBS) assessed with Alizarin Red S staining and adipogenic differentiation staining assessed with Oil Red O staining after 28 days of culture in differentiation media.

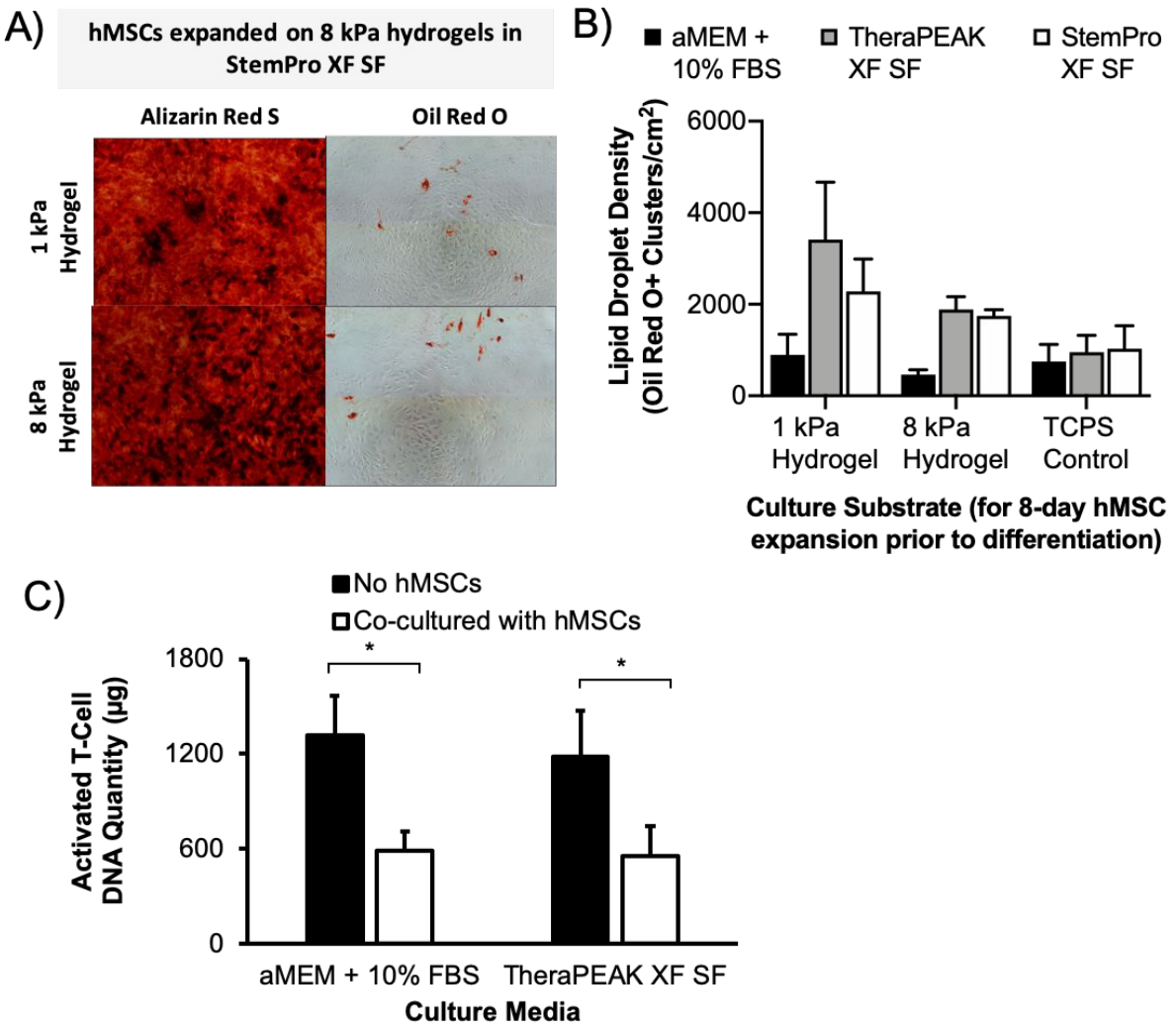


Figure S9. Confirmation of functional hMSCs after 8-days of expansion on hydrogels in SC and SF media. A,B) Directed differentiation and C) immunomodulatory activity by controlling T-cell proliferation.

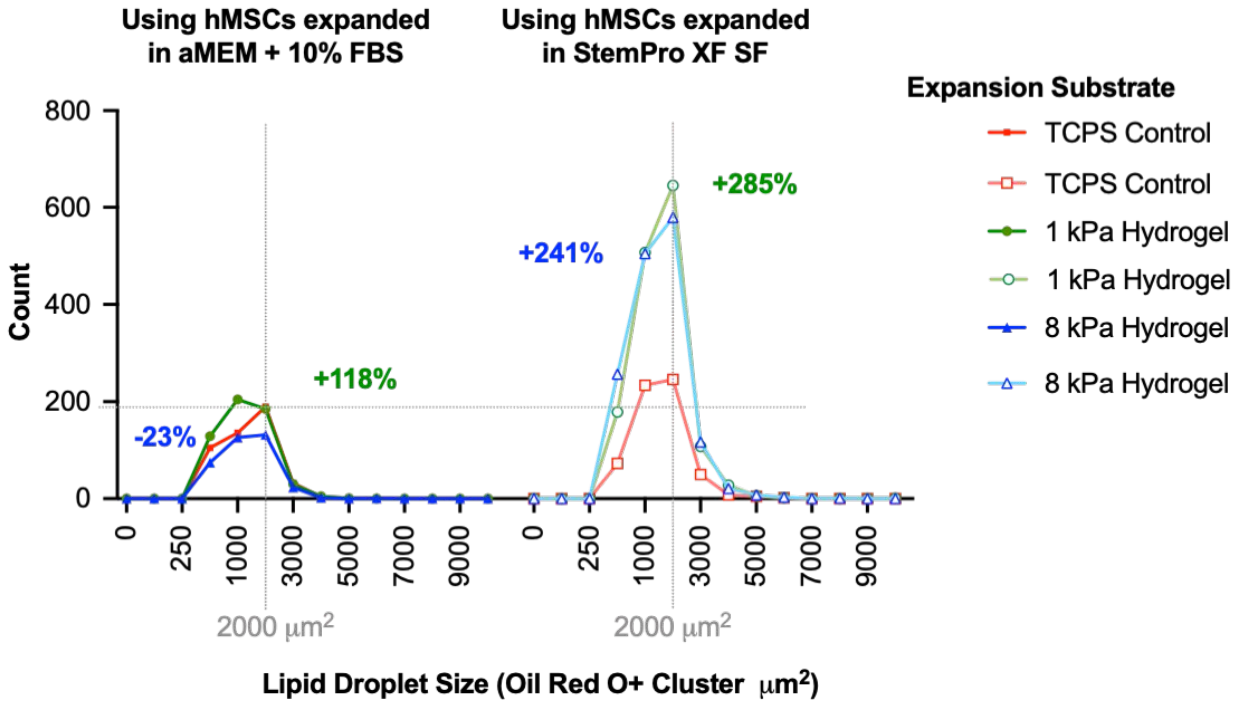


Figure S10. Directed hMSC adipogenic differentiation and Oil Red O+ quantification of hMSCs expanded for 8 days in αMEM + 10% FBS or StemPro XF SF media on TCPS controls or hydrogels of varying stiffness. Note TCPS control for αMEM + 10% FBS is uncoated TCPS and for StemPro XF SF is CellStart-coated TCPS.

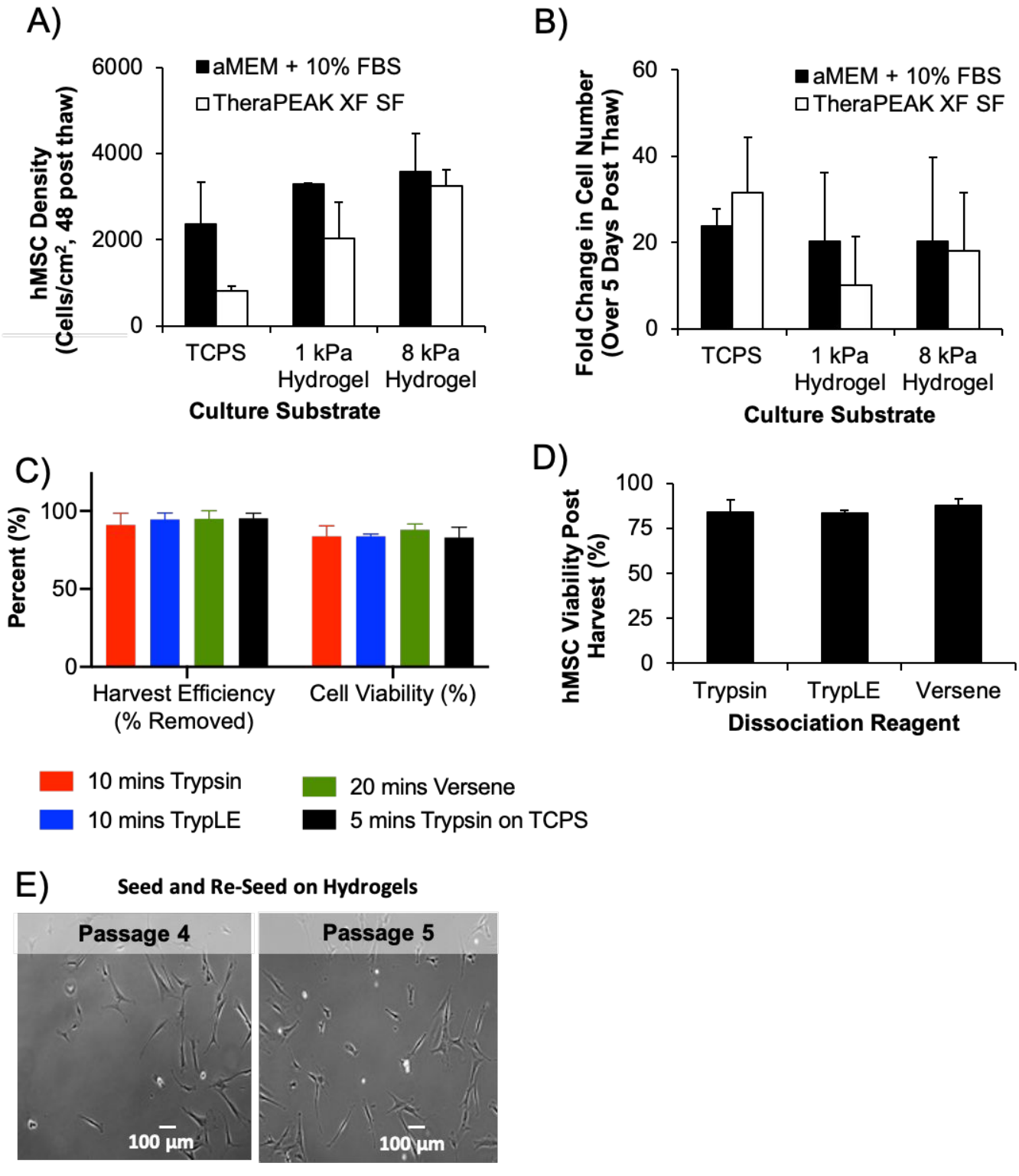


Figure S11. Integration of "hit" hydrogel substrates into standard hMSC culture workflow. A) hMSC adhesion and B) expansion following thaw directly onto hydrogels. hMSC C,D) viability following harvest with varying enzymatic and non-enzymatic dissociation reagents and E) after re-seeding onto new hydrogel substrates for continued expansion.

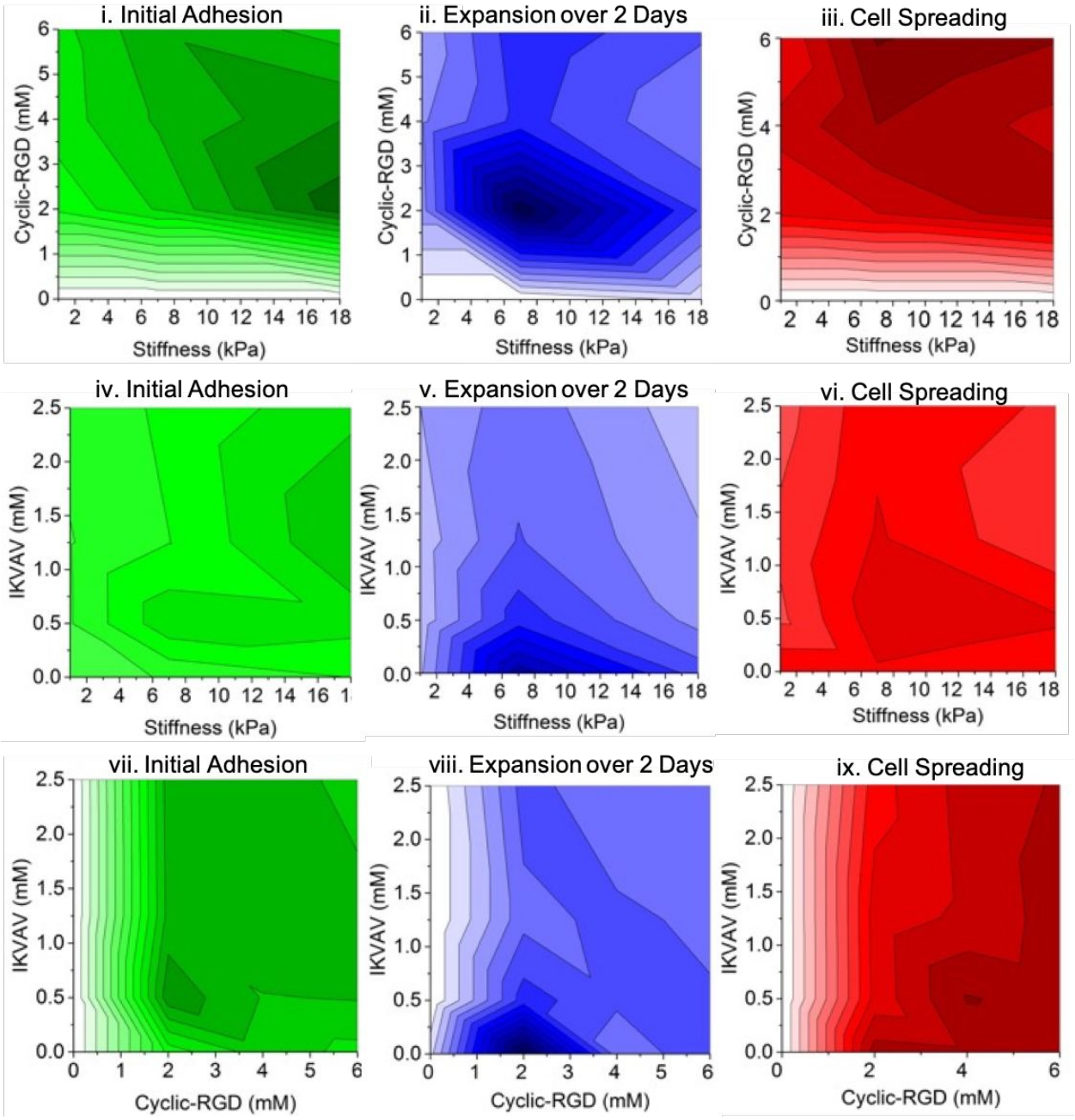
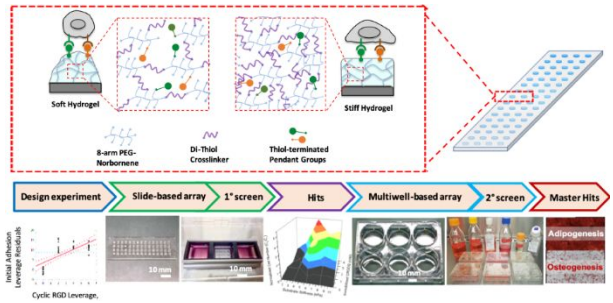


Figure S12. Heat map of hMSC i, iv, vii) adhesion, ii, v, viii) expansion and ii, vi, ix) spreading in TheraPEAK chemically-defined XF SF medium. Increasing color intensity indicates increasing adhesion, expansion, or spreading.



TOC Caption: Synthetic hydrogel arrays combined with a design of experiments approach identified hydrogel compositions for media-agnostic human mesenchymal stromal cell culture.