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Polystyrene-coated micropallets for culture and separation of primary muscle cells

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Abstract

Despite identification of a large number of adult stem cell types, current primary cell isolation and identification techniques yield heterogeneous samples, making detailed biological studies challenging. To identify subsets of isolated cells, technologies capable of simultaneous cell culture and cloning are necessary. Micropallet arrays, a new cloning platform for adherent cell types, hold great potential. However, the microstructures composing these arrays are fabricated from an epoxy photoresist 1002F, a growth surface unsuitable for many cell types. Optimization of the microstructures' surface properties was conducted for the culture of satellite cells, primary muscle cells for which improved cell isolation techniques are desired. A variety of surface materials were screened for satellite cell adhesion and proliferation and compared to their optimal substrate, gelatin-coated Petri dishes. A 1-μm thick, polystyrene copolymer was applied to the microstructures by contact-printing. A negatively charged copolymer of 5% acrylic acid in 95% styrene was found to be equivalent to the control Petri dishes for cell adhesion and proliferation. Cells cultured on control dishes and optimal copolymer-coated surfaces maintained an undifferentiated state and showed similar mRNA expression for two genes indicative of cell differentiation during a standard differentiation protocol. Experiments using additional contact-printed layers of extracellular matrix proteins collagen and gelatin showed no further improvements. This micropallet coating strategy is readily adaptable to optimize the array surface for other types of primary cells.

Keywords

Bioanalytical methods; Biomaterials; Cell systems/Single cell analysis

1. Introduction

Stem cells hold the promise of revolutionizing tissue engineering and other areas of regenerative medicine. Satellite cells, which are muscle progenitor cells, are a stem cell of great interest to the research community surrounding the family of diseases known as muscular dystrophy [1–4]. These diseases lead to a loss of muscle strength and/or function. The most severe form of muscular dystrophy, Duchenne muscular dystrophy (DMD),

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presents a progressive loss of strength in skeletal muscle and leads to muscle atrophy. Complications from progressive muscle deterioration limit the lifespan of affected individuals to two to three decades. Several animal models for DMD have been developed, with the two most influential being the mouse (MDX – muscular dystrophy X-linked) and the canine (GRMD - Golden retriever muscular dystrophy). The mouse model has been used extensively to examine the underlying disease physiology [5,6]. The canine model, GRMD, better mimics the human disease in severity and is a size relevant model [7,8]. Currently there are no clinically available therapies that correct, halt or limit the progression of the disease, though clinical trials are underway [9–11].

One therapeutic approach to treat DMD uses transplantation of satellite cells to correct or replace the cells responsible for muscle tissue regeneration. This approach, in principle, has the ability to restore lost muscle mass in late-stage patients. Currently, techniques to isolate and purify satellite cells and other muscle progenitor cells such as myoblasts have been based primarily on the preplate method and flow cytometry. The preplate technique is based on the adhesion of cells to polystyrene tissue culture dishes and involves repeated decanting and culture of the supernatant [12,13]. Quiescent satellite cells become activated only after a stimulus signals the need to repair damage. Thus in the initial platings, these cells are non-adherent and remain in the supernatant. Other undesired cell types, such as fibroblasts and macrophages, are programmed to actively perform functions within the muscle and adhere rapidly to polystyrene, remaining on the surface during the initial platings [14,12]. This preplating technique ultimately results in a satellite-cell-enriched but nonetheless heterogeneous mixture of cells.

Alternatively, flow cytometry protocols are capable of generating populations of increased purity, but require functional antibodies specific to cell surface markers. Particularly for canine cells, which at present lack an adequate repertoire of antibodies for selection, the technique's ability to sort and purify the desired cells remains limited and requires further cell characterization. Thus, new technologies are needed to more effectively sort and purify primary canine satellite cells (PCSCs).

The advent of microfabricated devices has enabled novel investigations of biological properties. Micropallet arrays have provided a means to clonally culture and isolate cells based on a wide range of characteristics, including the presence or absence of fluorescently tagged proteins, and additional criteria not available to flow cytometry, including cell morphology, growth rate, and other dynamic behaviors [15,16]. With the large number of microstructures available on an array, the cloning and isolation of moderate to large numbers of cells is greatly simplified over standard tissue culture cloning techniques. Micropallet arrays have previously been used to clone and sort tumor cells, murine embryonic stem cells, and other cell lines [17,18,16,15]. While numerous generations of these cell lines have been adapted to cell culture conditions, more physiologically relevant primary cells are not so adept at adhesion to artificial surfaces. This creates the need for a tailored culture surface to meet the requirements of these primary cells.

In this research, we have optimized the surface of micropallets for PCSCs, the cells responsible for maintenance and regeneration of skeletal muscle. Contact printing of the micropallets was evaluated for its capacity to generate a suitable surface for the culture of PCSCs and to lay the groundwork for developing procedures applicable to other primary cell types [19–21]. The long-term goal of this work is to utilize the micropallet arrays to sort PCSCs using a variety of parameters and shorter timescales not available through traditional cloning techniques or flow cytometry. This is expected to enable more efficient characterization of these cell types than is currently possible, as well as identify new cell subsets not previously identified.

2. Materials and Methods

2.1 Polymer and Copolymer Synthesis

Various polymers were synthesized, including neutral polystyrene (PS) and positive and negative copolymers. Briefly, components (Table 1) were weighed and mixed in a fume hood and placed in a 60 °C water bath overnight to polymerize. Negatively charged copolymers containing > 2.5% acrylic acid (AA) precipitated out of the toluene solvent forming a solid layer on the bottom of the reaction vessel. After reactions using these concentrations and polymers, the remaining solvent was decanted and replaced with an equal amount of tetrahydrofuran (THF) (Sigma, St Louis, MO) to solubilize the copolymer. For the 2.5% AA in PS copolymer, only a partial copolymer layer precipitated from the toluene, thus the toluene was evaporated from the mixture on a 60 °C hotplate to recover any non-precipitated copolymer. Once the toluene was removed, an equal volume of THF was added to dissolve the 2.5% AA in PS copolymer completely. Positively charged copolymers containing 4-vinyl pyridine (4VP) were fully soluble in toluene, so replacing toluene with THF was not necessary.

2.2 Cell Isolations and Culture

PCSCs were isolated from muscle biopsies of the vastus lateralis of a normal dog in the GRMD colony at University of North Carolina at Chapel Hill (UNC-CH). Cells were isolated from biopsies as previously described with minor modifications [14,12]. Briefly, biopsy material was finely minced and digested with collagenase in growth media, 16.5% FBS in Dulbecco's Modified Eagle Media (DMEM), for 6–8 h. Material was rinsed and digested with 0.05% trypsin for 1 hr with agitation every 15 min. Material was the passed through a 100 μm screen followed by a 40 μm screen and plated on 0.1% gelatin (Millipore, Billerica, MA) coated tissue-culture-treated polystyrene (TC) Petri dishes (BD Falcon, Franklin Lakes, NJ). Six successive platings with the preplate procedure resulted in enriched populations of PCSCs, with cells from plates 4, 5, or 6 used in the current experiments [13,12]. To confirm the presence of PCSCs, 1000 cells from passage two of preplate 5 were fixed and stained with anti-desmin antibodies and counterstained with Hoechst 33342 dye. Desmin, a marker for PCSC, was detected in 94% of the cells. The enriched cell populations were further cultured in uncoated TC dishes in 16.5% fetal bovine serum in Dulbecco's Modified Eagle Medium (DMEM), with 1% penicillin-streptomycin defined as standard growth conditions [22]. To differentiate the PCSCs, the cells were cultured in 2% horse serum (Invitrogen, Carlsbad, CA) in DMEM with 1% penicillin-streptomycin, defined as standard differentiation conditions [22].

2.3 Photoresist and Polymer Film Fabrication

Photoresist, 1002F, was prepared as previously described (see also Figure 1A) [23]. Approximately 1.5 mL of 1002F was poured into the center of a plasma-cleaned glass slide (75 × 25 × 1 mm, Corning, Corning, NY). The photoresist was spin-coated onto the slides by spinning at 500 rpm for 10 s, followed by 2200 rpm for 30 s. Photoresist-coated glass slides were placed in a 95 °C oven (Fisher Scientific, Dubuque, IA) for a 50 min soft bake, removed and allowed to cool to room temp. Soft baked photoresist was placed on a UV exposure system (Oriel, Newport Stratford, Inc., Stratford, CT) and illuminated with 1500 mJ. The photoresist-coated surfaces were returned to the 95 °C oven for a 10 min post-exposure bake (PEB), removed and permitted to cool to room temp. PEB-photoresist was placed in a photoresist developer (1-methoxy-2-propyl acetate, Sigma-Aldrich, St. Louis, MO) on a rotary shaker for 4 min. Developed photoresist was rinsed with 2-propanol (VWR, West Chester, PA), blown dry with nitrogen, and placed on a hotplate at 95 °C for 10 min, followed by 70 min at 120 °C. To add the polystyrene film, photoresist films were again placed on the spin coater, coated with 3 mL of the desired polystyrene mixture by spinning

at 500 rpm for 10 s. Polystyrene-coated photoresist films were then placed in a 60 °C vacuum oven (VWR, West Chester, PA) for at least 48 h to evaporate any remaining solvent.

2.4 Micropallet Array Fabrication and Contact Printing of Polystyrene and Extracellular Matrices (ECMs)

Micropallet arrays were fabricated as previously described (see also Figure 1A) [15,23]. Briefly, a mask outlining numbered micropallets was used to photolithographically define a 50 × 50 array of 150 × 150 × 50 m (L×W×H) micropallets possessing a 50 μm gap between micropallets. Polyacrylic acid (PAA) (Polysciences Inc., Warrington, PA), 25% in aqueous solution (MW:~50,000) diluted to 8% in DI water, was applied to the upper micropallet surfaces via contact printing [19]. This deposited PAA would serve later as a sacrificial layer to remove the organosilane ([heptadecafluoro-1,1′, 2, 2′-tetrahydrodecyl] trichlorosilane, Gelest, Morrisville, PA) from the top surface of the micropallets. Only arrays possessing ≥90% fully PAA-coated micropallets were used in subsequent steps. 80% of the arrays met this criterion. The organosilane was applied by vapor-deposition in a vacuum chamber as previously described [15]. Arrays were removed from the chamber, incubated in deionized water for 30 min and rinsed with deionized water to remove the sacrificial PAA layer. Micropallet surfaces were then contact-printed with the desired polystyrene coatings (Electronic Supplementary Material Table S1), again only arrays possessing ≥90% fully polystyrene-coated micropallets were used in subsequent steps [19]. 80% of the arrays met this criterion. Once printed with polystyrene, arrays were placed in a 60 °C vacuum oven for 48 h to remove any remaining solvent. Arrays were sterilized with 75% ethanol and allowed 30 min to dry.

To contact-print ECMs, 5μL of 1 mg/mL collagen or 3 μL of 1 mg/mL gelatin was added to a sterile glass slide and spread with the side edge of a pipette tip to cover a 1 cm^2 area. Arrays were then inverted and pressed against the protein-coated slide and removed to create a single-layer coating. This procedure was repeated to create a double-layer coating. In experiments to determine the persistence of the gelatin layer contact printing, gelatin was labeled with a fluorescent Alexa Fluor®568 (Invitrogen, Carlsbad, CA) per manufacturer protocol. Labeled gelatin was printed in two layers onto the micropallets, allowed to dry, placed in standard growth conditions and imaged. Cells were plated onto the array and images were taken again at 24 and 96 h.

2.5 Measuring Contact-Printed PAA and Copolymer Thickness

Micropallets contact-printed with copolymers were observed using an environmental scanning electron microscope (ESEM) (Quanta 200, FEI Company, Hillsboro, OR). The ESEM was performed in low vacuum (0.75 Torr) mode and a backscattered electron detector was used to acquire images. Contact-printed micropallets were also removed from the glass surface and imaged from the side (Figure 1D). The thickness of the contacted-printed copolymer layer, in addition to the thickness of the contact-printed PAA layer, was measured using a profilometer (P6 Stylus Profilometer, KLA Tencor, San Jose, CA). Copolymer and PAA thickness were determined by measuring micropallet height before and after contact-printing.

2.6 Experiments Studying Cell Adherence and Proliferation

Cell chambers were created from poly(dimethylsiloxane) (PDMS) reservoirs (10 × 10 × 8 mm) glued with uncured PDMS onto the 1002F and copolymer films or micropallet arrays. Before use, the cell chambers were sterilized with 75% ethanol and allowed to dry 30 min in a tissue culture hood under sterile conditions. Reservoirs were rinsed twice with 1 mL of phosphate buffered saline (PBS). Before seeding cells, 500 μL of media was added and

allowed to sit for 10 min. Cells were then loaded into the reservoir as 500 μL of an 8000 cells/mL suspension added drop wise into the reservoir in a grid-like pattern to spread the cells evenly over the array. Cells were then placed in a 37 °C incubator (5% CO_2, ~95% RH) for up to 96 h during the course of the experiment.

2.7 Cell Imaging and Counting

Cells grown on films of copolymer, photoresist, or micropallet arrays were stained with 1 μg/mL Hoechst 33342 (Sigma, St. Louis, MO) for 10 min in a 37 °C incubator. Cells were imaged with an epifluorescence microscope (IX81, Olympus, Center Valley, PA) using a Coolsnap HQ^2 charged coupled device camera (Photometrics, Tucson, AZ). For cells on films, six independent images were obtained at 4× magnification, and cells were counted using ImageJ software (NIH, Bethesda, MD). Cells grown on micropallet surfaces were imaged at 10× and the numbers of cells per micropallet were counted using a Matlab script (Mathworks, Natick, MA).

2.8 mRNA Analysis

Four aliquots of 150,000 cells were placed in 1.5 mL microcentrifuge tubes. Tubes were centrifuged at 600 g for 2.5 min and media was then removed, leaving the cell pellet. Pellets were rinsed with 1 mL of 1× PBS and centrifuged again. The supernatant was removed leaving the cell pellet in 100 μL PBS. A 100 μL quantity of 2× nucleic acid lysis buffer (Applied Biosystems, Carlsbad, CA) was then added to the tube. The suspension was mixed and placed in a −20 °C freezer. These tubes were denoted as Day 0. Additional aliquots of cells were plated on the experimental surfaces as follows. Glass slides (75 × 25 × 1 mm) were coated with 1002F photoresist and 5% AA in PS films as described above and PDMS reservoirs were applied. Samples were sterilized with ethanol and rinsed with PBS, followed by 4 mL of warmed growth media. A cell suspension (37,500 cells/mL, 2 mL) was added drop wise to each plate, applied in a grid-like pattern. Plates were then placed in a 37 °C incubator (5% CO_2, ~95% RH). On Days 3, 5, and 7, cells were collected for analysis. To collect, samples were rinsed with PBS and cells were removed with 500 μL of trypsin-EDTA (Invitrogen, Carlsbad, CA) followed by addition of 500 μL of PBS. Samples were then prepared in the same fashion as Day 0. Once all trials were complete, samples were submitted to the UNC-CH Animal Clinical Chemistry and Gene Expression Laboratories for RNA analysis using TaqMan® probes on an ABI PRISM® 770 Sequence Detection System (Applied Biosystems, Carlsbad, CA) using primer probe sequences developed for *Pax7* and *Utrophin*. *Pax7* primers/probe: Forward (AGT ACG GCC AGA CTG CTG TT), Reverse (AAT GCT CCC CGA GCT TCA TA), Probe (Fam AC CTG GCC AAA AAC GTG AGC CTC TCTamra). *Utrophin* primers/probe: Forward (CTG ACA GCA GCT CTA CCA CA), Reverse (CCT CCA AGC GTC TGA CAG TA), Probe (Fam TG TGG AGG ACG AGC ATG CCC TCA TC Tamra).

2.9 PCSC Separation and Pax7 Verification

A heterogeneous population of cells derived from a canine muscle biopsy was obtained from the earlier stages of the preplate procedure described above, specifically preplate 4. 2000 cells were seeded onto an array of 2500 micropallets of dimensions 150 × 150 × 50 μm (L×W×H). Cells were allowed 48 h to adhere and micropallets were examined for cellular adhesion. Micropallets containing cells with a spindle-like morphology were released from their glass substrate using an ACL-1 532 nm frequency-doubled Q-switched Nd:YAG laser (New Wave Research, Fremont, CA) generating ~7 μJ laser pulses with a 5 ns pulse width as previously described with minor modifications [15]. The glass slide containing micropallets was placed inverted atop a 15 × 15 × 4 mm PDMS reservoir affixed to a glass slide containing culture media (described above). The laser was focused at the base of the micropallet through a Nikon Eclipse E800 upright microscope (Nikon, Melville, NY) using

a Nikon 20× extra-long working distance objective (Nikon, Melville, NY). The microscope was fully enclosed in a 37°C incubated environment with humidity and temperature controls provided by an Air-Therm ATX-H Controller (World Precision Instruments, Sarasota, FL) and CO_2 control provided by a ProCO$_2$ Controller (Biospherix, Lacona, NY) (4% CO_2, ~75% humidity). After being released into the media-filled tissue culture dish, cells were placed in a 37°C incubator (5% CO_2, ~95% RH) and allowed to proliferate for 48 hrs. After this time, cells were stained via a modified protocol for the transcription factor and intracellular marker Pax7 [24,25]. Briefly, cells were rinsed in PBS and fixed in a 4% paraformaldehyde solution in PBS for 10 min. Cells were permeabilized for 15 min with 0.5% Triton X-100™ in PBS to permit antibody access to the nucleus. Primary mouse anti-Pax7 antibody (DSHB, Iowa City, IA) used at 2μg per mL was incubated with cells for 12 h. Secondary anti-mouse antibody labeled with AlexaFluor®594 (Invitrogen, Carlsbad, CA) was incubated at 4 μg/mL with cells for 1 h. Cells were imaged with the previously described epifluorescence microscope using DAPI and Texas Red filters (Olympus, Center Valley, PA).

3. Results and discussion

3.1 Surface Modifications for Growth of PCSCs

The 1002F photoresist from which the micropallet arrays were fabricated has been shown to be suitable for culturing tumor cell lines [15,21]. In some instances, the 1002F required an additional coating such as collagen or fibronectin for cell attachment and growth. When PCSCs were cultured on the arrays, cells initially adhered to the 1002F surface, but did not proliferate. The addition of an adsorbed ECM to the micropallet surfaces also failed to support cell proliferation. The 1002F beneath the ECM may have leached a component toxic to the overlying cells or the ECM may not have exhibit the same properties as those on the polystyrene surfaces of tissue culture dishes. For this reason, a variety of surface coatings designed to mimic either the glass or polystyrene culture surfaces on which these cells are traditionally grown were placed onto the arrays. Layers of microbeads (22 nm and 500 nm silica glass or 50 nm polystyrene) contact-printed on to the array surfaces exhibited a non-uniform coating, with cracking of the printed layer and surface-detachment after 4 days. [19]. Since these defects were not compatible with light microscopy and may have also permitted leached materials from the 1002F to contact the cells, these modifications were not tested further.

Polystyrene is a standard and well-accepted surface for cell culture; therefore, polystyrene in an organic solvent was contact-printed onto the micropallets [19]. The polystyrene layer exhibited a uniform surface coverage, unambiguous transparency, and no visible cracking, Figure 1B, C. The coating also remained on the micropallet surface for two weeks in culture, the maximum time examined, making the contact-printing of thin layers of polystyrene a convenient method of modifying micropallet surfaces for microscopy applications.

Standard TC dishes are the accepted culture vessel for PCSCs, and were therefore used as the gold standard for comparison of cell adhesion and proliferation [13,12]. Commercial TC dishes are oxidized, imparting a negative charge to the surface [26,27]. Direct oxidation of the polystyrene-coated micropallet surfaces was not possible since the hydrophobic organosilane coating on the intervening glass surface was not stable to oxidants. This hydrophobic coating is used to entrap air between the micropallets, blocking cell access to the inter-pallet regions. Since these virtual air walls are critical to direct cells to the micropallet surfaces, another strategy was required to impart a charge to the polystyrene coating. For this reason, the charged monomers acrylic acid (negative charge) or 4VP (positive charge), were mixed into the styrene monomer at different concentrations during polymer synthesis to form polystyrene copolymers with varying charge densities. To

determine whether PCSCs could be cultured on these polymers, flat films comprised of the copolymers were assessed for PCSC adherence and growth over 4 days, Figure 2. PCSCs did not efficiently adhere to or proliferate on the uncharged polystyrene or the 4VP in polystyrene. Negatively charged copolymers of acrylic acid in polystyrene (AA in PS) supported greater cell adhesion and proliferation than any of the other synthesized surfaces at all time points. Within the first 24 h, the negatively charged AA-in-PS coating showed no significant differences for any AA concentration when compared to standard TC dishes. However, by 96 h, PCSCs on the 5% AA-in-PS surface exhibited equivalent growth properties to that on the TC dish. In contrast, the surfaces with 2.5, 10, 15 and 20% AA in PS demonstrated significantly lower rates of proliferation as revealed by the lower cell numbers at 96 h. Cells grown on the TC dish and 5% AA in PS showed similar morphology, long slender cells, Figure 3. Cells grown on the bare 1002F were scattered and more rounded in morphology. These data demonstrated that PCSCs adhere to and proliferate on the 5% AA in PS copolymer as effectively as on the “gold standard” surface of the TC dish, making 5% AA in PS atop 1002F micropallets an effective growth surface for these primary cells.

3.2 Characterization of Contact-Printed PAA and Copolymer

To determine the thickness of the 5% AA-in-PS layer contact printed on to the micropallets, ESEM was used to image individual micropallets. The apparent polymer thickness was 1–2 μm, Figure 1D. Since the exact orientation of the pallet was difficult to ascertain, more precise measurements of the layer thickness could not be obtained using ESEM. For a more precise measurement, a stylus profilometer was used to measure the height of the micropallet above the glass substrate before and after contact printing with 5% AA in PS. The same procedure was completed for the contact-printed PAA. The copolymer thickness was 1.0 m ± 0.3 μm (n=50), while the PAA thickness was 0.63 μm ± 0.2 μm (n=50).

3.3 ECM Protein Coatings

PCSCs are commonly cultured on collagen or gelatin-coated TC flasks [13,12]. For this reason, gelatin was contact-printed onto the surface of the micropallet arrays composed of 1002F micropallets with a 5% AA in PS top layer. To determine whether the contact-printed gelatin was stable over time, AlexaFluor®568-labeled gelatin was utilized and two layers of fluorescent gelatin were contact-printed onto the array. Arrays with or without cultured cells were incubated for 4 days under standard tissue culture conditions. Images were taken immediately after the adhesion of cells at 4 h and again at 24 and 96 h. The gelatin fluorescence intensity on the micropallets decreased from 1.00 at 4 h to 0.90 ± 0.05 at 24 h. The fluorescence intensity then remained unchanged through 96 h, indicating that the gelatin remained attached to the surface of the pallet for the duration of the culture period. Pallets contact-printed with fluorescent gelatin and cultured with cells also demonstrated a drop in fluorescence from 1.00 at 4 h to 0.90 ± 0.11 at 24 h. The fluorescence intensity was then unchanged through 96 h. In this instance the fluorescence of the cell’s cytoplasm plus that of the micropallet surface was measured since the two fluorescence sources could not be separated. Cells growing on the gelatin demonstrated bright red punctate spots suggesting that they were able to take up the fluorescent dye. This phenomenon was most likely due to the enzymatic degradation of the fluorescent gelatin by the cells and subsequent uptake of the labeled protein [28].

Cells on 5% AA-in-PS-coated micropallets with either a single or double layer of contact-printed gelatin did not demonstrate significant enhancement in initial cell adhesion or proliferation compared to those on the 5% AA-in-PS-coated micropallet alone, Figure 4A&B. Similar results were obtained when collagen was contact-printed onto micropallets in either single or double layers. These data suggested that the ECM coating was not

necessary for PCSC adherence and growth on the micropallets containing a 5% AA-in-PS surface.

3.4 Monitoring Growth and Differentiation of PCSCs on Standard and Optimized Surfaces

A risk in culturing cells on novel surfaces is the potential to alter cellular properties, such as the induction of PCSC differentiation toward terminally differentiated multi-nucleated myotubes [29,30,22,31]. This process is regulated by the transcription factor Pax7, which maintains the stem cell state. Utrophin, a structural protein, is indicative of PCSC differentiation [32,33]. Differentiation patterns of the PCSCs cultured on commercial tissue culture dishes versus films composed of the 5% AA in PS were assayed for their ability to remain undifferentiated as well as their ability to respond to a differentiation signal. The relative mRNA expression levels of *Pax7* and *Utrophin* corresponding to undifferentiated and differentiated states, respectively, were measured [34,32,33,35,36]. Under standard growth conditions, cells cultured on 5% AA in PS and TC dishes remain undifferentiated, maintaining relatively high stable mRNA levels of *Pax7*, Figure 5A, and low levels of *Utrophin*, Figure 5B [22]. When subjected to standard differentiation conditions (addition of horse serum), the rate of differentiation of PCSCs was similar on both 5% AA-in-PS surfaces and TC dishes, as shown by a decrease in *Pax7* mRNA quantity over time, Figure 5A, and an increase in *Utrophin* mRNA levels over time, Figure 5B [22]. The samples cultured on the TC dishes or the 5% AA-in-PS surfaces under either the standard growth or differentiation conditions did not demonstrate statistically distinct mRNA levels of *Pax7* or *Utrophin* using a one tailed student T-test.

3.5 PCSC Separation and Pax7 Verification

Satellite cells have been demonstrated to grow with a spindle-like morphology in culture (characterized by two to three points of surface attachment) [37,38]. Satellite cells also express the transcription factor and internal marker, Pax7 [24,25]. To demonstrate that satellite cells could be sorted based on their morphology, micropallet arrays coated with 5% AA in PS were seeded with a heterogeneous mixture of cells from a canine muscle biopsy [39]. Micropallets containing cells with a spindle-like morphology were identified and released from the array onto a plasma-treated glass slide, Figure 6A. After 48 h in culture, cells were stained with the DNA-binding dye, Hoechst 33342 and then examined by brightfield and fluorescence microscopy, Figure 6B and C. The presence of the transcription factor Pax7 was examined using immunochemistry, Figure 6D. From this experiment, it was found that 88% of the collected spindle-shaped cells on micropallets were also Pax7+. Thus the 5% AA in PS coating enabled PCSCs to separated based on their morphology.

4. Conclusions

A suitable copolymer surface has been developed for the culture of PCSCs atop micropallets. Our results show that the 5% AA-in-PS copolymer mimicked the ability of tissue culture polystyrene in supporting the adhesion and proliferation of the PCSCs. Importantly, cells cultured on this surface showed neither induced differentiation under standard growth conditions nor a rate of spontaneous differentiation greater than that seen with the control TC dish under standard differentiation conditions as indicated by mRNA levels of *Pax7* and *Utrophin*. This copolymer material was readily and consistently applied to the surface of the micropallets using the described contact printing procedure. Experiments using additional contact-printed layers of the ECM proteins collagen and gelatin on the 5% AA-in-PS layer showed no increase in either initial PCSC adhesion or proliferation rates relative to surfaces without the extracellular matrices. The contact-printing method developed in this study is readily applicable to screen thick coatings of almost any polymer matrix for the growth and well being of primary cells including stem

cells. More importantly this should enable, as we have demonstrated, the sorting of primary cells cultured on the arrays based on a number of cellular attributes (morphology and other spatial properties, growth rate and other temporal behaviors) not accessible by current cell separation methods such as preplating and flow cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Rando TA, Pavlath GK, Blau HM. The fate of myoblasts following transplantation into mature muscle. Exp Cell Res. 1995; 220 (2):383–389. [PubMed: 7556447]
2. Partridge T. The current status of myoblast transfer. Neurol Sci. 2000; 21 (5 Suppl):S939–942. [PubMed: 11382193]
3. Partridge T. Myoblast transplantation. Neuromuscul Disord. 2002; 12(Suppl 1):S3–6. [PubMed: 12206788]
4. Quattrocelli M, Cassano M, Crippa S, Perini I, Sampaolesi M. Cell therapy strategies and improvements for muscular dystrophy. Cell Death Differ. 2010; 17 (8):1222–1229. [PubMed: 19876070]
5. Wells DJ, Wells KE. What do animal models have to tell us regarding Duchenne muscular dystrophy? Acta Myol. 2005; 24 (3):172–180. [PubMed: 16629050]
6. Vainzof M, Ayub-Guerrieri D, Onofre PC, Martins PC, Lopes VF, Zilberztajn D, Maia LS, Sell K, Yamamoto LU. Animal models for genetic neuromuscular diseases. J Mol Neurosci. 2008; 34 (3): 241–248. [PubMed: 18202836]
7. Kornegay JN, Tuler SM, Miller DM, Levesque DC. Muscular dystrophy in a litter of golden retriever dogs. Muscle Nerve. 1988; 11 (10):1056–1064. [PubMed: 3185600]
8. Khurana TS, Davies KE. Pharmacological strategies for muscular dystrophy. Nat Rev Drug Discov. 2003; 2 (5):379–390. [PubMed: 12750741]
9. Miyagoe-Suzuki Y, Takeda S. Gene therapy for muscle disease. Exp Cell Res. 2010; 316 (18): 3087–3092. [PubMed: 20580709]
10. Guglieri M, Bushby K. Molecular treatments in Duchenne muscular dystrophy. Curr Opin Pharmacol. 2010; 10 (3):331–337. [PubMed: 20434401]
11. DiPrimio N, McPhee SW, Samulski RJ. Adeno-associated virus for the treatment of muscle diseases: toward clinical trials. Curr Opin Mol Ther. 2010; 12 (5):553–560. [PubMed: 20886387]
12. Rando TA, Blau HM. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J Cell Biol. 1994; 125 (6):1275–1287. [PubMed: 8207057]
13. Jankowski RJ, Huard J. Myogenic cellular transplantation and regeneration: sorting through progenitor heterogeneity. Panminerva Med. 2004; 46 (1):81–91. [PubMed: 15238884]
14. Jankowski RJ, Deasy BM, Huard J. Muscle-derived stem cells. Gene Ther. 2002; 9 (10):642–647. [PubMed: 12032710]
15. Salazar GT, Wang Y, Young G, Bachman M, Sims CE, Li GP, Allbritton NL. Micropallet arrays for the separation of single, adherent cells. Anal Chem. 2007; 79 (2):682–687. [PubMed: 17222037]
16. Wang Y, Young G, Aoto PC, Pai JH, Bachman M, Li GP, Sims CE, Allbritton NL. Broadening cell selection criteria with micropallet arrays of adherent cells. Cytometry A. 2007; 71 (10):866–874. [PubMed: 17559133]

17. Shadpour H, Sims CE, Thresher RJ, Allbritton NL. Sorting and expansion of murine embryonic stem cell colonies using micropallet arrays. Cytometry A. 2009; 75 (2):121–129. [PubMed: 19012319]

18. Wang Y, Young G, Bachman M, Sims CE, Li GP, Allbritton NL. Collection and expansion of single cells and colonies released from a micropallet array. Anal Chem. 2007; 79 (6):2359–2366. [PubMed: 17288466]

19. Xu W, Luikart AM, Sims CE, Allbritton NL. Contact printing of arrayed microstructures. Anal Bioanal Chem. 2010; 397 (8):3377–3385. [PubMed: 20425106]

20. Gunn NM, Bachman M, Li GP, Nelson EL. Fabrication and biological evaluation of uniform extracellular matrix coatings on discontinuous photolithography generated micropallet arrays. J Biomed Mater Res A. 2010; 95 (2):401–412. [PubMed: 20648537]

21. Shadpour H, Sims CE, Allbritton NL. Enrichment and expansion of cells using antibody-coated micropallet arrays. Cytometry A. 2009; 75 (7):609–618. [PubMed: 19504569]

22. Burton NM, Vierck J, Krabbenhoft L, Bryne K, Dodson MV. Methods for animal satellite cell culture under a variety of conditions. Methods Cell Sci. 2000; 22 (1):51–61. [PubMed: 10650335]

23. Pai JH, Wang Y, Salazar GT, Sims CE, Bachman M, Li GP, Allbritton NL. Photoresist with low fluorescence for bioanalytical applications. Anal Chem. 2007; 79 (22):8774–8780. [PubMed: 17949059]

24. Kuang S, Charge SB, Seale P, Huh M, Rudnicki MA. Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. J Cell Biol. 2006; 172 (1):103–113. [PubMed: 16391000]

25. Michal J, Xiang Z, Davenport G, Hayek M, Dodson MV, Byrne KM. Isolation and characterization of canine satellite cells. In Vitro Cell Dev Biol Anim. 2002; 38 (8):467–480. [PubMed: 12605541]

26. Beaulieu I, Geissler M, Mauzeroll J. Oxygen plasma treatment of polystyrene and Zeonor: substrates for adhesion of patterned cells. Langmuir. 2009; 25 (12):7169–7176. [PubMed: 19505169]

27. Dhayal M, Alexander MR, Bradley JW. The surface chemistry resulting from low-pressure plasma treatment of polystyrene: The effect of residual vessel bound oxygen. Appl Surf Sci. 2006; 252 (22):7957–7963.

28. Coopman PJ, Do MT, Thompson EW, Mueller SC. Phagocytosis of cross-linked gelatin matrix by human breast carcinoma cells correlates with their invasive capacity. Clin Cancer Res. 1998; 4 (2): 507–515. [PubMed: 9516943]

29. Gardel M, Schwarz U. Cell-substrate interactions. J Phys Condens Matter. 2010; 22 (19):190301. [PubMed: 21386358]

30. Levy-Mishali M, Zoldan J, Levenberg S. Effect of scaffold stiffness on myoblast differentiation. Tissue Eng Part A. 2009; 15 (4):935–944. [PubMed: 18821844]

31. Lee JH, Jung HW, Kang IK, Lee HB. Cell behaviour on polymer surfaces with different functional groups. Biomaterials. 1994; 15 (9):705–711. [PubMed: 7948593]

32. Figarella-Branger D, Pellissier JF, Bianco N, Karpati G. Sequence of expression of MyoD1 and various cell surface and cytoskeletal proteins in regenerating mouse muscle fibers following treatment with sodium dihydrogen phosphate. J Neurol Sci. 1999; 165 (2):106–115. [PubMed: 10450794]

33. Gramolini AO, Jasmin BJ. Expression of the utrophin gene during myogenic differentiation. Nucleic Acids Res. 1999; 27 (17):3603–3609. [PubMed: 10446253]

34. Deasy BM, Jankowski RJ, Huard J. Muscle-derived stem cells: characterization and potential for cell-mediated therapy. Blood Cells Mol Dis. 2001; 27 (5):924–933. [PubMed: 11783957]

35. Kuang S, Kuroda K, Le Grand F, Rudnicki MA. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell. 2007; 129 (5):999–1010. [PubMed: 17540178]

36. Olguin HC, Olwin BB. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol. 2004; 275 (2):375–388. [PubMed: 15501225]

37. Cossu G, Zani B, Coletta M, Bouche M, Pacifici M, Molinaro M. In vitro differentiation of satellite cells isolated from normal and dystrophic mammalian muscles. A comparison with embryonic myogenic cells. Cell Differ. 1980; 9 (6):357–368. [PubMed: 7438216]

38. Schultz E, McCormick KM. Skeletal muscle satellite cells. Rev Physiol Biochem Pharmacol. 1994; 123:213–257. [PubMed: 8209136]

39. Jankowski RJ, Haluszczak C, Trucco M, Huard J. Flow cytometric characterization of myogenic cell populations obtained via the preplate technique: potential for rapid isolation of muscle-derived stem cells. Hum Gene Ther. 2001; 12 (6):619–628. [PubMed: 11426462]

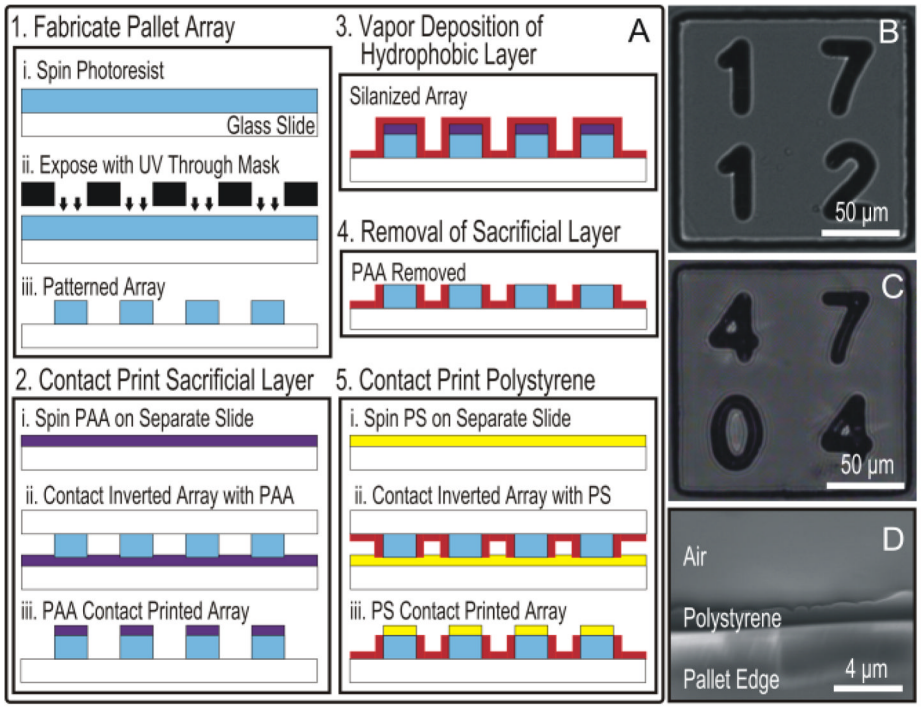

Fig. 1.

Pallet array fabrication. a) Schematic showing patterning of array (step 1), poly(acrylic) acid (PAA) contact printing (step 2), deposition of hydrophobic silane layer (step 3), Removal of PAA layer (step 4), and polystyrene-copolymer contact-printing (step 5). b) Brightfield image of pallet with no coating. c) Brightfield image of pallet contact-printed with 5% AA in PS. d) ESEM image of pallet viewed on edge showing polystyrene thickness.

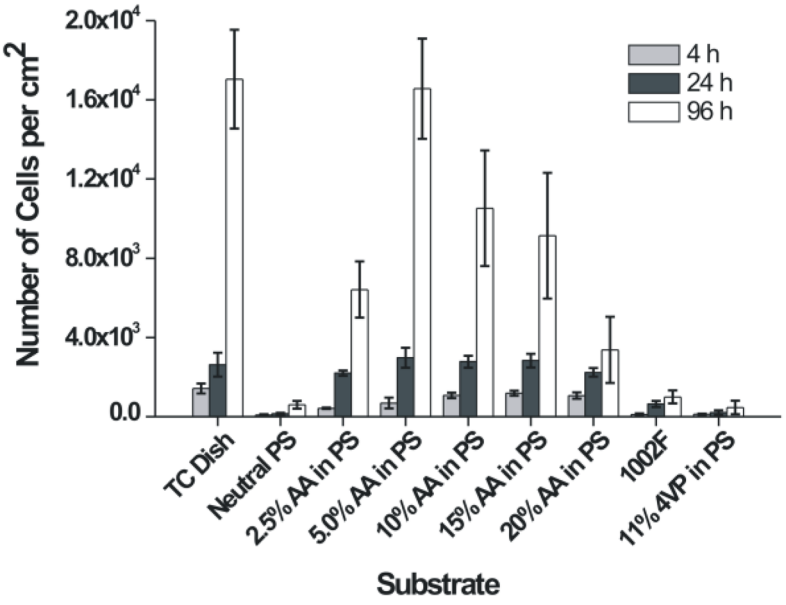

Fig. 2.

Cell adhesion and proliferation on various thin film substrates. PCSCs purified with the preplate technique were cultured on TC dishes, neutral PS, various percentages of AA in PS, 1002F photoresist (1002F), or 4VP in PS. Cells were stained with Hoechst 33342 dye, imaged and counted at 4, 24 and 96 h.

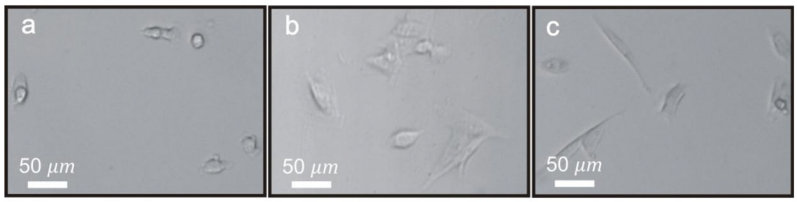

Fig. 3.

PCSCs cultured on: a) bare 1002F photoresist film, b) photoresist film coated with 5% AA in PS and c) TCPS. Magnified images of PCSCs grown on these surfaces are shown in d–f.

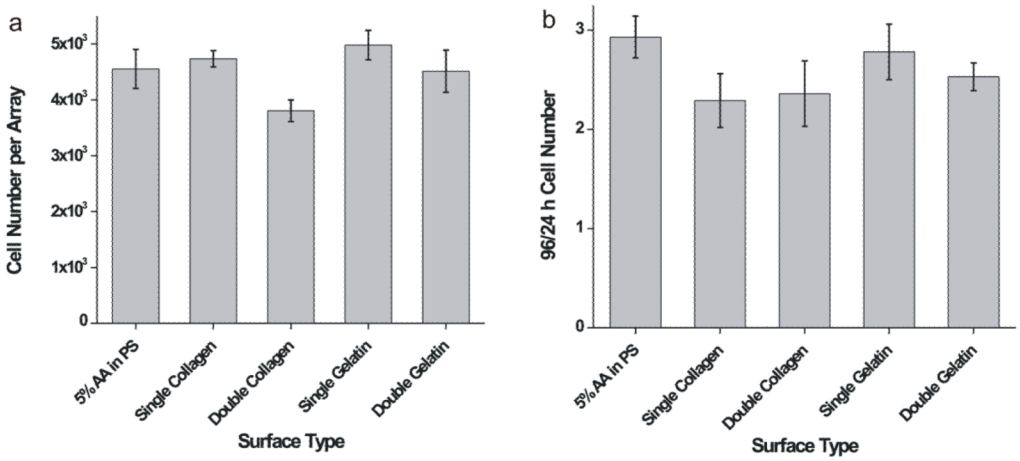

Fig. 4.

Adhesion and proliferation of cells on micropallets contact-printed with selected proteins. a) Number of PCSCs adhering at 24 hours to micropallet arrays contact-printed with 5% AA in PS alone, with 5% AA in PS followed by contact printing with collagen or gelatin (single) or with 5% AA in PS gelatin followed by two-sequential, contact printings with collagen or gelatin (double). b) Ratio of the number of PCSCs counted at 96 hours to 24 hours on micropallet arrays contact-printed with 5% AA in PS alone or with an additional single or double layer of collagen or gelatin. Micropallet arrays for both (a) and (b) consisted of 2500 micropallets of dimensions 150 μm × 150 μm× 50 μm (L × W × H).

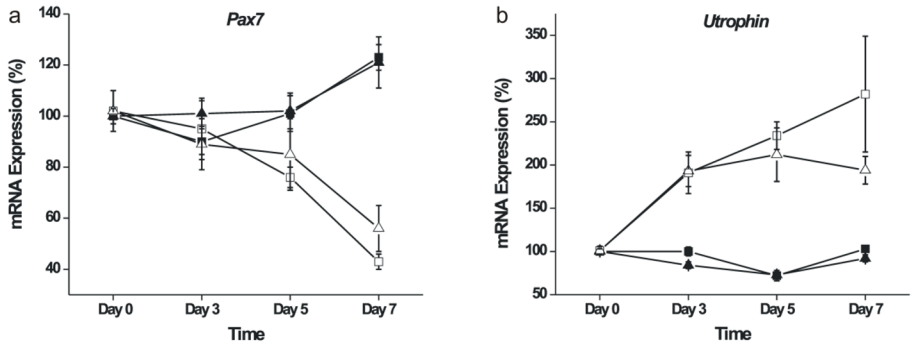

Fig. 5.

mRNA expression levels under standard growth and differentiation conditions. a) *Pax7* mRNA expression in PCSCs grown on TC dishes (squares) or 5% AA in PS-coated films (triangles) in either standard growth (solid symbols) or differentiation (open symbols) conditions. b) *Utrophin* mRNA expression in PCSCs grown on TC dishes (squares) or 5% AA in PS (triangles) in either standard growth (solid symbols) or differentiation (open symbols) conditions. For both (a) and (b) mRNA expression levels are normalized to Day 0.

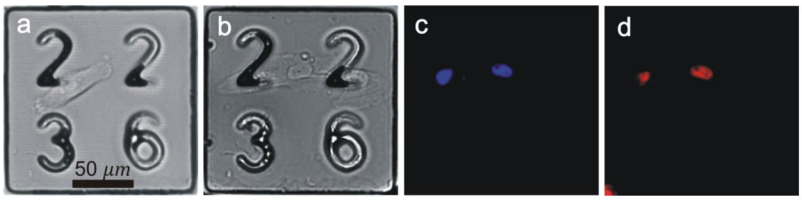

Fig. 6.

Separation of spindle-shaped cells. a) Brightfield image of a spindle-shaped cell growing on 5% AA in PS-coated micropallet before release from the array. b) Brightfield image of 5% AA in PS-coated micropallet with cells 48 h after release from array. c) Fluorescence image of cells from (b) stained with Hoechst 33342 dye (blue). d) Fluorescence image of cell from (b) stained with antibody against Pax 7 (red).

Table 1

Composition of copolymers by wt%

Copolymer Type	Toluene	Styrene	Acrylic Acid	Dibenzoyl Peroxide	4-Vinyl- Pyridine
Neutral	69.8%	29.9%	-	0.3%	-
11% 4VP in PS	67.6%	29.0%	-	0.3%	3.1%
2.5% AA in PS	69.8%	29.2%	0.7%	0.3%	-
5.0% AA in PS	69.8%	28.4%	1.5%	0.3%	-
10% AA in PS	69.8%	26.9%	3.0%	0.3%	-
15% AA in PS	69.8%	25.4%	4.5%	0.3%	-
20% AA in PS	69.8%	23.9%	6.0%	0.3%	-