Developing a high-throughput platform to direct adipogenic and osteogenic differentiation in adipose-derived stem cells

Short title: Developing a high-throughput platform to direct stem cell differentiation

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Abstract

The popular reductionist approach towards stem cell differentiation has identified an array of factors that can induce lineage-specific differentiation. Whether these variables direct differentiation in a multi-variable setting in the same way as a single variable setting is a question of interest. Polyacrylamide hydrogels of specific stiffness were microcontact printed with fibronectin in specific shapes and sizes to construct 54 different extracellular matrix types of defined stiffness, shape and area. Three media types were also used to investigate the effect of both biomechanical and biochemical inducers of differentiation on adipose-derived stem cells. Stiffness was found to be a significant regulator of both adipogenic (3kPa) and osteogenic (35kPa) differentiation across all conditions. Biochemically-induced osteogenic differentiation occurred as well as increased osteogenesis on larger ECM areas (above 5,000 μm^2). The absence of clear trends for all variables demonstrates the atypical expression patterns that arise when variables that may work competitively or synergistically are considered together in a single, high-throughput system.

Keywords

Stem cell differentiation; mechanotransduction; mechanobiology; extracellular matrix; microcontact printing; stiffness; hydrogel

1. Introduction

The unique regenerative capacity of stem cells and their potential for use in a clinical setting has made them incredibly exciting in regenerative medicine. Deepening our understanding of the factors inducing stem cell behaviours such as differentiation may allow us to produce lineage-specific terminally differentiated cells (Dalby *et al.*, 2007; Engler *et al.*, 2006; Underhill & Bhatia, 2007).

Previous studies have identified a variety of biochemical factors that can direct stem cell differentiation into specific lineages including the adipogenic (Choi *et al.*, 2006; Zuk *et al.*, 2001), myogenic (Mizuno *et al.*, 2002) and osteogenic (Dragoo *et al.*, 2003; Zuk *et al.*, 2002). More recently, biomechanical cues have also been shown to direct stem cell differentiation. Mimicking *in vivo* extracellular matrix (ECM) stiffness can induce lineage-specific differentiation; among other lineages, cells sensing an ECM with the same stiffness as neurogenic, adipogenic, myogenic and osteogenic tissues undergo neurogenic (Engler *et al.*, 2006; Georges *et al.*, 2006), adipogenic (Winer *et al.*, 2008), myogenic (Engler *et al.*, 2006) and osteogenic (Engler *et al.*, 2006; Shih *et al.*, 2011; Wen *et al.*, 2014) differentiation, respectively. Controlling ECM size, which regulates cell size, can also induce differentiation. Stem cells cultured on smaller ECM areas undergo adipogenic differentiation while larger ECM areas induce osteogenic differentiation (Kilian *et al.*, 2010; McBeath *et al.*, 2004; Peng *et al.*, 2011). Mimicking cytoskeletal arrangement unique to lineages *in vivo* has also been shown to induce lineage-specific differentiation; for example, culturing cells on rounded ECM shapes induces adipogenic differentiation through a less assembled cytoskeleton while pointed ECM shapes induce osteogenic differentiation through the development of concentrated actin bundles along the edges of the cell (Guilak *et al.*, 2009; Kilian *et al.*, 2010; McBeath *et al.*, 2004).

The reductionist approach has contributed greatly to our understanding of how single factors can drive lineage-specific differentiation; models are becoming increasingly sophisticated to study how these factors work in combination with one another (Kourouklis, Kaylan & Underhill, 2016). A combinatorial, high-throughput approach considering an array of factors can identify how known factors could work synergistically or competitively as well as bypass undesirable variables that may constrain single variable approaches. A combinatorial approach may also provide a closer representation of how a cell interacts with the complex, multi-faceted *in vivo* environment. In this study, a high-throughput platform was fabricated to control both biochemical and biomechanical (ECM stiffness, shape and size) inducers of differentiation. This platform was then used to yield data in 54 different conditions and identify ideal conditions for differentiating adipose-derived stem cells (ASCs) into the adipogenic and osteogenic lineages.

2. Materials and methods

2.1 Hydrogel fabrication

Polyacrylamide hydrogels of 3kPa (4% acrylamide/0.3% bis-acrylamide; Bio-Rad) and 35kPa of stiffness (10% acrylamide/0.3% bis-acrylamide) were fabricated (Tse & Engler, 2010). Circular 25mm diameter coverslips were exposed to one minute of UV radiation (UV/Ozone ProCleaner™, BioForce Nanosciences) per side, immersed in ethanol with 0.3% acetic acid and 0.005% 3-(trimethoxysilyl)propylmethacrylate (Sigma) for five minutes then transferred to ethanol for one minute before being air-dried. Hydrogel polymerization was initiated with addition of 10μL of 10% ammonium persulphate (Sigma) and 1μL N,N,N',N'-tetramethylethylenediamine (Bio-Rad) into

1 mL of polyacrylamide solution, and 25 μ L was pipetted onto a glass slide treated with dichlorodimethylsilane (Sigma). Coverslips were placed over the solution and polymerization was allowed to occur for 15 minutes before hydrogels were placed into sterile 6-well plates in 1x phosphate buffered saline (PBS; Sigma).

2.2 Microcontact printing hydrogels

The stamp designed for the microcontact printing (μ CP) process was designed in AutoCAD 2015 and consisted of three shapes (circle, square, rectangle) in three sizes ($1,000\mu\text{m}^2$, $5,000\mu\text{m}^2$, $10,000\mu\text{m}^2$) with minimum separations of $150\mu\text{m}$ between patterns within a $16\times 16\text{mm}^2$ area. Photolithography was done to produce a silicon wafer for stamp fabrication (Figure 1A-F). Briefly, photoresist was spin coated over a silicon wafer and a chrome photomask with the described pattern was placed over the top before exposure to UV light through the photomask to polymerize the photoresist. Non-polymerized photoresist (blocked by UV; the pattern design) was then washed away to leave a silicon wafer patterned with the inverse of the described patterns that were $25\mu\text{m}$ in height. A 10:1 ratio of silicon to curing agent (total weight = 44g; Sylgard[®] 184 Silicon Elastomer kit, Dow Corning) was combined and vacuumed. The mixture was poured over the wafer, vacuumed again then set in an oven at 60°C overnight.

Hydrogel functionalization and the μ CP process are outlined in Figure 1G-L. Hydrogels were functionalized for protein adsorption with $200\mu\text{g/mL}$ sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (Thermo Fisher) in 50mM pH 8.5 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES; Sigma) and exposed to UV light for ten minutes. Hydrogels were then washed with HEPES (all μ CP washes and solutions used HEPES). Hydrogels were dried in an oven at 60°C for ten minutes before stamping. Stamps were coated with $200\mu\text{g/mL}$ fibronectin (Gibco) and a

coverslip was placed over the patterned area for 30 minutes to prevent evaporation. The stamp was then wiped with the coverslip to remove excess solution and firmly stamped onto the hydrogel by hand for five seconds then by a 50g weight for five minutes. Hydrogels were then incubated in PBS overnight at 37°C and prior to cell seeding were placed in 1% Pluronic F127 (Sigma) for ten minutes then washed in PBS. μ CP efficacy was verified with immunofluorescence using mouse anti-human fibronectin (1:100; ab2413) with goat anti-mouse Alexa Fluor 488 (1:200; ab150113).

2.3 Cell culture and experimental design

Human ASCs were purchased from Lonza (donor age 26-31 years, female) and cultured in flasks to passage three in growth media (10% fetal bovine serum, 1% antibiotic-antimycotic, Dulbecco's Modified Eagle Medium; Gibco). Cells were seeded at 15,000 cells/well onto microcontact printed hydrogels of 3kPa and 35kPa of stiffness to represent adipogenic and osteogenic tissue stiffness, respectively (Engler *et al.*, 2006). Cells were incubated in growth media for five hours before media was replaced to wash excess cells. Samples were incubated for 24 hours before one third of samples were placed in adipogenic differentiation media (Gibco), another third were placed in osteogenic differentiation media (Gibco) while the remaining third were placed in growth media. Samples were incubated for a further three days before analysis. A population of undifferentiated ASCs acting as a control population were also cultured for five hours (long enough to adhere to hydrogels without changing expression as a result of inducing conditions).

2.4 Immunocytochemistry

Immunocytochemistry was done to determine expression of mechanosensitive proteins (to verify ASC mechanosensation) and four differentiation markers. Samples were fixed in 4% paraformaldehyde (Sigma), washed with PBS (all immunocytochemistry washes used PBS), permeabilized with 1% Triton X-100 (Sigma) for 15 minutes and then washed. All antibodies were purchased from Abcam and Santa Cruz unless otherwise stated, and all were used in 2% bovine serum albumin (Sigma). The following markers and primary antibodies (1:100) were tested: PPAR γ (C26H12; Cell Signalling Technologies), CEBP α (ab15047), RUNX2 (ab76956) ALP (ab108337), YAP (sc-101199) and MRTF-A (sc-390324). Hydrogels were coated with 200 μ L primary antibody solution and incubated at 4°C overnight. The following secondary antibodies were used (1:400): goat anti-mouse Alexa Fluor 594 (ab150116), goat anti-rabbit Alexa Fluor 594 (ab150080), goat anti-mouse Alexa Fluor 647 (ab150115). After washing, hydrogels were incubated with secondary antibodies and Alexa Fluor 488 Phalloidin (1:400, Gibco) for one hour at 37°C and washed. Nuclei were visualized using DAPI with Fluoroshield mounting solution (Sigma). Samples were imaged using a Nikon C2+ confocal microscope using NIS Elements Advanced (4.0) software (Nikon).

2.5 Data collection and analysis

Expression of mechanosensitive markers was observed by taking three images per sample and analyzed per stiffness. A 4x4 frame slide scan image per sample was taken for observation of differentiation marker expression. Differentiation samples were categorized into 54 conditions based on ECM stiffness (3kPa, 35kPa), ECM shape (circle, square, rectangle), ECM size (1,000 μ m², 5,000 μ m², 10,000 μ m²) and

media type (growth, adipogenic, osteogenic). Images were analyzed in CellProfiler using a custom pipeline. Fluorescence intensity of undifferentiated ASCs was used as a baseline to assess fold change in differentiation marker expression resulting from inducing conditions per cell.

One-way ANOVA tests followed by post-hoc Dunnett tests revealed significant changes in differentiation marker expression per set of conditions relative to undifferentiated ASCs. One-way ANOVA tests followed by post-hoc Tukey HSD tests revealed significant expression changes between three-variable datasets (e.g. differences between ECM sizes) while unpaired t-tests revealed significant changes in expression between two-variable datasets (i.e. per stiffness).

3. Results and discussion

3.1 High-throughput platform fabrication and observation of mechanosensitive cell behaviour

Fibronectin staining (Fig. 1L) revealed successful transfer of fibronectin patterns onto hydrogels using the described microcontact printing method. Platform functionality and results are summarized in Fig. 2, Fig. S1 and Table S1. As expected, mean cell size increased as ECM area increased (Fig. S1A; $p < 0.01$) and the number of cells adhering to a single pattern increased with increasing pattern size (Fig. S1B). Number of cells analyzed per condition (Table S1) increased with increasing ECM area with an average of 11, 42 and 80 cells analyzed for areas of $1,000\mu\text{m}^2$, $5,000\mu\text{m}^2$ and $10,000\mu\text{m}^2$, respectively. Previous studies looking at single cell patterns only have demonstrated ECM shape-specific actin organization (Kilian *et al.*, 2010). Pattern recognition by cells was observed in this study (representative images in Fig. 2A), which confirmed the ability of the platform to direct cell morphology. However, although

cells recognized ECM shape, actin organization was varied within shapes, which is thought to be due multicellularity on some patterns (Fig. S1C). Additionally, this study investigated ECM sizes up to $10,000\mu\text{m}^2$, which is much larger than previous studies and cells did not perfectly occupy the entire ECM area on every pattern (Fig. S1A).

Expression changes in mechanosensitive proteins verified typical ASC mechanotransduction occurring in response to ECM stiffness. Increased nuclear localization of YAP was observed on stiffer hydrogels (Fig. 2B) in agreement with previous findings (Dupont *et al.*, 2011; Hadden *et al.*, 2017; Swift *et al.*, 2013) and MRTF-A followed the same trend (Fig 2B; $p < 0.05$) as expected from previous findings (Shiwen *et al.*, 2015).

3.2 Differentiation marker expression

Lineage-specific marker expression was analyzed to determine extent of osteogenic and adipogenic differentiation in response to changing ECM conditions. PPAR γ (Chalwa *et al.*, 1994) and CEBP α (Ntambi & Young-Cheul, 2000) are both transcription factors necessary for adipocyte gene expression and their expression was measured in these experiments. RUNX2 is an essential regulator of bone mineralization (Deng *et al.*, 2008) so was used to assess osteogenic differentiation along with ALP – a marker also important in mineralization (Granéli *et al.*, 2014).

Data were presented primarily as fold change in marker expression compared to expression in undifferentiated ASCs per condition or with conditions pooled based on the similarity of conditions (e.g. pooling all samples with a 3kPa stiffness together and comparing to all those with a 35kPa stiffness). Intensity per set of conditions was also presented, as were comparison between sets of conditions differing by just one

variable (e.g. 3kPa circle ECM, 5,000 μm^2 ECM, osteogenic media vs 35kPa, circle ECM, 5,000 μm^2 ECM, osteogenic media), which are shown in supplementary figures.

The following sets of conditions were found to increase PPAR γ , CEBP α , RUNX2 and ALP expression, respectively: 35kPa, 5,000 μm^2 , circle, osteogenic media (PPAR γ), 3kPa, 5,000 μm^2 , circle, adipogenic media (CEBP α), 35kPa, 5,000 μm^2 , circle, osteogenic media (RUNX2) and 35kPa, 5,000 μm^2 , circle, osteogenic media (ALP).

When pooling data to observe effect on differentiation per inducing factor (i.e. stiffness, shape, size, and induction media), stiffness was found to be an important regulator of both adipogenic and osteogenic marker expression. PPAR γ showed an increase in expression (Fig. 3A&E) and CEBP α significantly increased on soft ECMs (Fig. 3B&F; $p < 0.05$). Significantly increased osteogenic marker expression was observed for both RUNX2 (Fig. 3C&G; $p < 0.01$) and ALP (Fig. 3D&H; $p < 0.01$) on stiffer ECMs. These trends are indicated by an upward shift in fold change on the scatterplots. This stiffness-mediated differentiation is further emphasized when average fluorescence intensity per condition is observed (Fig. 4). It is particularly noticeable in RUNX2 and ALP expression with a dampening of expression to below baseline levels on soft ECMs (Fig. 4C&D, circles) compared to stiff ECMs (Fig. 4C&D, squares). Even when comparing between single conditions where there is only one dissimilar condition (i.e. non-pooled), significant trends in lineage-specific stiffness is observable in PPAR γ , CEBP α and ALP expression (Fig. S3A, B, D).

ECM size was also found to significantly regulate osteogenic differentiation in particular. RUNX2 expression significantly increased on larger ECM sizes when pooled (Fig. 3K; $p < 0.05$) and even when comparing single conditions (Fig. S3C; $p < 0.05$). ALP similarly increased with increasing size (Fig. 3L). Similar ECM size

trends were not observed in pooled data for PPAR γ and CEBP α expression. Biochemically-induced differentiation was also observed with osteogenic media significantly increasing RUNX2 (Fig. 3S and Fig. S3C; $p < 0.05$) and ALP (Fig. 3T; $p < 0.05$) expression compared to adipogenic and growth media. Biochemical trends were less clear for adipogenic markers with osteogenic media significantly increasing PPAR γ expression (Fig. 3Q; $p < 0.01$) but adipogenic media significantly increasing CEBP α expression in some single condition sets (Fig. S3A; $p < 0.05$).

Exposing cells to lineage-specific tissue stiffness positively influenced adipogenic and osteogenic differentiation - an observation consistent with single variable studies (Engler *et al.*, 2006; S. H. Oh *et al.*, 2016; Qiu *et al.*, 2015; Shih *et al.*, 2011; Wen *et al.*, 2014). This platform also demonstrated the pronounced effect of ECM area on osteogenic differentiation with larger ECM sizes facilitating increased osteogenic differentiation (Kilian *et al.*, 2010; McBeath *et al.*, 2004; S. Oh *et al.*, 2009).

Trends in ECM shape were not observed for both adipogenic and osteogenic differentiation in all markers (Fig. 3I-L; Fig. S3). As noted, a combination of single and multicellular patterns, very large patterns (up to 10,000 μm^2) and submaximal spreading on larger patterns (Fig. S1A) could have prevented the actin organization seen in previous studies that looked at single cell behaviour (Kilian *et al.*, 2010). Improvements in technologies that would allow for single cell per pattern delivery would vastly improve the ability to create a homogenous platform of single celled patterns and more consistent actin organization trends.

When analyzing all inducing factors collectively (Fig. S2), it is clear that expression patterns of lineage-specific markers are not as categorical and predictable as they might be if a reductionist approach is taken and just a single inducing factor is manipulated. Some trends are clear despite the presence of potentially competing

inducers such as stiffness-mediated differentiation, as mentioned above. However, where single variable studies might identify a small ECM area as the best inducer of adipogenesis, for example (McBeath *et al.*, 2004; Peng *et al.*, 2011), the trends are less clear in this more complex model. When considering RUNX2 expression, a clear ‘three step’ increase in fluorescence intensity can be observed with increasing ECM area for cells on soft ECMs in osteogenic media (Fig. 4C, red circles; Fig. S3C). However, when cells sense a stiff ECM in the same media condition, the ‘third step’ in expression increase with increasing ECM area diminishes and the difference between expression on medium and large ECMs is negligible (Fig. 4C, red squares). As such, it appears the osteogenic predisposition generated from the stiff ECM ‘masks’ the effect of ECM size. Similar multi-variable setting effects on cell differentiation have recently been documented. For example, cholangiocyte differentiation has recently been shown to occur on higher stiffness ECMs, however, the extent of stiffness-mediated differentiation and traction force can be dependent on the ECM protein used in substrate coating (Kourouklis, Kaylan & Underhill, 2016).

When considering the biochemical inducers of differentiation, inducers from osteogenic media increased osteogenic marker expression even when cells sensed low ECM stiffness (Fig. 4C&D, red circles), which suggests biochemical inducers play a prominent role in directing osteogenic differentiation. Despite this, many expressing condition combinations per lineage shared similarities (Fig. S2); lineage-specific stiffness produced consistently high differentiation, many of the highest adipogenesis conditions featured small-to-medium ECM areas while the inverse ECM area trend was observed for osteogenesis. Biochemically-induced osteogenesis was also observed in many of the highest expressing conditions.

Overlapping of the four variables between conditions was done to determine whether changes in a single variable could significantly affect differentiation despite the presence of potentially co-inducing or competing factors. However, with this overlap in conditions came a challenge to finding a single set of conditions that could significantly direct differentiation. As such, this study rather acted as a 'screening process' to identify trends in expression by analyzing similarities between conditions inducing similar expression. Notably, lineage-specific stiffness and media type appear important for both adipogenic and osteogenic differentiation while larger ECM size (above 5,000 μm^2) promotes osteogenic differentiation in this platform. However given the masking effect of osteogenic stiffness on ECM size, large ECM size (10,000 μm^2) may not be essential in future models of differentiation.

On a practical level, generating large amounts of data from a single, high-throughput platform is both time and resource-efficient. This platform has concurrently identified ideal as well as non-ideal conditions for differentiation in a multi-variable system. This work can be used to inform future experiments delving further into these ideal conditions for differentiation. The efficacy in promoting lineage-specific differentiation has been shown in the short-term. Long-term culture could be done in the future and more success of long-term differentiation could be analyzed (e.g. Oil Red O and Alizarin Red S staining beyond a week). Additionally, this platform is very flexible and modifiable; different patterns can be designed and printed easily to manipulate ECM shape and size, stiffness can be changed, cell types can be changed as well as the lineage-specific markers that have been investigated in this instance.

4. Conclusion

This study employed a multi-variable, combinatorial approach towards differentiation to address the current inability to achieve terminal differentiation of stem cells *in vitro*. This high-throughput micropatterned hydrogel platform has demonstrated the capability to modify multiple inducers of differentiation to identify optimal conditions for adipogenic and osteogenic differentiation in ASCs. It has been shown that trends in expression when considering multiple variables are not always as clear-cut as they appear to be in single-variable studies. However, despite often-conflicting inducing factors, some factors such as ECM stiffness still have pronounced effects on both adipogenic and osteogenic differentiation.

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6. Declaration

The authors declare no conflict of interest for this work.

7. References

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Figure 1: High-throughput platform fabrication process.

A silicon wafer is spin-coated with photoresist (A) before a chrome photomask is placed over the top (B) with transparent areas in the specific design. UV light is shone through the photomask (C) and un-polymerized photoresist is washed away to give a 25µm high silicon wafer (D) with an inverse of the desired stamp pattern. PDMS is set on the wafer (E) at 60°C overnight and cast out with raised features in the desired pattern (F). Polyacrylamide hydrogels 50µm high are functionalized with sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH; G), the stamp is coated with fibronectin (H) and stamped onto the hydrogel (I). The stamp is removed and fibronectin patterns with specific shape and size remain crosslinked to the hydrogel (J). Non-patterned areas are blocked with Pluronic F127 (K) leaving the microcontact printed hydrogel (L). Fibronectin shapes (circle, square, rectangle) in three sizes (1,000µm², 5,000µm², 10,000µm²) were microcontact printed onto hydrogels using a PDMS stamp. Immunofluorescent verification of printing efficacy

was visualized with Alexa Fluor 488 (L) on a confocal microscope using a slide scan technique.

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Figure 2. TOP OF FIGURE.

Figure 2: Cell behaviour observed on microcontact printed polyacrylamide hydrogels.

ASCs were cultured on hydrogels with specific stiffness microcontact printed with fibronectin to control cell shape and size. (A) Shape-recognising single cells on patterns with an area of 10,000µm². (B) Analysis of expression of mechanosensitive markers showed increased YAP and MRTF-A expression with increasing stiffness. Scale bar = 50µm. Data shown as percentage of marker localized to the nucleus. * = p<0.05.

Figure 3. TOP OF FIGURE.

Figure 3: Biomechanical- and biochemical-dependent adipogenic and osteogenic marker expression in ASCs.

Cells were cultured on microcontact printed polyacrylamide hydrogels with specific ECM stiffness, shape and size in growth, adipogenic or osteogenic media for four days. Fluorescence intensity of PPAR γ and CEBP α (adipogenic markers), and RUNX2 and ALP (osteogenic markers) within conditions was compared to that of undifferentiated ASCs to indicate fold change in marker expression (calculated per cell). Fold change in marker expression per cell size on 3kPa (blue) and 35kPa (red) hydrogels (A-H), per ECM size (1,000 μm^2 , 5,000 μm^2 , 10,000 μm^2 ; indicated by small, medium and large circles) (I-L), per ECM shape (circle, square, rectangle; indicated by shapes) (M-P) and per media type (GM = growth media, AM = adipogenic media, OM = osteogenic media) (Q-T). Bar graph data is expressed as mean $\pm$ SEM. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Figure 4: Differentiation marker fluorescence intensity in ASCs under various biomechanical and biochemical inducers of differentiation.

Cells were cultured on microcontact printed hydrogels of defined ECM stiffness (kPa), shape and size (μm^2) in either growth (GM), adipogenic (AM) or osteogenic media (OM) for four days. Intensity of PPAR γ (A; adipogenic), CEBP α (B; adipogenic), RUNX2 (C; osteogenic) and ALP (D; osteogenic) are shown. The 54 combinations of inducing factors are divided along the x-axis: the left half of data used 3kPa hydrogels (soft) and the right half used 35kPa hydrogels (stiff); stiffness is divided into the different media types (GM = growth media, AM = adipogenic media, OM = osteogenic media); media type is then divided into respective ECM shapes (circle, square, rectangle) and sizes (1,000 μm^2 , 5,000 μm^2 , 10,000 μm^2) represented by the shapes on the axis. Shaded area along the bottom of the graph = expression in undifferentiated ASCs. Data expressed as mean $\pm$ SEM.