Supplemental Methods:

Cell lines & Culture Conditions: Human ASCs were obtained from raw human lipoaspirates under an approved IRB protocol and were cultured as in a previous study [1, 42] . Lipoaspirates were taken from female donors undergoing elective cosmetic surgery. The donors were anonymous and, as such, specific information about the depot site and the age of the donor was not recorded. However, only lipoaspirates obtained from thighs, flanks or abdomen and from women between 30 and 65 years of age were used in this study. The raw lipoaspirates were washed extensively with sterile phosphate-buffered saline (PBS) and then treated with 0.1% collagenase (type I; Sigma, St. Louis, MO) in PBS for 30 min at 37°C with gentle agitation. The collagenase was inactivated with an equal volume of DMEM supplemented with 10% FBS. The infranatant was removed and the sample was centrifuged for 10 min at 1500 rpm. The cellular pellet was resuspended in DMEM, 10% FBS, and filtered through a 100-µm mesh filter before being centrifuged as above. The ASCs were expanded on conventional tissue culture plates in non-inductive Control Medium/CM (DMEM, 10% FBS, 1% antibiotic/antimycotic) [1]. Cultures were passaged at 80-90% confluency. Studies were performed using early passage cultures (P1 to P3).

Confirmation of osteogenic potential: ASCs at 50 to 70% confluency were osteo- induced by culturing in Osteogenic Medium (OM), containing 0.1 uM dexamethasone, 50 ug/ml ascorbic-2-phosphate and 10 mM β-glycerophosphate (BME) [10]. Osteogenic capacity was confirmed in each ASC population using a biochemical calcium assay previously published [10]. For this, each ASC population (n=15) was plated in triplicate into 12-well dishes and induced in OM for up to 35 days or maintained in CM as controls. The cultures were harvested at various time points and the extracellular matrix (ECM) extracted in 0.1 N HCl. Mineral content was quantified spectrophotometrically and normalized to total protein content. Because 21 days of OM induction resulted in significant mineralization in many of the ASC populations analyzed (see **Supplemental Figure S2** for a representative population), OM induction was limited to this time point in these populations. For those

populations showing negligible mineralization capacity, induction was increased to 35 days. To further confirm osteogenic capacity, alkaline phosphatase activity was quantified as previously published [10].

Microarray analysis of osteogenic differentiation: ASCs (n=1) were maintained for 7 or 21 days in CM and OM. Harvested ASCs were washed in 1X PBS and DNA-free total RNA prepared using the Clontech Altas Array Total RNA Kit (Clontech, Palo Alto, CA). Oligo dT-primed cDNA was prepared from total RNA. cDNA from CM-maintained ASCs were labeled with Cy3 and cDNA from OM-induced cells were labeled with Cy5 (Clontech Fluorescent Labelling kit and Amersham Pharmacia Fluorolink^{TM} Cy3 or Cy5 monofunctional dye; Piscataway, NJ). Excess label was removed (PCR Extraction Kit, Qiagen, Valencia, CA). The two labelled cDNA samples were hybridized to the Atlas Human 1.0 array (Clontech), containing 1089 pathway-focused genes, according to the manufacturer. Hybridized array slides were scanned using a Virtek Scanner (Virtek Vision Corp, Waterloo, Ontario). Scans were analysed and adjusted signal intensities calculated for each spot. A list of signals above background was created and denoted as “calls”. Those spots with signals lower than background were denoted as “absent”. Each “call” was normalized using the housekeeping genes provided on the array (9 housekeeping genes). The normalized calls were compiled using Excel and the resulting gene lists were denoted as an ASC-CM array (ASC-CM7, ASC-CM21) or an ASC-OM array (ASC-OM7, ASC-OM21). To determine changes in expression between an ASC-CM and an ASC-OM array, normalized expression levels for each ASC “call” on the ASC-CM array was compared with its corresponding gene on the ASC-OM array using Excel. For each OM/CM array comparison, two gene lists were generated: those ASC-OM genes expressed at a higher level vs. ASC-CM (over 2.5 fold) and those ASC-OM genes expressed at a lower level vs. ASC-CM arrays (below 2.5 fold).

Real-time confirmation of microarray analysis: CM and OM-induced ASC samples (n=8) were harvested after 7 and 21 days and cDNA was synthesized from 1 ug total RNA (Taqman Gold reagents; Applied Biosystems). cDNA was also prepared from the individual ASC population used for microarray analysis. Real-time PCR

reactions were set-up using the Quantitect Probe PCR Kit (Qiagen) and run on an ABI 7700 Fastcycler. Customized primer/probe mixes to several MAPK pathway genes were purchased from Applied Biosystems (FAM-labelled probes, TAMRA quencher). For each primer/probe mix, regression lines were calculated using serial dilutions prepared from known cDNA amounts. Expression levels were determined using these regression values (ΔCt method) and were normalized using GAPDH and 18S rRNA. Normalized ASC-OM gene expression levels were then compared with ASC-CM controls.

Supplemental Results & Discussion:

ASC osteogenic capacity is linked to time-dependent increases in alkaline phosphatase activity, matrix mineralization and augmented osteogenic gene expression

The ASC populations chosen for this study were assessed for osteogenic potential through real-time PCR quantitation of osteogenic gene expression and biochemical analysis of alkaline phosphatase (AP) activity and matrix mineralization. Osteogenesis was initially confirmed through observation of increasing amounts of extracellular mineral deposition and AP staining as induction time increased (**Supplemental Fig. S2A**). Those ASC populations with augmented AP activity and mineralization also showed increased expression of several osteogenic genes with respect to non-induced controls (**Supplemental Fig. S2B**). Increased expression of the transcription factor Runx-2, together with AP and osteocalcin (OC) was measured throughout the entire induction period with increased expression of osteonectin (ON) and osteopontin (OP) occurring only within the first week of osteo-induction. This was followed by significant decreases in these markers at 3 and 4 weeks and may be a possible consequence of the ASC being terminally differentiated at the point of analysis. Finally, osteogenic induction did not significantly impact ASC proliferation versus CM controls (**Supplemental Fig. S2C & D**).

Microarray analysis of osteo-induced ASCs – detection of differentially expressed MAPK genes

In order to determine those pathways critical to ASC osteogenesis, an ASC population with confirmed osteogenic capacity was induced in OM for either 7 or 21 days (*OM7ASC, OM21ASC*) or maintained in non-inductive CM (*CM7ASC, CM21ASC*) were analysed using a simple microarray consisting of 1089 genes. Analysis of our results identified multiple genes with differential expression, including many of the MAPK cascade, suggesting that this complex signaling pathway may play a role in ASC osteogenesis. Because array analysis was performed on only one ASC population, real-time PCR analysis of multiple ASC populations (including the one used for microarray analysis) was used to confirm our microarray results. With a few exceptions, the majority of microarray results could be confirmed (**Supplemental Fig. S4)**. Overall, one-week OM induction was associated with modest changes in the expression of most MAPK cascade genes. However, both microarray and real-time analysis did detect a small, but statistically significant, increase specifically in the ERK2 isoform and an increase in the MEK3 kinase of the p38MAPK pathway.

When OM induction was increased to 3 weeks, changes in expression became more apparent in the downstream MAPK kinases and targets, while the expression of the upstream kinases remained relatively unchanged. Real-time PCR was able to confirm significant increases in the terminal kinases ERK1, p38MAPK, JNK1 and JNK2, together with their downstream targets c-fos, MAPKAP-2 and c-jun, respectively. In contrast, insignificant changes in the upstream kinases raf-1, MEK1, MEK3 and MEK6 were measured. While the MEKK, MEK and MAPK families signal in a linear fashion, this arrangement also allows for amplification, with each upstream kinase phosphorylating multiple copies of its downstream target. The observed increases in the expression of the terminal ERK and p38MAPK kinases and their targets c-fos and MAPKAP-2 measured after 3 weeks induction are consistent with this arrangement. However, real-time PCR did measure increased expression in JNK1/2 and their upstream kinases MEK4 and MEK7 at this time point.

Despite much of the microarray results being confirmed by real-time PCR, there were a few exceptions. While microarray analysis measured decreased expression in the ERK2 isoform after 21 days induction, only a slight

trend towards decreased expression was measured by real-time PCR was used. This trend was not statistically significant. Furthermore, when real-time PCR quantitated ERK2 expression in the ASC population used for microarray analysis, no significant change in expression was measured (1.29±0.26 fold vs. controls), calling the array results for ERK2 into question. Three week microarray results could also not be confirmed for JNK1, MEK6 and MEK7. As a result, microarrays alone are insufficient for drawing conclusions about expression levels. However, based on our real-time PCR results, it appears that significant changes in the terminal kinases and targets of the MAPK cascade are associated with osteogenic induction in ASCs.

Supplemental Figure Legends

Supplemental Figure S1: The MAPK Cascade. The MAPK signaling cascade is comprised of a tiered series of cytosolic kinases that are activated indirectly through the action of extracellular ligands and stress (dashed arrows). The penultimate MAPK kinases, which are activated in the cytoplasm, can be translocated into the nucleus, where they mediate the activity of numerous transcription factors (Targets), controlling gene expression. The kinases of each MAPK cascade arm are capable of interacting with one another (thin arrows) via additional kinases and signaling pathways, increasing the complexity of signaling through this cascade. Phosphorylation of the MAPK can be inhibited through the action of MAPK phosphatases (MKPs), regulating activation at the level of the MAPK.

Supplemental Figure S2: ASC Osteogenic potential. Panel A – Osteogenic differentiation of a representative ASC population from two weeks (d14) to 4 weeks (d28) shows a time-dependent increase in both AP activity (red stain) and matrix calcification (black stain). Panel B – Expression of the osteogenic genes Runx-2, alkaline phosphatase (AP), osteopontin (OP), osteonectin (ON) and osteocalcin (OC) upon induction in OM for 7 days, 3 weeks and 4 weeks. Expression levels are shown relative to those measured in ASCs maintained for up to 4 weeks in non-inductive CM (-fold change vs. CM). Panel C – ASC proliferation was measured during 5 weeks of

osteogenic differentiation (black circles) or maintenance in CM (black diamonds). Total ASC cell number was determined using a hemacytometer and expressed as #ASCs x 10^5. The time point at which mineralization is first observed is shown with an arrow. Panel D – ASC proliferation over 3 weeks as measured through a metabolic MTT assay. Best fit curves are shown.

Supplemental Figure S3: MAPK inhibitors significantly decrease MAPK phosphorylation. Kinase activation levels in ASC populations (n=9) cultured for 24 hours or 21 days with OM containing the indicated inhibitors and concentrations of the ERK1/2 (Panel A), JNK1/2 (Panel B) or p38MAPK (Panel C) signaling pathways was confirmed through western blotting using phospho-specific antibodies. Activation was compared to levels measured in OM controls treated with inhibitor "vehicle" (Kinase phosphorylation, -fold change vs. OM/vehicle). 21 day administration and analysis was not performed for ERK2 Activation Inhibitor (ERK Inhibitor). Statistical significance ($p<0.05$) is shown with an "*".

Supplemental Figure S4: Real-time confirmation of ASC microarray results. The expression of the indicated MAPK genes (top of the graph) was measured via real-time PCR in several ASC populations (n=8; including the population used for microarray analysis) induced in OM (OM/ASC) for 7 or 21 days. Normalized expression levels in these populations were compared with controls maintained in non-inductive CM (Expression level, -fold change vs. CM). Gene expression measured by real-time (solid bars) was then compared with levels obtained through microarray analysis (patterned bars). Statistical significance versus controls ($p<0.05$; t-test) is indicated with a "*". See supplemental text for discussion of results.

Supplemental Figure S5: p38MAPK activation does not appear to correlate with ASC mineralization capacity. Activation of p38MAPK was measured in ASC populations with negligible mineralization capacity (Category 1) and high mineralization capacity (Category 3) and levels compared with non-induced controls (-fold change vs. CM). Average mineralization levels ±SD versus controls are given for both categories below the graph.

Supplemental Figure S6: Inhibition of MAPK activity decreases AP activity. Osteo-induced ASC treated with the indicated MAPK inhibitors for the first 24 hours of induction or throughout the 21 day induction period (24hrs – 21 days, 21 days, respectively) were assessed for Alkaline Phosphatase activity and then compared with OM/vehicle controls. Those inhibitors not significantly affecting alkaline phosphatase activity are not shown. The inhibitor SP600125 was administered during the first 24 hours or seven days of OM induction. Statistical significance ($p<0.05$) is shown with a "*". Continuous administration of the ERK1/2 or p38MAPK inhibitors PD98059, SB203580 or SB202190 resulted in significant declines in AP activity after 3 weeks whereas MEK1/2 or JNK1/2 inhibition (U0126, SP600125, respectively) did not have a significant impact. Restricting inhibitor treatment to the first 24 hours of osteogenic induction significantly affected AP activity in U0126, and ERK2 Activation Inhibitor Peptide samples, both inhibitors of MEK/ERK signaling.

Supplemental Figure S7: Schematic for ERK1/2 siRNA production through shRNA lentiviruses. For the knockdown of ERK1/2 using short-hairpin RNA/shRNA lentiviruses, an ERK1/2 oligo is designed containing both sense (red) and anti-sense (green) sequences (see **Supplemental Table S2** for sequence information). The oligo contains a short loop of four nucleotides (blue) found between these sequences that will produce the hairpin structure in the shRNA particle. A reverse complement oligo is synthesized and a double stranded oligo (ds oligo) produced. This ds oligo contains overhangs for its insertion into a lentivirual shuttle vector (pENTR™/U6 – Invitrogen Technologies Corp.) shRNA-producing lentiviruses are made by transfecting human embryonal kidney (HEK) 293 cells containing the SV40 large T antigen (293FT cells) with the resulting recombinant shuttle vector and a proprietary ViraPower™ Lentiviral Packaging mix containing plasmids for the lentiviral gag/pol and rev proteins. Lentiviruses within the culture medium are collected with 48 hours and are used to transduce ASCs. shRNA particles produced within ASCs are processed by the cell into siRNA. siRNA-RISC complexes bind to mRNA target sequences and result in silencing.