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Disruption of cell-matrix interactions by heparin enhances mesenchymal progenitor adipocyte differentiation

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Abstract

Differentiation of marrow-derived mesenchymal progenitors to either the osteoblast or adipocyte lineage is reciprocally regulated. Factors that promote osteoblastogenesis inhibit adipogenesis, while adipogenic factors are inhibitory to osteoblast differentiation. Heparin, a soluble glycosaminoglycan, inhibits bone formation in vivo and osteoblast cell differentiation and function in vitro, and has been shown to promote adipocyte differentiation. To elucidate the role that heparin plays in the adipogenic induction of murine mesenchymal progenitors, we studied immortalized marrow stromal cells (IM-MSC), the MSC cell line, ST2, and 3T3L1 pre-adipocytes. Heparin alone was not sufficient to induce adipogenesis, but enhanced the induction under a variety of adipogenic cocktails. This effect was both dose- and time-dependent. Heparin showed a positive effect at concentrations > 0.1 µg/ml when applied before day 3 during the induction course. Heparin's effect on adipogenesis was independent of cell proliferation, cell density, and extracellular lipid. This effect is likely related to the unique structure of heparin because another polyanionic glycosaminoglycan, dextran sulfate, did not promote adipogenic differentiation. Heparin treatment altered morphology and adhesion characteristics of progenitor cells, resulting in cell rounding and aggregation. As well, heparin counteracted the known inhibitory effect of fibronectin on adipogenesis and decreased basal focal adhesion kinase and paxillin phosphorylation. We conclude that heparin-mediated disruption of cell-matrix adhesion enhances adipogenic potential.

INTRODUCTION

A variety of studies have demonstrated reciprocal effects of growth factors on adipogenic and osteoblastic differentiation of marrow-derived mesenchymal progenitors. For example, the administration or over-expression of BMP has been shown to promote osteoblast

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differentiation, while inhibiting adipogenesis [1]. Similarly, *wnt10b* inhibits adipogenesis while promoting osteoblastogenesis [2, 3]. In general these reciprocal effects are molecularly-linked, so that downstream transcriptional cascades which are activated to promote differentiation to one phenotype are inhibitory to the other [2, 4-8]. Indeed, overexpression of Ppar gamma, a transcription factor that has previously been shown to be required for adipocyte differentiation, inhibits osteoblast differentiation [5], while *Cbfa1*, a transcription factor required for osteoblast differentiation, is associated with decreased adipocyte differentiation [4]. Furthermore, knocking down Ppar gamma and *Cebp-beta* in ST2 cells promotes osteoblast differentiation [8].

Osip et al. (2004) have shown that the extracellular proteoglycan heparin and other sulfated proteoglycans, including dermatan sulfate, dextran sulfate, and low molecular weight heparin, have reciprocal effects on differentiation of primary murine marrow stromal cell cultures [9]. Heparin promotes adipogenesis while inhibiting osteoblastogenesis [10]. However, it should be noted that in other studies heparin has also been shown to enhance osteoblastogenesis secondary to BMP stimulation [11]. Importantly, heparin related molecules, heparan sulfated proteoglycans, are produced by bone marrow stromal cells [12] and function to regulate the adhesion of hematopoietic cells to the stroma [13, 14].

Heparin and heparan sulfate are related glycosaminoglycans (GAGs) with various levels of sulfation [15]. Heparin prevents coagulation by binding to antithrombin and inhibiting serine proteases involved in the coagulation cascade [15]. Cell-surface heparan sulfated proteoglycans have high affinity binding sites for lipoprotein lipase (LPL), while heparin binds and releases it from the cell surface. This greatly enhances the LPL hydrolysis of triglyceride [16]. Heparin also directly binds to a variety of growth factors and can regulate cell signaling and down-stream effects of these factors [10, 15, 17]. As an example, heparin can alter cell proliferation by interacting with PDGF, bFGF and aFGF [17-19]. Heparin and heparan sulfate also interact with ECM components such as fibronectin and collagen. Through these interactions, heparin can modulate cell-ECM interactions, matrix assembly, and cytoskeletal organization [20, 21].

In this study, we characterized the regulation of mesenchymal progenitor adipogenesis by heparin. We utilized three different murine cell types including, two marrow-derived mesenchymal cell lines, and the preadipocyte line, 3T3L1. We show that heparin affects the early stages of adipocyte differentiation. However, heparin is not sufficient for inducing adipogenesis in pure mesenchymal progenitor cultures. Modulation of adipogenesis by heparin is dose-dependent and saturable, but is not dependent on the presence of extracellular lipid. Finally, we show that heparin alters cell morphology and adhesion. Heparin counteracts the adipo-inhibitory effect of pro-adhesive substrates, such as fibronectin. Heparin also decreases focal adhesion kinase (FAK) and paxillin phosphorylation. We conclude that heparin induces a de-adhesive state, regulating adhesion-related signaling, and thereby promoting adipogenic differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture

Immortalized marrow stromal cells (IM-MSC) were harvested from the femur and tibia of transgenic mice carrying a temperature sensitive SV40 large T antigen. Bone marrow was plated in MSC media, MEM supplemented with antibiotic-antimycotic (Gibco 154240-062), 2mM L-glutamine, and 20% FBS (Define Fetal Bovine Serum, Hyclone SH30070.03). Primary cells were harvested after 14 days in 37 °C and then shifted to MSC media with 10% FBS plus 20 U/ml interferon gamma (Calbiochem) at 32 °C for immortalization. ST2 cells, a bone marrow stromal cell line from BC8 mice [22], were grown in MSC media

supplemented with 10% FBS (Gemini 100-106). Preadipocyte 3T3L1 cells from American Type culture Collection (ATCC) were grown in DMEM (Gibco 11965-092) supplemented with antibiotic-antimycotic, 2 mM L-glutamine, 1 mM MEM sodium pyruvate (Gibco 11360-070) and 10% calf serum (Gibco 16170-070). For adipogenic differentiation a specific lot of FBS was used for ST2 and 3T3L1 cells, but basal media composition remained the same.

Adipogenic differentiation

For adipogenic differentiation, cells were plated in 24-well plates, unless noted otherwise, at 40×10^3 , 10×10^3 , and 20×10^3 per well for IM-MSC, ST2 and 3T3 L1 respectively. Induction of adipogenic differentiation was initiated 1 day after plating for immortalized MSC and ST2, and 3 days after plating for 3T3 L1 (at confluence). Three different adipogenic induction treatments were utilized for the first three days, MDI (26 μ M isobutylmethylxanthine, 0.5 μ M dexamethasone, 0.085 μ M insulin), or TZD (5 μ M Thiazolidinedione, Troglitazone, Cayman 71750) the Ppar gamma agonist, or DT (0.5 μ M dexamethasone and 5 μ M Thiazolidinedione). MDI and TZD were used for IM-MSC and ST2, and MDI and DT for 3T3L1. Following the initial three days of induction the cells were shifted to basal media plus 0.085 μ M insulin alone until complete differentiation was reached at day 9. Media were changed every 3 days. These protocols were modified based on established differentiation protocol [23].

For experimental treatments during adipogenic differentiation, 20 μ g/ml heparin (porcine intestinal mucosa, Sigma), 20 μ g/ml dextran sulfate sodium (Sigma), 100 μ g/ml protamine sulfate (American Pharmaceutical Partners Inc), and 50 ng/ml bFGF (Scios) were used. These reagents were added to the media from day 0 - 9 throughout the induction course, except where noted otherwise.

For serum-free or lipid-free experiments, supplemented McCoy's 5a medium (Invitrogen) (20 μ g/ml asparagine, 10 μ g/ml serine, 0.75x MEM vitamins, 0.38x MEM amino acids, 75 μ M non-essential amino acids, 100 u/ml pen/strep, 2.3 mM L-glutamine, 1.3 mM sodium pyruvate, 0.06% sodium bicarbonate, 50 μ M BME) was used as basal medium. For serum lipid add-back, 750 μ g/ml lipoprotein (lipid cholesterol rich adult bovine serum, Sigma) or 675 μ g/ml BSA (Sigma) as control were added. For the delipidated serum experiment, 10% delipidated calf serum or normal calf serum was added to the basal serum-free media.

To achieve 50 μ g/cm² adhesive coatings, 50 μ g/ml human plasma fibronectin, type I collagen, or poly-L-lysine (Sigma) were added to 24 well plates at 250 μ l per well, incubated at room temperature for 1 hour, and rinsed twice with PBS.

Staining and quantification of adipogenic differentiation

Differentiated adipocytes were stained with Oil red O (Sigma) as described [24]. To quantify the total lipid amount and overall adipocyte differentiation level, Oil red O dye was extracted with isopropanol and absorbance was measured at 540 nm.

Cell proliferation

Cell proliferation was assessed using the CyQUANT® cell proliferation assay kit (Molecular Probes). Briefly, cells were grown and induced in a 96-well low fluorescence profile tissue culture plate (Costar 3603) and samples were collected at day 3. At the same time, samples with designated cell densities for standard curves were plated separately and harvested after overnight adhesion. Fluorescence dye working solution was added, and plates read by a fluorescence plate reader with filters appropriate for ~480 nm excitation and

~520 nm emission maxima. 4X CyQUANT GR was used to extend the standard curve to a higher cell number range.

Quantitative RT-PCR Analysis

Total RNA was isolated using Qiaquick RNA isolation kit (Qiagen). 1 µg of total RNA was used in cDNA reverse transcriptase reactions with Oligo dT (Invitrogen), M-MLV reverse transcriptase, and associated buffers (Invitrogen). Quantitative PCR was carried out with a DNA engine Opticon 2 (MJ Research) and Sybr green fluorescence. Primer sequences used include LPL, F: TGGAGAAGCCATCCGTGTG, R: TCATGCGAGCACTTCACCAG; Cebp alpha, F: GGACAAGAACAGCAACGAG, R: TCACTGGTCAACTCCAGCAC; Ppar gamma, F: AGTGGAGACCGCCCAGG, R: GCAGCAGGTTGTCTTGGATGT; Fabp4, F: TGGAAGCTTGTCTCCAGTGA, R: AATCCCCATTACGCTGATG; β-Actin, F: AAGAGCTATGAGCTGCCTGA, R: TGGCATAGAGGTCTTTACGG. Annealing temperature was 68 °C for 34 cycles.

Western blotting

Twenty-four hours following treatment cells were lysed with RIPA buffer, protein quantified using Bradford assay, and equal quantities of protein loaded on SDS-PAGE gels. Protein was transferred to nitrocellulose by Western blotting and protein detected using primary antibodies against FAK, phospho-FAK, Paxillin, and phospho-paxillin.

Statistics

Data were analyzed using either one-way ANOVA or two-way ANOVA with post-hoc Tukey analysis.

RESULTS

Heparin promotes adipogenic induction of mesenchymal progenitors

Adipogenesis of three different mesenchymal precursor lines treated with heparin was evaluated under varying inductive conditions. Heparin induced an increase in Oil-red-O staining for all three cell types under varying adipogenic conditions, but was not sufficient to induce adipogenesis (Figure 1). Oil-red-O was extracted with isopropanol and absorbance at 540 nm was read to quantify total lipid (Figure 1C-E). Depending on the cell type and inductive condition evaluated, heparin increased Oil-Red-O staining by 20 to 150%. The most pronounced effects were seen with MDI treated IM-MSC and ST2 cells and DT treated 3T3L1 cells. The increase in adipogenesis was secondary to both an increase in lipid content per cell (adipocyte size) and to an increased percentage of cells developing into adipocytes (results not shown).

Heparin treatment increases expression of genes associated with adipogenesis

To verify that the increased Oil-red-O staining reflected true adipogenic induction, expression of the transcription factors Cebp alpha and Ppar gamma, and the late stage adipocyte markers lipoprotein lipase (Lpl) and fatty acid binding protein 4 (Fabp4) were evaluated using quantitative RT-PCR. ST2 and 3T3L1 (results not shown) cells were induced in the presence or absence of heparin, and samples were harvested 1, 4, 7 days after induction. Although heparin was not sufficient for adipogenic induction (Figure 1), heparin alone induced small but statistically significant increases in Cebp-alpha at day 7 (Figure 2A); Ppar-gamma at day 4 (Figure 2B); and Fabp4 at day 7 (Figure 2D). When heparin was administered with the adipogenic treatments, there were increases in both Ppar gamma (Figure 2B) and Cebp alpha (Figure 2A) over non-heparin treated cultures, depending on the time point evaluated or the adipogenic protocol. The largest magnitude changes were

observed in combination with MDI at Day 7. Similarly, Lpl and Fabp4 levels showed the highest degree of difference between heparin treated and non-heparin adipogenic cultures at day 7 (Figure 2C,D). In particular, Fabp4 levels were four-fold greater in MDI + heparin treated cultures than without heparin.

The effect of heparin on adipogenesis is dose-dependent and saturable

Hausser et al (2004) reported a dose-dependent biphasic effect of heparin on osteoblast-like cells. There was inhibition of osteoblast activity at a high dose but stimulation at low dose. To explore whether or not there may be a biphasic dose response of adipocyte induction to heparin and to determine if the effect was saturable, ST2 and 3T3L1 (results not shown but similar) cells were treated with different concentrations of heparin. Cells induced by MDI or TZD responded similarly to increasing doses of heparin (Figure 3A). At low levels, less than 0.1 µg/ml, heparin had no effect on adipocyte differentiation. With heparin levels greater than 0.1 µg/ml, adipogenesis increased with heparin dose. A plateau was reached at 100 µg/ml heparin, with no further increase through 1000 µg/ml. Heparin can promote adipocyte differentiation at a dose range over four orders of magnitude from 0.1 to 100 µg/ml, with a 50% effective dose being 3 µg/ml. In contrast to the case of osteoblast-like cells, no biphasic effect for adipocyte differentiation was observed.

Heparin is only required early during the induction phase to promote adipogenesis

To determine whether there were distinct temporal requirements for the action of heparin, treatment was initiated or ended at distinct time points during a 12 day adipogenesis assay in ST2 cells and 3T3L1 cells (results not shown but similar). Heparin was effective at increasing adipogenesis under both MDI conditions (Figure 3B) and under TZD conditions (results not shown) when present earlier than day 3 post-induction (Figure 3B). After day 3, heparin had relatively little effect on adipogenesis. Interestingly, just a single day exposure to heparin, initiated 24 hours before adipocyte induction (24 hour pretreatment), is effective at promoting enhanced differentiation.

Heparin effects are independent of cell density and cell proliferation

The effect of heparin on promoting adipogenesis could be due to an overall increase in cell proliferation, since heparin can increase the activity of mitogenic growth factors such as bFGF that may be present in serum or produced by the cells themselves. However, heparin failed to make a significant difference in ST2 cell number under either MDI or TZD induction (results not shown). These data suggest that heparin does not increase adipogenesis through an increase in total precursor cell number. To further differentiate heparin's effect on adipogenesis from cell number, cultures of varying plating density (sparse plating to confluent density) were induced to differentiate. Heparin increased adipogenesis independent of cell density under both MDI (Figure 4) and TZD conditions (data not shown).

Heparin effect on adipogenesis can be blocked with a competitive binding agent and can not be replicated with an additional sulfated GAG

Osip et al, (2004) showed that a variety of glycosaminoglycans could promote adipogenesis. This would suggest that the effect of heparin is not related to the heparin core structure, but rather to sulfation or charge density. To address this possibility we evaluated adipogenesis in the presence of dextran sulfate, another glycosaminoglycan. Interestingly, rather than promoting adipocyte formation, 20 µg/ml dextran sulfate, strongly inhibited ST2 adipocyte induction by both MDI and TZD (Figure 5A). Furthermore, the positively charged protamine sulfate, which competitively binds heparin, has no effect on adipogenic differentiation at a dose of 100 µg/ml, but when 100 µg/ml protamine sulfate was added with

20 $\mu\text{g/ml}$ heparin, it completely abolished the heparin positive effect on adipogenesis (Figure 5B).

Extracellular lipid is not required for the promotion of adipogenesis by heparin

Lipoprotein lipase is an extracellular cell-bound enzyme that is involved in triglyceride hydrolysis and fatty acid uptake during adipogenesis. Heparin can bind to LPL and therefore we postulated that the effect of heparin could be to increase the extracellular activity of LPL. To investigate whether the uptake of lipid from the extracellular environment was required for the activity of heparin, we evaluated adipogenesis in lipid-free conditions. Adipocytes have the ability to synthesize lipid *de novo*, and do not require extracellular lipid. To create lipid-free media conditions, delipidated calf serum, or serum free medium were used. Under delipidated conditions (Figure 6) and serum-free conditions (data not shown) heparin increased adipogenesis.

Heparin treatment results in cell aggregation by disrupting cell-ECM interactions

In heparin-treated cultures we noted that there were distinct morphological differences in the appearance of the cell monolayers in the presence of heparin. Heparin induced cell aggregation and clumping (Figure 7A). In fact, in 3T3L1 cultures the clumping resulted in large spheroids of adipocytes. This observed aggregation was closely correlated with heparin dose and timing of application. The higher the heparin dose, the more pronounced the aggregation and heparin induced cell aggregation only occurred when added before day 3 (data not shown).

The effect on cell aggregation could be attributed to weakened cell-matrix interaction. Heparin has previously been shown to interfere with cell adhesion and focal adhesion formation [20, 21], and increased cell adhesion to fibronectin is associated with reduced adipogenesis [25, 26]. To investigate whether heparin could disrupt cell-matrix interactions, we studied the influence of heparin on adipogenesis following the plating of cells onto fibronectin, collagen (not shown), or poly-L-lysine (not shown). These adhesive coatings decreased adipogenesis and 20 $\mu\text{g/ml}$ heparin abolished the inhibition of these coatings (Figure 7B).

The effect of heparin on disruption of cell-ECM interactions was not just apparent in fully-differentiated cultures, but rather short-term heparin treatment (24 hours) clearly altered cell morphology and resulted in cell rounding, either in the presence or absence of adhesive coatings (Figure 7C).

Finally, addition of heparin to 3T3L1 cultures results in a loss of focal adhesion kinase activity within one hour (not shown) of treatment and extending for at least 24 hours (Figure 7D). With the addition of heparin the amount of phosphorylated focal adhesion kinase (FAK) and the focal adhesion associated protein, paxillin, were significantly decreased. MDI treatment also decreases phosphorylated FAK and paxillin, and the combination of MDI and heparin results in the most pronounced decrease in phosphorylation.

DISCUSSION

Heparin and heparan sulfate are involved in regulating a wide variety of physiological processes including development, cell proliferation, and differentiation [15]. Furthermore, heparin sulfates are produced by marrow stromal cells [12] and regulate hematopoietic cell adhesion to the stroma [13, 14]. Data presented herein demonstrate that heparin also promotes adipogenesis. Heparin regulation of adipogenesis is independent of mesenchymal progenitor type or induction conditions; however, heparin is not sufficient to induce a mature adipocyte phenotype. This contrasts with results reported by Osip et al. (2004),

which show that heparin can induce adipogenesis in primary murine marrow stromal cell cultures. Our results also contrast with Osip et al. (2004) in that we have found that the effect of heparin is not recapitulated by dextran sulfate. In fact, in our study, dextran sulfate inhibited adipogenesis. These differences might reflect the mixed cell environment of primary murine MSC [27, 28] that were used in the aforementioned study.

We believe that heparin is disrupting established cell-ECM interactions, resulting in enhanced adipogenesis. Thus, in cases where these adhesions are even stronger, for instance when plated on fibronectin, heparin actually counteracts pro-adhesion (Figure 8). Heparin has previously been shown to disrupt cell adhesion by disrupting focal adhesion assembly [21]. Heparin or heparin binding fragments of fibronectin can disrupt normal interactions of cell surface HSPG with fibronectin [21, 29]. In our study we have also found that heparin treatment alters cell adhesion resulting in cell rounding and cell clumping, with a loss of FAK and paxillin phosphorylation.

Recent work by McBeath et al. clearly shows an association between adhesion and adipocyte differentiation [30]. Plating cells on substrates in which cells can spread and form focal adhesions resulted in Rho kinase and ROCK activation and inhibition of adipogenesis. Substrates that limit adhesion, resulting in cell rounding, lead to Rho inhibition and adipogenesis. Consistent with these results our experiments also show heparin disrupts cell-ECM interactions and alters cell morphology. Although McBeath et al. did not explore how adhesive transition states were controlled in their system, our data supports the hypothesis that disruption of FA signaling through FAK may be a factor that alters mesenchymal progenitor adipogenic potential. Previous research has demonstrated that disruption of fibronectin-cell interactions with fibronectin fragments can promote adipogenesis [26]. Further, Liu et al have recently demonstrated that during adipogenic induction there is a transition in integrin expression, i.e. down-regulation of alpha5 integrin and up-regulation of alpha6 [31]. By manipulating levels of these adhesive molecules, adipogenesis can be regulated. For instance, alpha6 integrin overexpression leads to increased adipogenesis and also cell clumping [31]. One possibility that remains unexplored through these studies is that decreased cell-matrix interactions and resultant cell aggregation also may promote cell-cell interactions (eg., gap junction formation) which could enhance adipogenesis.

Our results, considered in conjunction with referenced studies, are important for several reasons. First, they demonstrate the importance that cell adhesion and cell-ECM interactions likely play in regulating adipogenesis. In vitro and in vivo results have demonstrated that there is a generalized down-regulation of adhesive extracellular matrix proteins during adipogenesis [32]. Clinically, if those interactions could be enhanced or signaling through FAK promoted, this could be a mechanism through which adipogenesis is decreased. Alternatively, inhibiting adhesive interactions, or promoting three-dimensional structures, could be beneficial for producing fat for tissue engineering applications [33, 34]. Finally, long-term systemic treatment with heparin results in osteoporosis in both human and animal models [35-38]. Osteoporosis is associated not only decreased bone but with increased intramedullary fat [39, 40]. It is possible that long-term heparin treatment influences bone mass through regulation of marrow adipogenesis.

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Figure 1. Heparin promotes adipogenic differentiation

Oil-Red-O staining of (A) IM-MSC and (B) ST2 cells at day 9 after adipogenic differentiation induction with (+Hep) or without (-Hep) 20 µg/ml heparin. Quantification of lipid accumulation following Oil-Red-O staining of (C) IM-MSC, (D) ST2, and (E) 3T3 L1 cells at day 9 after adipogenic induction with 20 µg/ml heparin (White bars) or without (Black bars). MDI: 0.26 µM isobutylmethylxanthine, 0.5 µM dexamethasone, 0.085 µM insulin for day 0-3. TZD: 5 µM Thiazolidinedione (Troglitazone) for day 0-3. DT: 0.5 µM dexamethasone and 5 µM Thiazolidinedione for day 0-3. For all treatment cells were shifted to basal media plus 0.085 µM insulin alone for days 3-9. Columns, Mean; Error bars, Standard deviation. Experiments repeated at least three times. *, p-value<0.01 comparing heparin treated to untreated.

Figure 2. Heparin treatment results in upregulation of adipogenic-associated genes

Quantitative RT-PCR for adipogenic differentiation marker genes in ST2 cells with 20 µg/ml heparin (White bars) or without heparin (Black bars). Samples were collected at day 1, day 4 and day 7 after induction. A. C/EBP alpha; B. PPAR gamma; C. LPL; D. FABP4. Columns, mean; Error bars, standard deviation of n=2 replicate tissue culture wells assayed in duplicate PCR reactions. Experiment repeated twice. *, p-value<0.01 comparing heparin treated to untreated for each adipogenic protocol at each time.

Figure 3. Heparin effect on adipogenesis is dose and time dependent

Quantification of lipid accumulation following Oil Red O staining of ST2 cells at day 9 after adipogenic induction with (A) different doses of heparin, or (B) 20 μg/ml heparin added at different time periods before or during adipogenic differentiation induction with MDI. In (A) white diamonds, TZD; black squares, MDI. Statistical significance was evaluated using ANOVA with Tukey post-hoc mean's testing. Differing letters (TZD) or numbers (MDI) indicate that values are significantly different at $p < 0.05$. If points have the same letter or number than differences are not statistically significant. In (B) columns, mean; error bars, standard deviation of $n=3$ replicate wells. Experiment repeated at least three times. *, $p < 0.05$ comparing each heparin treatment to the MDI, no heparin control.

Figure 4. The effect of heparin on adipogenesis is not dependent on cell density

Quantification of lipid accumulation following Oil-Red-O staining of ST2 cells at day 9 after adipogenic differentiation induction with MDI, and with 20 μ g/ml heparin (open diamonds) or without heparin (black squares). Cells were plated at different cell densities from 2500 (sparse) to 80000 (confluent) cells per well in 24 well plates. Symbol, mean; error bars, standard deviation of n=3 replicate wells. Experiment repeated at least three times. *, p<0.05 comparing each heparin treatment to no heparin control.

Figure 5. Negatively charged proteoglycan dextran sulfate can not recapitulate the effect of heparin

A. Quantification of lipid accumulation following Oil-Red-O staining of ST2 cells at day 9 after adipogenic differentiation induction with 20 µg/ml dextran (white bars) or without dextran (black bars). *, $p < 0.01$ comparing dextran treated to untreated. B. Quantification of lipid accumulation following Oil-Red-O staining of ST2 cells at day 9 after adipogenic differentiation induction with or without 100 µg/ml Protamine and/or 20 µg/ml heparin. Results analyzed for statistical significance using ANOVA and post-hoc analysis. Differing letters (MDI group) or numbers (TZD group) indicate values that are statistically significant at $p < 0.05$. Columns, mean; error bars, standard deviation of $n=3$ replicate wells. Experiment repeated at least three times.

Figure 6. Heparin effects on adipogenic differentiation are independent of extracellular lipid
Quantification of lipid accumulation following Oil-Red-O staining of 3T3 L1 cells at day 9 after adipogenic differentiation induction with or without 20 μ g/ml heparin, serum-free basal medium supplemented with 10% delipidated calf serum or normal calf serum. Columns, mean; error bars, standard deviation of $n=3$ replicate wells. Experiment repeated at least three times. Results analyzed for statistical significance using ANOVA and post-hoc analysis. Differing letters (MDI group) or numbers (DT group) indicate values that are statistically significant at $p<0.05$.

Figure 7. Heparin disruption of Cell-ECM interactions counteracts the pro-adhesive inhibition of adipogenic differentiation and disrupts focal adhesion signaling

(A) Bright field image of cells at day 9 during 3T3 L1 and ST2 cells adipogenic differentiation induced with MDI, with 20 μ g/ml Heparin or without heparin. 100X magnification. (B) Quantification of lipid accumulation following Oil-Red-O staining of ST2 cells at day 9 after adipogenic differentiation induction with or without 20 μ g/ml heparin, plated onto 50 μ g/cm² fibronectin. Results analyzed for statistical significance using ANOVA and post-hoc analysis. Differing letters (TZD group) indicate values that are statistically significant at $p < 0.05$ (C) Bright field microscope picture taken at day 3 during 3T3 L1 adipogenic differentiation induced with MDI under 10% calf serum condition on TC plates or fibronectin, with 20 μ g/ml heparin or without heparin. 100X magnification. (D) Western blots showing FAK and PAX phosphorylation 24 hours following heparin (hep), MDI, or heparin + MDI (M/H).

Figure 8. Model of heparin regulation of adipogenesis

Mesenchymal cells form mature focal adhesions through the binding of integrins and syndecan to fibronectin. Upon addition of heparin, focal adhesions are disrupted, but integrin interaction with fibronectin remains intact. This state of deadhesion results in a loss of FAK signaling.