Rho-ROCK signaling differentially regulates chondrocyte spreading on fibronectin and bone sialoprotein

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The growth plate is a dynamic region in endochondral bones responsible for the longitudinal growth of the mammalian skeleton. Chondrocytes within this region process through subsequent stages of proliferation, maturation, and terminal differentiation into hypertrophic cells before their death and removal. The sequential nature of this differentiation program is reflected in the way these cells are organized into discrete resting, proliferating, and hypertrophic zones within the growth plate. The cells are further organized into columns along the longitudinal axes of long bones with the columns separated by intimate associations with the cartilage extracellular matrix (ECM) (17). The ECM is required to ensure proper organization and coordination of growth plate development and turnover (4). Growth plate chondrocytes and the secreted ECM constitute a temporary cartilage template that is eventually replaced by bone in a process termed endochondral ossification (4). Interactions of chondrocytes with the ECM are mediated through adhesion receptors such as integrins and control chondrocyte shape, which is linked to essential roles in regulating chondrocyte proliferation, differentiation, and apoptosis (56).

Fibronectin (FN), a dimer consisting of two 220- to 250-kDa monomer subunits, is expressed throughout the growth plate (24). It stabilizes ECM structure by binding multiple matrix components such as collagens and proteoglycans. It mediates cell adhesion through a central RGD (Arg-Gly-Asp) motif and various non-RGD sequences (40). Although mice with cartilage-specific deletion of FN have no apparent phenotype under standard laboratory conditions (3), numerous studies have shown a role for FN (54) or FN-binding integrins, for example, α5β1- (11, 15) and β1-containing integrins (3), in regulating chondrocyte development.

Unlike FN, which is expressed ubiquitously, bone sialoprotein (BSP) expression is essentially restricted to mineralized tissues (14). BSP has extensive posttranslational modifications (PTMs) that add upwards of 23 kDa to the 34-kDa molecular mass of the core protein (59, 60). This monomeric protein has three well-characterized, functionally distinct domains that, similar to FN, stabilize ECM structure: an NH2-terminal collagen binding site (47), two unique central glutamic acid-rich regions capable of binding mineral hydroxyapatite (19, 44), and a COOH-terminal integrin-binding RGD domain (23, 33, 44) that binds α5β1- (26), αvβ3- (37) and αvβ5-integrins (46). BSP is synthesized by hypertrophic chondrocytes at a time when the hypertrophic zone of the growth plate undergoes mineralization, vascular invasion, and eventual replacement by a bone matrix (14). In vitro and in vivo assays have linked BSP to the aforementioned processes (5, 20, 49, 53), but it is not known whether growth plate chondrocytes are responsive to BSP. Furthermore, it is not known how cells use intracellular signaling intermediates to control their cellular responses to BSP and how these intracellular signaling molecules control similar responses to FN.

BSP and FN are examples of ECM proteins that bind different cell surface integrins and form functional units of cell adhesion termed focal adhesions. Signals emanating from focal adhesions determine the cellular response to ECM proteins (31). Focal adhesion kinase (FAK) interacts directly with the cytoplasmic tail of various transmembrane receptors and has been implicated in regulating many adhesion-related functions.
Many of these FAK functions are regulated by Src, which coactivates FAK and forms a dual FAK-Src signaling complex through the binding of SH2 domain of Src to a phosphorytrosine at Y397 of FAK (35). We have shown FAK-Src signaling as negative regulators of early chondrogenesis (7, 39), but it is not known how this signaling complex is regulated in growth plate chondrocytes by different integrin-binding proteins and whether chondrocyte adhesion to and spreading on different ECM proteins is regulated by FAK-Src signaling.

Members of the Rho family of small GTPases constitute another set of proteins involved in cellular responses to adhesion (6, 21, 41). Rho GTPases are major regulators of the cell cytoskeleton and act upstream and downstream of integrin ligands (6, 31). The Rho GTPases cycle between active GTP-bound forms and inactive forms following the hydrolysis of bound GTP into GDP (6). RhoA and Rac1 are common examples of Rho GTPases (6). Our in vitro studies have demonstrated important roles of RhoA and Rac1 in chondrocyte differentiation (51, 55, 57, 58). We have also shown that Rac1-null chondrocytes display reduced adhesion and spread on different ECM surfaces (52). However, it is not known how Rho and an effector of Rho, Rho kinase (ROCK), regulates chondrocyte adhesion and spreading on different ECM substrates.

Measuring cell adhesion and spreading on adsorbed ECM components in the two-dimensional context of tissue culture can be used as indicators to gauge the ability of these proteins to promote cell contact and consequently alter cell behavior (10, 36). Thus studying cell adhesion and spread to ECM components and signaling pathways affecting these processes in vitro are directly relevant to understanding how ECM proteins contribute to maintaining tissue architecture in vivo. With this in mind, the objectives of this study were fourfold: 1) determine to what extent actin polymerization controls cell adhesion to and contact with FN- and BSP-coated surfaces; 2) use adhesion and spreading dynamics to define chondrocyte sequence-function relationships with BSP; 3) determine how FAK-Src and Rho-ROCK signaling regulate chondrocyte adhesion and spreading on FN- or BSP-coated surfaces, and 4) determine differences in the manner that chondrocytes and fibroblasts use Rho-ROCK signaling to control cell adhesion and morphology.
density reduced cell clumping. After incubation at 37°C in a humidified atmosphere with 5% CO₂ for 60 min (unless stated otherwise), weakly adhered cells were removed by washing each well with adhesion medium. Cells were then fixed with 4% formalin in PBS for 20 min, permeabilized with 0.1% Triton-X100 in PBS for 10 min, and washed twice with PBS before storage for up to a month at 4°C in the dark with PBS containing 14 mM rhodamine-phalloidin and 86 mM DAPI. Before analysis, excess fluorescent stain was washed away with three PBS washes. Random micrographs of cells adhered to different coats were taken using an inverted Leica DMIRE2 microscope equipped for fluorescence detection attached to a Hamamatsu ORCA-ER digital camera. Each well had at least five micrographs taken representing a 5% sampling of total well area. Pictures were analyzed using OpenLab software. Binary masks were generated for each fluorescence dye (rhodamine-phalloidin and DAPI). Masks generated from pictures of DAPI-stained cells were used to count attached cells; each nuclei was counted as an attached cell. Masks generated from pictures of rhodamine-phalloidin-stained cells were used to measure average cell spread and average cell perimeter. Cell spread was computed as total area of spread cells per micrograph per number of DAPI-stained nuclei from corresponding micrograph (expressed as μm²/cell). Cell perimeter was computed as total perimeter of identified objects in rhodamine-stained cells per micrograph per number of DAPI-stained nuclei from corresponding micrograph (expressed as μm/cell). Although the cell perimeter measurements are not absolute measurements of cell perimeter, these values represent the protrusiveness of adhered cells and are values that are reflective of each treatment.

Quantification of adsorbed protein. To determine the amount of protein adsorbed to the plate surface, 96-well Falcon plates were coated with 10 μg/ml solutions of FN, rBSP, and nBSP. Each well was coated in triplicate at 150 μl/well. After 14–16 h at 4°C, the coating solution was removed and mixed with a single 150-μl PBS wash solution from the corresponding well used to remove loosely bound protein. Of this 300-μl mixture, 150 μl were mixed with 150 μl of Pierce Micro BCA working reagent in a microfuge tube, incubated at 60°C for 1 h, and absorbance measured at 562 nm. Individual standard curves were made using each protein under identical conditions. The protein content of the recovered solution used to coat each well was subtracted from the total protein initially plated to determine the amount of protein adsorbed to each well. The experiment was repeated three times.

In addition, an ELISA was used to determine relative levels of protein coating as modified from Tye et al. (47). Briefly, 24-well Falcon plates were coated with 2–20 μg/ml of FN or rBSP in triplicate wells as before. Wells were blocked with adhesion medium for 1 h at 37°C. Plates were then incubated overnight at 4°C with primary antibody (anti-His at 1/100,000 or anti-FN at 1/40,000) and subsequently detected with the corresponding horseradish peroxidase-linked secondary antibody (anti-mouse at 1/4,000 or anti-rabbit at 1/2,000) incubated for 1 h at room temperature. Addition of 0.4 ml/ml ortho-phenylenediamine in phosphate-citrate buffer (200 nM Na₂HPO₄, 100 mM citric acid at pH 5) to each well initiated a colorimetric reaction that was stopped 4 min later with 2.5 M H₂SO₄. The absorption of the resulting solution was measured at 492 nm using a Safire² Microplate Detection System. Relative coating indexes were obtained by dividing all absorbencies by the absorbance measured at the 2 μg/ml coating for each protein. The experiment was repeated three times.

Immunofluorescence. All steps up to and including cell fixation and permeabilization were followed as described in the cell adhesion protocol above. Washes, incubations with primary and secondary antibodies, rhodamine-phalloidin and DAPI, mounting with Vectashield antifade mounting media and sealing slides were as described (52). Pictures were obtained using an upright Leica DMRA2 microscope equipped for fluorescence detection with a Q-imaging Retiga Ex 12-bit camera. Images were processed using OpenLab and ImageJ (NIH) software. Each staining was performed on triplicate slides and repeated at least twice. No fluorescent staining was observed when staining with secondary antibody alone (data not shown) nor when slides were reacted with solutions containing primary antibody and target immunogenic peptide indicating specificity of the primary anti-phospho-FAK Y397 antibody for the target protein (data not shown).

Collection and preparation of cell lysate. As in the cell attachment assays in Cell adhesion assay, Falcon T-175 flasks were coated with protein solutions overnight and blocked with adhesion medium. After the incubation of 15 × 10⁶ chondrocytes per flask on various protein coats at 37°C for 1 h, adhered cells were scraped into 1 ml of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 2 mM PMSF, 8 mM Na₃VO₄, 2 mM NaF, and 1 protease inhibitor cocktail tablet (Roche) per 25 ml of buffer) on ice. Adhesion medium from each flask containing nonadhered cells was collected and centrifuged. These cell pellets were mixed with adhered cell lysates from the corresponding treatments. Lysates were stored at −80°C until use. Before use, two freeze-thaw cycles were used to further ensure cell lysis. This crude lysate was centrifuged to remove insoluble cellular debris, and the supernatant was used for subsequent experiments.

Immunoprecipitation and Western blot analyses. The Catch and Release Reversible Immunoprecipitation System (Upstate) was used to precipitate FAK with mouse anti-FAK antibodies from preclared cell lysates according to the manufacturer’s instructions. Lysates containing equal cell numbers were used for each immunoprecipitation. After this initial FAK pulldown, this same lysate was subjected to another round of immunoprecipitation with mouse anti-FAK antibodies to assess the efficiency of the original FAK pulldown. Captured FAK was resolved on 10% SDS-polyacrylamide gels and transferred to polyvinylidifluoride membrane (Amersham Biosciences) using the Bio-Rad Trans-Blot SD Semi-Dry electrophoretic transfer cell. For immunoblotting of β-actin in the immunoprecipitation loading control, 7.5 × 10⁶ cells were loaded per lane. After a 5% BSA block, membranes were probed with relevant antibodies and detected using ECL Western Blotting Detection kit (Amersham Biosciences). Blots were visualized using ChemiImager5500 (AlphaInnotech).

Statistical analyses. Numerical data were analyzed using Graphpad 4.0 software where possible. To determine statistical differences among means, one-way ANOVA was performed. If a statistically significant difference was determined (P < 0.05), a Bonferroni multiple contrasts test was performed to determine which pairs of means were statistically different.

RESULTS

Chondrocyte adhesion and spread to FN and rBSP are time and dose dependent. Multiple studies have attempted to compare the adhesion and subsequent signaling properties of different ECM proteins in a variety of cell types but have poorly characterized the interaction of their ECM ligand with the coating surface. In this study, there was no significant difference (P > 0.05) in the mass of protein bound to plates among surfaces coated with FN [345 ± 51 ng/cm² (mean ± SE)], rBSP (428 ± 33 ng/cm²), or nBSP (390 ± 23 ng/cm²) when coated with 10 μg/ml of protein. Most experiments described herein used FN or rBSP at concentrations (10 μg/ml) that saturated the plate surface as determined by ELISA (Fig. 1A). Despite this saturation, the adhesion and spreading dynamics of the cells varied with time, dose, and type of substrate coated.

Chondrocytes adhered (Fig. 1B) and spread (Fig. 1C) on FN- and rBSP-coated surfaces but not on the negative control coat of BSA over a 2-h time period. Maximal adhesion and spread-
ing were observed within 1 h of incubation. In fact, there were no statistical differences between 60 min and later time points for either the number of cells adhered or the average cell spread ($P > 0.05$). These studies were limited to adhesion and spreading within 1 h to avoid morphological changes due to ECM secreted by the chondrocytes themselves. Of note, there was significantly more cellular spread and thus more cellular contact with surfaces coated with FN when compared with cells bound to rBSP-coated surfaces at all time points ($P < 0.001$) (Fig. 1C).

To determine the dependence of adhesion and spread on coating concentration, chondrocytes were plated on a range (0.1–20 g/ml) of FN and rBSP concentrations for 1 h (Fig. 1, D and E). Maximal chondrocyte adhesion was observed starting at 2 g/ml for FN and 5 g/ml for rBSP. Maximal cell spread was only observed when coating with at least 5 g/ml for each protein. This cell spread was not statistically different from the cell spread measured at higher coating concentrations ($P > 0.05$). Consistent with the time-dependent data, more cell spread and thus more cell contact were observed on FN-coated surfaces than rBSP-coated surfaces. Of note, similar cell spread was observed for chondrocytes bound to rBSP at 10 g/ml and FN at 2 g/ml. These data establish that maximal adhesion and cell spread are observed within 1 h in chondrocytes plated on FN- or rBSP-coated at 10 g/ml.

Attachment to BSP is entirely dependent on the RGD domain. PTMs of proteins have been implicated in modulating cellular activities. Previous studies have shown that BSP extracted from bone has numerous PTMs (13, 43, 59, 60). We therefore examined whether PTMs present on native, bone-extracted BSP could regulate chondrocyte adhesion and spread. To ensure that nBSP and rBSP had similar binding properties to tissue culture plastic, we showed that there was no significant difference in coating between native and recombinant BSP (as discussed above). There was no difference in chondrocyte adhesion (Fig. 2A) to or spreading (Fig. 2B) on native or rBSP. These results suggest that PTMs on nBSP do not play a role in the adhesion or spread of chondrocytes to BSP.

BSP has been reported to contain other non-RGD binding sequences in promoting adhesion (33, 44). Having established that the adhesion and spread of chondrocytes on BSP are limited to the primary sequence of the protein, we investigated the role of the RGD sequence. Mutated rBSP, in which the RGD was altered to KAE (rBSP-KAE) (20), was coated onto plates. Based on negligible cell binding, we conclude that the
RGD sequence is the sole domain responsible in full-length BSP for chondrocyte (Fig. 2C) and fibroblast attachment (Fig. 2D).

Actin polymerization is essential for chondrocyte and fibroblast spreading on FN and BSP. Numerous experiments have shown that elements of the cell cytoskeleton can act downstream of integrins in response to ECM proteins (25, 32). We wanted to determine to what extent actin microfilaments mediate cellular contact with surfaces coated with FN and BSP. Our data demonstrated that chondrocytes attach to and spread on FN and rBSP with distinct F-actin structures but retain a rounded-shape on BSA (Fig. 3A). Note that the actin cytoskeleton clearly outlines the shape of the cell and can be used to perform morphometric analyses. Incubation with the F-actin capping toxin CD abolished chondrocyte spreading on FN (data not shown) and rBSP (Fig. 3A). Similar results were obtained with actin sequestering agent Latrunculin B (data not shown). Whereas chondrocyte (Fig. 3B) and fibroblast (Fig. 3D) adhesion were unaffected by repression of actin polymerization, spreading of both cell types (Fig. 3, C and E, respectively) were significantly reduced (P < 0.001) when compared with controls and similar to levels of spread observed for cells on BSA. These results indicate that whereas cell adhesion is independent of actin polymerization, cell spreading on FN or BSP is completely dependent on the ability of chondrocytes and fibroblasts to polymerize actin.

FAK-Src activation does not correspond to differences in chondrocyte spreading to FN and BSP. Nonreceptor protein tyrosine kinases are concentrated at focal adhesions in numerous cell types and are known to regulate cell spreading. We wanted to examine how adhesion of chondrocytes to FN or rBSP affects the localization of activated FAK. Phosphorylation of Y397 residue in FAK activates the central kinase domain and accelerates FAK autophosphorylation and transphosphorylation of other signaling targets (27). Interestingly, FAK phosphorylated at Y397 was detected most strongly at the periphery of chondrocytes that attached and spread to FN and rBSP but was not detected in cells bound to BSA (Fig. 4A). In particular, Y397-phosphorylated FAK was localized at vertices of the cell perimeter, regions that determine the extent of cell spread on matrix proteins.

To determine whether the level of FAK activation could be correlated to the degree of cell spreading, FAK was immunoprecipitated from cell lysates of chondrocytes plated on BSA, BSP, and two different coating concentrations of FN (Fig. 4B). Cells in suspension before plating and those plated on BSA for 1 h have negligible FAK activation. In contrast, those cells bound to BSP or FN for 1 h, displayed increased phosphorylation of FAK at Y397 and more phosphorylated tyrosine residues. However, no difference was evident in the levels of FAK activation and tyrosine phosphorylation in cells associated with different levels of cell spread or different adsorbed matrices. In parallel experiments, chondrocytes were incubated with the Src inhibitor PP2 before cells were plated on FN- and rBSP-coated surfaces. We have previously shown that PP2 treatment decreases FAK activation in chondrocytes (8). Src inhibition decreased chondrocyte adhesion (Fig. 4C) and spread (Fig. 4D) on both FN and rBSP. Since there were no differences in levels of FAK phosphorylation from FN- and BSP-bound cells and inhibition of Src/FAK activation decreased adhesion and spread on both ECM-proteins, activation of FAK-Src signaling is likely not responsible for the observed differential spreading of chondrocytes bound to BSP and FN as noted in Fig. 1C.

Inhibition of Rho alters cell morphology and increases cell spread on BSP. Since Rho GTPases have long been known to alter actin-based cytoskeletal responses, we were interested in determining whether Rho was modulating parameters of chon-
drocyte morphology (cell spreading area and cell perimeter) in response to surfaces coated with FN or BSP. To investigate this, cells were incubated with the Rho-inactivating toxin C3 before these cells were plated on substrate-coated surfaces. Regardless of the coating substrate, C3-treated chondrocytes lost intracellular actin fibers and formed prominent actin structures at the cell periphery (Fig. 5). Actin remodeling induced by C3 treatment did not significantly change the morphology of cells on FN and BSP. Whereas cell spread (Fig. 6A) did not significantly change on FN ($P > 0.05$), C3 did cause the formation of membrane protrusions that significantly increased the cell perimeter ($P < 0.01$; Fig. 6B). Chondrocytes treated with C3 and adhered to rBSP increased cell spread (Fig. 6A) and cell perimeter (Fig. 6B) to levels not statistically different ($P > 0.05$) from those observed for FN.

To determine whether the effects of Rho inactivation on different ECM substrates were specific to chondrocytes, the aforementioned experiments were repeated with NIH3T3 fibroblasts. Similar to chondrocytes, there was no statistical difference ($P > 0.05$) in adhesion of fibroblasts to FN and rBSP (Fig. 3D), and there was more cell spread ($P < 0.001$) on cells adhered to FN than on rBSP (Fig. 6C). C3-treated fibroblasts and chondrocytes also shared some common features;
most notables were a clearing of intracellular F-actin and increased F-actin staining at the periphery of the cell (Fig. 5).

Similar to chondrocytes, Rho inhibition in fibroblasts did not alter adhesion to FN or rBSP (supplementary Fig. 1B) but did increase cell spread (Fig. 6C) and perimeter (Fig. 6D) on rBSP-coated surfaces. However, unlike Rho-inhibited chondrocytes, Rho-inhibited fibroblasts adhered to rBSP were not able to increase cell spread and perimeter to levels observed on FN-adhered cells. Another obvious difference in C3-treated fibroblasts was the lack of membrane protrusions on FN as opposed to the F-actin rich protrusions in FN-adhered, Rho-inhibited chondrocytes. This was reflected in the actin organization of rhodamine-phalloidin-stained fibroblasts (Fig. 5) and the absence of changes in perimeter or spreading of FN-adhered fibroblasts treated with C3. Regardless of differences between cell types, these studies suggest that Rho activation is a generic signaling pathway that restricts cell spread on rBSP-coated surfaces.

ROCK inhibition has different consequences in chondrocytes and fibroblasts. ROCK is a serine-threonine kinase that is directly activated by GTP-bound Rho. To determine whether ROCK could act downstream of Rho and mimic the effects of Rho inhibition in chondrocytes, chondrocytes were incubated with the ROCK inhibitor Y27632 before cells were plated on coated surfaces. As noted with Rho inhibition, ROCK inhibition dramatically altered F-actin arrangement within cells in a FN- or rBSP-dependent manner (Fig. 7) but did not change the number of adhered cells (supplementary Fig. 2A).
chondrocytes treated with the ROCK inhibitor did not alter cell spread \((P > 0.05; \text{Fig. 8A})\) when compared with control cells but did develop lengthy actin-rich membrane protrusions that significantly \((P < 0.001)\) increased cell perimeter (Fig. 8B), similar to Rho inactivation. Another similarity with Rho inhibition in chondrocytes was the ability of ROCK inhibition in rBSP-adhered chondrocytes to increase cell spread to levels not statistically different from cells adhered on FN. Plating

![Fig. 5. Rho inhibition alters the actin cytoskeleton in chondrocytes and fibroblasts bound to FN and rBSP. Chondrocytes and NIH 3T3 cells were incubated with the Rho inhibitory membrane-permeable C3 toxin before being plated on FN- or rBSP-coated surfaces for 1 h. Bound chondrocytes (A–D) and fibroblasts (E–H) were stained for F-actin (red) and nuclei (blue). White arrowheads point to membrane protrusions from cells; bar = 50 µm.](image)

![Fig. 6. Rho inhibition increases spread in chondrocytes and fibroblasts bound to rBSP. Chondrocytes and NIH 3T3 cells were incubated with the Rho inhibitory membrane-permeable C3 toxin before being plated on FN- or rBSP-coated surfaces for 1 h. Chondrocyte spread (A) and perimeter (B) were subsequently assessed to find Rho-dependent regulation of chondrocyte spread on rBSP and membrane protrusiveness on FN. Similar analyses for fibroblast spread (C) and perimeter (D) found a dependence of Rho signaling in regulating fibroblast spreading on rBSP. Values for all graphs represent means ± SE for experiments performed in three independent trials, in triplicate wells each; NS at \(P < 0.05\); S by at least \(P < 0.05\).](image)
Y27632-treated chondrocytes on surfaces coated with less FN did not increase cell spread (supplementary Fig. 3), suggesting Rho-ROCK regulation of cell spreading is unique to chondrocytes plated on BSP. Thus, in terms of cell spread and perimeter, and for each ECM molecule, ROCK inhibition was able to mimic the effects of Rho inhibition in chondrocytes.

Y27632-treated fibroblasts still developed long dendritic F-actin-rich protrusions (Fig. 7) akin to Rho-inhibited fibroblasts and Rho- and ROCK-inhibited chondrocytes. ROCK inhibition also did not affect fibroblast adhesion to FN or rBSP (supplementary Fig. 2B). However, ROCK inhibition did not significantly (P > 0.05) increase cell spread (Fig. 8C) for fibroblasts on rBSP-coated surfaces compared with untreated controls, in contrast to Rho-inhibited chondrocytes and fibroblasts, and ROCK-inhibited chondrocytes. Instead, cell perimeter (Fig. 8D) was significantly increased in both FN- and rBSP-adhered, Y27632-treated fibroblasts when compared with controls (P < 0.001). Thus, in fibroblasts, ROCK signaling seems to regulate membrane protrusiveness (P < 0.001) in both FN- and rBSP-adhered cells but not cell spread.

DISCUSSION

The primary objective of this work was to delineate how growth plate chondrocytes differ in their adhesive interactions to FN- and BSP-coated surfaces and to determine the involvement of FAK-Src and Rho-ROCK signaling in these processes. To do this, we quantified the extent of cell contact with FN- and BSP-coated surfaces by measuring cell adhesion and other parameters of cell morphology (cell spread and perimeter). The major novel finding of our work is that despite similar adhesion to both FN and BSP, chondrocytes specifically use the Rho-ROCK pathway in regulating their contact with BSP-coated surfaces. This is different from chondrocytes on FN and different from fibroblasts on FN or BSP.

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The main difference noted in this study is decreased cell spread on BSP-coated surfaces compared with cells bound to FN, irrespective of time. A possible explanation for this is that FN-coated surfaces provide more adhesive contacts compared with BSP-coated surfaces, likely because FN has numerous well-characterized cell adhesion sites (40). Some studies have identified non-RGD regions within BSP that mediate cell adhesion (33, 44). BSP is a member of the small integrin ligand N-linked glycoprotein (SIBLING) family of ECM molecules (42). It has also been shown that the extensive PTMs within the SIBLING family regulate various cell functions including adhesion (42). However, we demonstrate that the PTMs of nBSP do not alter the adhesion or spread of chondrocytes. Instead, growth plate chondrocyte and fibroblast adhesion to BSP is entirely dependent on the RGD sequence. To rule out the possibility that decreased cell spread on BSP was a result of less BSP bound to surfaces, we showed that on a mole-per-mole basis, 16× more BSP adsorbed to surfaces compared with FN. Hence, the vast molar excess of BSP adsorbed on surfaces implies that other mechanisms restrict chondrocyte spreading and thus cellular contact with BSP-coated surfaces.

Establishing that a functional actin cytoskeleton is an absolute requirement for cell spread on FN and BSP independent of cell adhesion, we hypothesized that intracellular signaling molecules known to regulate actin organization are responsible for the lower level of cell spreading on BSP when compared with FN. The FAK-Src signaling complex was one prime candidate for such a function. The ability of FAK to regulate cell adhesion is not universal but is dependent on the cell type and the adhering matrix (22). We found that FAK-Src inhibition decreased chondrocyte adhesion to both FN and BSP. FAK controls focal adhesion dynamics and cell spread (34) consistent with the localization of activated FAK in the perimeter of adhered chondrocytes on both FN and BSP in our study.
Inhibition of FAK-Src signaling also decreased chondrocyte spread to both FN and BSP. However, FAK activation did not discriminate between different adhesive matrices nor did it correlate with the degree of cell spreading. Our results suggest that FAK-Src signaling act as positive regulators of chondrocyte adhesion and spreading to both FN and BSP and is likely a part of the basal adhesion and spreading machinery of chondrocytes but is not responsible for the difference in cell spread between FN and BSP.

In contrast to FAK-Src signaling, Rho signaling has been implicated as both a positive and negative regulator of cell spread in numerous models. In astrocytes, active Rho is responsible for spreading and flattening of cells in response to lysophosphatidic acid and thrombin (45), whereas multinucleated monocytes use active Rho for cell retraction and to negatively regulate cell spread by completely dissolving fibrillar actin structures (38). We identify Rho as a mediator of different spreading responses to particular ECM components. In astrocytes, active Rho is responsible for spreading and flattening of cells in response to lysophosphatidic acid and thrombin (45), whereas multinucleated monocytes use active Rho for cell retraction and to negatively regulate cell spread by completely dissolving fibrillar actin structures (38). We identify Rho as a mediator of different spreading responses to particular ECM components.

Inhibition of Rho increased spreading on BSP but not on FN, without affecting cell adhesion. Recent work by Flevaris et al. (12) have identified a molecular switch in which calpain-dependent cleavage of β3-integrin in fibrinogen-adhered cells switches the cellular response from cell spread to cell retraction by relief of Rho inhibition. Interestingly, studies by Chen et al. (9) have shown inhibition of Rho signaling to increase cell spread to cell-secreted and serum-adsorbed components independently of FAK signaling, which would agree with our findings.

ROCK inhibition in chondrocytes was able to corroborate our findings with Rho inhibition, namely, an increase in spreading to BSP and membrane protrusiveness to FN. However, ROCK inhibition was not able to mimic all effects of Rho inhibition. Rho inhibition disturbed the actin cytoskeleton in both chondrocytes and fibroblasts, leading to the loss of intracellular actin fibers and prominent filamentous actin structures at the cell periphery. This was not observed with ROCK inhibition in either cell type. ROCK inhibition in fibroblasts adhered to FN, or BSP increased membrane protrusions, which were not observed in Rho-inhibited fibroblasts. This is intriguing considering Rho inhibition increased cell spread in BSP-adhered fibroblasts while Rho and ROCK inhibition increased cell spread in BSP-adhered chondrocytes. Based on the more drastic phenotype observed with ROCK inhibition compared with Rho inhibition, we cannot rule out factors influencing inhibitor efficacy (for example, cellular uptake of inhibitors). However, the apparent differences in Rho and ROCK functions are not without precedent. Different cell types are known to use Rho effectors in different capacities (16). At least for some observed Rho-dependent phenotypes, other downstream effectors of Rho, for example mDia (6), may mediate these effects.

Our work has important implications for understanding chondrocyte behavior in physiological, pathological, and in vitro conditions."
tions. By identifying FAK/Src signaling as positive regulators of chondrocyte adhesion to matrix proteins, we provide a potential mechanism for enhanced chondrogenesis observed by reducing FAK/Src activity in chondrocyte precursors (39) and chondrocytes (7). We show that FAK/Src inhibition decreases chondrocyte-matrix interactions. Cell-matrix interactions negatively regulate early chondrogenic events where tight cell-cell adhesion is essential as opposed to later differentiation events, which depend on close cell-matrix interactions (56). In some forms of osteoarthritis, articular chondrocytes recapitulate the developmental processes of growth plate chondrocytes (1, 2). We have shown previously that during chondrocyte development, there is decreased RhoA activity (55) and increased expression of related proteins of the SIBLING family (28, 29). Our work implies that chondrocytes may preferentially increase their actin-based contact to matrices containing SIBLING proteins as these cells differentiate and mature. Furthermore, during dedifferentiation of chondrocytes to fibroblast-like cells in arthritis (57) or in prolonged in vitro culture (57), the basic mechanisms regulating cell shape may also change. Our work clearly demonstrates that chondrocytes and fibroblasts use Rho and ROCK in different capacities.

In summary, we show that similar to other cells [endothelial cells (5), osteoblasts (20, 53) and osteoclasts (49)] involved in endochondral ossification, growth plate chondrocytes can interact with BSP. However, chondrocytes and fibroblasts spread more on and thus have more contact with FN-coated surfaces compared with BSP-coated surfaces. We show FAK-Src signaling act as a positive regulator in controlling chondrocyte adhesion and spreading to FN and BSP. In contrast, inhibition of the Rho-ROCK pathway in chondrocytes and Rho in fibroblasts counteracts the reduced spreading on BSP, suggesting that the Rho-ROCK pathway inhibits chondrocyte spreading on BSP. Our work, to the best of our knowledge, is the first indication that classical integrin binding ECM molecules use different actin remodeling pathways to control cell shape and that these signaling pathways have unique consequences in controlling cell-matrix contact in different cell types.

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