The role of angiopoietin-2 in nucleus pulposus cells during human intervertebral disc degeneration

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Although evidence shows that intervertebral disc degeneration is generally characterized by angiogenesis, the role of angiopoietin has not been investigated. This study examined the presence of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) within the native intervertebral disc (IVD) and elucidated their functions in the regulation of nucleus pulposus (NP) cells. Initial investigation of uncultured NP tissue revealed that Ang-1 and Ang-2 were expressed by native NP cells. Ang-2 expression was significantly increased in infiltrated and degenerate samples relative to normal samples. The ratio of Ang-2/Ang-1 in tissues from patients increased markedly with increasing age and level of degeneration of the IVD. The ratio of both Ang-2/Ang-1 mRNA and protein increased over time when cells were subjected to constant pressure at 1 Mpa *in vitro*. Our findings indicate that Ang-2 plays a role in suppressing cell adhesion and viability, and promotes the apoptosis of NP cells and that Ang-2 can inhibit the pathways stimulated by Ang-1 and fibronectin. Ang-2 release during IVD degeneration causes higher ratio of Ang-2 to Ang-1, further inhibits NP cell viability and adhesion, promoting apoptosis by blocking PI3K/Akt signaling. The present study therefore provides new insights into the role of the angiopoietin-Tie system in the pathogenesis of IVD degeneration.

Low back pain, a major cause of disability, affects 632 million people globally,[1] and epidemiological studies showing that the incidence of this medical problem increases every year. Although complex multiple factors are involved in triggering lower back pain, the condition is generally believed to be associated with intervertebral disc degeneration (IDD). The progression of degeneration is accompanied by elevated levels of inflammatory cytokines, enhanced degradation of aggrecan and collagen, and changes in the phenotype of disc cells.[2] Angiogenesis represents an additional feature of disc degeneration; neovascularization enables the infiltration of macrophages into the intervertebral disc (IVD), which triggers inflammation and further accelerates disc degeneration.[3]

The angiopoietin-Tie system is not only crucial for angiogenesis, but also provides an important link between angiogenic and inflammatory pathways.[4] Two ligands of Tie2, namely angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), have been well characterized; Ang-1, which acts as an anti-inflammatory agent, is required for the stabilization of blood vessels, whereas Ang-2 acts as a functional antagonist of Ang-1.[5] Upregulation of Ang-2 expression may represent a critical step in the initiation of inflammatory processes.[6] Ang-2 activity is sufficient for recruitment of myeloid cells and induction of inflammation even in the absence of proinflammatory stimuli.[7] The expression of recombinant Ang-2 restores the induction of inflammation in Ang-2-deficient mice.[8] Ang-2 facilitates the expression of tumor necrosis factor and interleukin-1, which are key inflammatory cytokines involved in the upregulation of genes encoding matrix-degrading enzymes in IDD.[4,9–11] The stimulation of Tie2 results in activation of PI3K/Akt, which has been linked to cell growth, survival, and inhibition of apoptosis.[12] Previous studies have shown that Ang-1 is crucial for the survival of nucleus pulposus (NP) cells and that the numbers of Tie2^{+} NP cells decrease during ageing and IVD degeneration.[13] Thus, we postulated that in normal human

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NP cells that express the Tie2 receptor, pressure-induced IDD increases the Ang-2/Ang-1 ratio, which in turn reduces the cell vitality and induces NP cells apoptosis.

Immunoreactivity against the $\alpha5\beta1$ heterodimer has been observed in non-degenerated as well as degenerated IVDs.[14] The integrin receptor $\alpha5\beta1$ has been shown to be critical for interactions between IVD cells and fibronectin (FN), revealing functional integrin cell surface receptors that mediate interactions with extracellular matrix proteins in the IVD.[15] FN is a large extracellular glycoprotein that helps to organize the matrix protein; the main membrane receptor of FN in NP cells is a $\alpha5\beta1$ integrin subunit.[16] Stable interactions between the $\alpha5\beta1$ integrin and the Tie2 tyrosine kinase receptor, which regulate the response to Ang-1, are strengthened by FN in endothelial cells (ECs).[17] Thus, we hypothesized that Ang-2 induces NP cell apoptosis via the Tie2 tyrosine kinase receptor and $\alpha5\beta1$ integrin signaling.

In this study, we reveal that Ang-2 expression increased with increasing levels of degeneration. Our data demonstrate that the Ang-2/Ang-1 ratio increased in a time-dependent manner when cells were subjected to constant pressure *in vitro*. Ang-2 inhibits the viability and adhesion of NP cells and induces apoptosis of NP cells, enabling resistance to the effects of Ang-1 and FN. Finally, our data indicated that Tie2 and $\alpha5\beta1$ form a constitutive and specific complex, to which p85 and FAK are recruited. The results of this study show Ang-2 as a pro-degeneration molecule in NP cells.

MATERIALS AND METHODS
Human NP Tissues: Sample Collection and Staging
This study was approved by the Institutional Ethics Review Board of Tongji Medical College, Huazhong University of Science and Technology in accordance with the Declaration of Helsinki (2013) of the World Medical Association. NP tissues were collected from patients undergoing spinal surgery. T2-weighted magnetic resonance images were obtained at a resolution of 1.5 Tesla. Tissue samples were evaluated using the Thompson Grading System and Pfirrmann Classification System.[18] Thirty-five patients undergoing surgery for burst fracture ($n=4$), scoliosis ($n=6$), lumbar disc herniation ($n=12$), or spondylolysis ($n=13$) were enrolled in this study. The age of the patients ranged from 14 to 63 years. There were 6 patients in the grade I group, 8 in the grade II group, 8 in the grade III group, and 13 in the grade IV group. Grades I and II were assigned as non-degenerated disc groups.

Specimens were immediately sectioned for use in various experiments. One section was immediately fixed in 4% buffered formalin (pH 7.4) for eventual histological analysis. A second section was immediately immersed in RNA later (Invitrogen, Carlsbad, CA, USA) and frozen in liquid nitrogen for use in protein and RNA analysis. A third section was immediately immersed in phosphate-buffered saline (PBS) for cell isolation.

Immunohistochemistry
Tissue samples were embedded in paraffin and then cut into 4-μm-thick sections. The sections were dewaxed in xylene, then rehydrated in graded alcohol series. Antigen retrieval was performed by microwaving in Tris-EDTA pH 9 for 15 min. After washing with PBS, the endogenous peroxidase activity of the sections was blocked in 3% H_2O_2. Subsequently the samples were incubated with polyclonal anti-Ang-1 (ab8451, Abcam, Cambridge, MA, USA; 1:200 dilution) and anti-Ang-2 (sc-7015, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 dilutions) at 4 °C overnight. The negative controls included parallel sections treated with PBS solution. After washing, the sections were labeled for 40 min at 37 °C with a secondary antibody (1:2000) conjugated to horseradish peroxidase. After staining with diaminobenzidine (DAB), the sections were counterstained with hematoxylin, and images were captured using a microscope (Olympus IX71, Japan).

Isolation of Nucleus Pulposus Cells
Normal human NP cells were isolated from the discs in burst fracture patients and young scoliosis patients undergoing deformity correction surgery. The cells were isolated and cultured as previously described.[19,20] Briefly, NP tissues were minced into small fragments and enzymatically digested in 0.2% type II collagenase and 0.25% trypsin for 3 h. After being filtered and washed in PBS, the suspension was centrifuged and the isolated cells were cultured in growth medium (DMEM/F-12 supplied with 20% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin) in a 5% CO_2 incubator. The cells were passaged two to three times for use in the following experiments.

Pressure Treatment
A pressure of 1.0 MPa is widely used to induce IVD degeneration.[21] The sealed container used in the present study, assembled with a barometer and a thermometer, enabled introduction of compressed gas. The container was placed in an incubator at 37 °C. The cell culture dishes were placed on the clapboard in the container, which contained a small amount of double-distilled water to maintain a humidified atmosphere. A mixture of 5% CO_2 and 95% compressed air was charged into the container until the pressure reached 1.0 MPa.

Cell Viability Assay
In all experiments, 2×10^4 cells were seeded into 96-well plates. Then, the cells were treated with Ang-2 (100 ng/ml) or PBS. In another experiment, the stimulation condition consisted of Ang-2 (100 ng/ml) with PBS, Ang-2 (100 ng/ml) with Ang-1 (150 ng/ml), or Ang-2 (100 ng/ml) with the cells pre-plated on FN (5 μg/ml)-coated wells for 24 h. After treatment for 30 min, the cells were exposed to 1 MPa pressure for 0, 12, 24, 36, or 48 h. Cell viability was monitored using the 2-(4,5-dimethyltriazol-2-yl)-2, 5-diphenyl

tetrazolium bromide (MTT, Sigma) colorimetric assay. Three independent experiments were performed for each experimental condition. Recombinant human Ang-1 and Ang-2 were purchased from R&D Systems (Minneapolis, MN, USA).

Apoptosis Assay

Initially, 2×10^6 cells were seeded into 6-well plates. Then, the cells were treated with Ang-2 (100 ng/ml) or PBS. In another experiment, the stimulation conditions consisted of Ang-2 (100 ng/ml) with PBS, Ang-2 (100 ng/ml) with Ang-1 (150 ng/ml), or Ang-2 (100 ng/ml) with the cells pre-plated on FN (5 µg/ml)-coated wells for 24 h or Ang-2 (100 ng/ml) with 740Y-P (500 µg/ml). After treatment for 30 min, all samples were exposed to 1 MPa pressure for 0, 12, 24, 36, or 48 h. Then, the cells were harvested by trypsinization and centrifugation. After two washes in PBS, the resuspended cells were stained with propidium iodide and fluorescein isothiocyanate-conjugated Annexin-V (Beckman Coulter), and incubated in the dark for 15 min at room temperature. Subsequently, each sample was analyzed using a FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). The cells were counted and represented as a percentage of the total cell population using FCS express V2.0 software (De Novo Software, Los Angeles, CA, USA).

Cell Adhesion Assay

The wells were washed twice with PBS and coated with human plasma FN (5 µg/ml) or FN (20 µg/ml) diluted in PBS, with overnight incubation at 4 °C. After coating, the wells were blocked in PBS plus 3% BSA. The cells were exposed to 1 MPa pressure for 0, 12, 24, 36, or 48 h. Then the cells were treated with Ang-2 (100 ng/ml) and PBS control. In another experiment, groups contained Ang-2 (100 ng/ml) with PBS; Ang-2 (100 ng/ml) with Ang-1 (150 ng/ml); Ang-2 (100 ng/ml) with the cells adhesion on FN (20 µg/ml)-coated wells. After drug treatments for 30 min, the cells were harvested and plated at 20 000 cells per well and allowed to adhere (37 °C; 5% CO_2; 1 h; 5 µg/ml FN). In Ang-2 (100 ng/ml) with the cells adhesion on FN (20 µg/ml)-coated wells group, cells finally adhered on FN (20 µg/ml)-coated wells. Non-attached cells were removed by vigorous agitation and aspiration. Attached cells were fixed and stained with crystal violet (0.2% in H_2O). Cell adhesion was quantified by counting the total number of cells per image normalized to that in controls for each treatment.

Immunoprecipitation

Initially, 2×10^6 cells were seeded into 6-well plates and allowed to reach 100% confluence overnight. The cells were stimulated with Ang-2 (100 ng/ml) and then plated on FN (5 µg/ml), with Ang-1 (150 ng/ml) and then with Ang-2 (100 ng/ml); plated on FN (5 µg/ml) in the presence of varying concentrations of Ang-2; or treated with Ang-1 (150 ng/ml) in the presence of varying concentrations of Ang-2. After treatments for 30 min, the cells were washed

with ice-cold PBS and lysed in lysis buffer containing protease and phosphatase inhibitors. The cell debris was cleared by centrifugation, and the lysates were immunoprecipitated with anti-$\alpha5\beta1$ antibodies (2 µg) for 2 h at 4 °C. In another experiment, lysates were immunoprecipitated with anti-FAK antibodies (1 µg). The antibodies were collected on protein A/G beads (Santa Cruz Biotechnology) for 2 h at 4 °C. The beads were washed with ice-cold lysis buffer and resuspended in SDS sample buffer for blotting analysis specific for Tie2 (ab24859), $\alpha5\beta1$ (ab75472), anti-phosphotyrosine (ab8076) (Abcam), FAK (sc-271195), and p85 (sc-71891) (Santa Cruz Biotechnology).

Western Blotting

Whole cells from each culture dish were collected and lysed in RIPA buffer containing buffer protease and phosphatase inhibitors. Cells were lysed using membrane protein and cytoplasmic protein extraction kit (Beyotime, Nantong, China). Protein lysates were resolved by SDS–PAGE and transferred onto polyvinylidene fluoride membranes (Amersham Biosciences, USA). The membranes were incubated with primary antibodies (1:1000) at 4 °C overnight and secondary antibodies (1:5000) at room temperature for 1 h. The primary antibodies used were as follows: $\alpha5\beta1$ (ab75472) (Abcam), Na-K-ATPase (ab185210), anti-phosphotyrosine (ab8076), phospho-Akt (Ser-473, ab8932), phospho-Akt (T308, ab38449), Akt (ab8805), and tubulin (ab6160) (Abcam); beta actin (sc-47778) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-47724, Santa Cruz Biotechnology). β-actin, tubulin, Na-K-ATPase, and GAPDH were used as loading controls. Immunoreactive bands were detected using a DAB kit (Zhongshan Goldenbridge, Beijing, China). The protein signals were quantified by scanning densitometry using a FluorChem Q System (Alpha Innotech, CA, USA).

Immunostaining

Initially, 2×10^6 cells were seeded into 6-well plates and allowed to attach 100% confluence overnight. The cells were exposed to 1 MPa pressure for 0, 12, 24, 36, or 48 h. Subsequently, the cells were mounted on coverslips, washed with PBS, fixed with 4% paraformaldehyde for 15 min at 37 °C, permeabilized with 0.5% (v/v) Triton X-100 for 20 min, and blocked with 1% (w/v) goat serum albumin for 30 min. The samples were then washed, probed at 4 °C overnight with anti-Ang-1 (1:200) or anti-Ang-2 (1:200), and labeled for 40 min with a secondary antibody (1:2000). For DNA counterstaining, the samples were incubated in medium containing 4′-6-diamidino-2-phenylindole (DAPI) after washing with PBS. Images were captured using a microscope (Olympus IX71). Tie2 immunostaining was as described above without permeabilization with Triton X-100. Protein expression was quantified by integrated optical density using an Image-Pro Plus image analysis system (Media Cybernetics, Rockville, MD, USA).

ELISA

The supernatants were collected. The supernatant levels of Ang-1 and Ang-2 were measured using quantitative ELISA kit (R&D Systems) according to the manufacturer's instructions. Each sample was tested in duplicate. The results were expressed in pg/ml.

Real-time PCR

NP tissues were treated by liquid nitrogen grinding. Total RNA was isolated from NP tissues and cultured cells using TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA using a Transcriptor First Strand cDNA Synthesis Kit (TAKARA Biotechnology, Otsu, Japan). β-actin was used as an endogenous control. Real-time PCR was performed using SYBR Green Kit Master Mix (Applied Biosystems, Foster City, CA, USA), and the products were analyzed using an ABI 7500 Sequencing Detection System in accordance with the manufacturers' instructions. The primers used for PCR were: Ang-2 (F: 5′-ACTGTGTCCTCTTCCACCAC-3′, R: 5′-GGATGTTTAGGGTCTTGCTTT-3′); Ang-1 (F: 5′-CTCCGTAAGGGCTTCCAT-3′, R: 5′-CTGCGTCCTCTGTTGTCG-3′); Homo β-actin (F: 5′-AGCGAGCATCCCCCAAAGTT-3′, R: 5′-GGGCACGAAGGCTCATCATT-3′).

Statistical Analysis

All results are representative of three independent experiments consisting of three replicates per experiment and expressed as the mean ± s.e.m. Comparisons between groups were conducted by Student's unpaired *t*-test, one-way and two-way ANOVA. Correlations were analyzed using the Spearman correlation coefficient. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

The Ratio of Ang-2/Ang-1 in IDD Tissues Increases with Ageing and Degeneration

In NP tissues of the various extent of degeneration, we detected Ang-1 and Ang-2 expression by immunohistochemistry (Figure 1a). In order to elucidate the role of Ang-1 and Ang-2 in IDD, each NP tissues from surgical specimens with different grades of disc degeneration were analyzed three times by western blotting and RT-PCR (grade I, $n = 6$; grade II, $n = 8$; grade III, $n = 8$; grade IV, $n = 13$). Signal intensity of individual bands was quantified. Figure 1b shows a set of representative human NP protein extracts loaded in lanes representing surgical samples from patients with varying degrees of degeneration (14 NPs with Pfirrmann grades I–IV, the first normal sample as control). No significant differences in the protein levels of Ang-1 were observed with degree of degeneration (Figure 1c). Grade III IVD was found to be associated with a higher level of Ang-2 than grade II IVD ($P = 0.002$), and the positive bands for Ang-2 were strongest in samples from the patient with grade IV IVD (Figure 1d). The results coincided in transcription level (Figures 1e and f). Since Ang-1 and Ang-2 are antagonistic ligands, the Ang-2/Ang-1 ratio reflects the imbalance between them. The

increases in Ang-2/Ang-1 ratio correlated with the extent of disc degeneration (Figures 1g and h) in both protein and mRNA level. The ratio of Ang-2/Ang-1 in NP tissues was also found to increase with donor age (Supplementary Figure S1). These data strongly suggest that imbalance between Ang-1 and Ang-2 disorganized along with the degeneration.

Compression Mediates an Increase in the Expression of Ang-2 in NP Cells

Mechanical loading results in alteration of the ECM via exposure of NP cells to mechanical stress.[22] Numerous studies have reported that persistent mechanical overloading and exposure to compression and torsion induces IDD.[23–25] In the present study, cells were divided into five groups as follows: control group (0 h) and four pressure groups, in which cells were exposed to 1.0 MPa pressure for 12, 24, 36, and 48 h, and the expression of Ang-2 and Ang-1 was investigated. Results showed that compression promoted protein expression of Ang-2 in a time-dependent manner, but did not affect Ang-1 expression markedly (Figures 2a and b). The Ang-2/Ang-1 ratio increased with the duration of exposure to mechanical stress at the mRNA level (Figure 2c). As Ang-1 and Ang-2 are secreted proteins, their concentration in the supernatant of NP cell cultures was detected by enzyme-linked immunosorbent assay (ELISA). These results were consistent with the observed mRNA and protein expression levels of Ang-2 and Ang-1 (Figure 2d). In addition, an increase in the protein levels of Ang-2 was detected by immunofluorescence; however, no obvious change in Ang-1 protein levels occurred in response to compression in NP cells (Figure 2e).

Effect of Ang-2 on NP Cells

FN is thought to initiate matrix assembly by forming large aggregates in the matrix, resulting in the generation of a fibrillar meshwork[26] that provides binding sites for several cell membrane and matrix proteins involved in transmitting important information to NP cells.[15,27] We therefore examined the ability of Ang-2 to interfere with the adhesion of NP cells to FN. The cells were treated with 1.0 MPa pressure for 0, 12, 24, 36, or 48 h. Ang-2-mediated stimulation of NP cells inhibited binding to FN (Figure 3a). The differences between the two groups were significant when compression was applied for 24, 36, and 48 h. On addition of Ang-1 to the culture medium or adhesion to higher concentrations of FN-coated plates, the Ang-2-induced loss of adhesion of NP cells to FN was partially restored (Figure 3b). In addition, we investigated the possible role of Ang-2 in the degeneration of NP tissue by assessing cell viability using the MTT assay in NP cells subjected to mechanical stress. In the presence of Ang-2, cell viability declined more sharply on exposure to stress compared with the control group. The differences between the two groups were statistically significant after pressure was applied for 24, 36, and 48 h (Figure 3c). However, this decline was partially rescued by Ang-1 and FN (Figure 3d). Following treatment
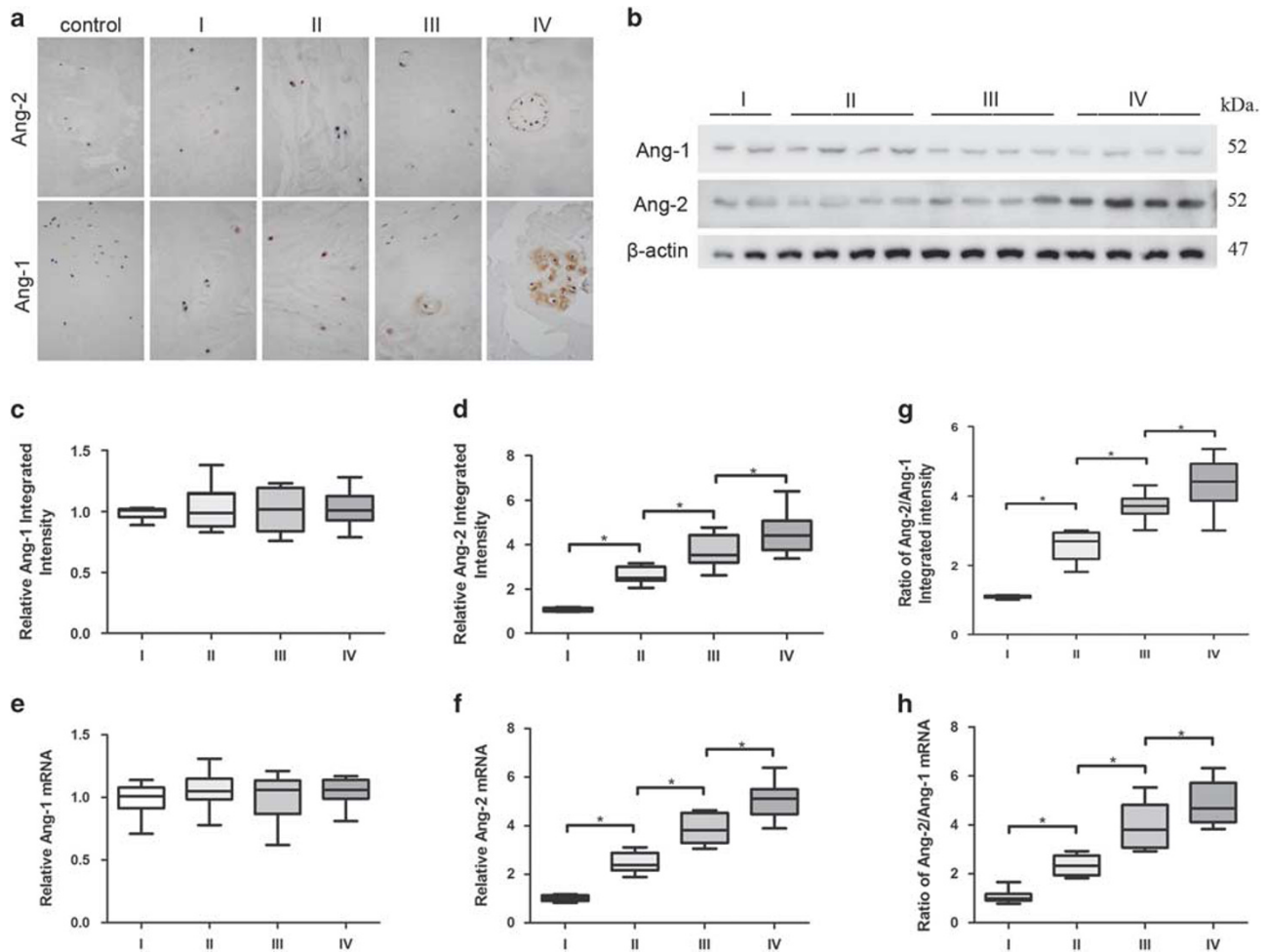
Figure 1 Ratio of Ang-2/Ang-1 is elevated in NP tissue. (**a**) Immunohistochemistry of collected NP samples in different extents of degeneration. (**b**) Western blot of collected NP samples using the Ang-1 and Ang-2 antibody. (**c**, **d**) Quantitative western blot analysis of Ang-1 and Ang-2 expression in NP samples. (**e**, **f**) Real-time PCR analysis of Ang-1 and Ang-2 expression in NP samples. (**g**, **h**) Correlation of Ang-2/Ang-1 ratio in NP tissues with intervertebral disc degeneration. *$P < 0.05$. Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; NP, nucleus pulposus.

with Ang-2, an increase in compression-induced apoptosis of NP cells was observed using flow cytometry (Figures 3e and f). As shown in Figures 3g and h, Ang-1 and FN exerted protective effects against apoptosis in NP cells under mechanical stress. PI3K-activating peptide (740Y-P) binds to the SH2 domain of the p85 regulatory subunit of PI3K to stimulate enzyme activity.[28] 740Y-P was found to block the Ang-2-induced apoptosis of NP cells, indicating that PI3K plays a role in Ang-2-induced outside-in signaling (Figures 3g and h). Our findings indicate that Ang-2 plays a role in suppressing cell adhesion and viability, and promotes the apoptosis of NP cells.

Ang-2 Antagonizes the FN- and Ang-1-Induced Formation of the Tie2/α5β1 Complex in NP Cells

In order to characterize the molecular signaling mechanisms underlying the apoptosis of NP cells, we investigated expression of Tie2 receptors in cultured normal NP cells initially.

Immunofluorescence studies detected Tie2 receptor in the cytomembrane (Supplementary Figure S2). Then the regulation of Tie2/α5β1 interactions in these cells was investigated. When NP cells were stimulated by Ang-1 or plated on FN, the amount of Tie2 co-immunoprecipitated with α5β1 integrin was remarkably higher than that when cells were grown in culture medium. This effect was partially abrogated by Ang-2 (Figures 4a and b). The ability of Ang-2 to antagonize the interaction enhanced by either FN or Ang-1 was dose-dependent (Figures 4c and d). The observed inhibitory phenotype of Ang-2 led us to examine the effect of Ang-2 on the induction of α5β1; our findings revealed that the expression of α5β1 is not affected by the presence or absence of Ang-2 (Figure 4e). However, translocation of α5β1 was detected under Ang-2 stimulation, and membrane α5β1 was found to accumulate in the cytoplasm (Figure 4f). To further confirm this finding, α5β1 translocation was assessed by western blot analysis using membrane and cytosolic extracts (Figure 4g).
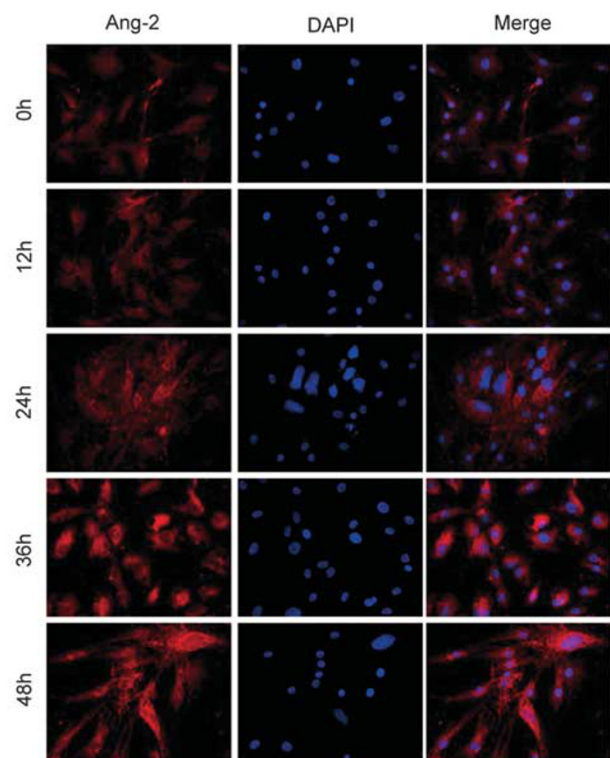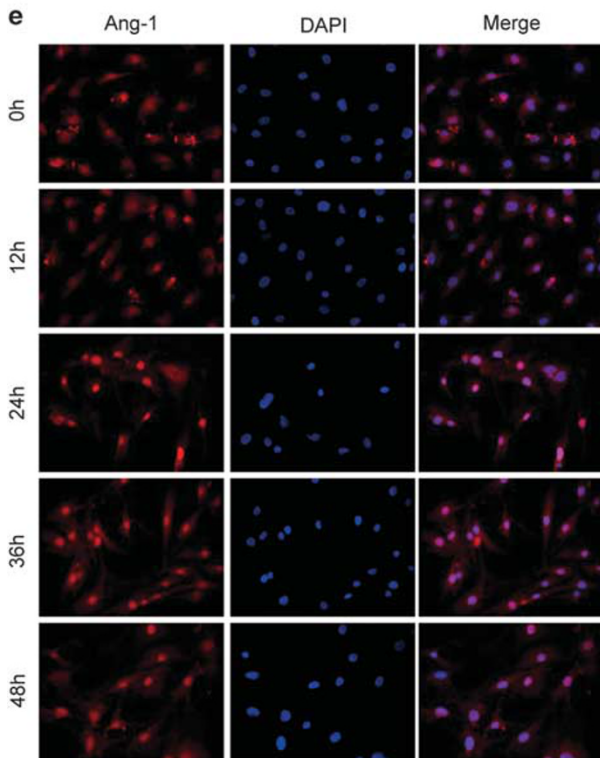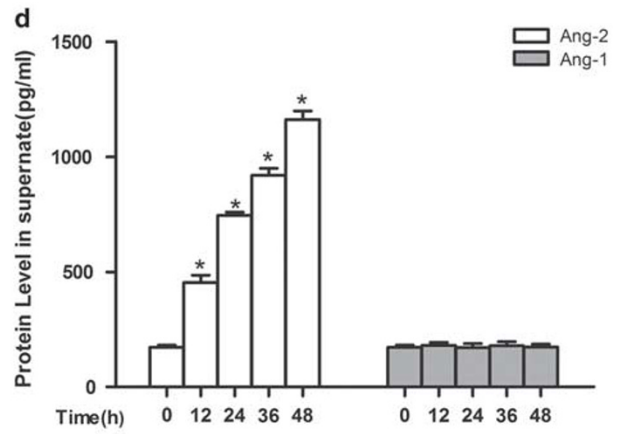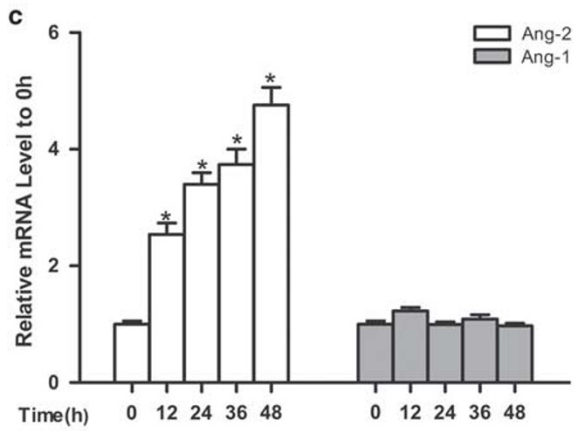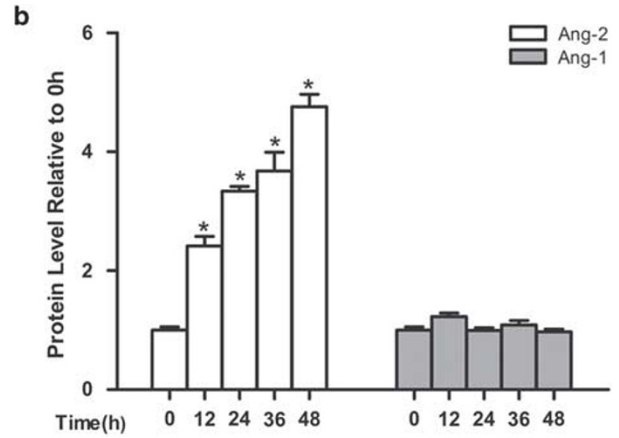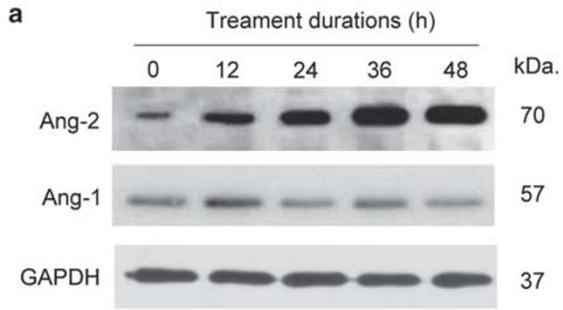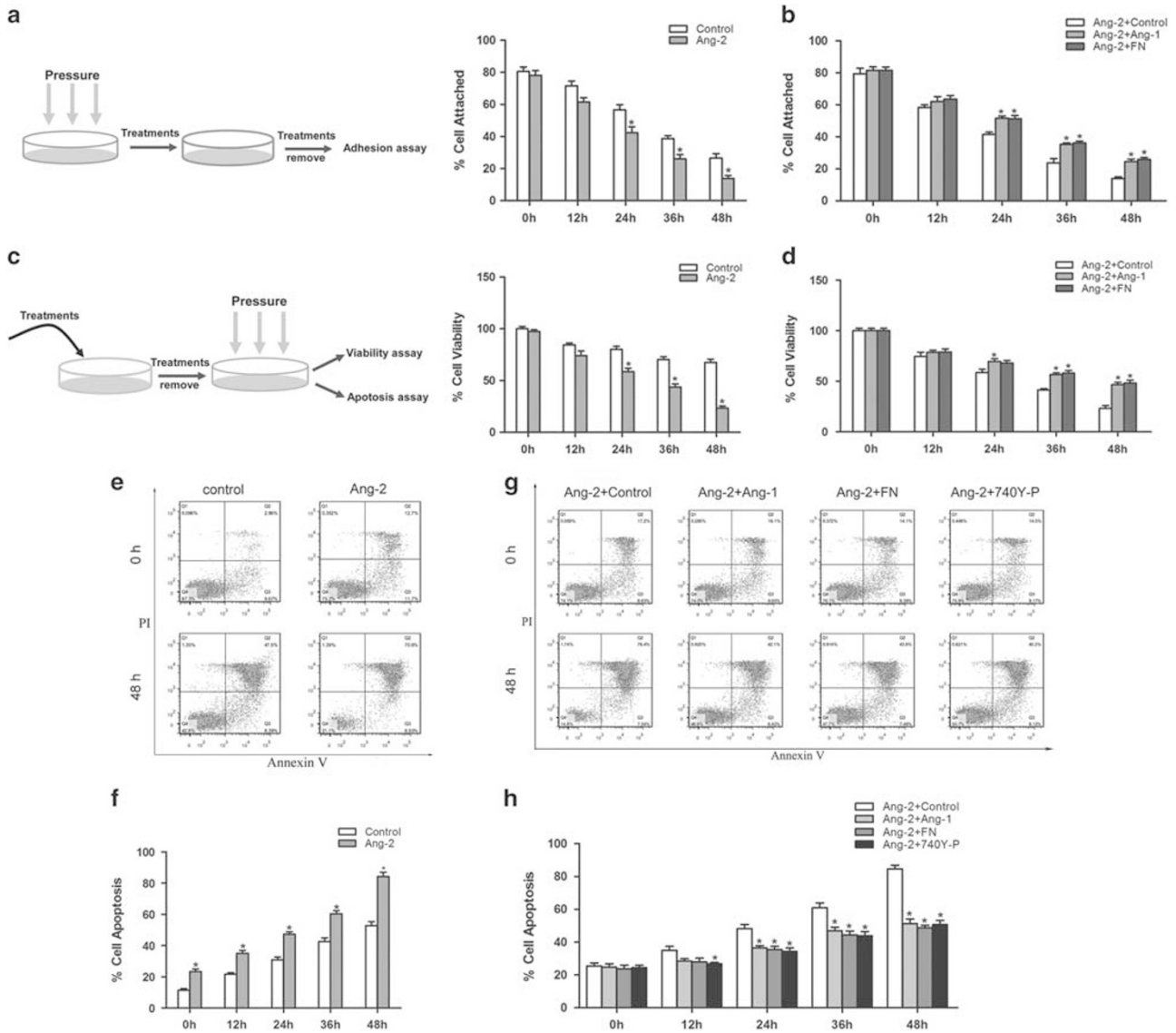
Figure 3 Function of Ang-2 in human NP cells. (**a**) A schematic of the treatment protocols for cell attachment assays. The adhesion of cells to FN (5 μg/ml) stimulated by Ang-2 (100 ng/ml) and control. (**b**) The adhesion of cells to FN (5 μg/ml) stimulated by Ang-2 (100 ng/ml) with Ang-1 (150 ng/ml); Ang-2 (100 ng/ml) with control and to FN (20 μg/ml) stimulated by Ang-2 (100 ng/ml). (**c**) A schematic of the treatment protocols for cell viability and apoptosis assays. The cells were stimulated by Ang-2 (100 ng/ml) and control to measure viability by MTT assay. (**d**) The cells were stimulated by Ang-2 (100 ng/ml) with Ang-1 (150 ng/ml); Ang-2 (100 ng/ml) with plated on FN (5 μg/ml); Ang-2 (100 ng/ml) with control to measure viability by MTT assay. (**e**) Representative flow cytometry data of 0 and 48 h cell apoptosis in two groups. (**f**) The cells were stimulated by Ang-2 (100 ng/ml) and control to analyze apoptosis by flow cytometry. (**g**) Representative flow cytometry data of 0 and 48 h cell apoptosis in different groups. (**h**) The cells were stimulated by Ang-2 (100 ng/ml) with Ang-1 (150 ng/ml); Ang-2 (100 ng/ml) with plated on FN (5 μg/ml); Ang-2 (100 ng/ml) with control to analyze apoptosis by flow cytometry. $n = 3$, $*P < 0.05$ *vs* control. Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; FN, fibronectin; NP, nucleus pulposus.

Figure 2 Effects of compression on mRNA and protein expression of Ang-1 and Ang-2. (**a**) The protein content of Ang-1 and Ang-2 detected in human NP cells subjected to compression at 1.0 MPa for various durations. (**b**) Densitometric analysis shows the relative amount of Ang-1 and Ang-2 detected in human NP cells. (**c**) The mRNA levels of Ang-1 and Ang-2 detected by RT-PCR in human NP cells subjected to compression at 1.0 MPa for various durations. (**d**) The levels of Ang-1 and Ang-2 in supernatant detected by ELISA in human NP cells subjected to compression at 1.0 MPa for various durations. (**e**) The fluorescent staining of Ang-1 and Ang-2 in human NP cells subjected to compression at 1.0 MPa for various durations. $n = 3$, $*P < 0.05$ *vs* 0 h group. Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; NP, nucleus pulposus; RT-PCR, real-time PCR.
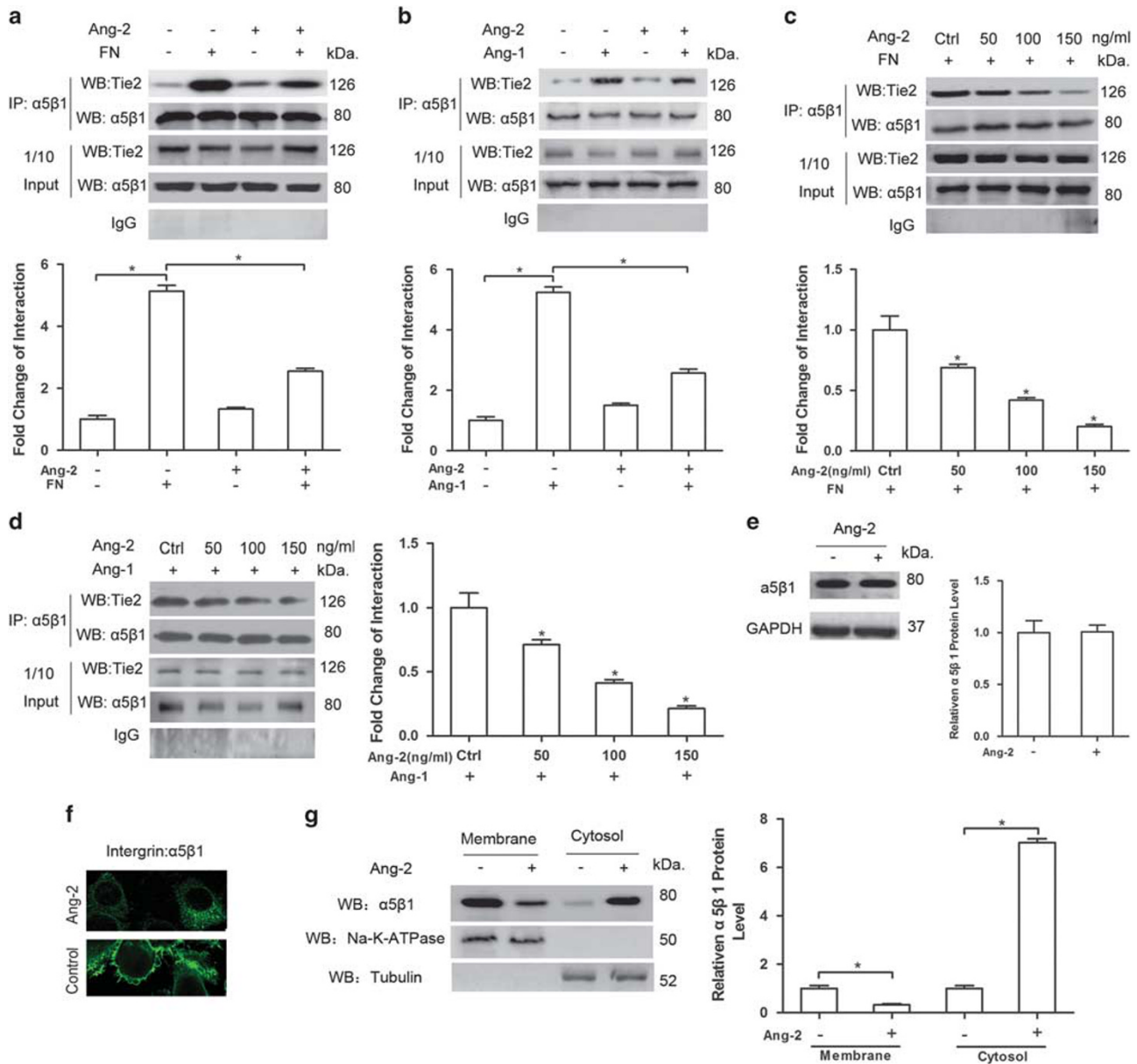
Figure 4 Ang-2 suppresses Tie2/α5β1 complex formation. (**a**) Immunoprecipitation assays using anti-α5β1 antibody were performed on lysates of NP cells that were cultured on FN-coated or uncoated plates and treated with or without Ang-2 (100 ng/ml). Densitometric analysis shows the relative amounts of Tie2 in α5β1 immunoprecipitates. *n* = 3, **P < 0.05. (**b**) Immunoprecipitation assays using anti-α5β1 antibody were performed on lysates of NP cells that were cultured on Ang-1-contained or uncontained plates and treated with or without Ang-2 (100 ng/ml). Densitometric analysis shows the relative amounts of Tie2 in α5β1 immunoprecipitates. *n* = 3, **P < 0.05. (**c**) NP cells were plated on FN (5 μg/ml) in the presence of varying concentrations of Ang-2 and then lysed for immunoprecipitation by anti-α5β1. Densitometric analysis shows the relative amounts of Tie2 in α5β1 immunoprecipitates. *n* = 3, **P < 0.05 *vs* Ctrl. (**d**) NP cells were cultured with Ang-1 (150 ng/ml) in the presence of varying concentrations of Ang-2 and then lysed for immunoprecipitation by anti-α5β1. Densitometric analysis shows the relative amounts of Tie2 in α5β1 immunoprecipitates. *n* = 3, **P < 0.05 *vs* Ctrl. (**e**) Representative western blotting images and quantification data displaying the levels of integrin α5β1 after Ang-2 stimulation. *n* = 3, **P < 0.05. (**f**) α5β1 translocation following Ang-2 stimulation was investigated by immunofluorescence. (**g**) The expression of α5β1 was assessed by western blotting and quantified using membranes and cytosolic extracts. *n* = 3, **P < 0.05. Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; FN, fibronectin; NP, nucleus pulposus.

Ang-2 Suppresses the Recruitment of FAK and p85 in NP Cells

Previous reports have suggested that PI3K plays a role in Ang-2-induced apoptosis (Figure 3f); recruitment of the α5β1 adaptor protein FAK results in p85 phosphorylation, thereby activating Tie2 to induce PI3K signaling.[29] To further examine the signaling activity resulting from the interaction between Tie2 and α5β1, we assessed the recruitment of FAK and the p85 subunit of PI3K to the complex. Compared to FN or Ang-1 stimulation alone, Ang-2 administration reduced
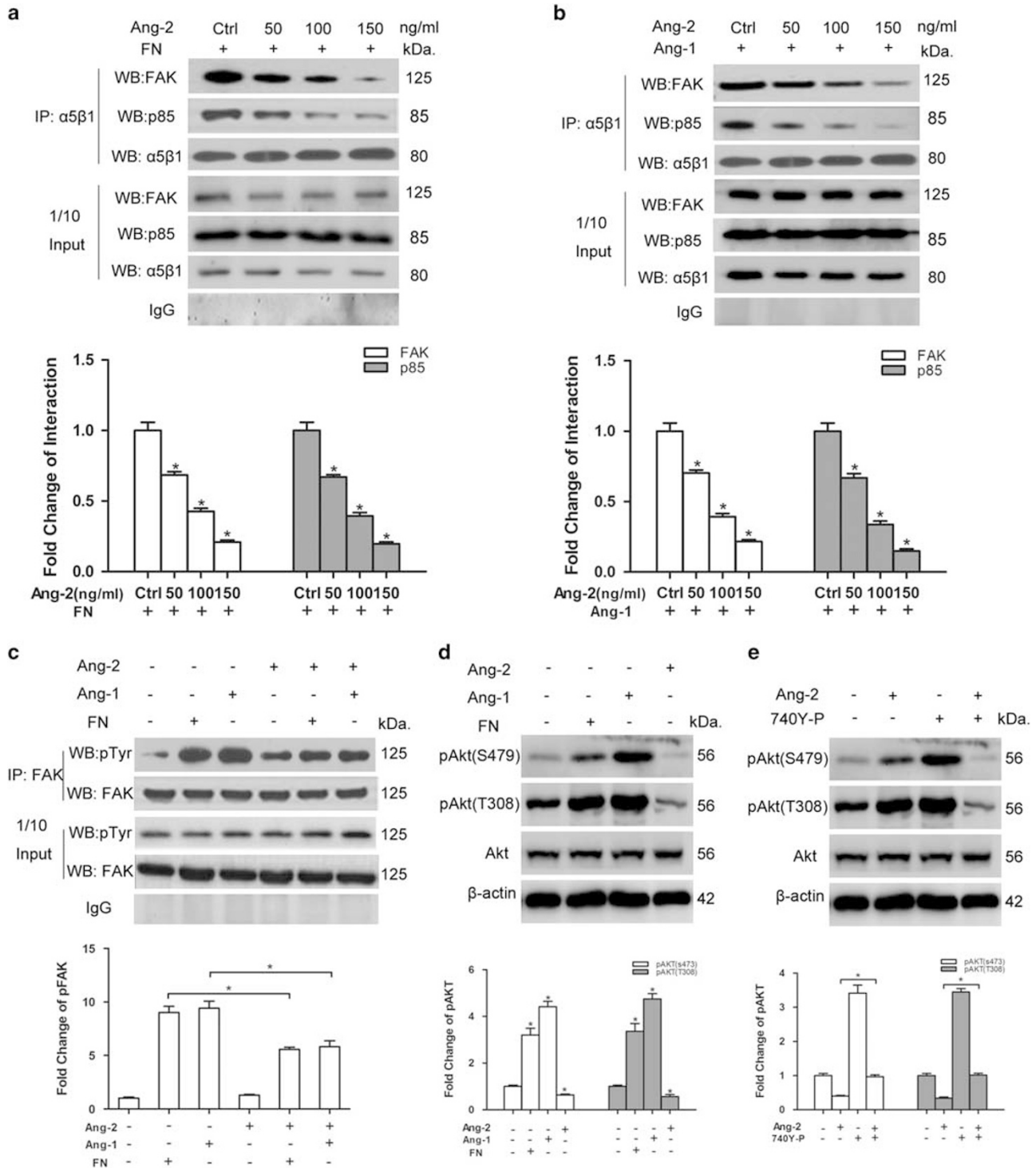
Figure 5 Ang-2 suppresses the recruitment of FAK and p85. (**a**, **b**) NP cells were cultured on FN (5 µg/ml) or with Ang-1 (150 ng/ml) in the presence of indicated concentrations of Ang-2 and lysed for immunoprecipitation, followed by immunoblotting and quantification for FAK and p85. $n = 3$, $*P < 0.05$ vs control. (**c**) pFAK was immunoblotted using anti-phosphotyrosine after immunoprecipitation with anti-FAK. The fold increase in FAK tyrosine phosphorylation was determined by densitometric analysis. (**d**) pAKT was immunoblotted and quantified to display the relative phosphotyrosine level of AKT in NP cells exposed to Ang-2, Ang-1, and FN, respectively. (**e**) pAKT was immunoblotted and quantified to display the relative phosphotyrosine level of AKT in NP cells exposed to Ang-2 and 740Y-P, respectively. $n = 3$, $*P < 0.05$. Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; FN, fibronectin; NP, nucleus pulposus.
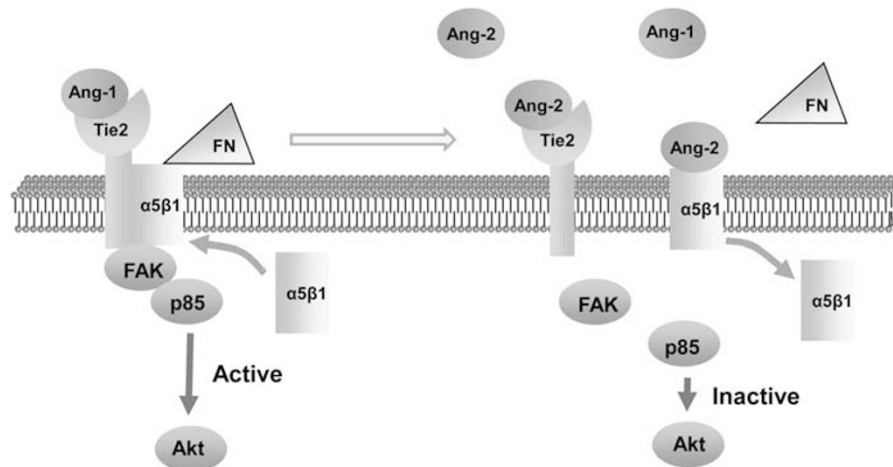
Figure 6 Schematic representation of Ang-2-regulated Tie2 tyrosine kinase receptor and $\alpha5\beta1$ integrin signaling in NP cells. Ang-1 acts as a ligand to activate Tie2 receptor. Integrin $\alpha5\beta1$ is assembled to Tie2 after FN stimulation and recruits FAK and p85, which further activate Akt signaling. When Ang-2 increases, Ang-2 interferes with Ang-1-induced Tie2 activation and Ang-2–$\alpha5\beta1$ interaction may induce $\alpha5\beta1$ to translocation, leading to disassemble the complex of Tie2 and $\alpha5\beta1$. The subsequent decreased recruitment of FAK and p85 ultimately brings down Akt activity. Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; FN, fibronectin; NP, nucleus pulposus.

the recruitment of FAK and p85 to $\alpha5\beta1$ in a dose-dependent manner after $\alpha5\beta1$ immunoprecipitation (Figures 5a and b). Stimulation of NP cells by Ang-2 not only reduced the association of Tie2 with $\alpha5\beta1$, but also decreased the recruitment of FAK and p85 to the complex. The FN- and Ang-1-stimulated phosphorylation of FAK was reduced by Ang-2 (Figure 5c). Next, we examined the phosphorylation of AKT; pAKT levels increased in the presence of FN or Ang-1, whereas treatment with Ang-2 reduced pAKT levels (Figure 5d). 740Y-P reduced the inhibition of the Ang-2-stimulated PI3K/AKT axis (Figure 5e). Taken together, these data indicate that Ang-2 suppresses the phosphorylation and function of the integrin adaptor protein FAK, as well as that of p85 to exert inhibitory effect via PI3K/AKT signaling in NP cells.

DISCUSSION

Ang/Tie signaling, which was initially found to occur in the endothelium-specific tyrosine kinase system, plays an important role in multiple cells and tissue systems. Ang-1 and Ang-2 are expressed at sites of endochondral bone formation in the growing skeleton.[30] Mesenchymal stem cells, which express Ang-1 and high levels of Ang-2, exhibit a strong ability to elicit remodeling of preexisting vasculature at the injured site and support new vessel formation.[31] Moreover, accumulating evidence shows that expression of Ang-2 by tumor cells is linked with invasive and metastatic phenotypes of gastric, colon, brain, prostate, and breast cancers.[32] To our knowledge, the present study is the first to report that Ang-2 is expressed by NP cells, and that the Ang-2/Ang-1 ratio in these cells increases in IDD. We showed that the acceleration of pressure-induced degeneration in NP cells, which is

exasperated by induction of Ang-2, decreased NP cell viability, adhesion, and promoted apoptosis.

The factors contributing to the strong correlation between the ratio of Ang-2/Ang-1 and degree of IDD were explored. The molecular phenotype of NP cells derived from normal discs was examined, and the expression of the Tie2 receptor in the cytomembrane was confirmed. Integrins participate in mechanotransduction in human NP cells by acting as mechanoreceptors that detect mechanical stimulation by dynamic loading.[33] Although Tie2 is a Ang-2 receptor, it is capable of forming a complex with integrins. Numerous studies have suggested that Ang-2 exhibits context-dependent functions, eg, It acts as a vessel-destabilizing molecule in Tie2-expressing ECs and as a directly pro-angiogenic molecule in Tie2-negative angiogenic ECs via integrin signaling.[34] Ang-2 interacts directly with $\alpha5\beta1$ integrins and activates down-stream effectors to promote tumor migration.[35] When acting alone, Ang-2 does not induce apoptosis; however, at high concentrations of glucose, such as in diabetic retinopathy, it induces apoptosis in pericytes via $\alpha3\beta1$ integrin signaling.[36] As seen in the ELISA experiments (Figure 2d), the physiological levels of Ang-2 may be at the 1 ng range, which contribute to NP cells survival. In the experiments, concentrations of Ang-2 were well above physiological (100 ng/ml) and these higher levels cause apoptosis as well as reduction of cell viability and adhesion. Our findings are consistent with those of previous studies. Following the activation of Tie2 signaling by Ang-1, $\alpha5\beta1$ binds to the complex; this interaction is enhanced by FN. Ang-2 weakens this Ang-1- and FN-induced interaction. Interestingly, the interaction between Tie2 and $\alpha5\beta1$ is mildly enhanced in the presence of Ang-2 alone. The basis for the discrepancy between these conditions is unclear but potentially could result from

differences in the method of Ang-2 delivery and require further investigation. Collectively, the data establish a contextual model according to which molecular interactions are differentially regulated by Ang-2 in response to specific conditions. Ang-2 and Ang-1 exhibited similarly high binding affinity for Tie2.[37] Ang-1 induces rapid autophosphorylation of Tie2 receptors to activate the downstream signaling pathway. Ang-2, which is 60% identical to Ang-1, binds to Tie2 without inducing phosphorylation.[38] When the Ang-2/Ang-1 ratio increases, Ang-2 acts as a competitive blocking antagonist via Tie2 receptor tyrosine kinase. The enhancement of the Tie2/$\alpha5\beta1$ signal by the combination of Ang-1 and fibronectin was inhibited by the Ang-2-induced Tie2 phosphorylation decrease.

It has been reported that the binding of FN to integrin $\alpha5\beta1$ results in elevated expression of the Bcl-2 protein, which is known to counteract apoptosis. Conversely, abrogation of FN-integrin $\alpha5\beta1$ interactions has been shown to induce apoptosis.[39] In this study, FN was found to exert positive effects on NP cells. Furthermore, we demonstrated that FN partly offsets, but not completely reverses, the negative effects of Ang-2 on NP cells; unexpectedly, we found that $\alpha5\beta1$ translocates from the membrane to the cytoplasm and that Ang-2 does not affect the total expression of $\alpha5\beta1$. The review by Huang *et al*[5] shows that Ang-2 binds to $\alpha5\beta1$ directly. The case, binding of Ang-2 to $\alpha5\beta1$, may cause the complex to internalize that conform to our findings (Figure 6). Some studies have indicated that the levels of FN are elevated in degenerate discs, and this protein is frequently observed as fragments. Elevated levels of FN reflect the response of disc cells to the altered environment. FN fragments result from normal or enhanced proteolytic activity, which may induce further degradation of the matrix.[27] Other findings support a pathological role for FN fragments in disc degeneration in the rabbit.[40,41] When FN is cleaved into fragments, FN–integrin $\alpha5\beta1$ interactions may be diluted leading to enhanced apoptosis.

Recent studies have shown that rat NP cells adapt for survival by the regulation of expression of critical genes, eg, the activation of the PI3K/Akt and MAPK survival pathways results in decreased apoptosis, as inhibition of the PI3K/Akt pathway impairs cell survival.[42] PI3K/Akt signaling mediated aggrecan gene expression in NP cells, suggesting it function as a positive pathway in the IVD.[43] FAK, a cytoplasmic tyrosine kinase involved in mechanotransduction, has been shown to regulate cell migration, proliferation, survival, and metabolism.[44] The recognition of an extracellular matrix ligand by an integrin typically results in the clustering of the integrin in the cell membrane and activation of FAK, with subsequent regulation of focal adhesion dynamics and cell movement via the formation of focal adhesions at the submembranous region of the cell.[45] In NP cells, FAK is activated by Ang-1 and FN to significantly promote cell adhesion. Furthermore, pFAK activates signaling pathways of mechanotransduction in NP cells, creating a potential binding site for the p85 receptor subunit of PI3K. It then activates the p110 catalytic subunit of PI3K, which leads to the phosphorylation of Akt, enhancing the viability of NP cells and reducing apoptosis. Ang-2 acts as a negative regulator in this process (Figure 6).

In summary, our data show that changes in the ratio of angiogenic factors Ang-2 and Ang-1 elicit NP cell apoptosis, likely initiating degeneration cascades. To our knowledge, our results demonstrate for the first time that NP cells express Ang-2 in an autocrine manner and respond to Ang/Tie2 signaling. Integrins respond to extracellular cues by modifying the avidity for Tie2 and $\alpha5\beta1$ signaling, enabling cross-talk between these pathways by acting at the level of the Tie2/$\alpha5\beta1$ complex. The strong correlation between the Ang-2/Ang-1 ratio and the degree of degeneration in IDD, observed in the present study, offers insights for IDD pathogenesis and indicates that blockade of Ang-2 function could be a potential therapeutic target to prevent NP cell loss in IVD.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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