Cyclic Hydrostatic Compress Force Regulates Apoptosis of Meniscus Fibrochondrocytes via Integrin α5β1

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Summary

Meniscus is a semilunar fibrocartilaginous tissue, serving important roles in load buffering, stability, lubrication, proprioception, and nutrition of the knee joint. The degeneration and damage of meniscus has been proved to be a risk factor of knee osteoarthritis. Mechanical stimuli is a critical factor of the development, maintenance and repair of the meniscus fibrochondrocytes. However, the mechanism of the mechano-transduction process remains elusive. Here we reported that cyclic hydrostatic compress force (CHCF) treatment promotes proliferation and inhibits apoptosis of the isolated primary meniscus fibrochondrocytes (PMFs), via upregulating the expression level of integrin α5β1. Consequently, increased phosphorylated-ERK1/2 and phosphorylated-PI3K, and decreased caspase-3 were detected. These effects of CHCF treatment can be abolished by integrin α5β1 inhibitor or specific siRNA transfection. These data indicate that CHCF regulates apoptosis of PMFs via integrin α5β1-FAK-PI3K/ERK pathway, which may be an important candidate approach during meniscus degeneration.

Key words: Integrin α5β1; Meniscus; Cyclic hydrostatic compress force

Introduction

Menisci are semilunar fibrocartilaginous tissues, which buffer load on the knee joint, including load transmission and shock absorption (Walker and Erkman 1975). They also serve important roles in knee joint stability, lubrication, proprioception, and nutrition of the articular cartilage (Fox *et al.* 2015). In 2015, it was estimated that there are approximately 1.7 million surgeries performed for menisci injuries worldwide and this number is rising rapidly (Aaron and Michael 2015). There are different injury patterns between different populations (Tandogan *et al.* 2004): acute tears due to trauma are predominantly found in young people, while degenerative tears are found mainly in older people (Englund *et al.* 2009) associated with aging (Aufderheide and Athanasiou 2004). The majority of aging-related meniscus tears are unsuitable for repair treatments (Rai *et al.* 2013), which often require partial or complete removal of the meniscus, namely meniscectomy. However, although meniscectomy helps to relieve pain and improve function, it does not protect against the development of osteoarthritis (Hall *et al.* 2014). It has been proved that by removing the meniscus, the average stress of the knee can be increased by 3 folds, with an even greater magnitude of the peak stress (Krause *et al.* 1976). Particularly, the aging-related meniscal injury has been reported to be risk factors of knee osteoarthritis (Englund *et al.* 2012). Thus it is critical to understand the initial stage of the meniscal degeneration.

The meniscus cells are described as fibrochondrocytes (Makris *et al.* 2011). In nature, they experience a combination of dynamic and static stress, including compressive, shear and tensile forces (Abdelgaied *et al.* 2015). Sufficient mechanical stimuli plays a critical role in maintaining the development, growth and functions of meniscus cells (McNulty and Guilak 2015). However, the transduction of mechanical signals into biology changes in these cells remains to be elusive.

Recent researches have begun to elucidate the role of integrin during this process. integrin is a family of cell-surface molecules, responsible for extracellular-intracellular

signaling transduction. Each integrin molecule is a heterodimer of α and β subunits. There have been 18 α and 8 β subunits identified in mammals (Humphries 2000), which are expressed in a tissue-specific manner in humans and mammals. Multiple integrins had been proved to be playing a role during mechano-transduction and sensing microenvironment in cartilage, however these integrins seem to act oppositely or complementarily. Among them, integrin α5β1 is a classic receptor for fibronectin (Woods Jr *et al.* 1994, Wright *et al.* 1997) in some tissues that are constantly exposed to mechanical stimuli, such as bladder smooth muscle, and nucleus pulposus of intervertebral disc (Xia and Zhu 2011).

Being consisted of fibrochondrocytes, menisci are structurally distinct from either the limb growth plate or the articular cartilage. They are unique in patterns of the cellular organization and antigenicity (Fox *et al.* 2015). The function of integrin α5β1 has not been identified during mechano-transduction in the meniscus, which is also a load transmission tissue. Here, in this study, we reported that cyclic hydrostatic compress force (CHCF) could promote cell proliferation and inhibit cell apoptosis in isolated primary meniscus fibrochondrocytes (PMFs) in vitro, via upregulating integrin α5β1 and the phosphorylation level of its downstream molecules, FAK, PI3K and ERK.

Materials and Methods

Isolation and Cell Culture of Primary Meniscus Fibrochondrocytes (PMFs)

The use of animals for this study was approved by the Animal Care Council of Nanfang Hospital. Menisci of 12-week Sprague-Dawley rats were harvested and cut into small pieces. Fibrochondrocytes were released by digestion with 0.22% (w/v) Type II collagenase (Sigma-Aldrich) and 0.25% trypsinase (Sigma-Aldrich) in PBS containing 100 mg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich) for 30 min at 37°C. The supernatant was removed and the remaining tissue was digested with Type II collagenase and trypsinase solution for an additional 3 hours and passed through a nylon cell strainer (70 mm, Corning). After rinsed with PBS for three times and prepared as a single cell suspension, cells were resuspended in growth medium

of DMEM supplemented with 10% FBS and 1% penicillin/ streptomycin. 4×10^6 cells were then seeded in a 60mm-dish and incubated in a humidified atmosphere at 37°C and 5% CO_2. For experiments, the PMFs were then passaged and seeded into 6-well plates in triplicates, at the density 1×10^6 cells/well.

Identification of PMFs

For immunochemistry staining, cells were fixed in 4% formalin solution for 15 min and permeabilized with 0.3% Triton X-100 in PBS for 10 min. The cells were then incubated with primary antibodies, including anti-Type-I collagen (ab34710, Abcam, 1:500) and anti-Type-II collagen (ab34712, Abcam, 1:300). After washing with PBS, cells were incubated with secondary antibody labelled with fluorescence (A11012, Gibco).

Application of CHCF on Cell Culture

CHCF was applied to cells by a computer-controlled pressure chamber (OTS, Taizhou, China), which allows sterile manipulation and up to 150 kPa of hydrostatic compress force. CHCF was applied on conluent cells for experiments at the level of 150 kPa for 12 hours, and then removed. It had been confirmed that the pH of the growth medium was constant at 7.5 and the temperature was maintained at 37°C.

Cell Proliferation Assay

The proliferation of the PMFs was detected over a seven-day period using CCK-8 solution according to the manufacturer's instructions. All experiments were performed in triplicates at least three times and representative results are shown.

qRT-PCR

Total RNAs were isolated using Trizol accroding to the manufacture's instructions, and then reversed transcribed using iScript cDNA Synthesis Kit and amplified by PCR (SYBR green) using primers for each integrin subunit (Ma *et al.* 2016, Wei *et al.* 2014) (Table 1).

qRT-PCR was assayed with Applied Biosystems® 7500 machine. Normalization of samples was achieved by measurement of the endogenous GAPDH. All reactions

were run in triplicates. A melting curve analysis was performed after the final PCR cycle, in order to check the presence of non-specific PCR products or primer-dimers. Efficiency of amplification was determined by a relative standard curve derived from serial dilutions. $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression level.

Cell Transfection

Specific siRNA for integrin α5 and β1 were transfected by Lipofectamine™ RNAiMAX (Invitrogen) reagent, according to the manufacturer's protocol. For each transfection, qRT-PCR was used to evaluate the expression level of target gene. The control groups were transfected by scrambled siRNA.

Western Blotting Analysis

PMFs were collected and washed twice in PBS. They were then lysed in RIPA buffer with proteinase and phosphatases inhibitor cocktail (ThermoFisher), for 20 min at 4 °C and centrifuged at 15,000 x g for 30 min at 4 °C. The supernatant was collected and the protein concentration was determined by BCA Protein Assay Kit (Invitrogen). Equal amounts of protein (15 μg) were separated by 10% SDS-PAGE and were transferred electrophoretically onto a PVDF membrane. Western blotting analysis was performed as standard protocol.

The primary antibodies used for western blotting were anti-integrin α5 (ab150361, Abcam), anti-integrin β1 (ab179471, Abcam), anti-FAK (sc-271195, Santa Cruz), anti-Pho-FAK (sc-81493, Santa Cruz), anti-PI3K (4249, CST), anti-Pho-PI3K (sc-1331, Santa Cruz), anti-ERK1/2 (9102, CST) and anti-Pho-ERK1/2 (4376, CST). The expression of βactin (sc-1615, Santa Cruz) was used as an internal control.

Flow Cytometry Analysis

The percentage of apoptotic cells was evaluated by staining cells with Annexin V-FITC (BD Biosciences). 1×10^6 PMFs were re-suspended in 1 × binding buffer, and then 5 μl of Annexin V-FITC was added and incubated for 15 min at room temperature in the dark. The samples were then examined using a BD Accuri™ C6 flow cytometer (BD Biosciences).

Statistical Analysis

All results were expressed as means ± standard deviation (Mean±SD). Statistical analysis was performed using Students *t* test, and $p<0.05$ was considered as statistically significant.

Results

Characterization of Primary Meniscus Fibrochondrocytes (PMFs)

In monolayer cell culturing, the PMFs exhibited the morphology of polygonal as fibroblasts (Fig.1A). They were strongly positive in Type I collagen staining (Fig.1B), and were weakly positive in Type II collagen staining (Fig.1C).

Cyclic Hydrostatic Compress Force (CHCF) Promotes Proliferation and Inhibits Apoptosis of PMFs

The cell viability was increased to around 1.5 folds with the presence of CHCF (Fig.2A). The PMFs showed a high apoptotic rate at about 63.81%±4.93% under regular culturing, which was decreased to 48.92%±6.92% when treated with CHCF (Fig.2B). Flowcytometric analysis demonstrated that CHCF treatment was able to decrease early apoptotic and late apoptotic cells (Fig.2C-G). Such effects of CHCF can be abolished by cilengitide, the integrin inhibitor (Fig.2A,B).

CHCF Increases Integrin α5 and β1 Expression Level of PMFs Cultured in Vitro

To identify which integrin subunits in cultured PMFs were changed by CHCF stimulation, the mRNA expression of integrin subunits α1, α3, α4, α5, αv, β1 and β3 were measured by qRT-PCR (Fig.3A-G). It was shown that among the tested subunits, the mRNA of integrin α5 and β1 were significantly increased by CHCF stimulation (Fig.3D,F), whereas other integrin subunits were not significantly affected. Consistently, at protein level, Western blot analysis found that integrin α5 and β1 expression were significantly increased by CHCF treatment (Fig.3H).

CHCF Modulates Downstream Molecules of Integrin α5β1

To further understand the mechanism of integrin α5β1 pathway in PMFs, the cells were transfected with integrin α5 and/or β1 siRNA. Compared with the controls, the

expression of integrin α5 and β1 were significantly suppressed, at both mRNA and protein levels (Fig.4A-C). Consequently, the enhanced cell proliferation and inhibited apoptosis from CHCF treatment were not observed (Fig.4D,E).

To investigate the downstream molecules that take part in the mechano-transduction progress, Western blot analyses were performed to evaluate the expression levels of focal adhesion kinase (FAK) and phosphorylated-FAK (Pho-FAK). Similarly as the expression pattern of integrin α5 and β1, Pho-FKA was increased by CHCF (Fig.4F). Consequently, Pho-PI3K and Pho-ERK1/2 were also increased. Instead, FAK, PI3K and ERK1/2 were not affected (Fig.4F). The protein level of caspase-3 was also decreased by CHCF (Fig.4F). These CHCF effects on protein expression and phosphorylation levels could be completely inhibited by the integrin α5β1 inhibitor or siRNA transfection (Fig.4F).

Discussion

The basic functions of the meniscus are to enable the complex movements of tibiofemoral articulation of the knee joint, protecting the articular cartilage. During these movements, mechanical forces, including compressive, shear and tensile stresses, are transmitted to the meniscus dynamically and cyclically. Mechanical stimuli has been established to be a major regulator of normal tissue morphology and function under physiological condition. Meanwhile, it is also an important determinant factor for cell fate during pathological processes (Kessler *et al.* 2001). The alteration of mechanical condition may lead to changes in osmotic pressure, streaming potentials and current, tissue pH, and hydrostatic pressure gradients, which can be sensed and responded by fibrochondrocytes of meniscus (Frank and Grodzinsky 1987, Mak 1986, Mow *et al.* 1984). Sufficient mechanical stimuli was important for preventing apoptosis (Pirttiniemi *et al.* 2004), and maintaining the metabolic activities including glycosaminoglycan (GAG) production and proteoglycan (PG) synthesis (Jung *et al.* 2014, Behrens *et al.* 1989, Jurvelin *et al.* 1989). However, due to different cell type and different tissue structure, the effects of CHCF on meniscus

fibrochondrocytes have not been identified. In this study, to mimic the real mechanical stimuli, CHCF was applied at the level of 150kPa and the interval of 12 hours according to previous study (McNulty and Guilak, 2015).

Integrin is a family of transmembrane adhesion molecules, composed of both α and β subunits. They have been proved to be the major cell-surface receptors for cell migration and adhesion (Widgerow 2013), involved in cell-extracellular matrix interaction. Growing evidence suggests that mechanical stimuli may be transduced by signaling pathways mediated by integrin, modulating various cellular functions, including cell survival, proliferation, gap junction and motility, and protein expression (Gerthoffer and Gunst 2001, Hood and Cheresh 2002). Exposure to mechanical stimuli has been found to be able to activate specific integrin family members. The expression of integrin α1β1, α2β1, α3β1, α5β1, αvβ3 and αvβ5 have been identified in chondrocytes (Kurtis *et al.* 2003, Salter *et al.* 1992), which can be altered by mechanical stimulation, local microenvironment and pathological process such as osteoarthritis (Kim *et al.* 2003, Lucchinetti *et al.* 2004). Integrin β1 alone was reported to be involved in the upregulation of aggrecan mRNA and suppression of matrix metalloproteinase-3 (MMP-3) mRNA levels by dynamic stretching of monolayer chondrocytes (Millward-Sadler *et al.* 1999, Millward‐Sadler *et al.* 2000). Meanwhile, blocking integrin α5β1 in articular chondrocytes abolishes chondrocyte responses to dynamic stretching (Holledge *et al.* 2008) and leads to enhanced cell apoptosis due to upregulated matrix metalloproteinase-13 (MMP-13). Indeed, the degeneration of meniscus has been reported to be an important risk factor of osteoarthritis. The cellular mechanisms accounting for these pathological changes may be the alterations in metabolic activities, such as proliferative activity and proteoglycan synthesis (Pirttiniemi *et al.* 2004). Our Western blot and qRT-PCT analysis found that, the expression of integrin α5β1 decreased as the degeneration of the meniscus processed (data not shown). This remains elusive as our study shows that CHCF is

able to upregulated integrin α5β1 expression. One of the possibilities is that other factors, such as shear stress or aging related changes play a more critical role during degeneration.

In the present study, FAK-PI3K/ERK pathway was found to be involved in CHCF induced mechano-transduction. FAK is a cytoplasmic tyrosine kinase located in the focal adhesion complex, transducing signals from integrins (Lal *et al.* 2007, Wen *et al.* 2009). Consistently, in other cell types (Diercke *et al.* 2011, Hong *et al.* 2010), FAK acts as an upstream regulator of p-ERK1/2 upon mechanical stimulation, which will translocate into the nucleus to active transcription (Ory and Morrison 2004). Specific inhibition of ERK1/2 activation could result in apoptosis of human chondrocytes (Shakibaei *et al.* 2001). Previous study suggested that the inhibited integrin-ligand interactions induced the conformational changes of the uncleaved caspase-3 molecule, followed by enhanced auto-cleavage and greater amount of active caspase-3 molecules (Buckley *et al.* 1999). Once treated with CHCF, the apoptosis of PMFs was inhibited by activation of integrin (Fig.2). On the other hand, FAK could also directly activate caspase by facilitating PI3K activation (Chen and Guan 1994, Kiyokawa *et al.* 1998, Sonoda *et al.* 2000). Consistent with these previous findings, the phosphorylation level of PI3K and ERK were elevated by CHCF treatment.

One of the limitations in this study is the monolayer culture system. It has been proved that a three dimensional (3D) culture system may perform better in simulating physiological microenvironments of chondrocytes (Grodzinsky *et al.* 2000, Sanz-Ramos *et al.* 2012). In the further study we will develop a 3D system to mimic the meniscus structure (Pingguan-Murphy *et al.* 2005). Furthermore, it is quite difficult to measure the exact mechanical loadings on meniscus during movements of the knee joint (Chen *et al.* 2018). We had tried to mimic the in-vivo stress pattern. However, although it had been confirmed that the pH of the growth medium was constant at 7.5, the major consern of the system applied in this study was the potential effect on cell culture medium. Thus the mechanical stimuli could be further

modulated, including treatment duration, magnitudes of the stress and cyclic patterns. In summary, the present study provides an insight into the role of integrin α5β1 during mechano-transduction of the PMFs. Further studies will be focused on different patterns of mechanical stress and culturing conditions.

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expression to degenerate nucleus pulposus cells. *Journal of Orthopaedic Research* **29**: 556-561, 2011.

Table1 Primers used for qRT-PCR analysis

Target gene	Primer sequence (5'-3')
Integrin α1	GATATTGGCCCTAAGCAGAC
	GCGATCGATTTTATTTCCTC
Integrin α3	GAATCACACCGAGGTCCACT
	GCATCTTCCCCAGCCCGTTG
Integrin α4	AAAGGCAGTACAAATCTATCC
	GAGCCCACCTAATCAGTAAT
Integrin α5	AGCGACTGGAATCCTCAAGACC
	AGTTGTTGAGTCCCGTCACCT
Integrin αV	TGTCAGCCCAGTCGTGTCTT
	GCTCAGCTCCCGTGTCATTC
Integrin β1	GGAGAAAACTGTGATGCCATACAT
	TGGGCTGGTACAGTTTTGTTCA
Integrin β3	CACAACACGCACCGACACCT
	CCCCGGTTGAACTTCTTACACT
GAPDH	TGATTCTACCCACGGCAAGTT
	TGATGGGTTTCCCATTGATGA

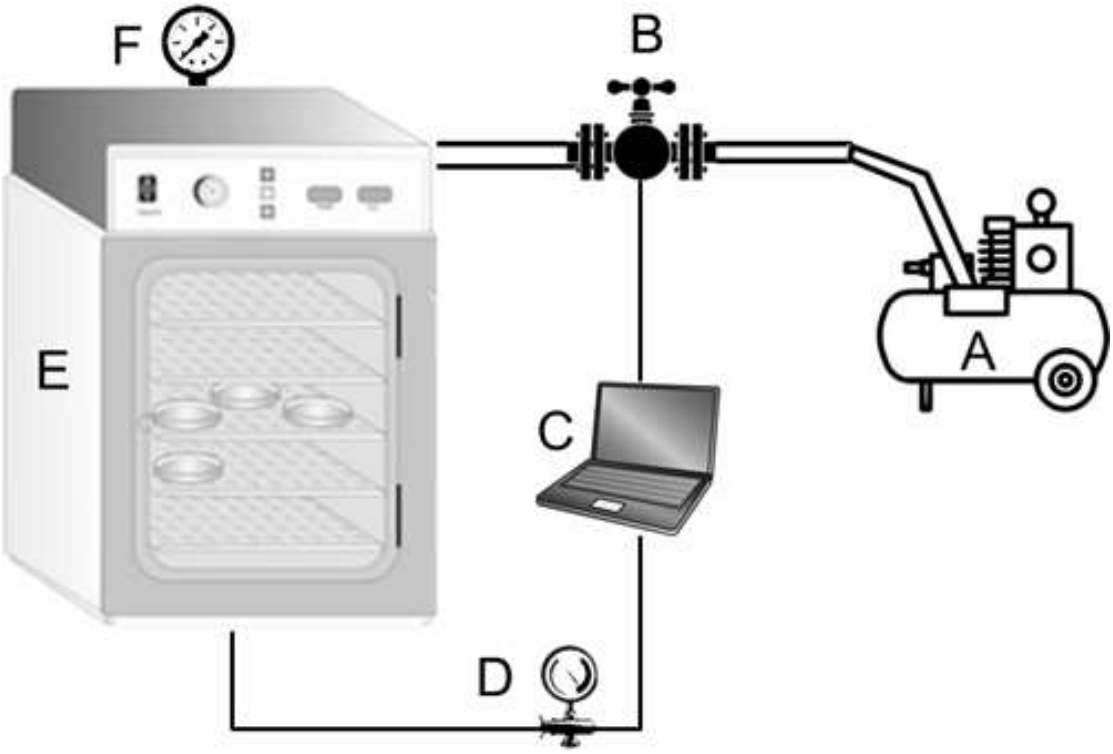

Figure 1 Schematic graph of computer-controlled pressure cell culture system

A, Air compressor. B, Computer-controlled valve. C, Computer. D, Pressure sensor. E, Cell incubator. F, Pressure gauge.

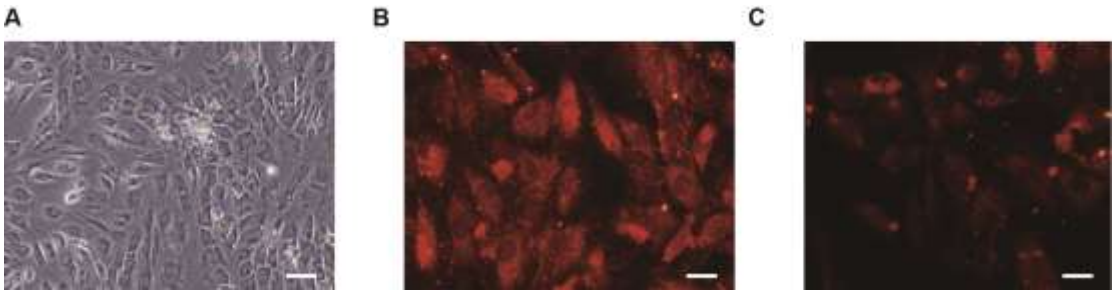

Figure 2 Characterization of Primary Meniscus Fibrochondrocytes (PMFs)

A, Morphology of PMFs in light micrograph. B, Immunochemistry staining of type I collagen. C, Immunochemistry staining of type II collagen. Scale bar=100 μm.

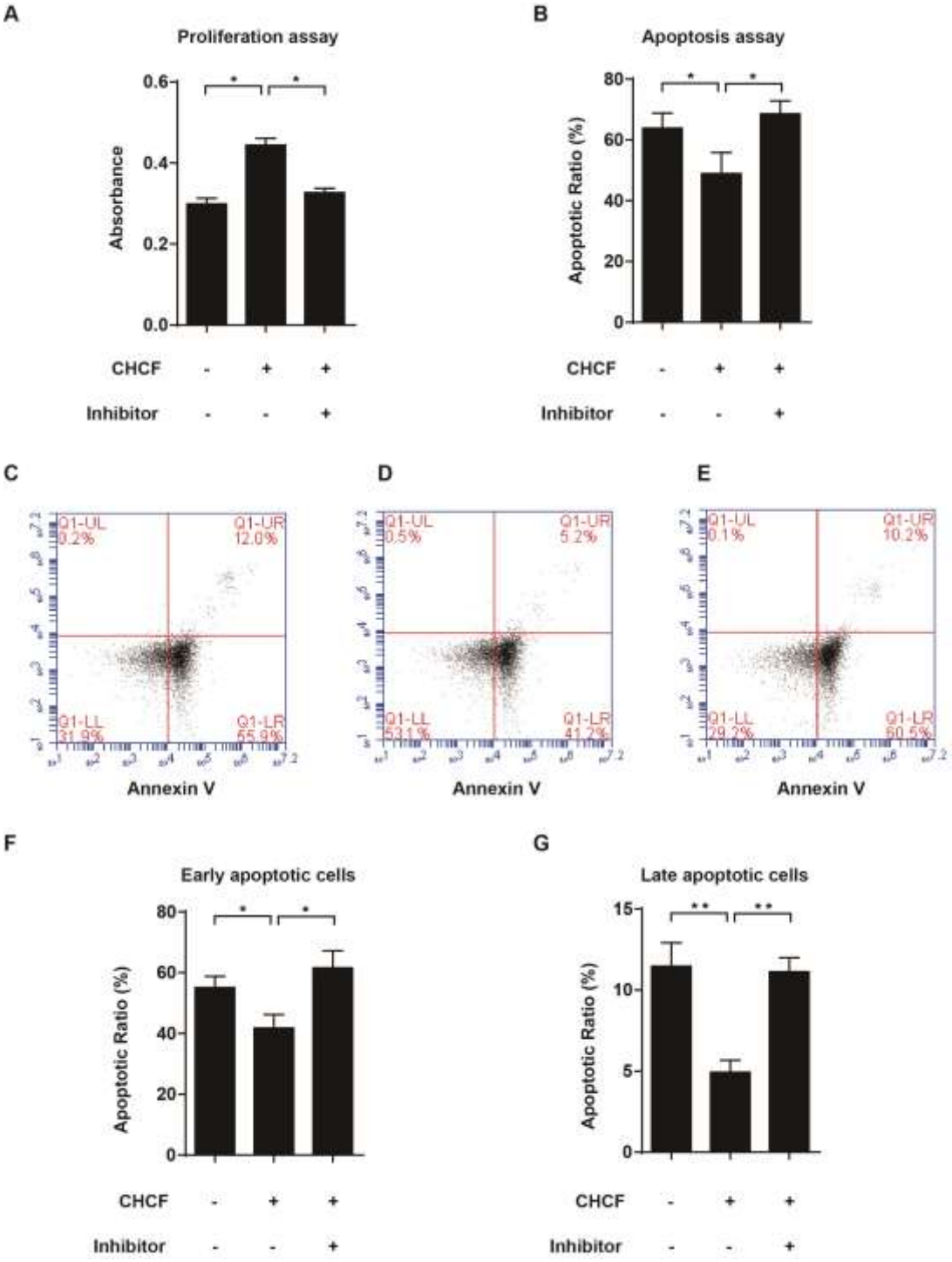

Figure 3 Cyclic Hydrostatic Compress Force (CHCF) Promotes Proliferation and Inhibits Apoptosis of PMFs

A, CCK-8 proliferation assay showing CHCF treatment increases cell viability, which can be abolished by integrin inhibitor. B, CHCF treatment decreases cell apoptosis, which can be abolished by integrin inhibitor. C-G, Flowcytometric analysis to detect

the apoptotic PMFs with or without CHCF treatment.

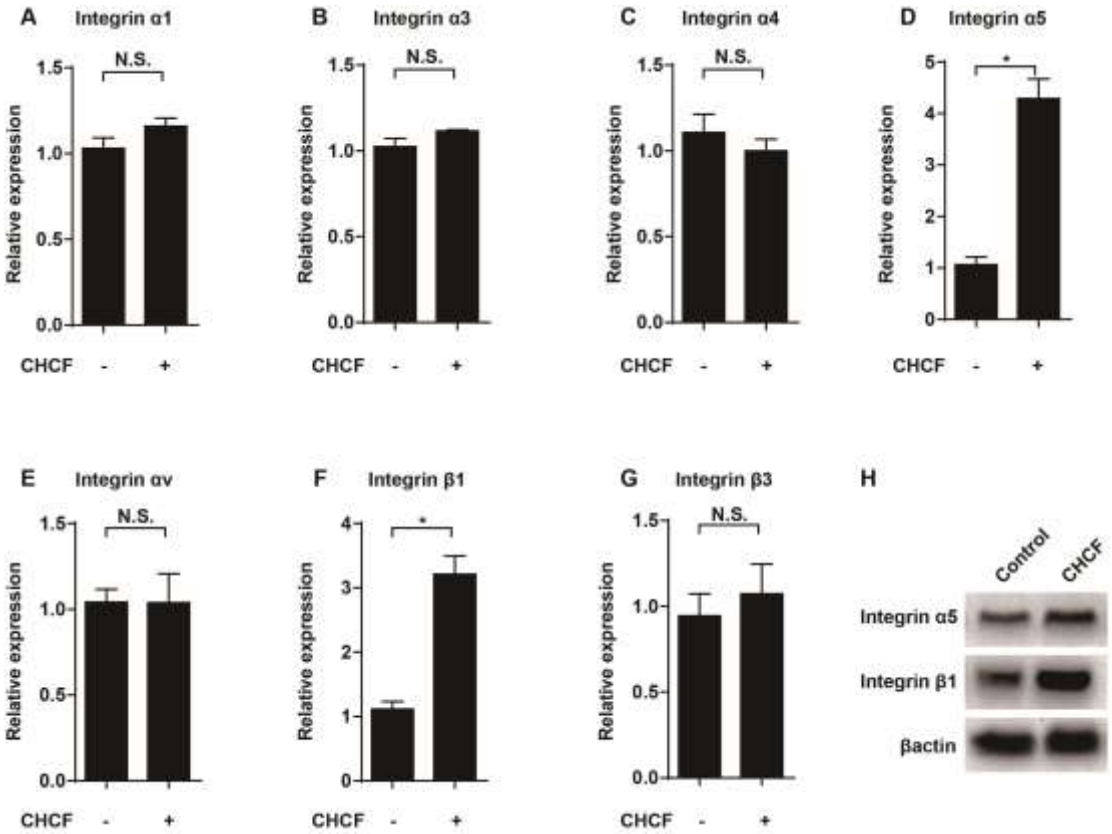

Figure 4 CHCF Increases Integrin α5 and β1 Expression Level of PMFs Cultured In Vitro

A-G, qRT-PCR analysis of mRNA expression level of different integrin subunits under with or without CHCF treatment. H, Western blot analysis showing the protein level of integrin α5 and β1 with or without CHCF treatment.

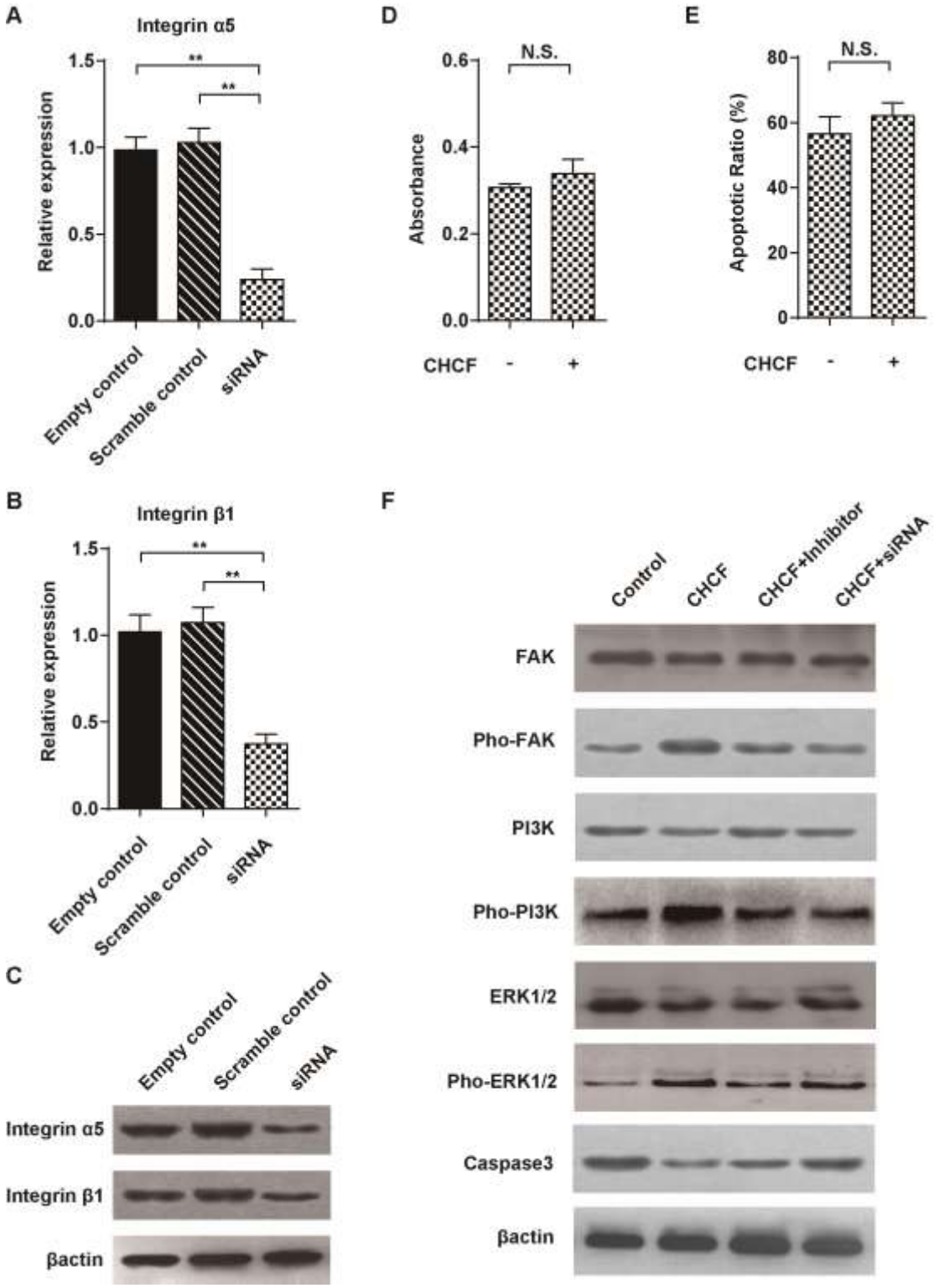

Figure 5 CHCF Modulates Downstream Molecules of Integrin

A, mRNA expression level of Integrein α5 after specific siRNA transfection. B, mRNA expression level of Integrein β1 after specific siRNA transfection. C, Protein expression level of Integrein α5 and β1 after specific siRNA transfection. D, CCK-8

proliferation assay on transfected PMFs, showing CHCF treatment does not affect cell viability. E, CHCF treatment does not affect cell apoptosis on PMFs transfected with siRNA. F, Western blot analysis of downstream molecules of integrin, showing that CHCF alters the phosphorylation level of FAK, PI3K and ERK1/2.