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# Binding of FAK and HIF-1 during mechanoperception

## Patricia Sanz-Ramos, Iñigo Izal-Azcárate

## ABSTRACT

**Aims: We have suggested the interaction of HIF-1α and FAK, as a mechanism for the influence of low oxygen concentrations in the mechanoperception in chondrocytes. We aimed here, to analyze the binding capacity for HIF-1α and FAK in protein extracts from rat chondrocytes in hypoxia. Methods: We have used protein extracts from chondrocytes subjected to changes in substrate stiffness for 30 minutes used them for immunoprecipitation and pull down assays. Results: We show the binding capacity for FAK and HIF-1α and demonstrate that it is independent on the phosphorylation of FAK. Conclusion: When in hypoxia, this interaction occurs in chondrocytes that suffer a change in the stiffness and plays a role in the mechanisms triggered by the mechanosensing.**

**Keywords: Chondrocyte, Hypoxia, Mechanosensing, FAK, HIF-1α**

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## INTRODUCTION

Aiming to give significant clues concerning the overall mechanism that allows a chondrocyte to behave differently when cultured on soft matrices, we have investigated several process during the mechanoperception cascade occurring in a chondrocyte. In a previous study, we described the participation of integrins (α1, ß1 and ß3), other focal adhesion (FA) members (Paxillin, RhoA or Src) and a special role for FAK and HIF-1 during hypoxia mechanosensing, suggesting the binding of these two proteins during the process [1]. FAK seems to be the recruiter of the signaling and structural molecules that assemble and form the focal adhesion complexes, functioning as a scaffold for the proteins that integrate them [2, 3]. HIF-1 on its own, has been demonstrated as a key factor in this response to low oxygen concentrations [4, 5], but nothing has been described concerning a possible implication in the mechanoperception of chondrocytes in hypoxia. We hypothesize that signaling cascades triggered by FAK and HIF-1α culminate in the modulation of cell behavior and in the losing or the maintenance of the final chondrocytic phenotype. We hypothesize here that FAK and HIF-1α, in hypoxic conditions, are able to interact directly and have the capacity to bind. We have proved this binding capacity and analyzed for its dependence on FAK phosphorylation.

## MATERIALS AND METHODS

### Immunoprecipitation and Pull down assay

Immunoprecipitation analysis was performed using *Dynabeads Protein-A* system (Invitrogen, Carlsbad,

CA, USA) following manufacturer's guidelines. Briefly, 20 µg of protein extract obtained using phosphosafe extraction reagent (Novagen, Madison, WI, USA) from chondrocyte embedded in collagen hydrogels for 30 minutes in a hypoxic environment were incubated with the beads that had been preincubated with 5 µL anti-FAK (Cell Signaling, Danvers, MA, USA) or HIF-1α (Sigma, St. Louis, MO, USA) antibodies. Binding of FAK and HIF-1α proteins were confirmed using pull down assay. HIF-1α was immobilized in a sepharose gel (Sigma, St. Louis, MO, USA) and the column washed with 10 volumes of PBS. Binded proteins were released using pHs 5, 3 and 1 in PBS. Selected fractions from every step were analyzed using western blot.

## Western Blot analysis

For the detection of FAK and HIF in the different fractions, we performed western blot analysis. Proteins were loaded in SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes and blocked using 5% milk in TBST. Except for actin, when a 1:10,000 dilution was used, all of the primary antibodies were diluted 1:300 in blocking buffer. After extensively washing the blot, secondary antibodies were used diluted 1:1,000 in blocking buffer and then washed again. Finally, the blots were visualized using Lumi light Plus western blotting substrate (Roche, Basilea, Suiza).

## RESULTS

Immunoprecipitation using FAK antibodies were used to determine the binding capacities of HIF and FAK proteins. Hypoxic chondrocytes were placed in collagen hydrogels for 30 minutes and then collected to obtain the total protein content. When HIF-1 antibody was immobilized on magnetic spheres, we detected the coprecipitation of FAK by using western blot analysis of fractions shown in (Figure 1A). We also performed immunoprecipitation using antibodies against different phosphorylated forms of FAK (on tyrosines 397, 576/7 and 925). In all cases, HIF-1 appeared on the precipitated fractions, suggesting the binding capacity independent on the phosphorylated state of FAK (Figure 1A). To confirm this binding, a pull down assay was performed immobilizing HIF-1 on a sepharose column by means of an antibody. The results in Figure 1B show the peaks of proteins measured by absorbance at 280 nm. Peaks selected in the figure were subjected to western blot analysis. We detected the presence of a band corresponding to FAK only after the elution using decreasing pH buffers. According to the figure, we assume peak B for non-specific binding of proteins to the column and D for other proteins that bind to HIF-1α with higher ionic strength than FAK and that have no role in our research.
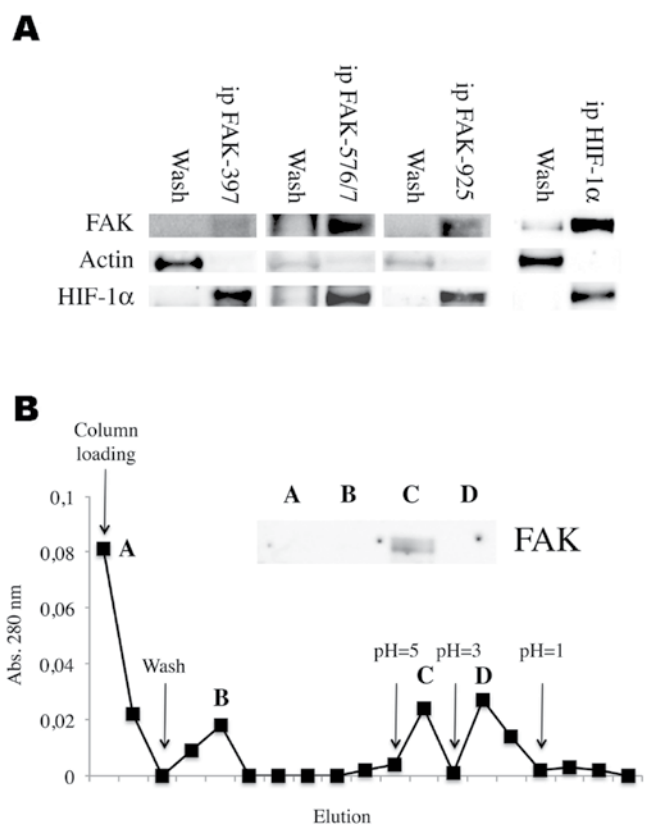


Figure 1: Interaction between FAK and HIF-1α. The binding of FAK and HIF-1α molecules was assayed by immunoprecipitation and pull down assay (A) Antibodies against HIF-1α was used, which showed the coprecipitation of both molecules. Immunoprecipitation using antibodies against FAK with different phosphorylation patterns, showing the coprecipitation of both proteins in all cases. (B) In the pull down assay, column was prepared with antibody against HIF-1α and subsequently protein sample loaded, washed and eluted using buffers with pH 5, 3 and 1. Western blot analysis showed the presence of FAK in elution fractions.

## DISCUSSION

Mechanisms that underlie the response to change in the substrate mechanics are not fully described and many data remain to be discovered in order to completely understand what makes a cell behave in the way it does after changes in the stiffness have been applied. This knowledge would give us the possibility to redirect their phenotype as desired. In a previous work, we described the participation of both FAK and HIF-1α in the mechanisms triggered by changes in the matrix rigidity and suggested the possibility of the interaction between these two molecules. Herein, we present data that supports the binding of FAK and HIF-1α during the process of mechanosensing. Results in Figure 1A–B prove the interaction of both molecules in protein extracts from cells 30 minutes after placing them in collagen hydrogels.

Moreover, our binding experiments showed the capacity of interaction of both proteins independently of the phosphorylated residues exhibited by FAK. We tested antibodies against FAK-397-P, FAK-576/7-P and FAK-925-P and all of them were capable of precipitating HIF-1α The phosphorylation of FAK is produced in a cascade beginning with the residue 397, which leads to the binding of Src and subsequently, to the phosphorylation in 576/7 and to the interaction with other signaling molecules [6–9]. A later phosphorylation in residue 925 releases FAK from the FA complex [10]. Our results identify then HIF-1α, as one of those signaling molecules that interact with the FA complex in hypoxia. In a previous work, we showed that FAK was upregulated during mechanoperception in normoxia, but downregulated while in hypoxia, suggesting its implication in the mechanoperception regulation by oxygen content. We also showed that HIF-1α is the responsible for changes in the response to mechanics with oxygen [11]. Despite the biological significance of this interaction remains to be fully described, putting it all together, it seems to take part in the regulation of the response to the mechanics in a hypoxic environment.

## CONCLUSION

FAK and HIF-1α bind when chondrocytes in a hypoxic environment are subjeceted to change in the substrate stiffness. This interaction does not depend on the phosphorylation of FAK residues Y397, Y576/7 and Y925.

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### Author Contributions
Patricia Sanz-Ramos – Substantial contribution to conception and design of the experiment, Data acquisition, Analysis and interpretation, Drafting the article, Final approval of the version to be published
Iñigo Izal-Azcárate – Substantial contribution to conception and design of the experiment, Data acquisition, Analysis and interpretation, Drafting the article, Final approval of the version to be published

### Guarantor
The corresponding author is the guarantor of submission.

### Conflict of Interest
Authors declare no conflict of interest.

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