Biochimica et Biophysica Acta 1843 (2014) 1111–1122

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

# ALK1 heterozygosity increases extracellular matrix protein expression, proliferation and migration in fibroblasts

José M. Muñoz-Félix [a,b], Nuria Perretta-Tejedor [a,b,c], Nélida Eleno [a,b], José M. López-Novoa [a,b], Carlos Martínez-Salgado [a,b,c,*]

[a] Unidad de Fisiopatología Renal y Cardiovascular, Instituto "Reina Sofía" de Investigación Nefrológica, Departamento de Fisiología y Farmacología, Universidad de Salamanca, Salamanca, Spain
[b] Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain
[c] Instituto de Estudios de Ciencias de la Salud de Castilla y León (IECSCYL), Unidad de Investigación, Hospital Universitario de Salamanca, Salamanca, Spain

ARTICLE INFO

*Article history:*
Received 4 September 2013
Received in revised form 19 February 2014
Accepted 23 February 2014
Available online 1 March 2014

*Keywords:*
ALK1
Fibrosis
Extracellular matrix
Smads
Proliferation
Migration

ABSTRACT

Fibrosis is a pathological situation in which excessive amounts of extracellular matrix (ECM) are deposited in the tissue. Myofibroblasts play a crucial role in the development and progress of fibrosis as they actively synthesize ECM components such as collagen I, fibronectin and connective tissue growth factor (CTGF) and cause organ fibrosis. Transforming growth factor beta 1 (TGF-β1) plays a major role in tissue fibrosis. Activin receptor-like kinase 1 (ALK1) is a type I receptor of TGF-β1 with an important role in angiogenesis whose function in cellular biology and TGF-β signaling is well known in endothelial cells, but its role in fibroblast biology and its contribution to fibrosis is poorly studied. We have recently demonstrated that ALK1 regulates ECM protein expression in a mouse model of obstructive nephropathy. Our aim was to evaluate the role of ALK1 in several processes involved in fibrosis such as ECM protein expression, proliferation and migration in ALK1$^{+/+}$ and ALK1$^{+/-}$ mouse embryonic fibroblasts (MEFs) after TGF-β1 stimulations and inhibitors. ALK1 heterozygous MEFs show increased expression of ECM proteins (collagen I, fibronectin and CTGF/CCN2), cell proliferation and migration due to an alteration of TGF-β/Smad signaling. ALK1 heterozygous disruption shows an increase of Smad2 and Smad3 phosphorylation that explains the increases in CTGF/CCN2, fibronectin and collagen I, proliferation and cell motility observed in these cells. Therefore, we suggest that ALK1 plays an important role in the regulation of ECM protein expression, proliferation and migration.



## 1. Introduction

Tissue fibrosis is an endpoint feature of several pathologies in kidney, liver, lung, heart and skin [1]. Extracellular matrix (ECM) protein synthesis, cellular proliferation, and migration are three important processes in the development of tissue fibrosis [2,3]. Transforming growth factor-beta 1 (TGF-β1) plays a relevant role in tissue fibrosis [4] inducing ECM protein expression proliferation and migration in cells such as stellate cells [5], fibroblasts [6,7] and keratinocytes [8]. TGF-β1 signals by binding to a receptor complex formed by one type I receptor, one type II receptor and, in some cases a type III co-receptor. Both type I and type II receptors are necessary for TGF-β to exert its biological functions [9]. Two type I receptors have been described for TGF-β1, activin receptor-like kinase type 1 (ALK1) and type 5 (ALK5) [10,11]. TGF-β1 binding to an ALK1-containing receptor promotes Smad1/5 phosphorylation whereas TGF-β1 binding to an ALK5-containing receptor promotes Smad2/3 phosphorylation, that is usually associated with increased ECM protein expression [12].

Goumans et al. [13] have described that ALK1 and its effectors (Smad1/5) exert a lateral antagonism of the ALK5 pathway. However, ALK5 is necessary for the activation of the ALK1 pathway by TGF-β. This mechanism has been described in endothelial cells [10,13–15]. Other authors have also demonstrated that ALK5 is necessary for TGF-β-induced activation of the Smad1/5 pathway in L6E9 myoblasts [16].

Although the involvement of ALK1-Smad1/5 signaling pathway has been described mainly for angiogenesis, there are some evidences that it is also involved in ECM regulation [17]. Thus, it has been described that the ratio ALK1/ALK5 regulates ECM protein degradation in osteoarthritis, due to a regulation mechanism through MMP-13 [18]. In addition, Finnson et al. [19] showed that ALK1 negatively regulates TGF-β1/ALK5-induced ECM protein expression in human chondrocytes. Silencing of ALK1 with siRNA leads to an increase in TGF-β1-induced connective tissue growth factor (CTGF/CCN2) expression [20]. However, in other experimental models of tissue fibrosis, it has been demonstrated that ALK1 behaves as a profibrotic receptor: In liver fibrosis, ALK1 induces hepatic stellate cell transdifferentiation into myofibroblasts [21]. On the other hand, ALK1 promotes skin fibrosis

* Corresponding author at: Department of Physiology and Pharmacology, University of Salamanca, Edificio Departamental, Campus Miguel de Unamuno; 37007 Salamanca, Spain.
*E-mail address:* carlosms@usal.es (C. Martínez-Salgado).

http://dx.doi.org/10.1016/j.bbamcr.2014.02.017
0167-4889/

through Smad1/5 pathway [6,22] and ALK1 heterozygosity reduces renal fibrosis induced by radiation, mainly due to reduced infiltration of inflammatory cells [23].

We recently demonstrated that ALK1 expression increases after 15 days of unilateral ureteral obstruction (UUO), an in vivo experimental model of renal fibrosis. This increase is observed mainly in interstitial myofibroblasts. Furthermore, ALK1 haploinsufficient mice develop more renal fibrosis which is explained by the effect of ALK1 in regulating ECM protein expression in renal fibroblasts [24]. These data suggest that ALK1 is involved in the regulation of renal fibrosis.

As fibroblasts play a major role in tissue fibrosis, our purpose is to analyze the role of ALK1 in ECM protein expression, proliferation and migration in fibroblasts. It should be noted that ALK1 knock out (KO) mice ($ALK1^{-/-}$) die at E.11.5 due to cardiovascular defects [25] and fibroblasts derived from $ALK1^{-/-}$ embryos are not viable, since they survive for only a few hours in culture conditions. Thus, we have cultured ALK1 mouse embryonic fibroblasts (MEFs) from heterozygous ($ALK1^{+/-}$) mice and their respective wild type controls ($ALK1^{+/+}$).

## 2. Materials and methods

### 2.1. Mice model of ALK1 haploinsufficiency

Generation of $ALK1^{+/-}$ mice was performed as previously described [25]. $ALK1^{+/-}$ mice were given by Dr. Peter ten Dijke (Leiden University, Netherland), and a breeding colony of adult $ALK1^{+/-}$ mice has been maintained in the pathogen-free facilities for genetically modified mice of the University of Salamanca, and backcrossed with C57Bl/6 mice for 9 generations. Routine genotyping of DNA isolated from mouse tail biopsies was performed by PCR using the primers previously reported [25]. Animals were kept under controlled ambient conditions (Animal Experimentation Service, University of Salamanca, Spain) in a temperature controlled-room with a 12 h light/dark cycle, and were reared on standard chow (Panlab, Barcelona, Spain) and water ad libitum. In all procedures, mice were treated in accordance with the Recommendations of the Helsinki Declaration on the Advice on Care and Use of Animals referred to in: law 14/2 007 (3 July) on Biomedical Research, Conseil de l'Europe (published in Official Daily N. L358/1-358/6, 18-12-1986), Spanish Government (Royal Decree 223/1 988, (14 March) and Order of October 13 1989, and Official Bulletin of the State b. 256, pp. 31349–31362, October 10 1990). All the procedures were approved by the Bioethics committee of the University of Salamanca.

### 2.2. $ALK1^{+/-}$ fibroblast generation, cell culture and TGF-β1 stimulation

$ALK1^{+/+}$ and $ALK1^{+/-}$ MEFs were subcultured and immortalized as previously reported [26]. Briefly, mouse embryos obtained from the mating of $ALK1^{+/-}$ mice were recovered at DPC 10, mechanically minced and treated with 0.25% trypsin solution for 30 min before plating on DMEM supplemented with 10% FCS, 0.66 μg/ml penicillin and 60 μg/ml streptomycin sulfate, in an atmosphere of 95% air/5% $CO_2$ at 37 °C. Immortalized cultures that survived crises after 15–20 passages were identified and cloned and their genotypes reconfirmed by PCR analysis as described previously [25]. For Western blot and PCR analysis, cells were seeded in 100 mm culture dishes, for total collagen measurements and proliferation studies cells were plated respectively at 20,000 or 9000 cells/well in 24 well plates. When cultures achieved 80–90% confluence, cells were serum-starved for 24 h and treated with active human recombinant TGF-β1 (1 ng/mL) or control vehicle during 30 min or 24 h in the absence of serum. When pharmacological inhibition was used, the ALK5 inhibitor SB431542 [27] (5 μM) or the Smad3 inhibitor SIS3 (4 μM) [28] were added 30 min before TGF-β1 stimulation. Cultures of similar percentage of confluence were used in every analysis performed.

### 2.3. Western blot

Total cell extracts were homogenized in magnesium lysis buffer (MLB, from Millipore, Billerica, MA, USA) supplemented with 80% glycerol, 1 mg/mL leupeptin, 1 mg/mL aprotinin, 10 mM PMSF, 1 mmol/L $Na_3VO_4$ and 25 mmol/L NaF, and centrifuged at 14,000 *g* during 20 min. Supernatants were recovered and the protein amount was quantified. Lysates (20 μg per lane) were loaded onto polyacrylamide gels and the proteins were transferred to nitrocellulose membranes (Millipore) by electroblotting. Next, membranes were blocked in bovine serum albumin (BSA) and were incubated overnight at 4 °C with the following antibodies: rabbit anti-collagen type I (dilution 1:1000) and rabbit anti-fibronectin (1:1000) from Chemicon International (Temecula, CA); rabbit anti-phospho-Smad3 (1:1000) and rabbit anti-phospho-Smad1/5/8 (1:1000) from Cell Signaling (Barcelona, Spain); goat anti-CTGF (1:1000), goat anti-Smad2/3 (1:1000), rabbit anti-ALK5 (TβRI) (1:1000), and mouse anti-Smad1 (1:1000) from Santa Cruz Biotechnology (Madrid, Spain), rabbit anti-ACVRL1 (ALK1) (1:1000) from Abgent (Derio, Spain), rabbit anti-phospho-Smad1 (1:1000) and rabbit anti-phospho-Smad2 (1:1000) from Upstate Biotechnology (Barcelona, Spain), and mouse anti-PCNA (1:1000) from Transduction Laboratories (Madrid, Spain). Membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:10,000) and were developed using ECL chemiluminescence reagent (Amersham Biosciences, Barcelona, Spain). Developed signals were recorded on X-ray films (Fujifilm Spain, Barcelona, Spain) for densitometric analysis (Scion Image software, Frederick, MD, USA). Erk1/2 was used as loading control.

### 2.4. RT-PCR analysis

Total RNA was isolated as described previously [2]. Quantitative RT-PCR was performed in triplicate. Each 20 μl reaction contained 1 μl of cDNA, 400 nM of each primer, and 1× IQ SybrGreen Supermix (Bio-Rad). Primers were designed for specific sequences and checked by the BLAST algorithm as previously described [29]. Primers used were: For mouse ALK1 (92 bp): forward 5′-CTGCTTTGAGTCGTACAAGT-3′ and reverse 5′-CCACAATGCCATTGATGATG-3. For mouse ALK5 (114 bp): forward 5′-CAGACAACAAAGACAATGGG-3′ and reverse 5′-ATCATTCCTTCCACAGTAACAG-3′. For mouse GAPDH (153 bp): forward 5′-GTCGGTGTGAACGGATTTG–3′ and reverse 5′-GAATTTGCCGTGAGTG GAGT-3′. Cycling conditions for ALK1, ALK5 and GAPDH: 95 °C, 5 min, 35 cycles of 1 min 95 °C, 1 min 59 °C and 1 min 72 °C, and an elongation cycle of 5 min 72 °C. Standard curves were run for each transcript to ensure exponential amplification and to rule out non-specific amplification. Gene expression was normalized to GAPDH expression. The reactions were run on an iQ5 real-time PCR detection system (Bio-Rad).

### 2.5. Wound-healing assay

In vitro scratched wounds were created on serum-starved confluent cell monolayers with a straight incision using a sterile disposable pipette tip. Cell migration into the denuded area was monitored over a time course using digital microscopy and cell movement was calculated as the reduction of the wound area over time (in percentage, initial area of the wound: 100%).

### 2.6. Cell proliferation assay

Cells in 24 well plates were serum-starved when cultures achieved 80–90% confluence during 48 h and 72 h and viable cell number was measured using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described [30].