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Barley β-glucan accelerates wound healing by favoring migration versus proliferation of human dermal fibroblasts

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ABSTRACT

β-Glucans are considered candidates for the medication in different human pathologies. In this work, we have purified β-glucan from a selected barley line and tested their effects in primary human dermal fibroblasts. Unexpectedly, we have observed that this compound promoted a short-transitory proliferation arrest at 24 h after its addition on the medium. We have determined that this transitory arrest was dependent on the cell-cycle regulator protein Retinoblastoma. Moreover, dermal fibroblasts increase their migration capacities at 24 h after barley β-glucan addition. Also, we have described that barley β-glucan strongly reduced the ability of fibroblasts to attach and to spread on cell plates. Our data indicates that barley β-glucan signal induces an early response in HDF cells favoring migration versus proliferation. This feature is consistent with our observation that the topical addition of our barley β-glucan in vivo accelerates the wound closure in mouse skin.

1. Introduction

β-glucans are carbohydrate polymers that can be found in the cell walls of many organisms such as bacteria, fungi, yeasts and some cereals like barley and oat (Ahmad, Anjum, Zahoor, Nawaz, & Dilshad, 2012). Their structure consists of a polymer with glucose monomers linked by glycosidic bonds in different positions according to the organism: β-glucans from yeasts and fungi mainly contain linear β-(1→3) chains or β-(1→3) with β-(1→6) linked side chains, whereas the polymers derived from cereals have a β-(1→4) backbone interrupted by separate β-(1→3) linkages. They show a great variability with respect to molecular mass, and the different linkages affect significantly water solubility, viscosity, gelation capacity as well as physiological properties.

Many types of β-glucans possess a broad spectrum of protective activities against adverse conditions and display important functions in the modulation of immunological, anti-inflammatory and wound healing responses. In general, they activate the immune system, and stimulate defensive responses against pathogen infection (Lundahl, Scanlan, & Lavelle, 2017), tumor development (Jin, Tang, Rong, & Zhang, 2018), or wounding (Majtan & Jesenak, 2018). These effects are mediated through pattern recognition receptors (Borchers, Krishnamurthy, Keen, Meyers, & Gershwin, 2008; Chan, Chan, & Sze, 2009; Ujita et al., 2009) located on target cells, predominantly monocytes, macrophages, neutrophils, natural killer cells (Brown & Gordon, 2003; Lundahl et al., 2017), and also on skin cells such as keratinocytes and fibroblasts (Wei, Williams, & Browder, 2002).

Concerning to cereal β-glucans, the best characterized attribute is the effectiveness to reduce serum cholesterol levels (Abumweis, Jew, & Ames, 2010) and post-prandial glycemic responses (Behall, Scholfield, Hallfrisch, & Liljeberg-elmståhl, 2006). Nonetheless, other systemic responses have been described such as their immune modulating

Abbreviations: HDFa, adult human dermal fibroblasts; FTIR, fourier transformed infra red; ECM, extracellular matrix; LSGS, low serum growth supplement; PBS, phosphate-buffered saline; PFA, paraformaldehyde; BSA, bovine serum albumin; Cdk, cyclin dependent kinase; GPC, gel permeation chromatography; RB1, retinoblastoma; Ccnd1, cyclin D1; BGN, β-glucan; FN, fibronectin; BrdU, bromodeoxyuridine

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capacity (Chanput et al., 2012; Rieder & Samuelsen, 2012). In this way, oat and barley β-glucans bind to dectin 1 receptor and interleukins and alter the expression of inflammatory-associated genes and macrophage activation (Tada et al., 2009; Volman et al., 2010). Also, barley β-glucan has been described as a moderate to strong inducer of cytokine production (Noss, Doekes, Thorne, Heederik, & Wouters, 2013). Additionally cereal β-glucans protect from microbial infections (Estrada et al., 1997; Yun, Estrada, Van Kessel, Park, & Laarveld, 2003), affect the viability of tumoral cells (Choromanska et al., 2018) and potentiate the activity of antitumor monoclonal antibodies (Cheung, Modak, & Vickers, 2002).

The protective effect of β-glucans applies also to the skin barrier, which is directly in contact with the environment. This property makes the skin prone to the presence of wounds. To safeguard survival, our organism has developed healing mechanisms to repair skin wounds and to elude infections. Wound healing involves complex and overlapping cellular events that can be assigned to any of three processes: inflammation, proliferation and remodeling (Stadelmann, Digenis, & Tobin, 1998). After wound, an immediate event is hemostasis and generation of inflammatory stimuli. Then, activated macrophages produce growth factors and cytokines promoting anti-inflammatory and antibacterial effects, and also, the migration of dermal fibroblasts to the wound. In turn, those fibroblasts proliferate in the wound and produce extracellular matrix (ECM) components, such as collagen, to initiate the remodeling process. In this scenario, β-glucans participate by activating both immune and non-immune cells to stimulate wound repair (Kougias et al., 2001; Majtan & Jesenak, 2018). For instance, (1→3),(1→6)-β-D-glucans from fungi promote proliferation, migration and procollagen secretion in human dermal fibroblasts (HDF) and keratinocytes (Majtán, Kumar, Koller, Dragúnová, & Gabriz, 2009; Son et al., 2007; van den Berg, Zijlstra-Willems, Richters, Ulrich, & Geijtenbeek, 2014; Wei et al., 2002; Woo et al., 2010).

The relevance of β-glucans on wound healing has also been tested in clinical trials. For instance, topical application of yeast β-glucans improves healing of diabetic and venous ulcers (Medeiros et al., 2012; Zykova et al., 2014). In particular, for this kind of treatments, different laboratories are developing the preparation of porous membranes of various biopolymers (Tamer et al., 2018; Woo et al., 2010).

Most effects on wound healing have been found using linear o branched β-glucans from fungi and yeast. However, up to date there is little information regarding skin cell response after cereal β-glucans exposure (Akkol et al., 2011; Du, Bian, & Xu, 2014). The purpose of this investigation is the evaluation of the effects of β-glucan (1→3),(1→4) from barley on adult human dermal fibroblasts in order to clarify the action on wound healing in vitro and in vivo.

2. Materials and methods

2.1. Sample material

A hulled, waxy-endosperm, two-rowed barley line with high β-glucan content (7.5%) has been used in this work as raw material. Seeds were provided by Semillas Batlle S.A (Bell.lloc d'Urgell, Lleida, Spain)

2.2. Barley β-glucan extraction

The extraction in the Pilot Plant of the University of Lleida was performed according to the procedure of Morgan 1998 (Morgan & Ofman, 1998) with minor modifications. Briefly, 2 kg of whole barley grain were milled in a laboratory disk mill DLFU from Bühler Group. Flour was suspended in 10 l of warm distilled water to reach 1:5 flour/solvent ratio. Mixture was stirred in a 50 l tank during 1 h with a constant temperature of 55 °C. The solution was centrifuged using a Beckman J2-21 centrifuge during 5 min at 3000 rpm at 4 °C in order to pellet the solid residue. The supernatant fraction was frozen at −20 °C during 24 h and then thawed at room temperature. This solution was filtered through a 75 μm nylon mesh and β-glucan gels recovered. The resulting extracts were rinsed with ultrapure water and freeze-dried.

2.3. Compositional analyses

Total β-glucan composition of dry powder extracts was determined by the method of McCleary and Mugford (1997)) using the mixed linkage β-glucan assay kit from Megazyme (Wicklow, Ireland) with modifications for samples with high content of β-glucan.

2.4. Molecular weight determination

The molecular weight of barley β-glucan extracts was determined by Gel Permeation Chromatography (GPC) using a chromatography system, which consists of an isocratic pump (Waters 600 Waters, Milford, MA), an automatic injector 717 Waters, a GPC column (PSS SUPREMA, Analytical Ultrahigh, 8 × 300 mm, 10 μm) and a differential refractive index detector Waters 2414. The column was kept at 35 °C and the flow rate of the mobile phase ($NaNO_3$ 100 mM, NaN_3 5 mM) was set at 0.6 ml min^{-1}. Six different molecular weight β-glucan standards from Megazyme (Wicklow, Ireland) in the range of 35–650 kDa were dissolved in ultrapure water to obtain the calibration curve.

2.5. FTIR-ATR spectroscopy

The FTIR-transmission spectra were recorded on a 6300 series Jasco FT-IR equipped with a TGS detector by attenuated total reflectance (ATR). Spectra were obtained by averaging 56 scans from 4000 cm^{-1} to 650 cm^{-1} at a resolution of 4 cm^{-1} and corrected for background absorbance by subtraction of the spectrum of the empty ATR crystal. FTIR spectra were compared with pure barley β-glucan standard from Megazyme (ref P-BGBL, 95% purity)

2.6. Cell culture

HDFa (adult Human Dermal Fibroblasts) were obtained from Thermo Fisher Scientific (GIBCO, C0135C). Cells were maintained at 37 °C in a 5% CO_2 incubator, and grown in Medium 106 (M106; GIBCO) supplemented with Low Serum Growth Supplement (LSGS), 100 μg ml^{-1} penicillin/streptomycin and 10 μg ml^{-1} gentamicin. Barley β-glucan was diluted in the medium at different final concentrations always from a freshly prepared stock (10 mg ml^{-1}). To prepare β-glucan stock, 50 mg of powder was solubilized in 5 ml of sterile water, and the solution was heated and stirred in a water bath (90 °C) for 5 min. Then, shaking was continued for 10 min without heating. Methyl cellulose (Sigma M7027) and Amylopectin (Sigma 10120) were solubilized in water at room temperature following manufacturer's instructions (stock 10 mg ml^{-1}).

2.7. Immunoblotting

For immunoblot, protein samples were resolved by SDS-PAGE, transferred to PVDF membranes (Millipore), and incubated with primary antibodies anti-CyclinD1 (monoclonal DCS-6, Santa Cruz #sc-20044, 1:200), anti-Cdk4 (polyclonal C-22, #sc-260, 1:250), anti-pho-p38 (Thr180/Tyr182, monoclonal 28B10, Cell Signaling #9216, 1:500), anti-p38 (polyclonal, Cell Signaling #9212, 1:500), anti-Rb (monoclonal G3-245, BD Pharmingen #554136, 1:500), anti-pho-Rb (S780, monoclonal J146-35, BD Pharmingen #558385, 1:500), anti-tubulin (monoclonal B-5-1-2, Sigma #T5168, 1:10000) and anti-actin (monoclonal C4, Millipore #MAB1501R, 1:1000).

Appropriate peroxidase-linked secondary antibodies (GE Healthcare UK Ltd) were detected using the chemiluminescent HRP substrate Immobilon Western (Millipore). Chemiluminescence was recorded with a ChemiDoc-MP imaging system (BioRad).

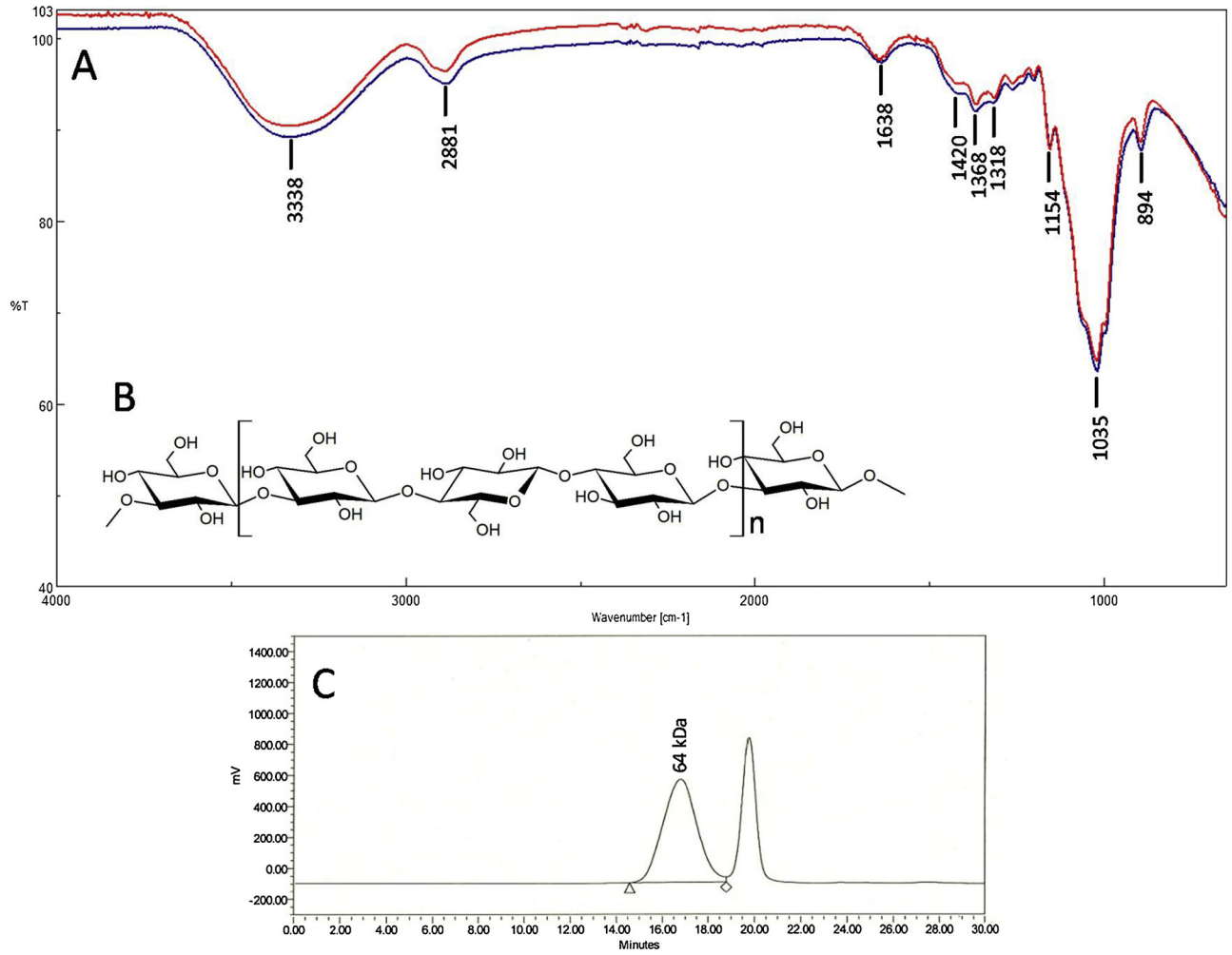

Fig. 1. A. FTIR of both pure β-glucan standard from Megazyme (blue line) and barley β-glucan extract (red line) showing the same spectra. **B.** Structure of mixed-linkage (1→3)(1→4) β-glucan. **C.** GPC plot of the β-glucan extract with a peak Mw of 64 kDa (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.8. Proliferation assay

Three thousand HDFa cells per well were seeded on a 24-well plate and treated with a final concentration of 400 μg ml^{-1} barley β-glucan added to the growth medium. Control cells were untreated. After 24, 48 and 72 h of treatment cells were trypsinized, diluted in 300 μl of media with Trypan Blue and counted in a Neubauer's chamber.

2.9. Cell adhesion and spreading assays

Petri dishes were coated overnight at 4 °C with a 5 μg ml^{-1} solution of fibronectin (Invitrogen) in PBS or with 400 μg ml^{-1} solution of barley β-glucan. HDFa were trypsinized and seeded in serum-free medium in uncoated, fibronectin-coated, barley β-glucan-coated or fibronectin-barley-β-glucan double coated 35-mm-well plates. Seeded fibroblasts were incubated for 30 or 80 min, and then, plates were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Total cells (adhered) were counted and the percentages of spread cells were determined. Round and bright cells were considered to be unspread.

2.10. Cell migration assay

HDFa cells were seeded on the bottom side of an 8.0-pore-size 6.5-mm filter (Transwell, Corning) for 4 h in an incubator. Afterwards, filters were loaded with M106 with LSGS and incubated in 24-well plates containing LSGS-free M106 for 24 h. This protocol was performed with or without 400 μg ml^{-1} barley β-glucan (final concentration) added to both media. Under these conditions, some cells migrate from the bottom to the upper side of the filter. The remaining cells at the bottom of the filter were removed with a cotton swab, and cells at the upper-side were fixed and stained with Hoescht. All cells throughout the filter were counted with Image J software.

2.11. Bromodeoxyuridine (BrdU) incorporation assay

HDFa cells were seeded in a 24-well plate and treated with 400 μg ml^{-1} barley β-glucan and control polymers for 24 h. For the last 8 h of treatment with β-glucan, 8 μg ml^{-1} BrdU were added. The same was done with untreated cells that were used as a control. After 24 h, fibroblasts were fixed with 4% PFA for 15 min at room temperature. Then, cells were permeabilized with 0.2% Triton-X-100 for 3 min at room temperature, washed with PBS, and successively treated with HCl 2 M for 30 min at 37 °C and with sodium tetraborate 0.1 M (pH 8.5) for 2 min. Finally, preparation was blocked with 3% BSA. Anti-BrdU (monoclonal BU1/75 (ICR1), Immunologicals Direct, #OBT0030, 1:200) was used with adequate Alexa488-labelled secondary antibody (Molecular Probes) in PBS with 0.3% BSA. Nuclei were stained with Hoechst (Sigma). Images were acquired using 40X and 60X objectives in an Olympus FV1000 confocal system. Percentage of BrdU

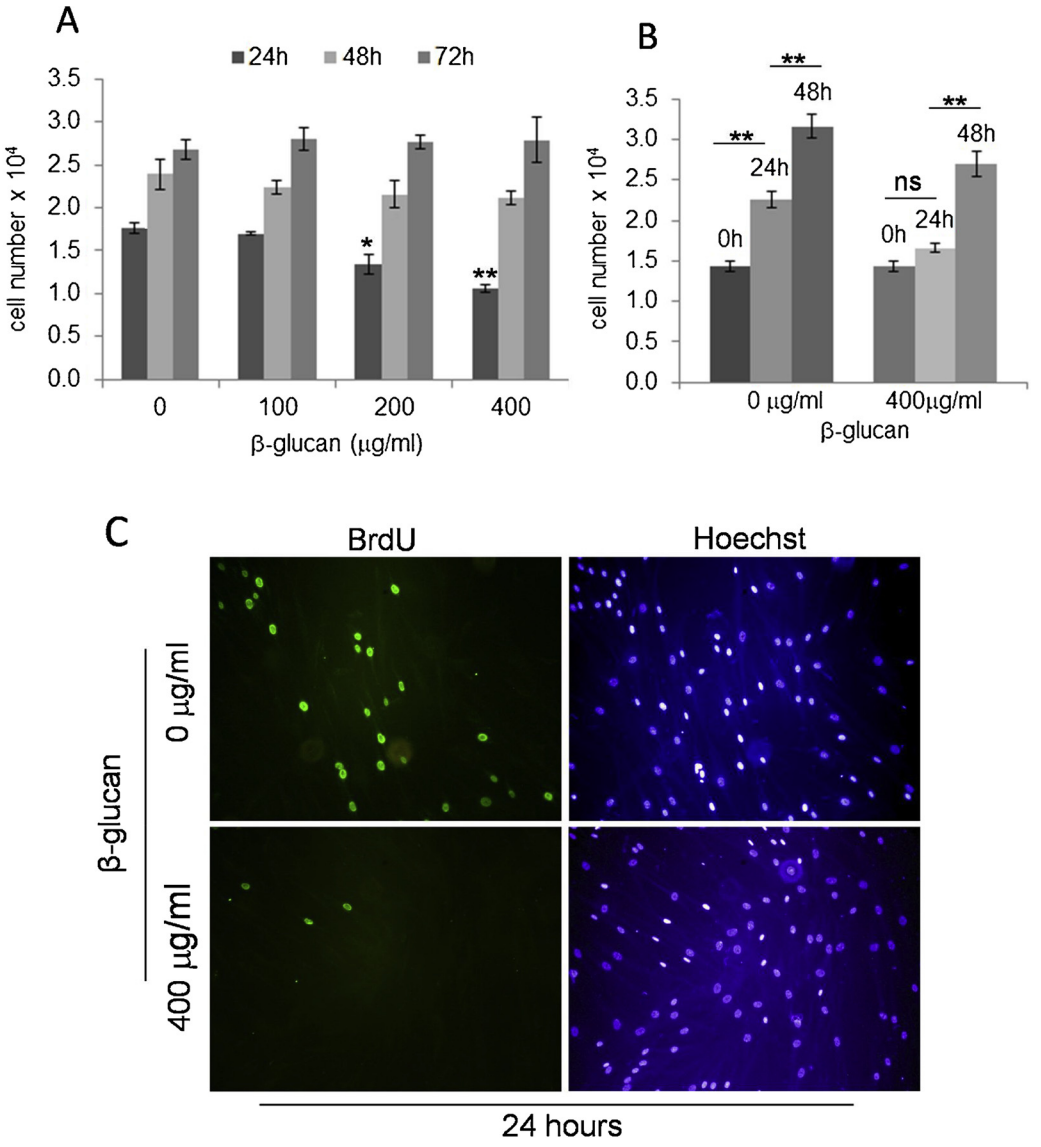

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Fig. 2. Barley β-glucan triggers a short arrest in the proliferation of human fibroblasts. A. The same number of HDF cells (10^4) was incubated with different concentrations of barley β-glucan, and cell count was determined at 24, 48 and 72 h after β-glucan addition. The values from three experiments are represented as the mean ± sem. The differences of growth among concentrations at 24 h were significant by one way ANOVA and Tukey-HSD post-test (*P < 0.05; **P < 0.01). **B.** The same number of initial HDF cells was incubated 48 h in the absence of β-glucan. Then, cells were treated with 400 μg ml^{-1} of β-glucan and viable cells (trypan-blue negative) were counted at 0, 24 and 48 h after β-glucan addition. Untreated cells were used as a control. The experiment was independently repeated three times. Cell number is represented as mean ± sem. Significance values were determined by one way ANOVA and Tukey-HSD post-test (**P < 0.01; ns, no significant). **C.** Representative images of BrdU staining. HDF cells were treated with 400 μg ml^{-1} of β-glucan and processed at 24 and 48 h after β-glucan addition. Untreated cells were used as a control. Eight hours before processing, 10 μM BrdU was added to the medium. Cells were fixed and processed for IF to detect BrdU incorporation. **D.** Quantification of BrdU staining of C. The percentage of BrdU-positive cells (mean ± sem) was obtained from three experiments. Significance values were determined by one way ANOVA and Tukey-HSD post-test (**P < 0.01). **E.** HDF cells were treated with 400 μg ml^{-1} of β-glucan, methylcellulose or amylopectin, and processed at 24 h after compounds addition. Untreated cells were used as a control. Eight hours before processing, 10 μM BrdU was added to the medium. Cells were processed as in C. The percentage of BrdU-positive cells (mean ± sem) was obtained from four independent experiments. Significance values were determined by one way ANOVA and Tukey-HSD post-test (*P < 0.05). BGN, β-glucan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

incorporation was obtained by counting green (BrdU-positive) and total nuclei (Hoescht).

2.12. Wound closure assay

The procedure performed in this study followed the European Union Guidelines for the Care and Use of Laboratory Animals, and it is according to the Law 5/1995 and the Decree RD53/2013, which regulate the use of animals for experimental and other scientific purposes (Catalonian Government), and it was certified by the Ethics Committee on Animal Experimentation from the University of Lleida (CEEA 03-03/13).

C57BL/6 wild type mice were punched in their lumbar skin and treated with water or a 30 mg ml^{-1} aqueous solution of barley β-glucan for two weeks. Photos of the wound were taken every three days, and the surface of the wound was estimated with Image J software.

3. Results

3.1. Characterization of the (1→3)(1→4)-β-D-glucan from barley grains

Total β-glucan content of lyophilized barley extract was determined using the Megazyme mixed linkage β-glucans assay kit. Purity was more than 95%, and similar to the Megazyme β-glucans standard.

Lyophilized barley β-glucan was analyzed by FTIR spectroscopy. Fig. 1A shows three broad absorption bands: one at the 3338 cm^{-1} region that can be assigned to the hydroxyl stretching vibration of the polysaccharide, indicating a strong O—H band; a second band at the region between 1,318–1,420 cm^{-1}, assigned to OH-bending; and a third broad band for polysaccharides at 1200–800 cm^{-1}. The peak between 2820 and 3000 cm^{-1} reflects a C—H stretching and, assigned to CH groups. The main peak at 1035 cm^{-1} can be assigned to C—O bonds of the alcohol groups. Peak shoulder at 1070 cm^{-1} and peak at 1154 cm^{-1} agree with the linear structure of β-glucan linked through 1–3 linkages (Wang, Yao, Guan, Wu, & Kennedy, 2005). Also, peak at 894 cm^{-1} is related to β(1–4) linkages (Zhang et al., 2018). These data agree with peaks present in carbohydrates (Limberger-Bayer et al., 2014).

When the spectrum of the purified β-glucan used in this study (red line) is compared with that of the pure β-glucan standard from Megazyme (blue line)they fully overlap, which agrees with the purity data obtained by spectrophotometric methods.

In Fig. 1C, the GPC plot of the β-glucan extract shows a main peak with a retention time of 16.7 min and a Mw of 64 kDa. This agrees with a low molecular weight β-glucan. There is also a second peak at 19.9 min, which corresponds to refractive index changes caused by dead volume.

3.2. Barley β-glucan triggers a temporary proliferation arrest in human dermal fibroblasts

We have tested the effects of purified barley β-glucan on the proliferation of primary HDFs. Cells were cultured in the presence of increasing concentrations of β-glucan, and cell number was determined at different time points. Unexpectedly, we observed a reduction in cell number after 24 h of incubation, but not at longer incubation times, in samples treated with a β-glucan concentration of 400 μg ml^{-1} (Fig. 2A). Cells recovered their proliferation rate 48 h after β-glucan addition (Fig. 2A and B). The number of dead cells was not significant (fewer than 1%) for all conditions independently of the treatment. These results suggested the existence of a short arrest in cell proliferation due to the β-glucan addition. We confirmed the existence of this arrest by analyzing

BrdU incorporation. Control and β-glucan-treated HDF cells were incubated with 10 μM BrdU for 8 h before processing the samples. The levels of BrdU incorporated in the nucleus of the cells were determined by immunofluorescence (Fig. 2C). The percentage of BrdU-positive cells was reduced at 24 h of β-glucan treatment as compared to untreated cells, and it was clearly recovered by 48 h (Fig. 2D). In a similar experiment, methylcellulose and amylopectin did not produce a significant reduction in the BrdU levels at 24 h treatment. Hence, barley β-glucan induces a specific short arrest in the proliferation of human fibroblasts.

Cell proliferation is finely regulated by the D-type cyclins (Ccnd) and their partners: the cyclin-dependent kinases 4 and 6 (Cdk4/6). Phosphorylation and subsequent inactivation of the retinoblastoma protein (RB) by Ccnd-Cdk4/6 promotes cell cycle entry and proliferation. We determined by immunoblot the levels of Ccnd1 and Cdk4 in barley β-glucan-treated fibroblasts. We did not observe downregulation of these proteins neither at 12 nor at 24 h after β-glucan addition (Fig. 3A). Moreover, the total and phosphorylated levels of RB at serine 780 were not altered at 24 h after β-glucan addition (Fig. 3B). Therefore, our results suggest that the proliferation arrest induced by barley β-glucan in HDF cells is not dependent on the Ccnd1-Cdk4-RB axis. However, Posas et al. (Gubern et al., 2016) have demonstrated that the inactivation of RB due to the Cdks can be blocked when RB is phosphorylated by the stress-activated protein kinase p38. Then, to study whether the proliferation arrest triggered by barley β-glucan was dependent on RB, we knocked-down the levels of RB in HDF cells by RNA interference (Fig. 3C). Cells expressing a shRNA against RB (shRB) or a scramble shRNA as a control (scr) were treated with β-glucan for 24 h. The proliferation rate was determined by BrdU incorporation. Treated cells with low levels of RB showed a percentage of BrdU incorporation similar to untreated cells (Fig. 3D). By contrast, treated cells expressing normal RB levels showed a proliferation arrest. Therefore, our results indicate that the proliferation arrest induced by barley β-glucan in HDF cells is dependent on RB protein. In spite of this, barley β-glucan does not produce a classical stress response in human fibroblasts. Osmotic stress triggers a rapid phosphorylation of the stress-activated protein kinase p38 (Gubern et al., 2016). In agreement with this, the sudden addition of NaCl to the medium caused a rapid phosphorylation of p38 in HDFa cells (Fig. 3E). However, we could not detect p38 phosphorylation after β-glucan addition (Fig. 3E and F).

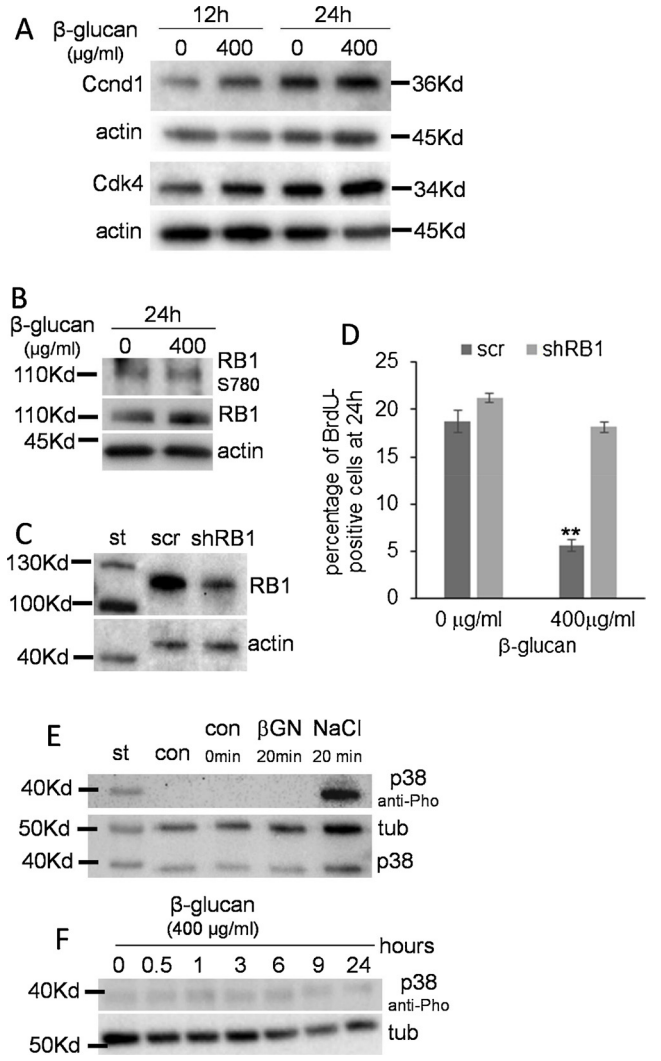

Fig. 3. The proliferation arrest induced by barley β-glucan is dependent on the retinoblastoma protein RB1. A. HDF cells were treated with β-glucan for 12 and 24 h. Total Ccnd1 and Cdk4 levels were determined by immunoblot. Untreated cells were used as a control. Actin was used as a loading control. **B.** In the same cells, after 24 h of β-glucan addition, the levels of total and phosphorylated RB at serine 780 were determined by immunoblot. **C.** Cells were infected with shRNA against RB (shRB1) or with a control shRNA (scramble; scr). After 72 h the levels of RB were examined by immunoblot. st, loading standard. **D.** The human fibroblasts from C were seeded and treated with β-glucan. After 24 h the proliferation rate was determined by BrdU incorporation. The percentage of BrdU-positive cells (mean ± sem) was obtained from three experiments. Untreated cells were used as a control. Significance values were determined by one way ANOVA and Tukey-HSD post-test (**P < 0.01). **E.** HDF cells were grown for 48 h under normal conditions (con). These cells were treated (con 0 min) with 400 μg ml^{-1} of β-glucan (βGN) or 500 mM NaCl. The levels of P-Thr-180 p38 were determined 20 min after treatment. Total p38 and tubulin (tub) were used as a loading control. st, loading standard. **F.** Time course showing the levels of P-Thr-180 p38 after β-glucan addition to human fibroblasts. Tubulin (tub) was used as a loading control.

3.3. Barley β-glucan promotes migration and hinders cell adhesion in human dermal fibroblasts

Our data show that in the first 24 h after barley β-glucan addition, HDF cells have a transitory arrest in proliferation. Following our study, we analyzed whether during this time β-glucan could induce other biological effects on these cells. First, we determined the contribution of β-glucan to the ability of human fibroblasts to migrate. To this purpose, HDF cells were seeded on the bottom side of a transwell membrane in

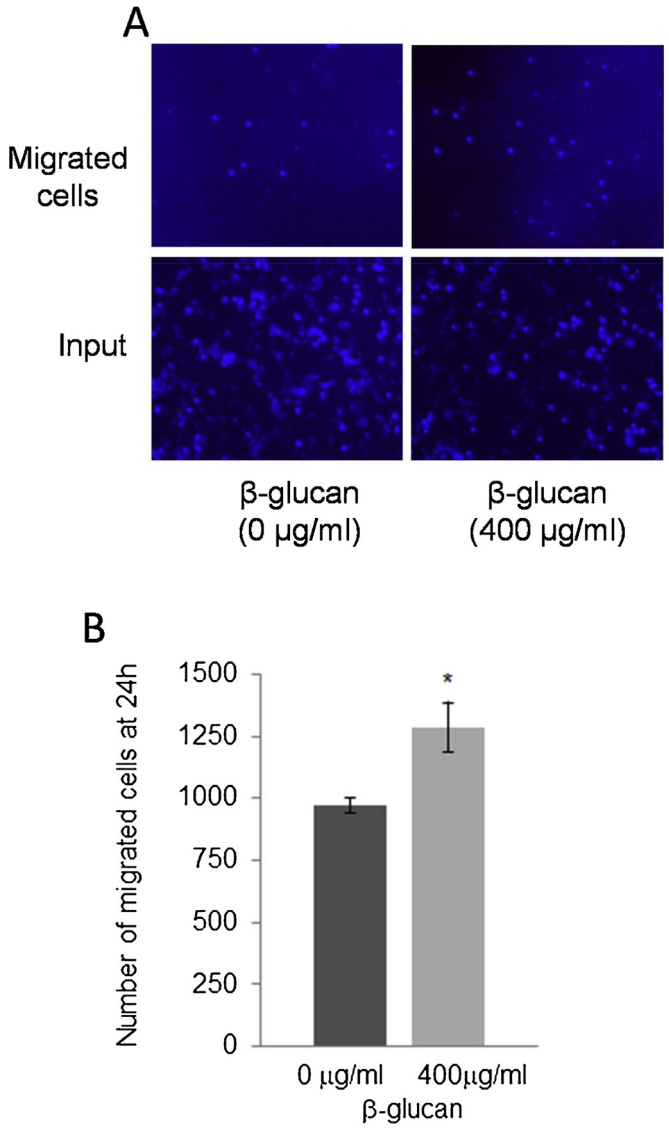

Fig. 4. Barley β-glucan promotes migration of human fibroblasts. A. HDF cells were seeded in Transwell filters and allowed to migrate for 24 h with and without β-glucan. To measure cellular migration, cells were fixed, stained with Hoescht and counted (migrated cells). Non-migrated cells were removed using a cotton applicator. Representative images of the experiments are shown. **B.** Quantification of the experiments in A. The values are expressed as mean ± sem. Data are from four experiments. Significance of the values were determined by the Mann-Whitney test (*P < 0.05).

the presence of medium without serum and with 400 μg ml^{-1} barley β-glucan. Serum-supplemented medium containing 400 μg ml^{-1} β-glucan was added to the upper side of the membrane. After 24 h of incubation, the cells that had crossed the membrane to the upper side were stained with Hoescht and counted (Fig. 4).

We observed that barley β-glucan treatment promoted fibroblasts migration (Fig. 4B). Thus, in the first 24 h after β-glucan addition, fibroblasts undergo a proliferation arrest but at the same time they improve their migration ability.

A characteristic frequently associated to the migratory potential is the ability of cells to disengage from the ECM (Gardel, Schneider, Aratyn-Schaus, & Waterman, 2010). Thus, we reasoned that β-glucan treatment could also affect the ability of fibroblasts to adhere and spread on external surfaces. To test this, we seeded HDF cells on barley β-glucan-coated plates and compared the spreading efficiency when seeded on uncoated plates or on fibronectin-coated plates. As expected,

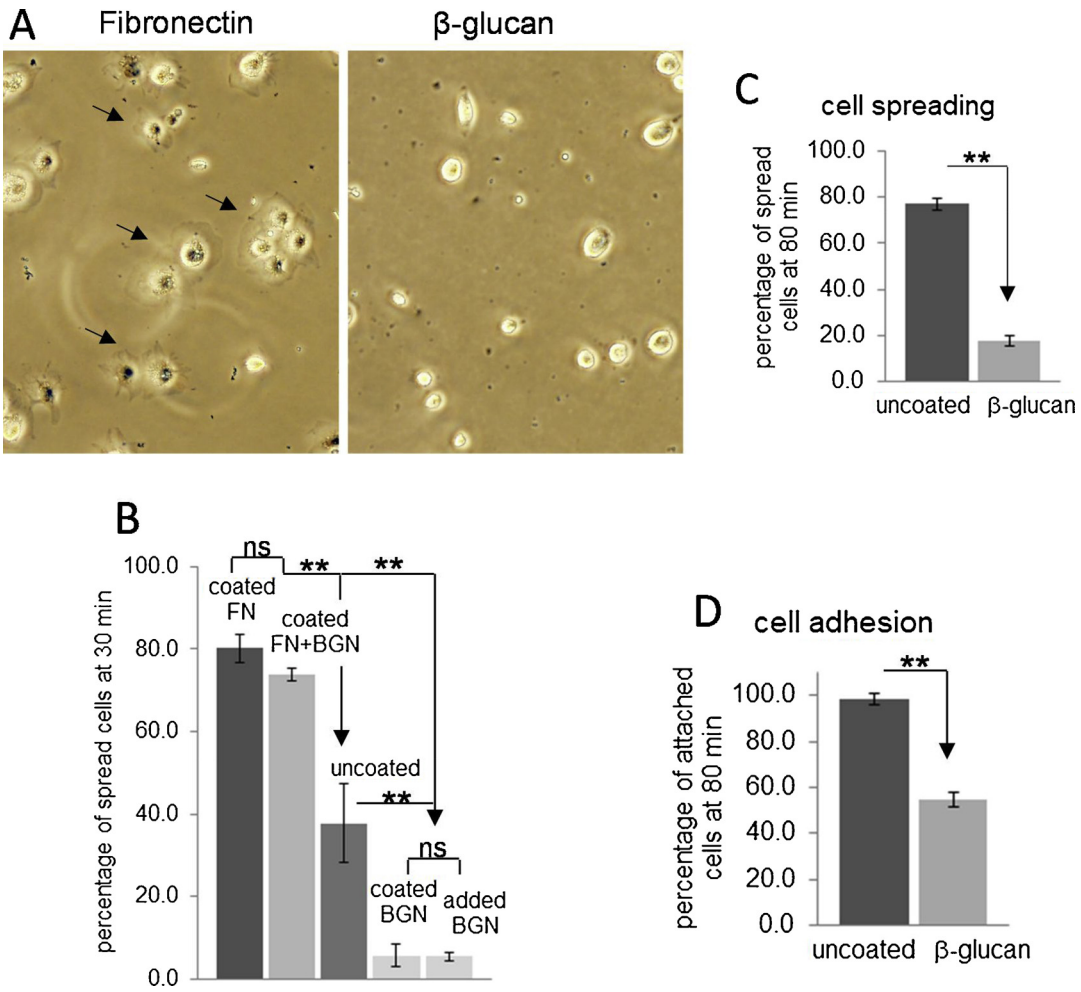

Fig. 5. Barley β-glucan hinders fibroblasts adhesion and spreading. A. HDF cells were trypsinized and seeded in serum-free medium on plates coated with 5 μg ml^{-1} fibronectin (FN) or 400 μg ml^{-1} β-glucan (BGN) or on uncoated plates. For double coating FN + BGN, a mixture of both solutions were used. For uncoated plates, BGN was also added to the medium before seeding (added BGN). Thirty minutes after seeding, the proportion of spread cells was determined. Representative images of HDFs seeded on fibronectin and β-glucan. Arrows show fibroblasts with spread morphology. **B.** Quantification of the experiments in A. Data (mean ± sem.) are from three experiments. Significance values were determined by one way ANOVA and Tukey-HSD post-test (**P < 0.01). **C.** Percentage of spread HDF cells at 80 min after seeding on β-glucan-coated or uncoated plates. Values are expressed as mean ± sem from three experiments. Significance of the values were determined by *t*-test (**P < 0.01). **D.** Percentage of HDF cells attached to the plates at 80 min after seeding on β-glucan-coated or uncoated plates. Values are expressed as mean ± sem from three experiments. Significance of the values were determined by *t*-test (**P < 0.01).

30 min after seeding most fibroblasts were spread on fibronectin-coated plates and a fraction of the cells on uncoated plates (Fig. 5A and B). By contrast, the percentage of spread fibroblasts in β-glucan-coated plates was extremely low (Fig. 5B). Most fibroblasts remained round and bright on β-glucan coated plates (Fig. 5A). The addition of β-glucan in the medium produced the same low efficiency of spreading after seeding cells on uncoated plates (Fig. 5B). To test whether β-glucan interfered in the interaction of cells with the ECM, we also analyzed the spreading ability of the fibroblasts in a double coating, fibronectin and β-glucan. This experiment showed that barley β-glucan does not interfere in the adhesion of cells to fibronectin (Fig. 5B). Next, we analyzed spreading and adhesion at longer times on uncoated and β-glucan-coated plates. In these experiments, the presence of β-glucan also reduced the efficiency of spreading (Fig. 5C) and adhesion (Fig. 5D) of human dermal fibroblasts.

3.4. Barley β-glucan accelerates wound closure in vivo

Our results suggest that barley β-glucan favors fibroblasts migration versus proliferation during an early period of the treatment. Since an early response in the wound healing process is the migration of fibroblasts to the wound, we expected that barley β-glucan would improve wound closure in vivo. To test this, a group of 10 C57BL/6 mice were used in a wound-healing test. Two full-thickness dorsal wounds were made on each mouse under general anesthesia and clean conditions. One group of mice was topically treated with barley β-glucan diluted in water (30 mg ml^{-1}) every three days, and another group (control) was treated in parallel but only with water. The wound remained uncovered during all the treatment. Wound size measurements were performed at five time points over 12 days. Fig. 6A shows representative images of wounds at different times. In both groups of mice, wounds were almost totally closed 12 days after their infliction. However, the speed at which wound closure took place was significantly increased in the barley β-glucan-treated group compared to the control group (Fig. 6B). Hence, β-glucan produced and purified from β-glucan-enriched barley grains is able to ameliorate the wound healing process in vivo.

4. Discussion

In this work we show that barley β-glucan triggers a short proliferation arrest in human dermal fibroblasts, which appears to be in

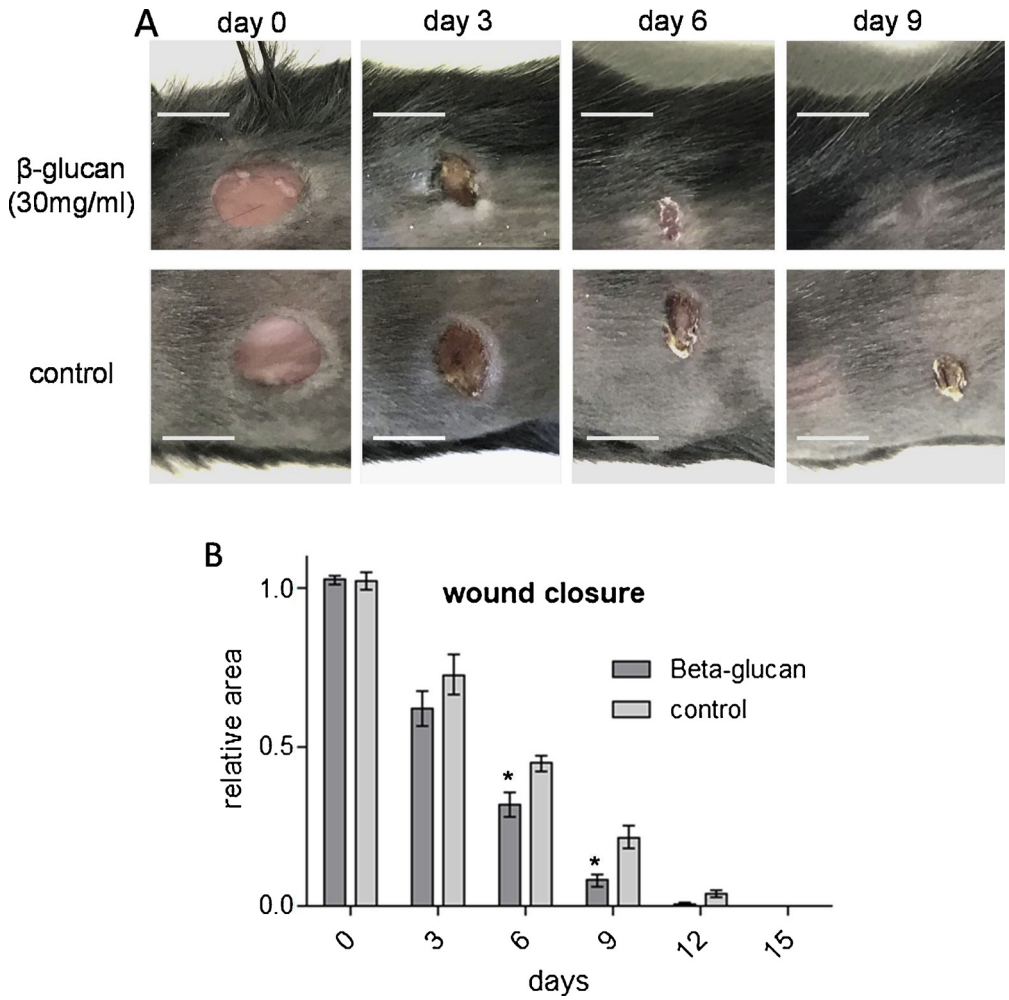

Fig. 6. Barley β-glucan improves wound closure in vivo. A. Appearance of the wounds in mice at 0, 3, 6, and 9 days after treatment with barley β-glucan or with water as a control. **B.** Quantification of the wound area. Values are expressed as mean ± sem from β-glucan-treated group (n = 9) and from untreated group (n = 6). Statistical significance was determined by a two-way ANOVA test (β-glucan treatment $p < 0.01$; days $p < 0.01$) and Bonferroni posttests (*$p < 0.05$).

contradiction with a previous report showing that β-glucans promote cell proliferation in different cell types (Majtan & Jesenak, 2018). To reconcile these observations, we need to consider that barley β-glucan induces time-dependent responses. At short term, 24 h after barley β-glucan addition, we observed an arrest in proliferation, but we also observed a complete recovery at 48 h. This β-glucan hallmark has not been reported before because most works in the field have analyzed β-glucans long-term proliferation responses. However, data from at least two different reports invite to consider a negative effect of fungal β-glucan on the proliferation of HDFs and rat cells after 24 h of treatment (Basha, Sampath Kumar, & Doble, 2017; Woo et al., 2010). At long term, from 48 h onwards, barley β-glucan does not significantly increase proliferation in primary HDFs. By contrast, several published works have shown that β-glucans promote long term cell proliferation when used in a similar range of concentrations (Basha et al., 2017; van den Berg et al., 2014; Woo et al., 2010). This contradictory result may be explained by the various β-glucans used as well as by cell-type differences. For example, it has been shown that (1→3),(1→6)-β-glucan promotes long term cell proliferation in mouse connective tissue cells (Son et al., 2005) but, as in our case, it does not change long term proliferation in primary HDFs (Son et al., 2007). Thus, our conclusion is that barley β-glucan can induce short term and long term responses on the control of cell proliferation. In our primary fibroblasts, barley β-glucan triggers an RB-dependent short-term response and has no significant effects on long term proliferation.

RB is a key negative regulator of cell cycle entry and proliferation. This protein inhibits E2F, a major transcriptional regulator of the cell-cycle gene network (Sherr, Roberts, Sherr, & Roberts, 2004). Cyclins D and cyclin E complexed with their respective Cdks phosphorylate and inactivate RB, releasing E2F function. Under osmotic stress conditions, the activated-p38 kinase phosphorylates RB and bypasses de effects of Cdks, producing a proliferation arrest (Gubern et al., 2016). However, this cannot account for the arrest we report here, because in our experiments barley β-glucan did not trigger p38 activation neither at shorter nor at longer times. Moreover, the proliferation arrest was not detected after addition of other control polysaccharides such as methylcellulose or amylopectin. Then, it is not a general shock response due to the polysaccharide addition. Even though we cannot rule out that other polysaccharides have the same effect as β-glucan, we assume that the response of HDFs to barley β-glucan depends on β-glucan-specific receptors. At the moment, the signal pathways involved are under study.

Barley β-glucan promotes cell migration in most cell types tested to date (Majtan & Jesenak, 2018). Treating fibroblasts with barley β-glucan we have also confirmed this phenotype. Cell movement requires the coordination of various processes, and among them, the control of cell adhesion is key. Concerning this, cells with better migration capacities show reduced cell–matrix attachment efficiencies (Li et al., 2006; Neumeister, 2003). Since barley β-glucan promoted cell migration in HDF cells, we questioned whether it was involved in the control of cell adhesion to the ECM. In our experiments, barley β-glucan did not alter the spreading efficiency of HDF cells on fibronectin-coated plates.

It is well known that the interaction of membrane proteins such as integrins with ECM proteins such as fibronectin triggers different signal pathways involved in the regulation of cell adhesion and spreading (Huveneers & Danen, 2009). Then, barley β-glucan does not seem to be a broad and major regulator of cell attachment to the ECM. However, barley β-glucan reduced adhesion and spreading efficiencies of HDF cells on treated-polystyrene plates. Then, this result suggests that β-glucan is somehow also connected to the cell-surface attachment and spreading processes. Further work will be required to elucidate whether these phenotypes are a consequence of the physical interaction of barley β-glucan with the plastic surface, or alternatively, β-glucan causes specific anti-adherent responses through receptor-dependent signaling.

5. Conclusions

β-glucans have a broad spectrum of effects on different cell types that can explain their proficiency on healing (Majtan & Jesenak, 2018). Moreover, different clinical trials report that topical application of fungal β-glucan ameliorates healing of diabetic and venous ulcers (Medeiros et al., 2012; Zykova et al., 2014). In this work we contribute new data showing that barley (1→3),(1→4) β-glucan induces an early response in HDF cells favoring movement versus proliferation, and accelerates wound closure in vivo. This is consistent with the wound healing process whereby fibroblasts initially migrate to the wound and later proliferate and produce ECM (Stadelmann et al., 1998).

Declarations of interest

None.

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