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Growth Inhibition of Mesenchymal Stem Cells by Laminarin: Impact on Chondrocyte Differentiation

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Abstract

Beta-(1 → 3)-glucans constitute highly promising biomolecules as evidenced by their immunostimulating ability which were first used as folk medicine in Chinese populations and further identified in fungi, yeasts and seaweeds. Previous investigations showed that glucans proteins interaction mediate cell growth pathways. We have adopted this approach to study the effect of laminarin, a beta-(1 → 3)-D-glucan, on Mesenchymal Stem Cells (MSCs) proliferation and differentiation. MSCs were cultured in MSC growth and chondrogenic differentiation mediums. Proliferation rate and apoptosis were explored by cell count, MTT assays and Annexin V staining. mRNA and protein expression of specifics markers for MSCs and chondrocytes were studied using qPCR and immunofluorescence. Results showed that laminarin treatment reduced MSCs proliferation in both growth and chondrogenic mediums ($p<0.05$). Annexin V staining showed no apoptosis. MSC cells in growth medium showed no impact of laminarin for Thy1, nucleostemin and endoglin mRNA analysis. Conversely, in chondrogenetic medium, laminarin had a negative effect on Thy1 levels and no change in nucleostemin and endoglin. Collagen II responded positively in chondrogenitic medium in absence of laminarin and significantly reduced when laminarin was added ($p<0.05$). These results indicate that laminarin inhibited both cells proliferation and chondrogenic differentiation suggesting potential clinical applications in MSC therapy.

Keywords: Arthritis; Cells differentiation; Cartilage; Cells proliferation

Introduction

Natural products, useful in preventing and treating various diseases, have been sought after for centuries. Since 1960, several studies highlighted the medicinal capacity of glucans [1-4] which are polysaccharides coming from natural sources including yeasts, mushrooms, algae, cereals or bacteria. Specifically the laminarin used in this study, a storage glucan found in brown algae, is a polymer composed by a (1 → 3) linear β-glycosidic chain core. Variations come from their length and branching structures [5,6]. These branching assignments appear to be specific; for example, β-glucans of fungus have (1 → 6) side branches whereas those of bacteria have (1 → 4) side branches. With regard to their different sources, they have different size, shape and viscosity making the rationalization of their biochemical effects complicated [7]. Likewise, glucans are involved in various biological processes such as immunostimulation effects, suppressing cancer cells development [8,9] and drug delivery [4]. It was shown that glucans modulated immunes effects regardless of their structural complexity such as β(1 → 3), β(1 → 4) or β(1 → 6) linkages Interestingly, various studies have pointed out that higher degree of structural complexity is coupled with higher immunomodulatory, anti-oxidant, and anti-cancer effects [3,6, 10-16]. However these effects are not the only ones. In a recent study, the impact of β-glucan gavage in a osteoarthritic (OA) rat model was evaluated [17]. It was shown that β-glucan treatment induced a decrease of OA severity associated with an increase in chondrocyte proliferation [17]. Similar results were obtained in a dog's model of OA [18]. Authors showed that the β-(1 → 3, 1 → 6)-glucan administration significantly improved the OA severity (decrease of stiffness, lameness and pain) [18]. Although these two last studies suggest that β-glucan is capable of decreasing OA severity [17], the cellular mechanisms of action of β-glucan and the impact of β-glucan stimulation on MSC, the chondrocyte progenitor, are still unknown.

Several in vitro and in vivo studies have suggested that MSCs from various adult tissues including bone marrow [19], adipose tissue [20-24] and synovium [25-27] restore functions, remodel injured tissue and can reverse cellular damage [28,29]. The use of MSCs in "tissue repair" is due to their ability to self-renew, to adhere to plastic and to differentiate into mesenchymal lineages [21,28,30-32]. For this reason, MSCs are considered as a great potential in cartilage tissue engineering [33,34] and currently show the most encouraging results for the treatment of articular cartilage lesions [21,35-39]. Yet, the use of MSCs for making stable chondrogenic differentiation remains challengeable. Such constraint is partially due to the hypertrophic potential of MSCs and to the unclear profile in their gene expression compared to differentiated cells [40-43]. Thus, influencing MSCs phenotype and modulating MSCs responses remain an important task in research related to tissue repair. Various studies have shown that activation of Toll-like receptors (TLR) pathway, scavenger receptor pathway or growth factor pathway can influence the capacity of these cells to differentiate into bone, fat or cartilage by increasing or decreasing their capacity [44-47]. Therefore understanding how different agonists of

these pathways may influence the MSCs responses could help to identify new molecules with a beneficial impact on MSCs, which can be used in the repair of tissues. Hence, we have questioned whether laminarin could have an effect on MSCs proliferation and differentiation. In this study, we specifically investigated the MSCs response to four difference doses of laminarin under growth and chondrogenetic mediums.

Materials and Methods

Ethics information

The study was approved by the Ethical Committee of Val de Loire, Tours, France (CEEA Vdl: 2011-11-2). Two male Wister rats (six-week old) were purchased from JANVIER-LABS, then immediately sacrificed and bone marrow (from tibia and femur) was harvested. All experimental procedures were in accordance with the guidelines of the French national committee for consideration of ethics in animal experimentation.

Rat mesenchymal stem cells

Six-week old male Wistar rats (JANVIER-LABS) were euthanized by intra-peritoneal (IP) injection of pentobarbital (40 mg/kg; Ceva Sante animal, Libourne, France). MSCs were isolated from the bone marrow as previously described [51]. Cells were collected by flushing the marrow of femur and tibia with α-MEM medium (Gibco™, Courtaboeuf, France). Then cells were washed twice in PBS and seeded in α-MEM medium supplemented with FBS 10%, glutamine 1%, PS 1%, and fungizon 0.1% at 5000 cells/cm^2 (Gibco™, Courtaboeuf, France). At 80% confluence, cells were dissociated by trypsin treatment (Gibco™, Courtaboeuf, France) and cultured in order to obtain sufficient numbers of cells. Media were changed three times per week. To demonstrate that the population, we purified, was MSCs in nature, the markers expression of MSCs namely CD90 and Nucleostemin was tested. This was also tested before each experiment to ensure the phenotype of the MSCs. We also tested markers for hematopoietic stem cell.

Laminarin treatment and Chondrogenic differentiation protocol

Laminarin (Carbosynth, Berkshire, UK) was used to stimulate cells. Typically, MSCs were dissociated by trypsin treatment and seeded in 6 wells at 1000 cells / cm^2 in α-MEM medium supplemented with FBS 10%, glutamine 1%, PS 1%. Forty eight hours after, laminarin (10, 100, 1000 µg/ml) was added into the α-MEM medium supplemented with FBS 10%, glutamine 1%, PS 1% or in chondrogenic medium (DMEM/F-12 supplemented by FBS 10%, PS1% 1; 10^{-7}M dexamethasone, 1 µM ascorbate-2-phosphate, L-proline (Sigma-Aldrich®, St. Quentin Fallavier, France); 1% sodium pyruvate, ITS 1X and 10 mg/ml TGF-β1 (Gibco™, Courtaboeuf, France). Cells were maintained in culture up to 21 days. Media were changed three times per week.

MTT assay (3-[4,5-Dimethyl-2 thiazoyl]-2,5-diphenyl-2H-tetrazolium bromide)

Cells were plated in 96-well multiplates at a density of 10^4 cells/well. Twenty-four hours after the initial seeding, cells were either treated with laminarin (10, 100 and 1000 µg/ml) for 24 hrs or left untreated. Then 5mg/ml of MTT (Sigma-Aldrich®, St. Quentin Fallavier and France) was added to the culture medium and cells were incubated for 4 h more. Following incubation, each well was emptied and 100 µl SDS-acidic-isopropanol buffer (0.5% SDS, 80 mM HCl; Sigma-Aldrich®, St. Quentin Fallavier, France) were added. After an incubation of 15 minutes at room temperature under smooth agitation, the plate was read immediately in a plate reader (Multiscan Go, Thermo Scientific, Courtaboeuf, France) at a wavelength of 540 nm using a wavelength of 620 nm as background reference.

NIR epifluorescent microscopy of MSCs

MSCs were cultured in α-MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% of L-glutamine (GlutaMAX) and 1% of streptomycin/penicillin antibiotics. Then cells were seeded in an 8-well Lab Tek Chamber coverglass (Nunc, Dutsher S.A, Brumath, France) at a density of 5×10^4 cells/well and cultured at 37°C in 5% humidified CO_2 atmosphere. After 24 hours, cell culture media were removed and cells were incubated with a 100 µM solution of etoposide and 0, 10, 100 or 1000 µg/ml of laminarin during the next 24 hrs. Further, cells were washed and incubated with Annexin V-FITC fluorescent probe (APOAF, SIGMA) in binding buffer for 15 min, followed with additional washing with OPTIMEM media. The cells were observed with a Zeiss Axio Observer Z1 fluorescence inverted microscope (Zeiss, Le Pecq, France) equipped with an ORCA-R2 high resolution CCD camera. The light source, Zeiss HXP 120, was combined with the FITC filter cube.

Nucleostemin and Collagen II staining

Immunofluorescence was performed on cells after laminarin treatment. Cells were fixed in paraformaldehyde 4% for 20 min or in methanol for 5 min and permeabilized for 10 min at room temperature with PBS-Triton 0.1% (Triton 100x, Acros organics, Geel, Belgium). Cells were incubated with anti-Nucleostemin (1:100; ab 70346, Abcam®, Paris, france) or anti-Collagen II (1:50; sc-28887, Santa Cruz Biotechecnology®, Inc) overnight at 4°C in a humid chamber. To detect the presence of antigens, cells were incubated for 1h at room temperature with the 488 or 549- conjugated anti-rabbit antibodies (1:2.000; Rochland, Limerick, Ireland). Nucleus were stained with DAPI (Life technologies, Courtaboeuf,France). Pictures were taken under the x40 magnification with a BA400 microscope (Motic® Wetzlar, Germany). The cells incubated with a control rabbit or mouse immunoglobulin showed no staining.

mRNA expression/quantitative real-time PCR

Total RNA were extracted using RNeasy mini kit (Qiagen, Paris, France) according to the manufacturer's instructions. The extracted RNA was then reverse-transcribed into single stranded cDNA, using Quanti Tect Reverse Transcriptase kit (Qiagen, Paris, France) according to the manufacturer's instructions. Quantitative RT-PCR amplification was carried out with the CFX96 Real Time System (Bio-Rad, Marnes-la-Coquette, France).

Cells were tested on the expression of CD105 (Endoglin), CD90 (Thy1), Nucleostemin (Gln3), PECAM1/CD31, CD34, CD45, Collagen II, Aggrecan (Table 1). Following their design, the primers were synthesized by Invitrogen™ (ThermoFisher Scientific, Courtaboeuf, France). The expression of the housekeeping gene RPL13a (QT00178675, Qiagen, Paris, France) was used to normalize gene expression in the analysis. The $2^{-\Delta\Delta Ct}$ method was used for relative gene expression analysis.

Primers	Forward	Reverse
Eng	CCCTGATTCAGCCAAAGTGT	GGTGCTACTCAGGACAAGGTG
Gnl3	AGCCCTGATGATGAGCAATC	ACTTGAGGACACCTGCAACC
Thy1	AGCTATTGGCACCATGAACC	AGGCTGAACTCATGCTGGAT
CD31	GAAGGTTTCTGAGCCCAGTG	CATCTGCCTTGGCTGTCTTA
CD34	GGGAGACATCAAATGTTCAGG	GGCCTCAGTCTCCTCCTTTT
CD45	CTTTGCAAGCAATACCACCA	AGCCATTGGAGAGAGTGACG
Col2a1	GTTCACGTACACTGCCCTGA	GGCCCTATGTCCACACCAAA
Acan	TCTTTGTGACTCTGCGGGTC	AGTGGTCACAGGATGCATGC

Table 1: List of gene specific primers used for quantitative RT-PCR.

Results

Characterization of MSCs

Cells obtained from the bone marrow of six-week-old male wistar rats were purified and isolated. After 4 passages, quantitative RT-PCR experiments showed that Nucleostemin, Thy1 and Endoglin were expressed positively (FigureS1A) whereas endothelial (CD31) or hematopoietic (CD45 and CD34) antigens expressions were negative (Figure S1B). In addition, Nucleostemin and Thy1 by immunofluorescence stained positively (Figure S1C).

Effect of laminarin on the proliferation of MSCs

In order to explore MSCs potential under laminarin stimulation in vitro, cells were seeded in 25 cm^2 flasks at a number of 105 then treated with 0, 10, 100 or 1000 µg/ml of laminarin for 7 days. Laminarin concentrations were chosen according to previous studies [8]. Our results showed that after 24 hours of laminarin treatment cells number significantly decreased compared to untreated cells (Figure 1). The rates of treated cells with 1000 µg/ml of laminarin have decreased significantly about 4 times compared to untreated ones and about 2 times for cells treated with 100 µg/ml of laminarin compared to untreated ones. In addition we also observed a slight decrease for cells treated with 10 µg/ml of laminarin compared to untreated ones (Figure 1).

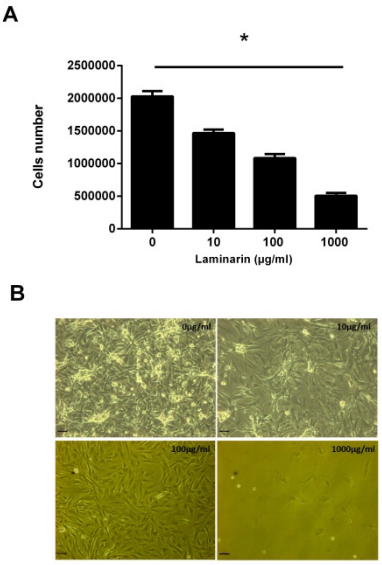

Figure 1: MSCs numbering after 7 days of laminarin treatment. (A) Counting of cells at day 7. Data (n=3 biological replicates) are presented as mean ± SEM (*p<0.05). (B) Microscope imaging of MSCs at day 7. Cells were cultured in MSC medium and treated with laminarin (0, 10, 100 and 1000 µg/ml) for 7 days. (Scale bar 20 µm).

Effect of laminarin on the viability of MSCs

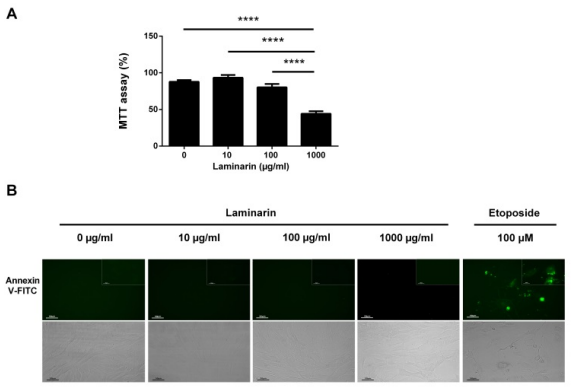

Figure 2: MSCs viability and proliferation. (A) Effect of difference concentration of laminarin on the cellular proliferation of BMSCs. Data (n=6 biological replicates) are presented as mean ± SEM (*p<0.05). (B) Effect of difference concentration of laminarin on the cellular apoptosis of MSCs. Cells were cultured in MSC medium then treated with laminarin (0, 10, 100 and 1000 µg/ml) and ectoposide 100 µM for 24h. (Scale bar 50 µm for 20x magnification and 20 µm for 63X magnification).

In order to explore the effect of laminarin on MSCs, the viability/ proliferation rate was explored by MTT assay (Figure 2A). After 24

hours of laminarin treatment (1000 µg/ml), cells number significantly decreased compared to untreated cells. As the MTT test does not indicate only the cell viability but also the proliferation rate, we investigated the cells death under laminarin supplementation. Trypan blue staining showed no significant difference in MSC cells in growth medium with laminarin compared to without (data not shown). Apoptosis was explored using an Annexin-FITC fluorescent probe. Results from epifluorescence microscopy showed no staining for cells in growth medium supplement with laminarin compared to the positive control, cells treated with ectoposide (Figure 2B).

Effect of laminarin on MSCs markers after treatment for 7 days

Expression analysis of mRNA of nucleostemin, Thy1 and endoglin showed no significant difference between untreated and laminarin treated cells (Figure 3A). These results were mirrored for the immunofluorescence. Yet, most of laminarin treated cells (all concentrations) were Nucleostemin positive in the nuclei (Figure 3B).

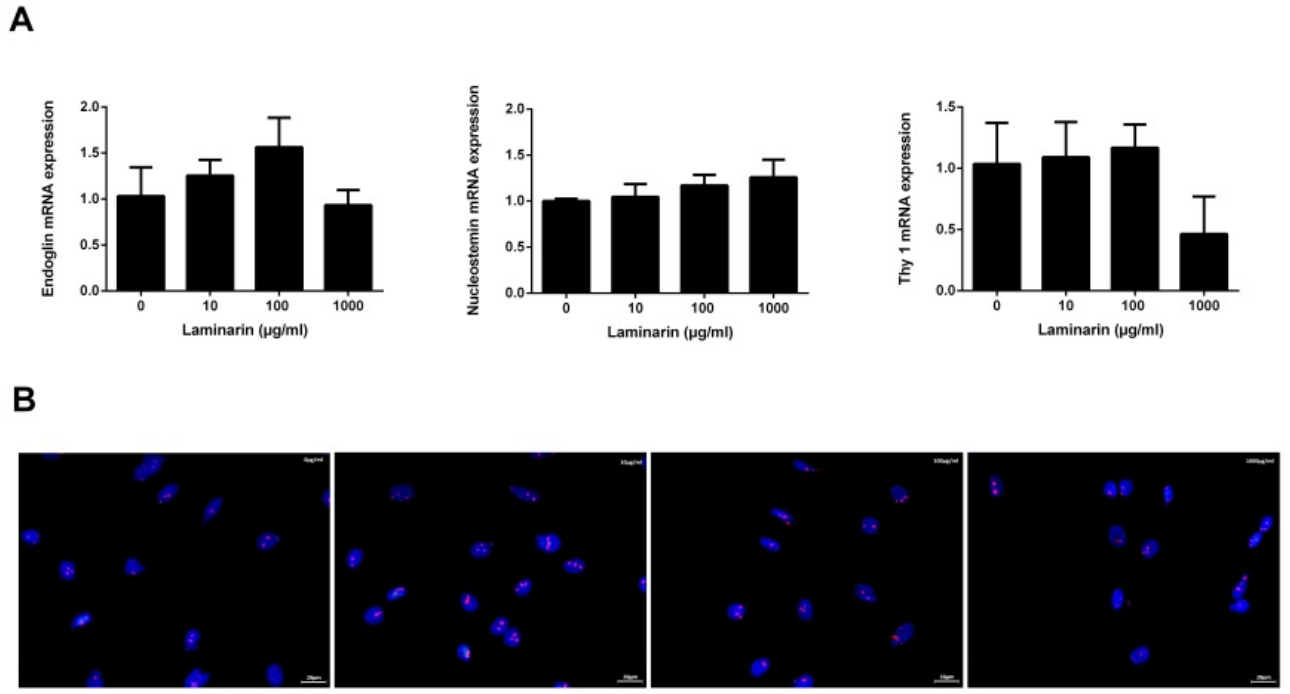

Figure 3: MSCs markers under laminarin treatment. (A) Effect of laminarin on MSC-defining markers (Nucleostemin, Endoglin and Thy1) of MSCs. Cells were cultured in non-differentiation medium and treated with laminarin (0, 10, 100 and 1000 µg/ml) for 7 days. Data (n=3 biological replicates) are presented as mean ± SEM. (B) MSCs immunophenotyping at day 7. Red: Nucleostemin; blue: DAPI.

Effect of laminarin on MSCs chondrogenic differentiation and proliferation

7 days: Under chondrogenic cell culture conditions, MSCs were seeded in 6 wells plates at a number of 104 then treated with 0, 10, 100 or 1000 µg/ml of laminarin. After 7 days of chondrogenesis, the number of treated cells with laminarin 1000 µg/ml was significantly decreased by 4 times compared to untreated ones ($p<0.05$). Similarly when cells were treated with laminarin at 100 µg/ml compared to untreated ones, they decreased only by 3 times without reaching the level of significance (Figure 4A and 4B). Compared to untreated cells, quantitative RT-PCR assay showed a significant decrease in the mRNA level of Thy1 for all conditions of laminarin concentration. Whereas Nucleostemin and Endoglin mRNA expression for all treated cells did not show a significant difference (Figure 4C). However, cartilaginous markers Collagen II and Aggrecan were significantly higher for all laminarin concentrations ($p<0.05$) (Figure 4D). Immunophenotyping microscopy showed a markedly higher positive Nucleostemin staining for cells treated with laminarin 100 and 1000 µg/ml compared to untreated and 10 µg/ml laminarin treated cells (Figure 4E).

14 days: After 14 days of cell chondrogenic culture process, the number of treated cells with laminarin at 1000 µg/ml was significantly decreased by 8 times compared to untreated cells ($p<0.05$). However the number of treated cells with laminarin 10 or 100 µg/ml decreased slightly compared to untreated cells without showing a significant difference (Figures 5A and 5B).

For MSCs markers, quantitative RT-PCR assay showed a significant decrease in the mRNA level of Thy1 for all conditions of laminarin concentration. Whereas Nucleostemin and Endoglin mRNA expression for all treated cells did not show a significant difference (Figure 5C). However, chondrogenic markers Collagen II and Aggrecan were significantly higher for all laminarin concentrations ($p<0.05$) (Figure 5D). Immunophenotyping microscopy showed a higher positive Nucleostemin staining for cells treated with laminarin 100 and 1000 µg/ml compared to those untreated and with 10 µg/ml laminarin treated cells. However Collagen II signal was markedly

higher in untreated and 10 µg/ml treated cells compared with laminarin at 100 and 1000 µg/ml respectively (Figure 5E).

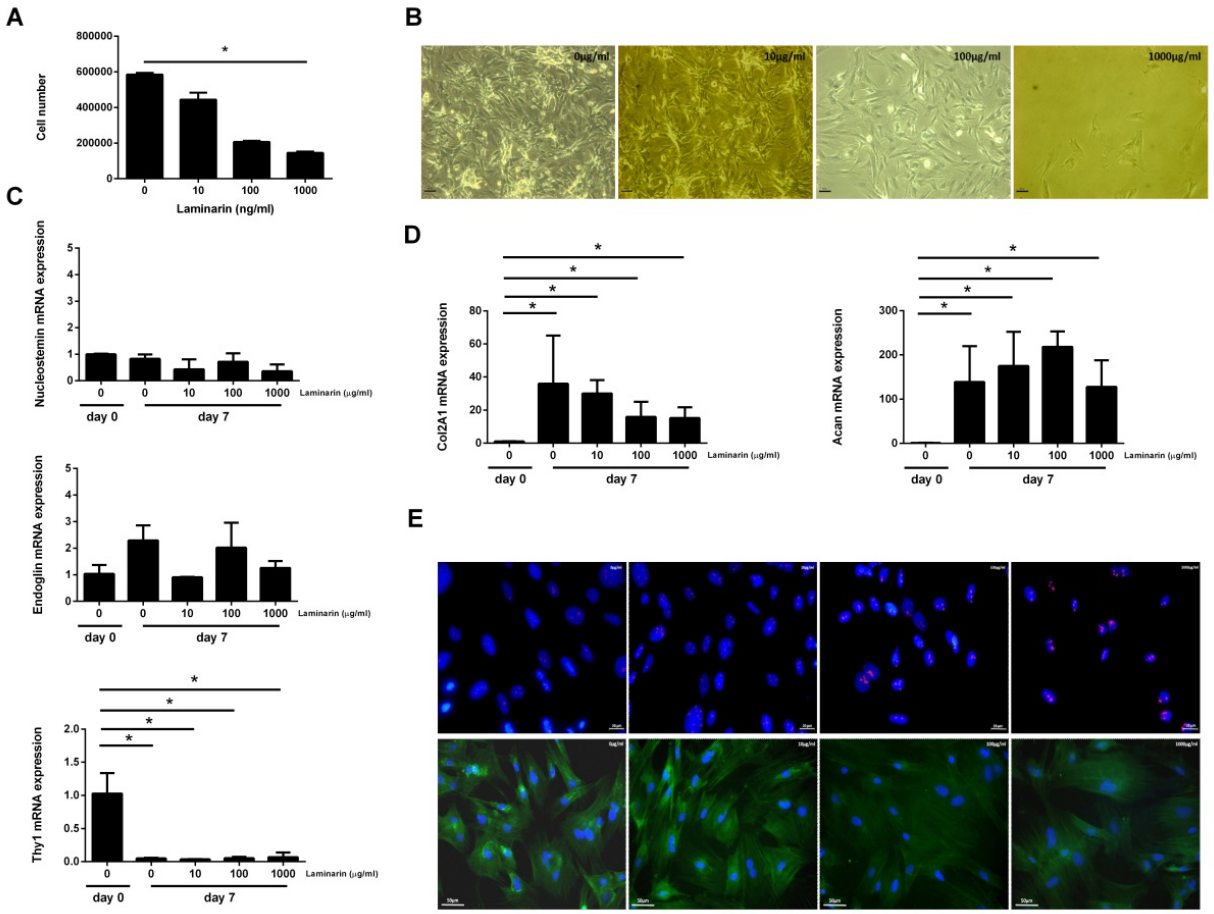

Figure 4: MSCs differentiation after 7 day under laminarin treatment. (A) Counting of cells at day 7 of chondrogenic differentiation. Data (n=3 biological replicates) are presented as mean ± SEM (*p<0.05). (B) Microscope imaging of MSCs at day 7 of chondrogenic differentiation (scale bar 20 µm). (C) Effect of laminarin on MSC-defining markers (Nucleostemin, Endoglin and Thy1) of MSCs during chondrogenic differentiation. Cells were cultured in chondrogenic medium and treated with laminarin (0, 10, 100 and 1000 µg/ml) for 7 days. Data (n=3 biological replicates) are presented as mean ± SEM (*p<0.05). (D) Effect of laminarin on chondrocyte markers (Collagen II and Aggrcan) of MSCs during chondrogenic differentiation. Cells were cultured in chondrogenic medium and treated with laminarin (0, 10, 100 and 1000 µg/ml) for 7 days. Data (n=3 biological replicates) are presented as mean ± SEM (*p<0.05). (E) MSCs immunophenotyping at day 7 of chondrogenic differentiation. Nucleostemin, red; Collagen II, green; DAPI, blue.

21 days: Microscopic visual observations suggested a huge difference in cellular proliferation numbers between laminarin treated and untreated cells especially among 0 and 1000 µg/ml concentration (Figure 6A). Unfortunately, these observations could not be confirmed quantitatively due to the high number of cells at day 21 and also because cells formed multiple layers. Quantitative RT-PCR of MSCs markers expressions showed that the mRNA level of Thy1 significantly decreased for all laminarin concentrations compared to day 0. Whereas Nucleostemin and Endoglin mRNA expression for all treated cells did not show a significant difference ($p<0.05$) (Figure 6B). In cells treated with 0, 10 and 100 µg/ml mRNA Expression of Collagen II showed a significant increase but no significant difference was found for 1000 µg/ml laminarin treated cells. Whereas Aggrecan mRNA level increased significantly for all treated cells compared to control ($p<0.05$) (Figure 6C). Immunophenotyping microscopy showed a higher positive Nucleostemin staining for cells treated with laminarin 100 and 1000 µg/ml compared to both untreated and 10 µg/ml laminarin treated cells. However Collagen II signal was markedly higher in untreated and 10 µg/ml treated cells compared to laminarin 100 and 1000 µg/ml treated cells (Figure 6D).

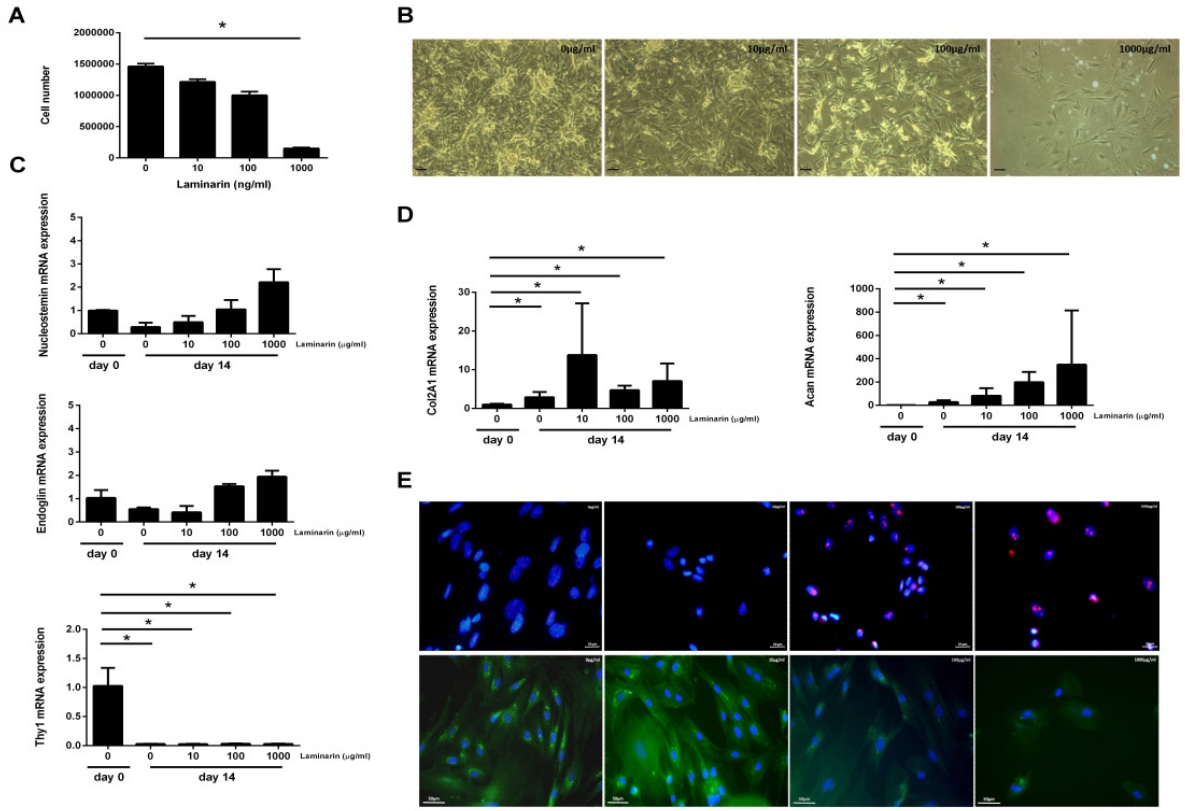

Figure 5: MSCs differentiation after 14 day under laminarin treatment. (A) Counting of cells at day 14 of chondrogenic differentiation. Data (n=3 biological replicates) are presented as mean ± SEM ($^{*}p<0.05$). (B) Microscope imaging of MSCs at day 14 of chondrogenic differentiation (scale bar 20 µm). (C) Effect of laminarin on MSC-defining markers (Nucleostemin, Endoglin and Thy1) of MSCs during chondrogenic differentiation. Cells were cultured in chondrogenic medium and treated with laminarin (0, 10, 100 and 1000 µg/ml) for 14 days. Data (n=3 biological replicates) are presented as mean ± SEM ($^{*}p<0.05$). (D) Effect of laminarin on chondrocyte markers (Collagen II and Aggrecan) of BMSCs during chondrogenic differentiation. Cells were cultured in chondrogenic medium and treated with laminarin (0, 10, 100 and 1000 µg/ml) for 14 days. Data (n=3 biological replicates) are presented as mean ± SEM ($^{*}p<0.05$). (E) MSCs immunophenotyping at day 14 of chondrogenic differentiation. Nucleostemin, red; Collagen II, green; DAPI, blue.

Discussion

MSCs have the remarkable potential to develop into different specialized cells in the body [21,28,30-32]. Because stem cell-based cartilage repair requires an optimum proliferation rate and stable phenotype status, this subject became the focus of several research protocols [34,38,48]. Therefore, we have explored the potential effect of laminarin on MSC proliferation and differentiation. MSCs in growth medium showed a marked decline in proliferation rate notably when laminarin was supplemented at high concentration (1000 µg/ml) and no apoptosis was observed. Thy1, nucleostemin and endoglin mRNA analysis showed no significant difference in MSC cells in growth medium with laminarin compared to without. MSC in chondrogenic medium mirrored the results obtained in growth medium but the mRNA expression of nucleostemin, endoglin, collagen II, Aggrecan showed significant difference in the laminarin treated cells compared to untreated. In the present study, we provided a new insight into the impact of laminarin stimulation on MSCs proliferation and differentiation.

First, we showed that, after 4 passages in growth medium, cells isolated from rats bone marrow expressed endoglin, nucleostemin and Thy1, three MSCs markers [49]. Note that Thy1 is a marker of MSCs whereas coll II is a chondrocyte marker. In addition, we observed that these cells lacked in mRNA expression of CD31 (an endothelial marker), CD45 and CD34 (two hematopoietic markers) as previously described [50,51]. In growth medium supplemented with laminarin, we observed a significant decrease in MSCs quantity compared to without and the higher dose of laminarin seems to have the more pronounced impact. Currently, it is not an easy task to study the cellular effect of β-(1 → 3)-glucans stimulation because they are able to interact with a variety of structures including polymers and small molecules, but also with biological receptors such as Dectin-1, TLRs, scavenger or CR3 receptors [7].

To further explore the effect of laminarin, we have performed an MTT assay for the viability or proliferation rate, a blue trypan for cell death and Annexin V staining for apopotosis. Results showed that, whatever the dose, laminarin treatment did not induce apoptosis of MSCs confirming its growth inhibiting role. To date one study has shown that β(1 → 3,1 → 6) glucan administered intravenously to mice

has the ability to stimulate stem cell proliferation, differentiation and activation via the CR3 receptor [52]. Stem cells used in this former study expressed CR3 receptor or Dectin-1 as hematopoietic stem cells which are not the case of the MSCs of the current study. Our findings also demonstrated no significant difference in Thy1, nucleostemin and endoglin mRNA level in MSCs cells in growth medium with laminarin compared to without. We also observed that cells have retained their characteristic to adhere on the plastic. These observations showed for the first time that laminarin supplementation inhibits MSCs growth without inducing their apoptosis or without modifiying the MSCs markers expression or their capacity to adhere to the plastic which are two of the three criteria that define the MSCs [54].

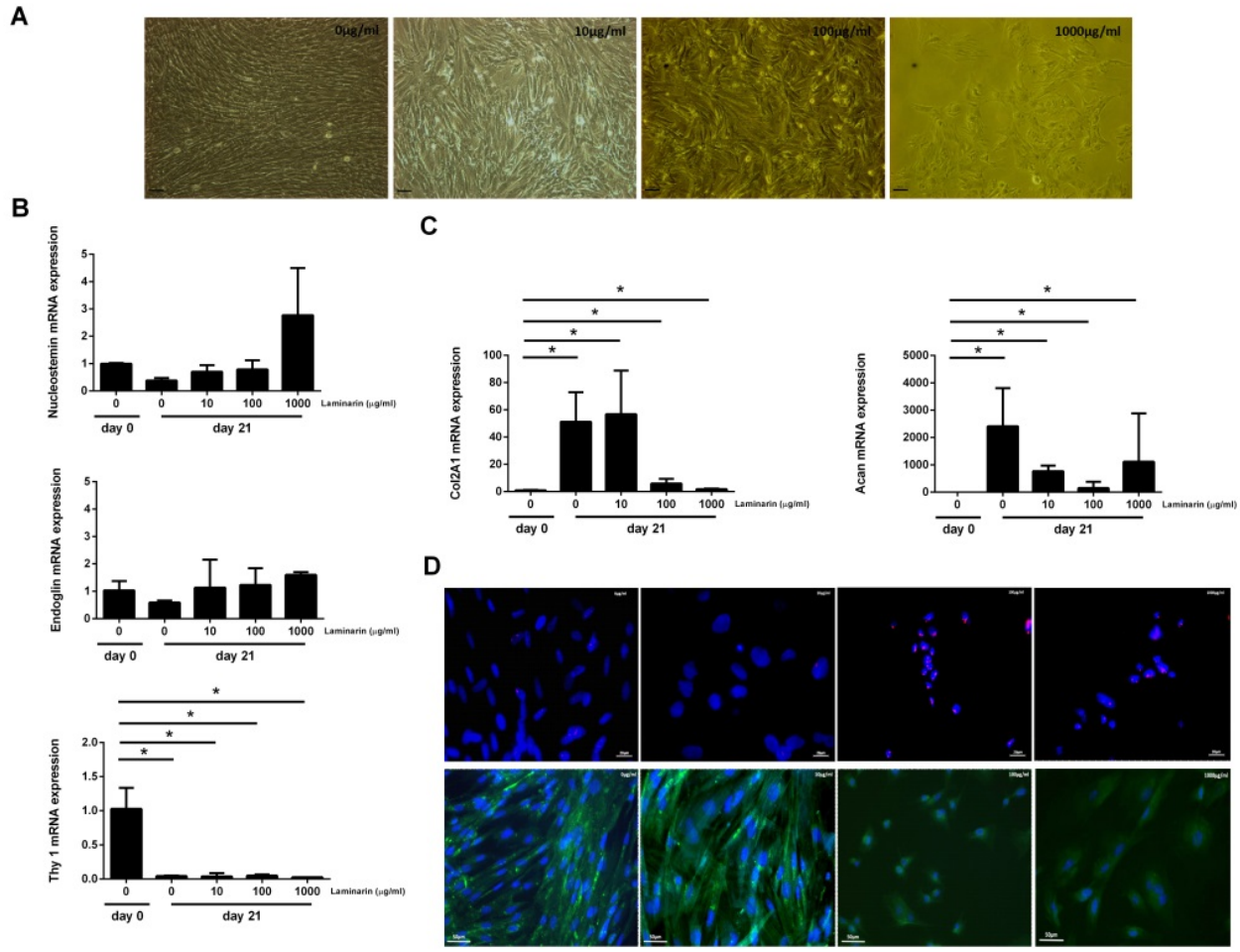

Figure 6: MSCs differentiation after 21 day under laminarin treatment. (A) Microscope imaging of MSCs at day 21 of chondrogenic differentiation (scale bar 20 µm). (B) Effect of laminarin on MSC-defining markers (Nucleostemin, Endoglin and Thy1) of MSCs during chondrogenic differentiation. Cells were cultured in chondrogenic medium and treated with laminarin (0, 10, 100 and 1000 µg/ml) for 21 days. Data (n=3 biological replicates) are presented as mean ± SEM ($^{*}p<0.05$). (C) Effect of laminarin on chondrocyte markers (Collagen II and Aggrecan) of MSCs during chondrogenic differentiation. Cells were cultured in chondrogenic medium and treated with laminarin (0, 10, 100 and 1000 µg/ml) for 21 days. Data (n=3 biological replicates) are presented as mean ± SEM ($^{*}p<0.05$). (D) MSCs immunophenotyping at day 21 of chondrogenic differentiation. Nucleostemin, red; Type 2 Collagen, green; DAPI, blue (scale bar 20 µm).

The third criterion is the capacity to differentiate into mesenchymal lineage cells [54]. Therefore in the second part of the study we have investigated the impact of laminarin supplementation on MSCs differentiation. After 21 days in chondrogenic medium, we observed an inhibition of cells proliferation in the conditions with laminarin compared to without. Cells number has decreased for all laminarin condition, but it was only statistically significant for the higher concentration. These results seem to contradict those obtained by Kim in 2012 [17]. In the latter study, authors reported that rat gavage with polycan, which is a purified Aureobasidium pullulans β-glucan, increased the proliferation rate of chondrocytes in the surface articular cartilage of the tibia and femur in osteoarthritic rats. However, several points can explain this difference. Foremost, in mammals, the responsible enzymes for degrading glucans namely the laminarinase are not present [64] and therefore poorly digested by the animals. Thereby the glucans used in this study and in our study are strongly different in term of molecular weight. Further in the study on osteoarthritic rat model, rats are force-fed with polycan. In addition, authors have only made a histological analysis of cartilage tissues andthe mechanistic process was not studied. As the polycan could

stimulate different cell types or influence some cell interaction, mechanistic study could highlight the pathway involved. Herein, we were interested in the direct effect of laminarin supplementation on cells.

In order to determine if the chondrocyte differentiation was successful, we investigated by RT-qPCR the collagen II and aggrecan expression, two chondrocyte markers, and confirmed by immunofluorescence the collagen II protein level. After 21 days, we observed an increase of collagen II and aggrecan mRNA expression collagen II for cells in chondrogenic medium compared to MSCs in growth medium. Further, by immunofluorescence, cells responded positively with the collagen II staining. These results are in accordance with those obtained by Helrosfen et al in 2010 [55]. Authors demonstrated that collagen II and aggrecan mRNA were greatly elevated already at day 7 of in vitro chondrogenic differentiation of human MSCs. Furthermore, it was shown that Collagen II and Aggrecan were dispersed throughout the extracellular matrix at day 21 [55]. However, in our study we observed a reduction in collagen II and aggrecan mRNA level in cells treated with laminarin (1000 µg/ml) compared to cells without. These results for collagen II were mirrored to those obtained in immunofluorescence. Collagen II staining was higher in condition without laminarin and this since the day 7 compared to the condition under laminarin stimulation. For the condition with the higher concentration of laminarin, we observed a really low expression of collagen II after twenty one days of culture.

In parallel of chondrocyte markers, we analysed the mRNA expression of nucleostemine, endoglin and Thy1 as well the protein expression of nucleostemine. In chondrogenic medium, Thy1 mRNA level decreases deeply since day 7 of differentiation. These results are in accordance to the literature, where it was demonstrated that many MSCs markers among them Thy1 were decreased during 7 days of chondrogenic differentiation [56]. For nucleostemin and endoglin, mRNA level analysis did not show difference in cell in chondrogenic medium compared to MSCs. However by immunofluorescence, we observed no staining for nucleostemin in cell in growth medium without laminarin. These results are in accordance with a high expression of collagen II in these differentiate cells. On the contrary in chondrogenic medium supplemented with laminarin (100 and 1000 µg/ml), cells exhibits in their nuclei high staining for nucleostemin. The presence of nucleostemin in nuclei demonstrated that these cells preserve their capacity for self-renewal as previously demonstrated [57]. Cells in chondrogenic medium supplemented with laminarin (100 and 1000 µg/ml) showed a high expression of nucleostemin together with a very low expression of collagen II suggesting that laminarin inhibited chondrogenic differentiation.

Several studies have highlighted the effect of TLRs on MSCs differentiation, It has been demonstrated that MSCs have low immunogenicity and have the capacity to respond to various stimuli including cytokines such as TNFα, growth factors such as FGF2, polysaccharides such as LPS or double-stranded RNA such as Poly (I:C) by secreting in turn various molecules like cytokines or growth factors which can change their environment and influence their capacity to differentiate [44,45,58-60]. In 2010, Waterman et al demonstrated that TLR stimulation by LPS or Poly (I:C) block the MSCs differentiation into bone or fat lineage [44]. Chondrogenic differentiation of human bone marrow MSCs has not been reported to be altered by activation through LPS, Poly (I:C) or R848 [61], but was increased by TLR2 activation on human UCB-MSCs [62]. Interestingly, some reports in mouse model link TLR signaling pathways with MSCs multipotency [63-65]. It has been suggested that laminarin interacted with various receptor including the TLRs receptor, the Dectin-1 and the Complement Receptor 3 (CR3) [7]. However MSCs doesn't express CR3 [53] and Dectin-1 but expresses the TLRs [66]. Yet, the signalling pathways related to the effects of laminarin require further investigations.

Statistical Analysis

MTT results and cells counting were compared using one-way ANOVA followed by Kruskal-Wallis multiple comparison test of the means. mRNA expressions were compared using a one-way ANOVA followed by the Mann-Whitney test (Prism6, GraphPad Software Inc. CA). A p-value<0.05 was considered statistically significant. Experiments have been performed three times for mRNA expression and six times for MTT assays.

Limitation

The limitation of the current study is that our experiments were performed only with one type of beta-glucan originating from brown algae. Yet, currently in our laboratory other derivative of polysaccharides e.g. from cereals, bacteria, or fungi, are planned to be tested. Also we recommend to perform the differentiation of MSCs into osteocytes and adipocytes under the laminarin treatment and compare it to the current results. In addition, we recommend to do some functional studies in order to identify the specific receptor (s) of laminarin in MSCs.

Conclusion

Here we demonstrated that laminarin (1 mg/ml) supplemented in growth and chondrogenic mediums inhibited cells proliferation without inducing cells death. Furthermore, under chondrogenic medium, laminarin at a similar dose prevented chondrocyte differentiation. Yet, a low concentration (10 µg/ml) didn't have any significant impact on chondrogenic differentiation. Collectively, our results indicate that laminarin is effective in cells proliferation and chondrogenic differentiation and empowers researchers to translate these insights into MSCs therapy clinical research programs.

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