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## TIEG1-null tenocytes display age-dependent differences in their gene expression, adhesion, spreading and proliferation properties

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### Abstract

The remodeling of extracellular matrix is a crucial mechanism in tendon development and the proliferation of fibroblasts is a key factor in this process. The purpose of this study was to further elucidate the role of TIEG1 in mediating important tenocyte properties throughout the aging process. Wildtype and TIEG1 knockout tenocytes adhesion, spreading and proliferation were characterized on different substrates (fibronectin, collagen type I, gelatin and laminin) and the expression levels of various genes known to be involved with tendon development were analyzed by RT-PCR. The experiments revealed age-dependent and substrate-dependent properties for both wildtype and TIEG1 knockout tenocytes. Taken together, our results indicate an important role for TIEG1 in regulating tenocytes adhesion, spreading, and proliferation throughout the aging process. Understanding the basic mechanisms of TIEG1 in tenocytes may provide valuable information for treating multiple tendon disorders.

### Keywords

Tenocytes; KLF10; TIEG; Adhesion; Spreading; Proliferation

### Introduction

Tendons are force-conducting tissues primarily composed of extracellular matrix (ECM) proteins and tenocytes. In tendons, force is conducted through a dense network of type I collagen fibrils, supplemented with various proteoglycans and other noncollagenous proteins. Tendon cells (tenocytes) are fibroblast-like cells that are responsible for maintenance, repair, and remodeling of tendons [1]. However, their characteristics are different from fibroblasts from other tissues, such as skin fibroblasts which display a different membrane protein expression pattern (e.g., integrins) [2–4]. Cellular responses to different types of tendon injury involve the remodeling of ECM [5–7] leading to variations in its composition during wound healing. Indeed, fibronectin has been shown to be deposited during the granulation phase of tendon healing [8] allowing fibroblast cells to move into the

wound site [9]. Moreover, Molloy et al. have developed a wound healing model and have demonstrated that expression of proteoglycans such as decorin and biglycan are upregulated whereas the cell adhesion molecule, laminin- $\beta_2$ , is downregulated [10]. However, in order to repair a physical disruption in tendon, several other processes are activated such as macrophage recruitment [11], as well as proliferation and migration of fibroblastic cells to the injured site [12]. Interactions between fibroblasts and the ECM are central events in tendon remodeling. It is known that collagen type I promote fibroblast adhesion via integrins [13], and that these interactions between cells and ECM components such as fibronectin, fibrinogen or vitronectin, modulate the behavior and the inflammatory responses of these cells [14–16].

In addition to the above processes, TGF $\beta$  modulates the proliferation of many cell types [17] which is also considered to be an important mechanism in several disease processes where TGF $\beta$  plays a central role in part by regulating extracellular matrix (ECM) metabolism [18,19], epithelial and endothelial cell growth, proliferation, differentiation, and apoptosis [20]. The members of the TGF $\beta$  superfamily have major biological effects in musculoskeletal tissues including tendon by regulating a diversity of developmental processes [17] and by controlling the differentiation and regeneration of tissue. TGF $\beta$  is considered to play a central role in several disease processes and contribute to both normal and pathological ECM turnover [8,18,19,21], tissue growth as well as cell proliferation, differentiation, and apoptosis [20,22]. These mechanisms are in part controlled by DNA binding transcriptional regulators such as the Krüppel-like family of transcription factors (KLFs) [23–25]. KLFs are expressed in a large variety of tissues, and function as transcriptional activators and/or repressors [26–28].

TGF $\beta$  inducible early gene-1 (TIEG1), also referred to as KLF10, was identified as an immediate response gene following TGF $\beta$  stimulation in osteoblasts [29] and plays a role in osteoblast-mediated mineralization and expression of osteoblast marker genes [30]. TIEG1 is also known to regulate gene expression through the modulation of Smad 2 and Smad 7 which ultimately enhances the TGF $\beta$  signal transduction pathway [31,32]. The originality of this study is based on the use of TIEG1 knockout (KO) mice which were developed by our group [30] as a model to investigate the involvement of this transcription factor in tendon. Through the use of this mouse model system, we have previously identified a number of tendon phenotypes including defects in resistance to mechanical strain as well as changes in tendon fibril size [33]. Additionally, we have shown that these structural and functional defects are age-dependent, with the most pronounced changes occurring at 3 months of age [33].

The purpose of the present study was to expand upon our previous work in an effort to further elucidate the role of TIEG1 in tenocyte development as a function of age. Furthermore, we sought to identify differences in the effects of important EMC components on various cellular processes such as adhesion, spreading and proliferation which were dependent upon expression of TIEG1.

## Materials and methods

### Animals

TIEG1 KO mice were developed in a C57BL/6 background as described previously [30]. Mice were housed in a temperature controlled room (22 $\pm$ 2 °C) with a light/dark cycle of 12 h. All mice had free access to water and were fed standard laboratory chow (Laboratory Rodent Diet 5001; PMI Feeds, Richmond, VA) ad libitum. To reduce variability among experiments, wild type (WT) and TIEG1 KO female littermates were utilized in all of the

experiments performed in these studies. The Institutional Animal Care and Use Committee (IACUC) approved all animal care and experimental procedures.

### Cell culture

Flexor tendons were harvested from the paws of WT and TIEG1 KO female mice at 1 ( $N_{WT}=3$ ,  $N_{KO}=3$ ), 3 ( $N_{WT}=3$ ,  $N_{KO}=3$ ) and 8 ( $N_{WT}=3$ ,  $N_{KO}=3$ ) months of age. About 18 tendons per mouse were extracted and primary tenocytes were isolated by overnight digestion in collagenase type I (0.5 mg/mL). The next day, cells were plated and cultured in  $\alpha$ MEM medium containing 10% FBS (Invitrogen, France), 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml Fungizone (Invitrogen, France). The cells were grown at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. Replicate cultures were obtained by trypsinization and only early passages (3 to 5) were used in these studies.

### Adhesion assay

Tenocytes isolated from 9 WT and 9 TIEG1 KO mice, as described above, were used for adhesion experiments. To study the implication of the extracellular matrix proteins on cell adhesion, 96-well plates (Falcon, Becton Dickinson, France) were either left untreated or precoated with fibronectin (10  $\mu$ g/mL, Invitrogen, France), collagen type I (25  $\mu$ g/mL, GIBCO, France), gelatin (2%, GIBCO, France), or laminin (10  $\mu$ g/mL, Invitrogen, France) for 30 min. Plates were then rinsed twice with phosphate buffered saline and tenocytes were seeded at a density of  $1.5 \times 10^4$  cells/well in 200  $\mu$ L of complete culture medium and adhered for 1 h (37 °C and 5% CO<sub>2</sub>). Following 1 h of incubation, wells were washed with culture medium in order to remove nonadherent cells and 200  $\mu$ L of 10% (v/v) Alamar Blue dye (Biosource International, USA) was added to each well as previously described [34] and incubated for 4 h at 37 °C and 5% CO<sub>2</sub>. The percentage of adherent cells was determined by measuring the increase in fluorescence at 590 nm, after excitation at 530 nm (Spectra FLUOR Plus plate reader, Austria). Three independent experiments were performed for each age group and each experiment was carried out in duplicate.

### Spreading assay

Four-well Labtek Chamber-Slide culture systems (Labtek, Becton Dickinson, France) were either left untreated or precoated with fibronectin (10  $\mu$ g/mL), collagen type I (25  $\mu$ g/mL), gelatin (2%), or laminin (10  $\mu$ g/mL) for 30 min. Wells were rinsed twice with phosphate buffered saline, and tenocytes were seeded at a density of  $2.0 \times 10^4$  cells/well in 500  $\mu$ L of complete culture medium. The chamber-slides were placed on the stage of a phase contrast microscope (Leica DMI 6000B, Germany) coupled to a CCD camera (Leica DFC 300FX, Germany) for visualization of cell spreading in vitro under 37 °C and 5% CO<sub>2</sub> conditions. Phase-contrast images of four randomly selected fields (1.3  $\times$  1 mm) per well were acquired every 30 min for a total of 6 h. Preliminary data revealed that the large majority of cells exhibited maximal spreading by the 4 h timepoint. Therefore, the 6 hour time duration was chosen for this study. A cell was considered to be completely “spread” when it displayed surrounding lamellipodia and had lost its ability to refract light under phase contrast microscopy (Fig. 1). Images were analyzed using ImageJ software (NIH Image, Bethesda, USA). Rounded and spread cells were counted separately and between 30 and 80 cells were evaluated per field. The percentage of spread cells relative to the total cells per field was calculated. The time course of spreading was represented as the percentage of spread tenocytes as a function of time. Three spreading parameters were analyzed: 1) the “spreading velocity” obtained from the slope of the curve, 2) the “plateau” representing the maximum percentage of spread cells and 3) the lag time required by the cells to reach this plateau. Spreading experiments were performed on tenocytes isolated from 9 WT and 9 TIEG1 KO mice as described above. Three independent experiments were performed for each age group and each experiment was carried out in duplicate.

### Proliferation assay

Tenocytes isolated from 9 WT and 9 TIEG1 KO mice (as described above) were used to determine differences in proliferation rates. 24-well plates (Falcon, Becton Dickinson, France) were either left untreated or precoated with fibronectin (10 µg/mL), collagen type I (25 µg/mL), gelatin (2%), or laminin (10 µg/mL) for 30 min. Wells were washed twice with phosphate buffered saline and tenocytes were seeded at a density of  $1.0 \times 10^4$  cells/well in 1 mL of culture medium. During the course of 8 days, a portion of the cells (2 wells from each condition) were detached every 24 h with 300 µL of trypsin. Cells were mixed with 200 µL of  $\alpha$ MEM/10% serum and subsequently counted using a hemocytometer counting chamber (Malassez slide). A proliferation curve was created by plotting cell number as a function of time. Three parameters were calculated from these curves: the maximum cell density upon confluence measured at the plateau (P) of the curve, with its corresponding time (T) and the maximal growth or spreading velocity (V) measured as the slope of the curve. Three independent experiments were performed for each age group and each experiment was carried out in duplicate.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time PCR was performed on tenocyte cultures using the TaqMan Gene Expression Cells-to-Ct kit as specified by the manufacturer (Applied Biosystems). Briefly, cultured tenocytes were detached from the flask with trypsin, counted and resuspended at a concentration of 200 cells/µL in lysis solution containing 1% DNase I. The lysis reaction was stopped using stop solution and cDNA was synthesized via reverse transcription PCR using the supplied master mix with the following conditions: a first step of 37 °C for 60 min, followed by a second at 95 °C for 5 min and a final holding step of 4 °C. A total of 9 WT and 9 TIEG1 KO mice ( $N_{1\text{month}}=3$ ,  $N_{3\text{month}}=3$ ,  $N_{8\text{month}}=3$ ) were used for qRT-PCR experiments.

Real-time PCR was subsequently performed using gene specific primers and a MiniOpticon Real-Time PCR Detection System (Biorad, France) with the following thermal cycling conditions: an initial step of 2 min at 50 °C, an activation step of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min using a PCR cocktail composed of 2X TaqMan gene expression master mix (Cells-to-Ct kit, Applied Biosystems) and 20X TaqMan gene expression assay (Applied Biosystems). Gene specific primers were purchased from Applied Biosystems (France) as follows: 1) collagen 1a2 (Col1a2): Mm00483888\_m1, 2) decorin (Dcn): Mm00514535\_m1, 3) fibromodulin (Fmod): Mm00491215\_m1, 4) TIEG1 (Klf10): Mm00449812\_m1, 5) laminin beta1-1 (Lamb1-1): Mm00801853\_m1, 6) lysyl oxydase (Lox): Mm00495386\_m1, and 7) lumican (Lum): Mm01248292\_m1. The housekeeping gene used was beta-2 microglobulin (B2m): Mm00437762\_m1. Quantitative values were calculated based on the threshold cycle (Ct) obtained during the real-time PCR reaction. The ratio of each target gene was calculated using a relative quantification method:  $2^{-\Delta\Delta C(T)}$  (Livak, 2001). Two independent experiments were performed in duplicate.

### Statistical analysis

Unpaired t-tests were performed using software R (Lucent Technologies, USA) to determine statistical significance ( $p < 0.05$ ) between WT and TIEG1 KO tenocytes for all of the experiments described here. Data is expressed as the mean  $\pm$  SD obtained from three independent experiments for each condition. In addition, unpaired t tests were performed with the software Statgraphics 5.0 (Sigma Plus, Maryland, USA) to compare the gene expression differences between tenocytes isolated from 1, 3 and 8 month old animals.

## Results

### Adhesion of WT and TIEG1 KO tenocytes to various substrates throughout age

**Effect of genotype on tenocytes adhesion to various substrates**—For tenocytes isolated from 1 month old animals in the absence of an adhesion substrate, the percentage of adherent cells was significantly lower for WT (~58%) than for TIEG1 KO tenocytes (~70%) (Fig. 2A). However, the average percentage of adherent cells was significantly ( $P<0.05$ ) higher for WT (~80%) than for TIEG1 KO cells when plated on collagen I (~64%) and laminin (~58%) coated culture plates. No effect of genotype was found with fibronectin or gelatin coated plates. At 3 months of age, in the presence and absence of all substrates, the percentage of adherent cells was significantly ( $P<0.05$ ) lower for WT cells (between 60% and 70%) relative to TIEG1 KO cells (between 78% and 85%) (Fig. 2B). Opposing results were observed at 8 months of age where the percentage of adherent cells was significantly ( $P<0.05$ ) higher for WT cells (between 85% and 99%) relative to TIEG1 KO tenocytes (between 70 and 80%) across all culture conditions (Fig. 2C). These data are summarized in Table 1 (Supplementary data).

**Effect of substrates on WT and TIEG1 KO tenocyte adhesion**—For WT cells, collagen I substrate significantly increased ( $P<0.05$ ) the extent of cell adhesion at 1 month (~48%), 3 months (~17%) and 8 months (~21%) of age compared to no substrate culture conditions (Fig. 2). Similarly, the laminin substrate induced an increase ( $P<0.05$ ) of WT cell adhesion at 1 month (~39%), 3 months (~12%) and 8 months (~13%) of age (Fig. 2), while gelatin induced a significant ( $P<0.05$ ) increase in WT cell adhesion at 1 month (~16%), 3 months (~10%) and 8 months (18%) of age (Fig. 2). For TIEG1 KO cells, fibronectin, collagen I and laminin substrates induced a significant ( $P<0.05$ ) decrease in cell adhesion at 1 month of age by 14%, 9% and 17% respectively (Fig. 2A). At three and 8 months of age, TIEG1 KO tenocytes exhibited no substrate-dependence with regard to their adhesion properties (Figs. 2B and 1C). These data are summarized in Table 2 (Supplementary data).

### Spreading of WT and TIEG1 KO tenocytes throughout age on various substrates

**Effect of genotype on the spreading parameters**—Fig. 3 represents the time course of the percentages of cell spreading for WT and TIEG1 KO tenocytes. Spreading of cells was defined by visualization of lamellipodia and loss of light refraction via phase contrast microscopy (Fig. 1). The velocity of spreading revealed that WT cells spread faster than TIEG1 KO cells in the absence of an exogenously added substrate regardless of age. Nearly identical behavior was observed for cells isolated from 1 month old animals (WT and KO) in the presence of collagen I substrate. On the contrary, WT cells were determined to spread slower than TIEG1 KO cells when plated on laminin regardless of age. The same results were found for cells isolated from 3 month old WT mice relative to TIEG1 KO cells on gelatin substrate. Similar spreading profiles were displayed between WT and TIEG1 KO cells for fibronectin (at 1, 3 and 8 months), collagen (at 3 and 8 months) and gelatin (at 1 and 8 months).

Our data also indicate that the spreading velocity for WT cells plateau at a higher level than TIEG1 KO cells on collagen I substrate at 3 months of age. Conversely, WT cells plateau at a lower level than TIEG1 KO cells when plated on laminin regardless of age. Equal plateaus were obtained at 1 and 8 months age between WT and TIEG1 KO cells on collagen I substrate. Moreover, no effect of genotype was found at 1, 3 or 8 months of age for the plateau parameter either without substrate or with fibronectin or gelatin substrates.

The time required by WT cells to reach the plateau was longer than TIEG1 KO cells on collagen I substrate at 3 months of age (2 h for WT vs. 1h30 for TIEG1 KO), on gelatin



substrate at 1 month of age (3h30 for WT vs. 3 h for TIEG1 KO) and 3 months of age (3h30 for WT vs. 2h30 for TIEG1 KO) and on laminin at 1 month of age (2 h for WT vs. 1h30 for TIEG1 KO). The lag time for WT cells was lower than TIEG1 KO cells in the absence of substrate regardless of age (4 h for WT vs. 5h for TIEG1 KO), as well as on collagen I substrate at 1 month of age (2 h for WT vs. 2h30 for TIEG1 KO) and on laminin substrate at 3 months of age (1h30 for WT vs. 2 h for TIEG1 KO). Moreover, no effect of genotype was detected at 1, 3 and 8 months for the lag time parameter when plated on fibronectin, as well as for gelatin, laminin and collagen I at the 8 month timepoint. These data are summarized in Table 3 (Supplementary data).

#### **Effect of substrates on WT and TIEG1 KO tenocytes spreading parameters—**

For WT cells, the collagen I, gelatin and laminin substrates induced a decrease in the time required to reach the plateau (lag time) at 1 and 3 months of age (Figs. 3A and B), whereas at 8 month of age, only collagen I and laminin substrates induced a decrease in this lag time (Fig. 3C). Regardless of age for WT mice, the collagen I, gelatin and laminin substrate induced an increase in spreading velocity, whereas fibronectin induced a decrease in velocity (Figs. 3A–C). Moreover, the laminin substrate induced a decrease in the spreading plateau (lag time) at all ages examined (Figs. 3A, B, C).

For TIEG1 KO cells, laminin substrate induced a decrease in the upper asymptote at all ages (Figs. 3D, E and F). However, collagen I substrate induced a decrease in the plateau only at 3 and 8 months of age (Figs. 3E and F). No effect was observed in the presence of fibronectin and gelatin in the time taken to reach the spreading plateau at all ages. Furthermore, all substrates induced a decrease of lag time as well as an increase of spreading velocity for the TIEG1 KO tenocytes at 1, 3 and 8 months (Figs. 3D–F). These data are summarized in Table 4 (Supplementary data).

#### **Proliferation rates of WT and TIEG1 KO tenocytes through age on various substrates**

**Effect of genotype on cell proliferation rates—**Wildtype cells were determined to proliferate faster than TIEG1 KO cells at 1, 3 and 8 months of age regardless of substrate (Fig. 4). The proliferation plateau revealed an effect of the genotype on cell proliferation rates in the absence of substrate and in the presence of fibronectin, which resulted in increased density of WT cells (about 1.5 fold) compared to TIEG1 KO cells, throughout age (Fig. 4). Moreover, the collagen I, gelatin and laminin substrates induced equal rates of proliferation and cell density (about  $1.2 \times 10^5$  cell/cm<sup>2</sup>) at 1 and 3 months of age (Figs. 4 A, B, D, E) for both WT and TIEG1 KO tenocytes. Interestingly, at 8 months of age, the cell density for WT and TIEG1 KO cells was lower (about  $7 \times 10^4$  cell/cm<sup>2</sup> for WT and  $5 \times 10^4$  cell/cm<sup>2</sup> for TIEG1 KO) than those measured at 1 and 3 months respectively (Figs. 4C and F) with higher cell densities observed for the WT tenocytes compared to TIEG1 KO tenocytes.

The time required for WT cells to reach the plateau in the absence of substrate, and in the presence of fibronectin, was higher (1 day) than that of TIEG1 KO cells at 1 and 3 months of age (Figs. 4A, B, D and E). On the contrary, the lag time of WT cells was lower (1 day) than TIEG1 KO cells when plated on collagen I, laminin and gelatin substrates at 1 and 3 months of age (Figs. 4A, B, D and E). Finally, WT and TIEG1 KO cells displayed equal times (6 days) to reach the plateau at 8 months of age regardless of substrate (Figs. 4C and F). These data are summarized in Table 5 (Supplementary data).

**Effect of substrates on WT and TIEG1 KO tenocytes proliferation rates—**For WT cells at 1 and 3 months of age, the laminin substrate stimulated proliferation rates while all other substrates had no significant effects compared to the no substrate controls. The

collagen I, gelatin and laminin substrates decreased the time taken to reach confluence at 1 and 3 months of age. The fibronectin substrate had no effect on WT cell proliferation at 1 and 3 months of age as no significant differences in the velocity, time to confluence or cell density were observed relative to controls (Figs. 4A and B). At 8 months of age, all substrates induced an increase in WT cell proliferation rates compared to control conditions, whereas the plateau parameter, and its corresponding time to confluence, were equivalent for all conditions (Fig. 4C).

For TIEG1 KO tenocytes at 1 and 3 months of age, the fibronectin substrate induced no changes in proliferation kinetics compared to the control condition. However, at 1 and 3 months of age, the collagen I, gelatin and laminin substrates induced an increase in cell density as well as a lengthening of the time taken to reach confluence. In addition, regardless of substrate, no effect was observed on TIEG1 KO cell proliferation velocity at 1 and 3 months of age. Indeed, the proliferation profiles describe similar proliferation curves until 5 days of cell culture (Figs. 4D and E). At 8 months of age, fibronectin, laminin and collagen I induced an increase in TIEG1 KO cell density, and only the collagen I substrate induced an increase in cell proliferation velocity (Fig. 4F). These data are summarized in Table 6 (Supplementary data).

### Quantitative real-time polymerase chain reaction (qRT-PCR)

In order to determine if alterations in the expression levels of genes known to be involved in tendon biology were affected by loss of TIEG1 expression, we examined the expression profiles of lumican, laminin beta1-1, decorin, collagen I, lysyl oxydase and fibromodulin between WT and TIEG1 KO tenocytes at 1, 3 and 8 months of age. For tenocytes isolated from 1 month old animals, the expression of all of these genes was determined to be significantly lower in TIEG1 KO tenocytes relative to WT controls (Fig. 5).

At 3 months of age, lumican, laminin beta1-1 and decorin gene expression levels were lower in TIEG1 KO tenocytes relative to WT cells while significant increases in the expression levels of collagen I and lysyl oxydase were observed. Fibromodulin gene expression was similar between WT and TIEG1 KO tenocytes (Fig. 5). At 8 months of age, fibromodulin, lumican, laminin beta 1-1, decorin and lysyl oxydase were determined to exhibit decreased expression levels in TIEG1 KO cells relative to WT tenocytes, while the expression levels of collagen type I were unchanged (Fig. 5). In addition, the expression of TIEG1 was undetectable in KO tenocytes attesting to the absence of the TIEG1 gene in the KO mice (data not shown).

### Discussion

We have previously demonstrated that tendons isolated from TIEG1 KO mice exhibit significant defects in their mechanical properties [25] as well as alterations in their microarchitecture [35]. The data presented in this manuscript are an extension of these past studies and are aimed at beginning to understand the cellular and molecular basis behind our previous observations. Here, we reveal significant changes in the cellular properties of tenocytes extracted from TIEG1 KO mouse flexor tendons relative to their WT littermates. Furthermore, we demonstrate alterations in the expression profiles of multiple genes known to play important roles in tendon physiology. The present work further reveals an important role for TIEG1 in tenocytes growth and behavior, as well as in the regulation of tendon development and integrity through mice lifespan. Here, we have utilized three different age groups of mice: 1, 3 and 8 months old which correspond to juvenile, adult and aged mice, respectively. These age groups were chosen to determine if the aging process influenced the fibroblast cellular properties analyzed here and to determine if loss of TIEG1 expression had differential effects on the properties as a function of age.



In our study, cell adhesion experiments revealed age-dependent and substrate-dependent behaviors for both WT and TIEG1 KO tenocytes. More specifically, significant decreases in the adhesion of TIEG1 KO tenocytes were observed for collagen I and laminin at 1 month of age (juvenile mice) and for fibronectin, collagen I, gelatin and laminin at 8 months of age (aged mice) relative to WT controls. Interestingly, significant increases in the adhesion properties of TIEG1 KO cells were observed for all substrates at the 3 month timepoint (adult mice). Changes in the adherence of fibroblasts resulting from different substrates have previously been characterized by Koseki et al. [13] who compared the impact of three substrates (plastic, gelatin and collagen) as well as the coating condition (plastic, collagen fibrils and gelatin). Their work revealed that significant changes in the patterns of protein synthesis were induced by the different culture conditions. Moreover, many other studies have demonstrated that the interaction of cells with different extracellular matrix (ECM) component such as fibronectin, fibrinogen or vitronectin, modulate the behavior and the inflammatory responses of the cells [14–16]. Thus, it is likely that the absence of the TIEG1 transcription factor in TIEG1 KO tenocytes significantly affects intracellular mechanisms which lead to the disruption and/or inhibition of cell–collagen interactions.

The collagenous ECM forms a stress-tolerant network that is essential for proper tendon functions such as resistance to mechanical strain and stiffness. Our data revealed significant differences in the spreading properties of both WT and TIEG1 KO tenocytes, which exhibited substrate-dependent, genotype-dependent and age-dependent alterations in cell density, velocity, and lag time. These results are in agreement with the study provided by Ohno et al. which showed that collagen-associated protein significantly enhances the spreading of rabbit and human fibroblasts via integrin  $\alpha 1\beta 1$  [36]. Furthermore, Kato et al. showed that Swiss 3T3 fibroblasts cultured on three representative cell culture substrates (polystyrene, nontreated polystyrene, ethylene terephthalate), displayed substrate-dependent changes in cell morphology, adhesion and spreading [37]. Similar to the adhesion experiments, these present results revealed the appearance of an age-dependent disorder in cell-ECM interactions and cell spreading due to the lack of TIEG1.

WT cell adhesion and spreading were increased on the collagen I substrate at 1, 3 and 8 months of age, indicating a strong effect of this substrate during the growing process of tenocytes. This result is in accordance with the literature which suggests that collagen promotes fibroblast adhesion [13] and increases the spreading kinetic of fibroblasts [36]. Interestingly, TIEG1 KO cells display a different behavioral profile on collagen I substrate compared to WT cells through age, demonstrating the implication of the lack of TIEG1 expression in the cell–collagen I interaction processes which ultimately mediates cell adhesion and spreading.

Cell proliferation assays revealed that TIEG1 KO tenocytes also display decreased proliferation rates relative to WT cells regardless of the substrates used at 1, 3 and 8 months of age. Furthermore, the proliferation data indicate that collagen I and laminin substrates induced an increase in TIEG1 KO cell density at 1, 3 and 8 months of age. TIEG1 is classically thought of as a suppressor of proliferation in other cell types. Indeed, it was recently shown that TIEG1 was up-regulated under mechanical stress conditions and seemed to negatively regulate cell proliferation [38]. Surprisingly, our data suggest that under resting conditions, TIEG1 acts to stimulate proliferation of tendon cells.

Interestingly, tenocytes extracted from both WT and TIEG1 KO 8 month old animals displayed decreased rates of proliferation with a loss of velocity and density compared to 1 and 3 months. This result demonstrates that the proliferation ability decreases as a function of age. Similar results have already been found for rabbit aortic endothelial cells. Kunz et al.

showed a significant decrease in the cell turnover with age, caused by both a lengthening of the cell cycle of the proliferating cells and a reduction of the growth fraction [39].

In order to better understand the molecular basis for the observed difference in cell adhesion, spreading and proliferation between TIEG1 KO tenocytes and WT controls, we next performed real-time RT-PCR experiments to identify differences in the expression levels of genes known to play important roles in tendon biology. These data revealed significant decreases in the expression of fibromodulin, lumican, laminin beta1-1 and decorin in TIEG1 KO tenocytes relative to WT cells. Decreased expression of collagen I and lysyl oxydase was also observed in TIEG1 KO cells at the 1 and 8 month timepoint. Interestingly, increased expression of collagen I and lysyl oxydase was observed at the 3 month timepoint in TIEG1 KO cells which correlated with the decreased time needed for these cells to reach the plateau in our adhesion assays. Furthermore, TIEG1 KO tenocytes isolated from 3 month old animals also exhibited increases in their adhesion properties relative to WT controls. While the exact mechanisms are not yet clear, these data suggest that TIEG1 can either directly or indirectly regulate the expression of genes which in part make-up the extracellular matrix and highlight a potential role for alterations in collagen I expression in the tendon phenotype described previously for TIEG1 KO animals [25]. Future experiments at the protein level will allow us to figure out the extracellular matrix profiles and their functional roles in the cells derived from TIEG1 KO mice.

In summary, these data indicate that lack of TIEG1 expression results in significant modifications in the cellular properties of cells isolated from tendons. These alterations include significant changes in the adhesion, spreading and proliferation profiles of these cells with concomitant differences in their gene expression profiles. Furthermore, many of these changes are age and substrate dependent. While there is little known about the exact roles of TIEG1 in mediating cell adhesion, spreading, proliferation and gene expression, this transcription factor seems to modulate these processes in tendon cells which ultimately ensure normal tendon development and maintenance through age.

In perspective, knowing that collagen I, laminin and fibronectin substrates are the most implicated substrates in tendon healing and diseases, it would be of interest to determine the role of TIEG1 in controlling these processes. Indeed, implication of TIEG1 in tendon repair and dysfunction could be a crucial factor in the way that improves tendon healing and diseases related to aging in humans. In order to better address these particular issues, 3D cultures are necessary and are the focus of future studies relating to the role of TIEG1 in mediating these processes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

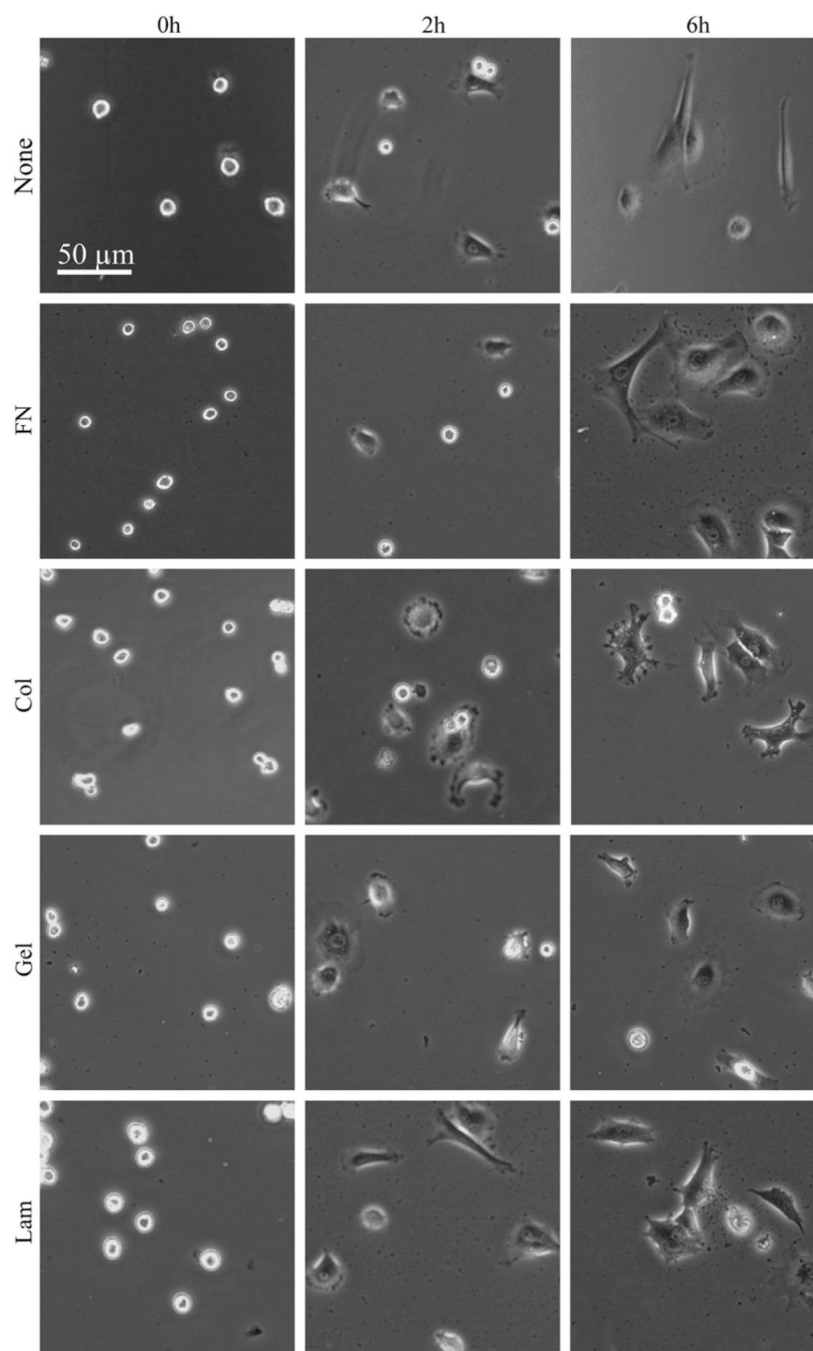
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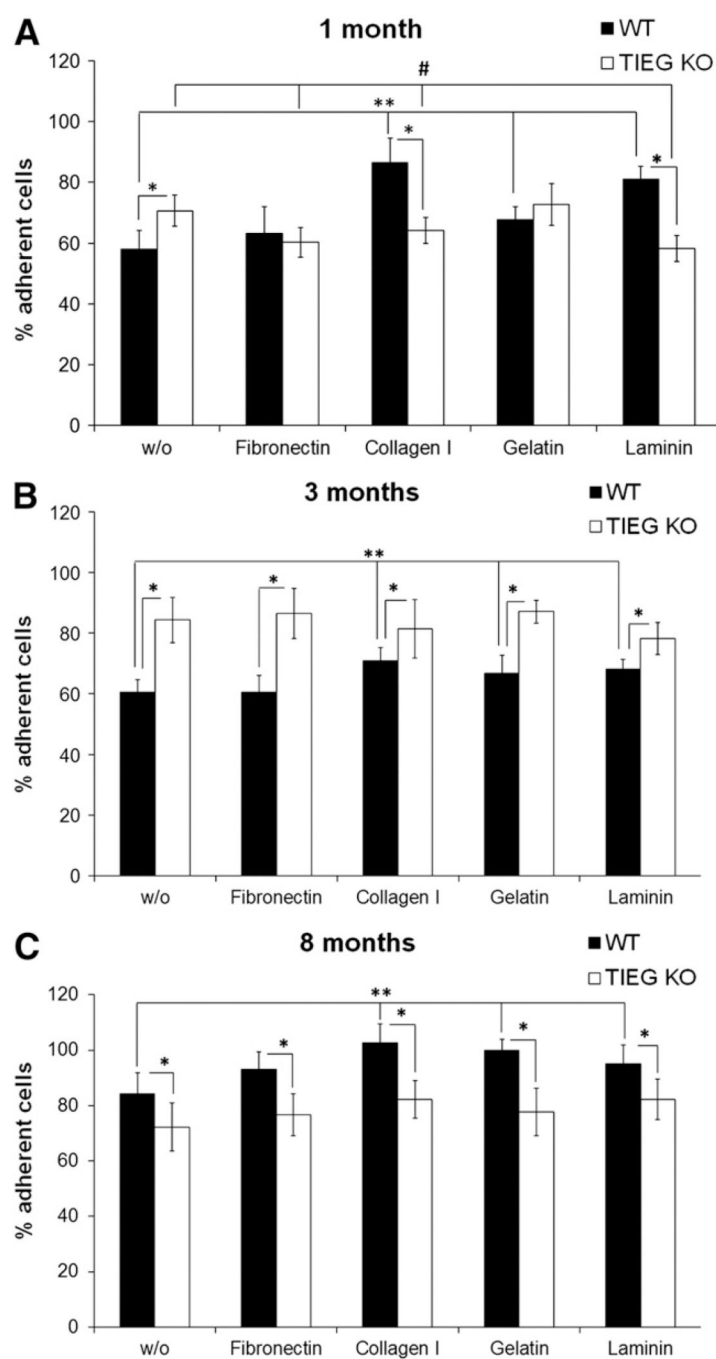
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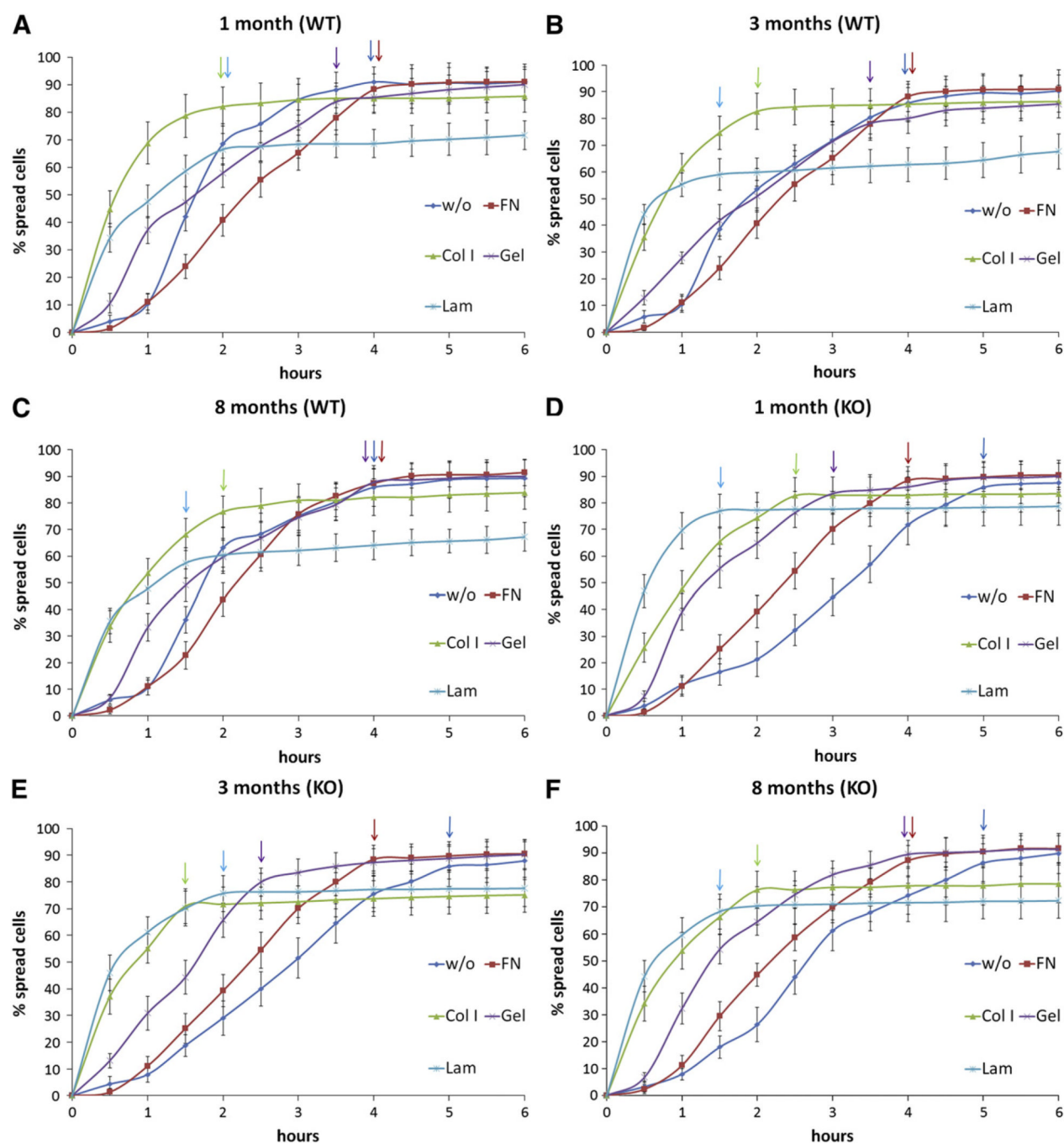


**Fig. 1.** Phase contrast images showing spreading of tenocytes extracted from 1 month old mice tendon grown on fibronectin, collagen I, gelatin and laminin substrates at 0, 2 and 6 h of spreading.

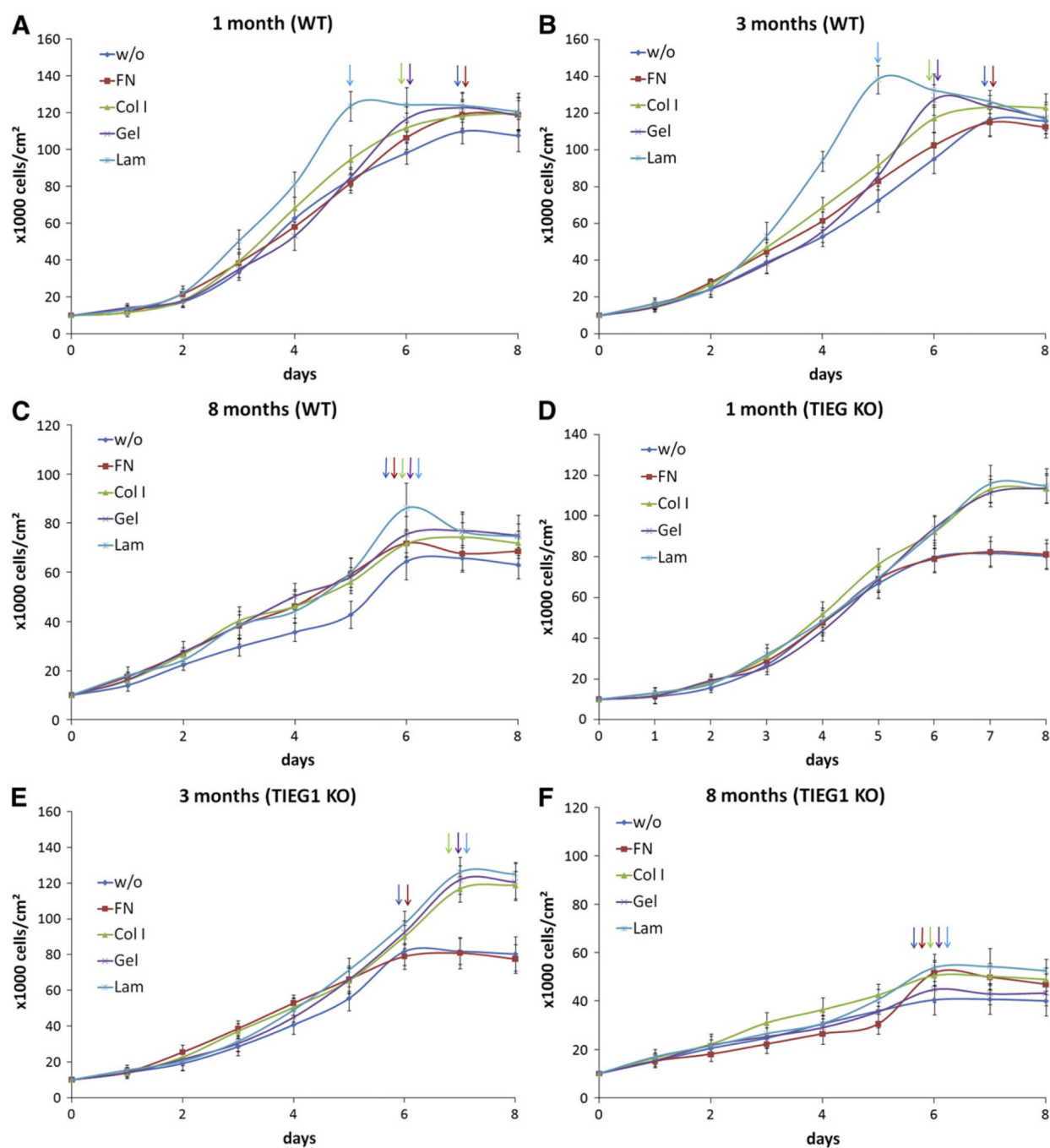


**Fig. 2.** Average percentage of adherent WT and TIEG1 KO tenocytes as a function of different substrates (fibronectin, collagen I, gelatin, laminin) at 1, 3 and 8 months of age. (\*p<0.001 WT vs. KO).

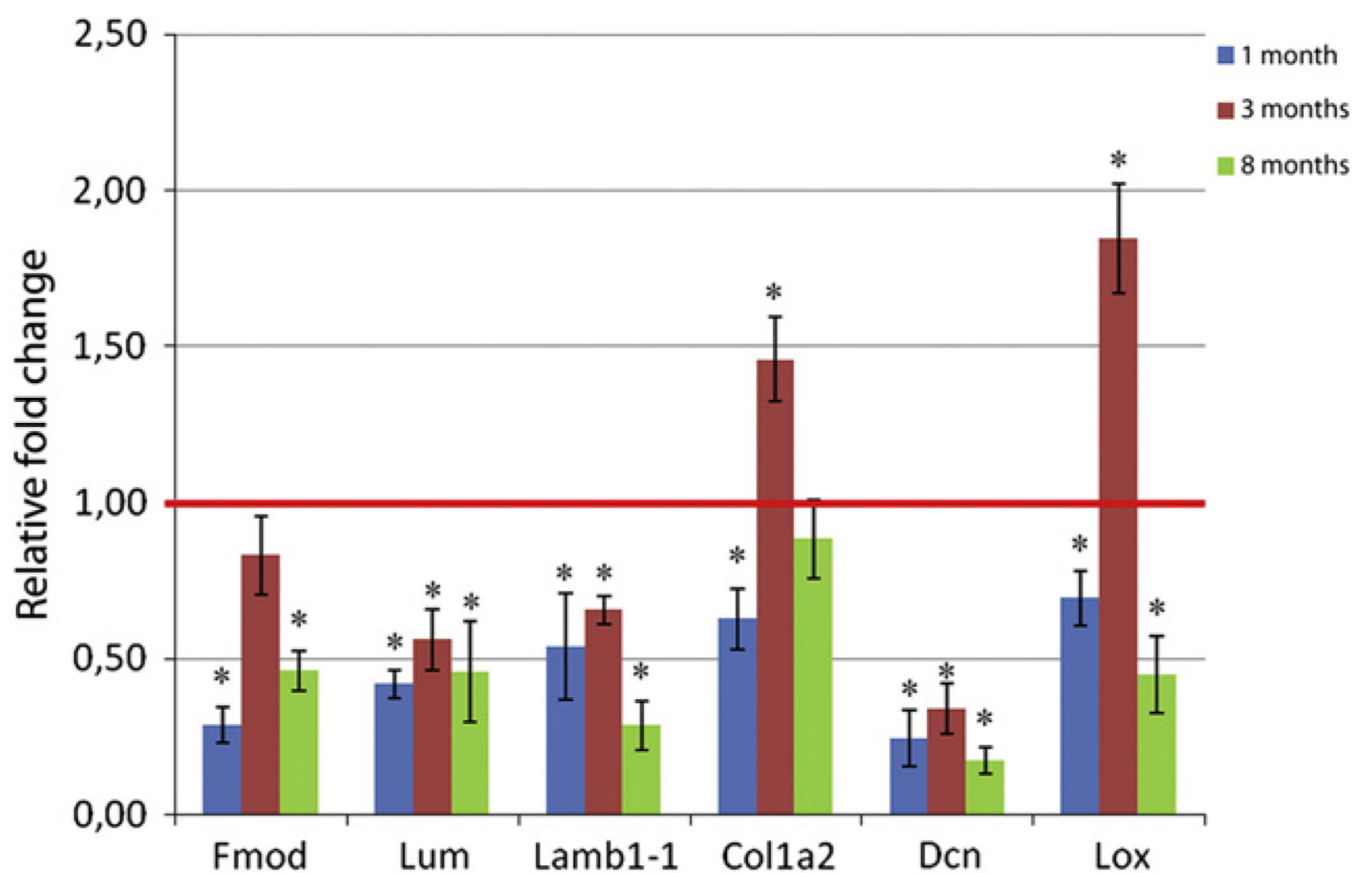




**Fig. 3.** (A–F) Percentage of spread WT and TIEG1 KO tenocytes as a function of substrate (fibronectin, collagen I, gelatin, laminin) at 1, 3 and 8 months of age. Colored arrows indicate the initiation of the plateau phase for each curve. Each point on the curves represents the mean $\pm$ SD of three independent experiments carried out in duplicate.



**Fig. 4.** Average values $\pm$ SD of WT and TIEG1 KO tenocytes proliferation rates as a function of substrate (fibronectin, collagen I, gelatin, laminin) at 1, 3 and 8 months of age. Colored arrows indicate the initiation of the plateau phase for each substrate.



**Fig. 5.**

Ratio of indicated gene expression levels between WT and TIEG1 KO tenocytes as analyzed by quantitative real-time polymerase chain reaction at 1, 3 and 8 months of age. Lum: lumican, Lamb1-1: laminin beta1-1, Dcn: decorin, Fmod: fibromodulin, Lox: lysyl oxydase, Col1a2: collagen I. (\* $p < 0.05$  WT vs. KO).