ABSTRACT
One of the main challenges relating to tendons is to understand the regulators of the tendon differentiation program. The optimum culture conditions that favor tendon cell differentiation have not been identified. Mesenchymal stem cells present the ability to differentiate into multiple lineages in cultures under different cues ranging from chemical treatment to physical constraints. We analyzed the tendon differentiation potential of C3H10T1/2 cells, a murine cell line of mesenchymal stem cells, upon different 2D- and 3D-culture conditions. We observed that C3H10T1/2 cells cultured in 2D conditions on silicone substrate were more prone to tendon differentiation, assessed with the expression of the tendon markers Scx, Col1a1 and Tnmd as compared to cells cultured on plastic substrate. The 3D-fibrin environment was more favorable for Scx and Col1a1 expression compared to 2D cultures. We also identified TGFβ2 as a negative regulator of Tnmd expression in C3H10T1/2 cells in 2D and 3D cultures. Altogether, our results provide us with a better understanding of the culture conditions that promote tendon gene expression and identify mechanical and molecular parameters upon which we could act to define the optimum culture conditions that favor tenogenic differentiation in mesenchymal stem cells.

KEY WORDS: Mesenchymal stem cells, Cell confluence, cell cultures, Plastic substrate, Silicone substrate, Tendon differentiation, TGFβ2, Scleraxis, Tenomodulin

INTRODUCTION
Mesenchymal stem cells (MSCs) are multipotent cells that can be induced to differentiate in various tissue lineages upon specific molecular or mechanical cues. Based on specific lineage markers and identified master genes, established protocols are now recognized to drive differentiation towards osteocytes, chondrocytes and adipocytes (Caplan, 1991; Pittenger et al., 1999; Prockop, 1997). Although studies identify tendon cell differentiation upon molecular and mechanical cues from MSCs (reviewed in Nourissat et al., 2015; Zhang et al., 2018), tendon lineage is less studied than other tissue-specific lineages. There is no recognized/established protocol with external inducers to differentiate MSCs towards a tendon phenotype. In addition, there is no identified master gene that initiates the tenogenic program in cell cultures as for the cartilage (Sox9), bone (Runx2) and muscle (muscle regulatory factors) programs (Buckingham, 2017; Karsenty et al., 2009; Liu et al., 2017).

Another difficulty in studying tendon differentiation is the limited number of specific tendon markers. The main structural and functional component of the tendon, type I collagen, is not specific to the tendon and is expressed in many other connective tissues (reviewed in Gaut and Duprez, 2016). To date, the bHLH transcription factor Scleraxis (Scx) is the best marker for tendons and ligaments during development (Schweitzer et al., 2001, 2010) and in the adult (Mendias et al., 2012). Although it is a powerful tendon marker, the exact function of Scx in tendon development, homeostasis and repair is still not fully understood (Huang et al., 2015; Murchison et al., 2007). The type II transmembrane glycoprotein tenomodulin, encoded by the Tnmd gene, is recognized to be a tendon differentiation marker with potential roles in tenocyte proliferation and differentiation in addition to type I collagen fibril adaptation to mechanical loads (Alberton et al., 2015; Dex et al., 2016, 2017; Docheva et al., 2005). Scx is required for Tnmd expression in mouse tendons during development (Murchison et al., 2007; Yoshimoto et al., 2017). Scx gain- and loss-of-function experiments combined with electrophoresis mobility shift assay (EMSA) in cell cultures indicate a direct regulation of Scx on Tnmd promoter (Shukunami et al., 2018; Yoshimoto et al., 2017). In addition to the well-studied tendon markers, Scx and Tnmd, a list of 100 tendon markers has been identified in limb tendon cells during mouse development via transcriptomic analysis (Havis et al., 2014).

The main extracellular signal known to promote tendon development is the TGFβ ligand (Havis et al., 2014, 2016; Maeda et al., 2011; Pryce et al., 2009). TGFβ ligands are recognized to have a generic tenogenic effect based on the increase of Scx transcription in cell cultures (Guerquin et al., 2013; Havis et al., 2014, 2016; Lorda-Diez et al., 2009; Pryce et al., 2009). The increase of Scx expression upon TGFβ2 exposure is abolished in the presence of TGFβ inhibitors, which block TGFβ signal transduction at the level of the receptors or at the level of the SMAD2/3 intracellular pathways in C3H10T1/2 cells (Guerquin et al., 2013; Havis et al., 2014).

In addition to chemical signals, mechanical signals are important parameters to consider when studying tendon cell differentiation. Because tendons transmit forces from muscle to bone in the...
Fig. 1. Tendon gene expression is not related to cell density in non-confluent conditions. (A) Representative pictures of cell density 16 h after plating 0.5×10⁵ (5555 cells/cm²), 10⁵ (11,111 cells/cm²) and 2×10⁵ (22,222 cells/cm²) C3H10T1/2 cells on 9 cm² plastic culture plates. (B) RT-qPCR analyses of the expression levels of tendon markers 

| Marker | 5555 cells/cm² | 11,110 cells/cm² | 22,220 cells/cm² |
|--------|----------------|------------------|------------------|
| Scx    | 26.5           | 17.6             | 21.6             |
| Tnmd   | 27.6           | 17.6             | 21.6             |
| Col1a1 | 23.1           | 17.6             | 21.6             |
| Aqp1   | 28             | 17.6             | 21.6             |

The relative mRNA levels were calculated using the 2^ΔΔCt method using the 11,110 cells/cm² plating condition as control. For each gene, the mRNA levels of the 11,110 cells/cm² plating condition were normalized to 1 (green spots). Graph shows means±s.d. of six biological samples. (C) RT-qPCR analyses of the expression levels for the tendon markers Scx, Tnmd, Col1a1 and Aqp1 and for lineage markers, Bglap (bone), Acan (cartilage), Myog (muscle) and Pparg (fat) in C3H10T1/2 cells 16 h after initial plating 11,110 cells/cm² on plastic culture plates. mRNA levels on the Y-axis are reported to the Rplp0 (36b4) gene (2^ΔCt×10⁴). Graph shows means±s.d. of six biological samples. The means of the initial Cts (obtained from 250 ng of mRNA) are indicated in brackets for each gene. Rplp0 (Cts=22.3 s.d.±1); Scx (Cts=26.2 s.d.±0.63); Tnmd (Cts=24.1 s.d.±0.62) and Col1a1 (17.9 s.d.±0.57).
musculoskeletal system, tendon cells are continuously subjected to variations in their mechanical environment (Schiele et al., 2013). Physical constraints subjected to the cells have been shown to be important for developmental processes and during the adult life (Mammoto et al., 2013). It is recognized that substrate stiffness controls many cellular processes such as cell fate, migration, proliferation and differentiation in culture systems of stem cells or progenitor cells (Bellas and Chen, 2014; Ivanovska et al., 2015; Kilian et al., 2010). MSCs are particularly responsive to matrix stiffness in terms of lineage commitment, ranging from neurogenic phenotype for soft substrates to osteogenic when cultured on rigid substrates (Discher et al., 2009; Engler et al., 2006; Humphrey et al., 2014). The forces transmitted through cell contacts upon confluence is another parameter that mechanically constrains cells in culture dishes and influences cell differentiation (Abo-Aziza and Zaki, 2017; Ren et al., 2015).

The tendon phenotype is not maintained in 2D-cultures of tendon cells over passages (Hsieh et al., 2018; Shukunami et al., 2018; Yao et al., 2006). 3D-culture systems in which tendon cells are embedded in hydrogels are recognized to provide an environment closer to that experienced by tendon cells in vivo (Kapacee et al., 2010; Kuo et al., 2010; Marturano et al., 2016; Yeung et al., 2015).

Fig. 2. The nature of the substrate does not modify gene expression profiles in C3H10T1/2 cells in non-confluent conditions. (A,B) Photographs of C3H10T1/2 cells cultured on plastic plates displaying a stiffness of 1 GPa (A) and on Uniflex culture plates made of silicon coated with type I collagen displaying a stiffness of 5 Mpa (B) in non-confluent conditions. (C,D) RT-qPCR analyses of gene expression levels in C3H10T1/2 cells. Scx, Tnmd, Col1a1, Aqp1 and representative genes for the bone (Bglap), cartilage (Acan), muscle, (Myog) and fat (Pparg) lineages in C3H10T1/2 cells in non-confluent conditions on plastic (C) and silicone (D) substrates. The means of the Cts (obtained from 500 ng of mRNAs) are indicated in brackets for each gene. (C) Plastic substrate: for each gene, the \( \Delta Ct \) was calculated using Rn18S as a reference gene. \( \Delta Ct = Ct \text{gene} - Ct \text{Rn18S} \). The mRNA levels were reported using the \( 2^{-\Delta Ct} \) method. In order to obtain values above 1, each \( 2^{-\Delta Ct} \) were multiplied per \( 10^6 \). Graph shows means±s.d. of four biological samples. (D) Silicone substrate: for each gene, the \( \Delta Ct \) was calculated using Rplp0 as a reference gene. \( \Delta Ct = Ct \text{gene} - Ct \text{Rplp0} \). The mRNA levels were calculated using the \( 2^{-\Delta Ct} \) method. For each gene, \( 2^{-\Delta Ct} \) were multiplied per \( 10^3 \). Graph shows means±s.d. of six biological samples.
Fig. 3. See next page for legend.
The mechanical environment provided to tendon cells homogeneously embedded within hydrogel in 3D-culture systems is recognized to act on tendon gene expression (Hsieh et al., 2018; Marturano et al., 2016). Most of the analyses of the effects of 2D and 3D environments have been performed with tendon stem/progenitor cells; however, the optimum culture conditions that drive tendon cell differentiation from MSCs have not been yet identified.

In the present study, we analyzed the tendon differentiation potential of C3H10T1/2 cells under different mechanical and molecular signals in 2D- and 3D-culture conditions.

RESULTS

In order to investigate tendon differentiation potential, we used C3H10T1/2 cells, a multipotent cell line established from mouse embryos (Reznikoff et al., 1973). C3H10T1/2 cells are known to differentiate into chondrocytes, osteocytes and adipocytes when cultured under appropriate cues (Guerquin et al., 2013). These cells have the ability to display a tendon phenotype under inductive molecular cues, such as the transcription factors EGR1 and MKX (Guerquin et al., 2013; Liu et al., 2015). The ability to differentiate into cell lineages related to the musculoskeletal system makes the C3H10T1/2 cells an ideal tool to study tendon commitment and differentiation under different mechanical and molecular cues in 2D- and 3D-culture conditions. To assess tendon differentiation, we used the mRNA levels of key tendon markers, Scx and Tnmd, in addition to Coll1a1, the main structural and functional tendon component. We also used tendon genes identified in the transcriptomic analysis of mouse tendon cells during development (Havis et al., 2014), such as aquaporin1 (App1) gene coding for a water channel protein and thrombospondin 2 (Thsb2) coding for an adhesive glycoprotein with antiangiogenic properties, both expressed in developing limb tendons.

Seeding density does not affect tendon gene expression in non-confluent conditions after 16 h of culture

We first determined whether the initial cell number interfered with the expression of tendon genes in non-confluent conditions. Different amounts of cells (0.5×10^5, 1×10^5, and 2×10^5) were seeded in 9 cm² culture plates (plastic substrate), corresponding to 5555 cells/cm², 11,110 cells/cm², and 22,220 cells/cm², respectively. After 16 h of culture, the expression of tendon genes, Scx, Tnmd, Coll1a1, and App1 did not display any change more than 20% upon different cell density seeding conditions (Fig. 1A,B). This shows that the initial cell number at seeding time does not have a major influence on tendon gene expression in expansion and non-confluent conditions.

We next compared the relative mRNA expression levels between tendon genes in C3H10T1/2 cells in non-confluent conditions on plastic substrate in the 11,110 cells/cm² seeding condition (Fig. 1C). The expression levels of each tendon gene were reported to the Rplp0 gene (Ct=19.8 for 250 ng of RNAs). We found that the Coll1a1 gene displayed high expression levels (Ct=17.6) compared to those of App1 (Ct=23.1), Scx (Ct=26.5) and Tnmd (Ct=27.6) genes in C3H10T1/2 cells in the non-confluent condition (Fig. 1C). Comparison with tendon gene expression in native adult mouse tendons indicated a similar tendency of high expression levels of Coll1a1 gene compared to Scx and Tnmd (Fig. 1D). Tnmd was also more expressed than Scx in adult mouse tendons (Fig. 1D), highlighting Tnmd as a potent tendon marker as already reported (Takimoto et al., 2012). Analysis of the mRNA expression levels for other lineage markers showed that Acan (cartilage) and Pparg (fat) genes were not expressed (Ct above 32), while Bglap (bone, Ct=28) and Myog (muscle, Ct=28.6) genes displayed low levels of expression in C3H10T1/2 cells in non-confluent conditions on plastic substrate (Fig. 1C). This shows that tendon genes are expressed in C3H10T1/2 cells seeded in non-confluent conditions on plastic substrate after 16 h of culture, with an expression level superior to that of other differentiation markers such as bone, cartilage, muscle and fat. We conclude that C3H10T1/2 cells display a fibroblastic phenotype.

Gene expression profiles are similar in C3H10T1/2 cells seeded on two different substrates on the rigid scale in non-confluent conditions after 16 h of culture

The same density of C3H10T1/2 cells (11,110 cells/cm²) was plated on classic culture plastic plates and Uniflex Flexcell plates made of silicone substrate coated with type I collagen (Fig. 2A,B). Plastic substrate displays a Young Modulus of 1 GPa magnitude and is considered as extremely rigid. Uniflex Flexcell plates display a stiffness estimated at 5 MPa by the company (Flexcell International Corporation). The silicon substrate is 200-fold less rigid (5 MPa) compared to the plastic substrate (1 GPa) but is still considered as rigid on the micro-stiffness scale for substrates (Discher et al., 2009). C3H10T1/2 cells were harvested 16 h after plating at a non-confluent state (Fig. 2A,B) and a similar amount of mRNA was analyzed for gene expression. Tendon and other lineage marker expression profiles were similar in both substrate culture conditions (Fig. 2C,D). This shows that two substrates with different levels of stiffness on the rigid scale do not affect gene expression profiles in C3H10T1/2 cells seeded in non-confluent conditions for 16 h.

Differentiation potential of C3H10T1/2 cells cultured on plastic substrate over time

We investigated the tendon differentiation potential of C3H10T1/2 cells cultured on plastic substrate over time. Cells were plated on plastic culture plates at 11,110 cells/cm² density and left for 16 h, defined as day 0. C3H10T1/2 cells were let to grow for 14 days with no passage. C3H10T1/2 cells were harvested at 1 day, 7 days, 10 days and 14 days of culture. The cell density of C3H10T1/2 cells was measured (Fig. 3A,B) at each time point. At day 0 we obtained 17,100 cells/cm² (s.d.±4885, N=12). Cells expanded until day 10 and reached a plateau from day 10 to day 14, defining two phases, one expansion phase until day 10 and a post-expansion phase after day 10 (Fig. 3B).

Lineage-specific gene expression analysis was conducted in order to assess the differentiation behavior of C3H10T1/2 cells cultured on plastic substrate over time. During the expansion phase (before day 10), Scx, Coll1a1 and App1 genes displayed a continuous decrease of mRNA levels, while Tnmd mRNA levels displayed a bell shape with a maximum of twofold increase between day 1 and day 7 (Fig. 3C). During the post-expansion phase, Scx and
Fig. 4. See next page for legend.
Myog Rplp0 substrate at different time points. Gene mRNA levels were normalized to the expression levels of tendon markers, C3H10T1/2 cells cultured on silicon substrate over time. Tnmd gene expression in C3H10T1/2 cells cultured on silicone substrate over time.

We conclude that the expansion phase has a positive effect on Tnmd gene expression in C3H10T1/2 cells cultured on silicone substrate over time.

Tendon differentiation potential of C3H10T1/2 cells in a 3D-culture system

We next investigated the differentiation potential of C3H10T1/2 cells in a 3D-culture system.

We used the 3D-fibrin gel method to produce in vitro-engineered tendons (Gaut et al., 2016; Guerquin et al., 2013, 2014, 2016; Lorda-Diez et al., 2010). This 3D-culture system is based on tension (Bayer et al., 2010) and has been extensively characterized for matrix production by tendon progenitor cells (Yeung et al., 2015). We engineered 3D-fibrin constructs with C3H10T1/2 cells (Fig. 5A–C). 3D-fibrin constructs took 5–7 days to fully form depending on the cultures (Fig. 5A). Day 0 was defined as when constructs were formed (Fig. 5A,B). Transverse sections to a 24-h construct show a homogeneous cell organization within the constructs (Fig. 5C). Longitudinal and transverse sections of 2-week-old constructs highlighted sustained homogeneous cell organization overtime (Fig. 5D). We compared tendon gene expression in C3H10T1/2 cells cultured in a 3D environment versus 2D plastic condition. The relative mRNA levels of Scx and Colla1 were significantly increased in C3H10T1/2 cells cultured in 3D versus 2D conditions, while those of Tnmd were not above 10 days of cultures (Fig. 5E).

Tendon and cartilage gene expression was analyzed at different time points (day 0, day 2, day 4, day 7, day 11 and day 18). The day 0 time point corresponds to the day when the constructs were formed (Fig. 5A) and was the reference time point. The expression profile of tendon genes at day 0 in 3D-fibrin constructs (Fig. 5F) was similar to that in 2D-cultures (Figs 1C and 2C,D), i.e. relatively high levels of Colla1 mRNAs compared to Scx and Tnmd. In contrast to a decrease in 2D-cultures (Figs 3 and 4), Scx and Colla1 displayed an unchanged expression in 3D-fibrin constructs over time following that observed in day 0 (Fig. 5G). Similarly to 2D cultures (Figs 3C and 4C), Tnmd expression displayed a bell shape with a maximum of twofold increase between day 0 and day 7 in 3D-fibrin constructs (Fig. 5G). The cartilage genes, Sox9 (progenitors), Acan and Col2a1 (differentiated cells) were expressed at day 0 (Fig. 5H) and increased over time in 3D-fibrin constructs, indicating that the potential of C3H10T1/2 cells to differentiate into cartilage is maintained in 3D-fibrin constructs (Fig. 5I). The expression of Ppar (early fat differentiation marker) was above 32 cycles at day 7, indicating an absence of adipocyte differentiation of C3H10T1/2 cells in 3D-fibrin constructs.

We conclude that the 3D-environment in fibrin gel maintains tendon gene expression in C3H10T1/2 cells over time.

TGFβ effect on tendon gene expression in C3H10T1/2 cells in 2D- and 3D-culture systems

The canonical TGFβ/SMAD2/3 pathway is recognized to have a pro-tenogenic effect in cell cultures based on Scx expression (Guerquin et al., 2013; Havis et al., 2014, 2016; Lorda-Diez et al.,
Fig. 5. See next page for legend.
conditions, we already showed that Sox9 expression was drastically decreased in TGFβ2-treated C3H10T1/2 cells (Havis et al., 2014). In order to test if the negative effect of TGFβ2 on Tnmd expression was inherent to the 2D-culture system, we also applied TGFβ2 in C3H10T1/2 cells cultured in 3D-fibrin gel. TGFβ2 was added in the culture medium of tendon constructs for 24 h and compared to non-treated constructs harvested at the same time. No apparent differences could be observed in the morphology of the TGFβ2-treated constructs when compared to controls (Fig. 6F). Consistent with the results obtained in 2D-cultures (Fig. 6D,E), we found an increase in the expression of Scx and a concomitant decrease in Tnmd expression in TGFβ2-treated 3D-tendon constructs compared to control constructs (Fig. 6G). Col1a1 expression was increased, as was that of Scx, while Agp1 and Thbs2 expression was decreased, as was that of Tnmd upon TGFβ2 exposure (Fig. 6G). The expression of cartilage genes was decreased (Sox9 and Acan) or not changed (Col2a1) upon TGFβ2 exposure (Fig. 6G), indicating an absence of cartilage differentiation upon TGFβ2 exposure. This shows that TGFβ2 has a negative effect on Tnmd expression, while having a positive effect on Scx expression in C3H10T1/2 cells cultured in 3D-culture conditions.

We conclude that TGFβ2 is a negative regulator of Tnmd expression in C3H10T1/2 cells in 2D- and 3D-culture systems.

DISCUSSION

In the present study, we analyzed the tendon differentiation potential of C3H10T1/2 cells cultured in different conditions. Our results show that C3H10T1/2 cells behave differently for tendon gene expression depending on the substrate on which they were seeded in 2D cultures and 3D environment. We also identified TGFβ2 as a potent negative regulator of the tendon differentiation marker Tnmd in C3H10T1/2 cells in 2D- and 3D-culture systems.

Tendon differentiation potential for C3HT101/2 cells cultured on plastic and silicon substrates

C3HT101/2 cells, although they express tendon genes in 2D-cultures (Fig. 1), are not preferentially committed to the tendon lineage as compared to primary tendon or ligament cells originating from native tissues. We found that the initial tendon gene profile was similar in C3H10T1/2 cells seeded on silicone and plastic substrate in non-confluent 2D conditions (Fig. 2). However, the silicone substrate was more prone to maintain the tendon phenotype of C3H10T1/2 cell cultures during the expansion and post-expansion phases over time compared to plastic substrate (Figs 3 and 4). A way to compare substrates of different chemical composition is to look at their stiffness. The design of our study allowed us to compare two substrates, plastic (1 GPa magnitude) and silicone (5 MPa) with a 200-fold difference in stiffness on the rigid scale. The extreme rigidity of plastic substrate (1 GPa) progressively decreases the expression of Scx, while a relatively less rigid substrate (5 MPa) decreases Scx and Col1a1 by twofold in 1 day but then maintains their expression over time. The silicone substrate favors the expression of the tendon differentiation marker, Tnmd during the expansion phase. Based on Scx and Tnmd expression, we conclude that a substrate of 5 MPa rigidity favors the tendon phenotype in C3H10T1/2 cells over time. Although the stiffness values of both substrates display a 200-fold difference, these two substrates are still in the rigid scale favorable for bone differentiation (Discher et al., 2009). Consistently, C3H10T1/2 meniscus stromal cells cultured on these two substrates (plastic and silicone) display a significant and drastic increase in the expression of the bone differentiation marker (bglap) over time. Because there was no addition of bone
differentiation medium in the culture conditions, we believe that cell confluence favors bone differentiation of C3H10T1/2 cells cultured on these two rigid substrates. The dramatic increase in the expression of the early differentiation fat marker Pparg in plastic substrate (high stiffness) is counterintuitive with the range of soft stiffness known to promote fat differentiation (Discher et al., 2009). We interpret the ability of C3H10T1/2 cells to differentiate towards the fat lineage under a stiff substrate by the fact that C3H10T1/2
cells make multilayers upon confluence. One obvious hypothesis is that cells expressing Pparg at 14 days of culture could be those in the superficial cell multilayer, not in contact with the plastic substrate and thus creating a soft environment.

**TGFβ is a potent negative regulator of Tnmd expression in C3H10T1/2 cells in 2D- and 3D-culture systems**

Our work identifies a striking inverse correlation between Tnmd expression and TGFβ activity (assessed with Smad7 expression) in C3H10T1/2 cells cultured in 2D conditions on both plastic and silicone substrates and in 3D fibrin gel systems over time. Consistently, TGFβ2 drastically decreases Tnmd expression, while promoting that of Scx in C3H10T1/2 cells cultured in 2D- and 3D-culture systems. The opposite behavior of Scx and Tnmd expression in cell cultures over time and upon TGFβ application could reflect different steps of tenogenesis, with a progenitor step revealed by Scx and a differentiation one by Tnmd. During development, Scx is expressed before Tnmd and it has been shown that Scx is required and sufficient for Tnmd expression in developing tendons (Murchison et al., 2007; Shukunami et al., 2006). Scx and Tnmd also display opposite expression profiles in primary tendon cells over time (Shukunami et al., 2018). Moreover, Scx has been recently shown to directly regulate Tnmd transcription in primary tendon cells (Shukunami et al., 2018). The absence of Tnmd activation concomitant with Scx increase upon TGFβ application (Fig. 6D–F) is unexpected but indicates that TGFβ inhibits Tnmd expression in C3H10T1/2 cells in 2D- and 3D-culture conditions. It has to be noted that TGFβ2 increased the expression of both Scx and Tnmd genes in chick and mouse limb explants (Havis et al., 2014, 2016), in high-density cultures of chick limb cells (Lorda-Diez et al., 2009) or in 3D-culture systems made of human tendon cells (Bayer et al., 2010). We cannot exclude that the negative regulation of TGFβ on Tnmd expression is cell-type specific and related to mesenchymal stem cells. The relevance to the in vivo situation of Tnmd inhibition by TGFβ2 in C3H10T1/2 cells requires further investigation.

**Conclusion**

This study shows that culture conditions such as expansion, confluence, substrates, and 2D and 3D environment affect the tendon differentiation potential of a murine cell line of mesenchymal stem cells, C3H10T1/2 cells. We also identify TGFβ2 as a negative regulator of Tnmd expression in C3H10T1/2 cells in 2D- and 3D-culture systems. The identification of the optimum conditions that induce tendon cell differentiation in vitro is of particular interest for optimization of tendon cell culture protocols from stem cells that can be used for tendon repair.

**MATERIALS AND METHODS**

**Tendon isolation from adult mice**

C57Bl/6 wild-type mice were purchased from Janvier (France). Achilles tendons were isolated from five wild-type mice at 5 months of age. The two Achilles tendons of the same mice were pooled to form one biological sample and processed for RT-qPCR analysis.

**Cell cultures**

The multipotent mouse mesenchymal stem cells, C3H10T1/2 cells (Renzikoff et al., 1973), were cultured on six-well TTP plastic culture plates (Merck) or six-well Uniflex Flexcell plates (FlexCell Int) made of silicone substrate coated with type I collagen, in Dulbecco’s Modified Eagles Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) 1% penicillin-streptomycin (Sigma-Aldrich), 1% glutamin (Sigma-Aldrich) and incubated at 37°C in humidified atmosphere with 5% of CO2. The culture medium was changed every 48 h.

To study the effect of cell number on tendon gene expression, 0.5×10⁵ (5555 cells/cm²), 10⁵ (11,110 cells/cm²), 10⁵ (11,110 cells/cm²) and 2×10⁵ (22,220 cells/cm²) C3H10T1/2 cells were seeded in 9 cm² six-well TTP tissue culture plates (plastic substrate), left for 16 h in culture and analyzed for tendon gene expression by RT-qPCR. 250 ng of RNA were extracted from each sample before proceeding with RT-qPCR. To study the effect of the initial cell number, six samples (N=6) were analyzed in each cell density condition. The Rplp0 gene was used as the reference gene. For the analysis of the differentiation potential of C3H10T1/2 cells seeded on plastic substrate, 10⁵ cells were seeded in six-well TTP culture plates (11,110 cells/cm²) and left for 16 h in culture. This defined day 0 (N=4) and then cells were cultured for another 24 h (1 day) (N=4), 7 days (N=4), 10 days (N=5) and 14 days (N=4). 500 ng of RNA were extracted from each sample before proceeding with RT-qPCR. For analysis of the differentiation potential of C3H10T1/2 cells seeded on silicone substrate coated with type I collagen, 10⁵ cells were seeded in six-well Uniflex Flexcell plates and left for 16 h in culture. This defined day 0 (N=5), then cells were cultured for another 24 h (1 day) (N=6), 48 h (2 days) (N=6), 7 days (N=6) and 11 days (N=6). 500 ng of RNA were extracted from each sample before proceeding with RT-qPCR.

**3D-engineered tendon constructs in fibrin gels**

3D fibrin-based tendon-like constructs made of mouse C3H10T1/2 cells were performed as previously described (Kapacee et al., 2008). Briefly, for each construct, 400 μl of cell suspension (7.5×10⁵ cells) were mixed with 20 mg/ml fibrinogen (Sigma-Aldrich) and 200 U/ml thrombin (Sigma-Aldrich). The fibrin gels containing cells were seeded in prepared SYLGARD-covered wells (DowChemical, Midland, MI, USA), in which two 8 mm sutures (Ethican, Sommerville, NJ, USA) were pinned 10 mm apart. Culture medium containing 200 μM of L-ascorbic acid 2-phosphate was added to the wells and gels were scored every day for a proper contraction into a linear construct. After 5–7 days, the C3H10T1/2 cells formed continuous tendon-like constructs between the two anchors. This was considered day 0. Each tendon construct was considered as a biological sample. The mRNA levels of each construct were analyzed by q-RT-PCR at 2 days, 4 days, 7 days, 11 days and 18 days after day 0.

**TGF-β treatment on 2D and 3D cultures**

10⁴ or 10⁵ C3H10T1/2 cells were plated on six-well TTP culture plates (plastic) and grown for 40 h. Then, human recombinant TGFβ2 (RD System) was applied at 20 ng/ml to C3H10T1/2 cells for 24 h. Cells were grown for another 24 h without TGFβ2 supplementation in the medium. Control cells were treated with Bovin Serum Albumin and HCl (BSA-HCl) in the same atmosphere with 5% of CO2. The culture medium was changed every 48 h.

To study the effect of cell number on tendon gene expression, 0.5×10⁵ (5555 cells/cm²), 10⁵ (11,110 cells/cm²), 10⁵ (11,110 cells/cm²) and 2×10⁵ (22,220 cells/cm²) C3H10T1/2 cells were seeded in 9 cm² six-well TTP tissue culture plates (plastic substrate), left for 16 h in culture and analyzed for tendon gene expression by RT-qPCR. 250 ng of RNA were extracted from each sample before proceeding with RT-qPCR. To study the effect of the initial cell number, six samples (N=6) were analyzed in each cell density condition. The Rplp0 gene was used as the reference gene. For the analysis of the differentiation potential of C3H10T1/2 cells seeded on plastic substrate, 10⁵ cells were seeded in six-well TTP culture plates (11,110 cells/cm²) and left for 16 h in culture. This defined day 0 (N=4) and then cells were cultured for another 24 h (1 day) (N=4), 7 days (N=4), 10 days (N=5) and 14 days (N=4). 500 ng of RNA were extracted from each sample before proceeding with RT-qPCR. For analysis of the differentiation potential of C3H10T1/2 cells seeded on silicone substrate coated with type I collagen, 10⁵ cells were seeded in six-well Uniflex Flexcell plates and left for 16 h in culture. This defined day 0 (N=5), then cells were cultured for another 24 h (1 day) (N=6), 48 h (2 days) (N=6), 7 days (N=6) and 11 days (N=6). 500 ng of RNA were extracted from each sample before proceeding with RT-qPCR.

**Conclusion**

This study shows that culture conditions such as expansion, confluence, substrates, and 2D and 3D environment affect the
volume applied for TGFβ2 treatment. TGF-β2-treated and non-treated C3H10T1/2 cells were then fixed and processed for qPCR assays to analyze gene expression. In each condition, four biological samples (N=4) were used. 3D tendon constructs were treated with TGFβ2 or with BSA-HCl (controls) at day 7 of culture for 24 h. In each condition, five biological samples (N=5) were used for qPCR analysis.

RNA isolation, reverse transcription and RT-qPCR

Total RNAs were extracted from 2D and 3D cell cultures: C3H10T1/2 cells were cultured on classic culture dishes at day 0, 1 day, 7 days, 10 days and 2 weeks; and TGF-β2-treated C3H10T1/2 cells were cultured in 2D and 3D conditions. Total RNA was isolated using the RNAeasy mini kit (Qiagen) with 15 min of DNase I (Qiagen) treatment according to the manufacturer’s protocol. For RT-qPCR analyses, 250 ng or 500 ng RNA was reverse-transcribed using the High Capacity Retroscript kit (Applied Biosystems). RT-qPCR PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) using the primers listed in Table 1. We used rnl18s (also named 18S) and Rppl0 (also named 36b4) as housekeeping genes. The rnl18s and Rppl0 genes did not show any variation in the different experimental conditions. The Rppl0 gene is detected around a Ct (threshold cycle) of 19.5 for 250 ng of RNAs and around a Ct of 18.5 for 500 ng of RNAs. This result is consistent with the log2-linear plot of the PCR signal. A decrease of one cycle corresponds to a twofold increase of RNA (Livak and Schmittgen, 2001; Schmittgen and Livak, 2001). ΔCt values were obtained by calculating the differences: Ct (experimental sample) - Ct (housekeeping gene). The relative mRNA levels were calculated using the 2^ΔΔCt method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2001). The ΔCt values were calculated by determining the differences between ΔCt (experimental condition) and the average of control ΔCt values. For the analysis of the relative mRNA levels of cells cultured over time in classic culture plates (plastic substrate), Uniflex Flexcell plates (silicone substrate) or 3D-fibrin condition) and the average of control cells in 2D or 3D conditions, the cells in the absence of TGFβ2 were normalized to 1. For the relative mRNA level analysis in TGF-β2-treated cells in 2D or 3D conditions, the cells in the absence of TGFβ2 supplementation were considered as controls and were normalized to 1.

For the absolute quantification of gene expression, 16 h after plating 10^5 cells, Y-axes correspond to 2^-ΔCt×10^3 against the Rppl0 housekeeping gene from 250 ng of RNA (Fig. 1C), to 2^-ΔCt×10^3 against the Rnl18s housekeeping gene from 500 ng of RNA (Fig. 1C) and 2^-ΔCt×10^3 against the Rppl0 housekeeping gene from 500 ng of RNA (Fig. 2D).

Statistical analyses

Results are shown as means±s.d. The exact number of independent biological samples (4–6) is reported for each experiment. RT-qPCR data were analyzed with the non-parametric Mann-Whitney test with Graphpad Prism V6. The asterisks in histograms indicate P-values that was considered significant, *P<0.05, **P<0.01, ***P<0.001.

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Competing interests

The authors declare no competing or financial interests.

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Author contributions

Conceptualization: M.M., D.D.; Methodology: L.G., M.-A.B., C.B., I.C., M.O.; Formal analysis: L.G.; Investigation: L.G.; Data curation: D.D.; Writing - original draft: D.D.; Writing - review & editing: M.M., D.D.; Supervision: M.M., D.D.; Project administration: D.D.; Funding acquisition: D.D.

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Table 1. Primers used for RT-qPCR

| Gene    | Forward primers                                           | Reverse primers                                           | Accession no. |
|---------|-----------------------------------------------------------|-----------------------------------------------------------|---------------|
| Acan    | 5′-GGCTGAGTATCGTCTCAGAAGAAG-3′                           | 5′-TCAGCTCAGTTAGTTGCTGATTG-3′                             | NM_007541.2   |
| Aqpl    | 5′-GGATCTGGTGCAGAACAGCAG-3′                              | 5′-TACCGCTGAGGGCCTGATTG-3′                                | NM_007541.2   |
| Cebpb   | 5′-GTGGTCACTTCCATGCAAGAC-3′                              | 5′-GAGAGAGGCTACAGAGGAG-3′                                 | NM_007541.2   |
| Col1a1  | 5′-AAGTGGCAGAGAGGTCTCAG-3′                               | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Col2a1  | 5′-AGTGGGAGGCTCAGAGGAG-3′                                | 5′-AGTGGGAGGCTCAGAGGAG-3′                                 | NM_007541.2   |
| Myog    | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Ppar    | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Rln18s  | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Rppl0   | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Runx2   | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Scx     | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Smad7   | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Sox9    | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Thbs2   | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
| Tnmd    | 5′-GGCCGAGGAGAGGCTCAGAGGAG-3′                            | 5′-ACCGAGGAGGCTCAGAGGAG-3′                                | NM_007541.2   |
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