Culturing Dupuytren Fibroblasts in Hypoxic Conditions Presents Different Levels of α-SMA, Collagen Type I and Collagen Modifying Enzymes

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Abstract

Dupuytren's disease (DD) is a benign condition characterized by excess deposition of collagen type I in the extracellular matrix in the palmar fascia and is associated with high presence of myofibroblasts. Transformation of fibroblasts to myofibroblasts is regulated by TGFβ. Myofibroblasts are subject to hypoxic conditions *in vivo* and therefore need to adapt, with the help of the HIF1-α transcriptional regulator. Previously, myofibroblasts cells have been cultured and studied under 20% oxygen (normoxia), whereas physiological conditions present 3-5% oxygen concentration (hypoxia). Here we set out to study and compare the varying gene expression of ACTA2, COL1A1/2, and collagen modifying genes in myofibroblasts in normoxic and hypoxic conditions with and without TGFβ presence. We show that gene expression varies based on the oxygen concentrations and TGFβ presence, as well as describing the differences observed when comparing DD cells to the controls under the different conditions. Taken together, we conclude that culturing under hypoxic conditions provides a more representative environment for Dupuytren cells, therefore leading to more accurate conclusions in future studies.

Introduction

Fibrosis can affect nearly every tissue in the body and is characterized by the accumulation of excess extracellular matrix components[1]. Different forms of fibrosis are responsible for approximately 45% of all deaths in the modern world[2]. The key to fibrosis is the excessive presence of the myofibroblast cell which is the primary collagen-producing cell[3]; overexpression of Collagen type I and III and α-smooth muscle actin (α-SMA) is closely associated with fibrotic tissues[4], as they are indicative of myofibroblast presence. The process of differentiation from fibroblasts to myofibroblasts is regulated by transforming growth factor (TGF)-β[5]. In order to replicate fibrotic conditions *in vitro*, TGFβ1 is added to the culture medium[6].

In fibrotic pathologies the quality of collagen, as well as the quantity, matter. Certain enzymes help determine the quality of collagen that is being produced by inducing modifications to it. The enzyme lysyl oxidase (LOX) is part of the LOX family of proteins that includes LOX, LOXL1, LOXL2, LOXL3 and LOXL4; it is primarily involved in the covalent crosslinking of collagens in the extracellular matrix (ECM) contributing to its tensile strength[7]. Lysyl hydroxylase is encoded by the PLOD1-3 genes and is abnormally activated in fibrosis. PLOD2 in particular requires the chaperone FKBP10 in order to exert its function[8]. Lysyl hydroxylase stabilizes collagen produced by converting lysine to hydroxylysine, making the collagen more resistant to degradation, therefore contributing to the irreversibility of fibrosis[9]. Finally, GLT25D1/COLGALT1 transfers galactose to the hydroxylysine residues of collagen. By doing this it aids in the process of the triple helix formation of the collagen[10].

Dupuytren's disease (DD) is a benign condition which is defined by excessive collagen deposition in the palmar and digital facia[11], resulting in impaired hand function of the patient due to the limited mobility of the fingers. DD is very common, affecting almost 3% of the general population[12]. Genetic factors are the biggest contributors to the cause of the disease, but other factors such as diabetes

mellitus as well as repeated physical injury/stress to the hand could also increase the chances of the development of DD[13]. DD initially presents as a nodule in the palm of the hand, as it progresses a chord extends into the finger, which then contracts, pulling the finger inward toward the palm[14]. No current treatments have proven to be permanently effective[15]; however recent advancements have led to less invasive and more effective treatments such as collagenase injections directly into the effected area[11].

Hypoxia is a common stress factor that can be seen as part of many normal physiological, but mostly as part of many pathological conditions[4]. Earlier, hypoxia has been shown to promote fibrosis and its progression[4,16,17]. Hypoxia-inducible factor 1-alpha (HIF1- α) is encoded by the HIF1A gene[18], it is a transcriptional regulator involved in the cellular adaptation to hypoxia[19]. Overexpression of HIF1- α is commonly seen in many fibrotic pathologies[20] such as in the liver[21] or, for example, in cancer[22]. Cells are usually cultured under 20% oxygen (atmospheric conditions), however these conditions are not representative of the in vivo conditions, where the oxygen concentration is 3-5% (hypoxia)[23].

In this study we aimed to investigate the effects of oxygen tension on collagen modification and collagen production by normal and activated fibroblasts, as well as explore the differences of DD cells compared to healthy controls.

Materials and Methods

Cell culture and treatment. Adult dermal fibroblasts obtained from nodules of DD patients, as well as healthy controls were seeded at 15,000 cells/cm^2 in 6-well plates and Lab-Tek slides. Both cell types were cultured in Dulbecco's modified Eagle medium (DMEM) with sterile fetal bovine serum (FBS) and Penicillin/Streptomycin Solution. Cells were placed at 37ºC in an CO_2 incubator with 5% CO_2. After 24 hours, cells were treated with a starvation medium DMEM (BE12-604F) + 0.5% FBS + P/S + 0.17 mM Ascorbic Acid, after 24h, starvation medium was refreshed. In order to induce hypoxia, cells were then treated with $CoCl_2$100mM stock solution 1000x diluted with sterile water and HCl (end concentration of stock solution is 100µM), a separate culture was treated with Deferoxamine 10mM solution, 1000x diluted with sterile PBS (end concentration of stock solution is 10µM), all with/without 5ng/ml TGFβ for another 24 hrs before harvesting.

Immunofluorescence staining. Cells were washed with PBS and fixed in 1:1 methanol/acetone for 10 minutes and

incubated with 0.5% Triton X-100/PBS for 3 min. Followed by blocking of endogenous biotin with a BIO-RAD Biotin Blocking System. Two chambers were then incubated with 10% serum (species secondary antibody) to check for non-specific binding (control). Subsequently, the remaining cells were then incubated with a primary antibody (Dako M0851- for asma binding, ab138492- for collagen binding) in 2,2% BSA at room temperature, Lab-Tek slides were then washed with PBS-Tween, and incubated with a secondary antibody (GαM IgG biotin (1:500) - for asma binding and GαRb IgG biotin (1:500)- for collagen binding) at room temperature. Finally, cells were washed again with PBS-Tween and then incubated with the tag (SA + Cy3 (1:500)) in DAPI and 2,2% BSA in the dark and at room temperature before being fixed in Citifluor and stored at -20℃. Fluorescent images were obtained with a fluorescence microscope.

SYBRGreen Real Time Polymerase Chain Reaction. TRIzol reagent kit (Invitrogen) was used for RNA isolation. RNA concentration of samples collected were measured using the NanoDrop™ spectrophotometer. Complementary DNA was then synthesized using the FERMENTAS cDNA synthesis kit using the PTC-200 Peltier Thermal Cycler (program: 5 min at 25℃, 60min at 42℃, 5min 70℃). For the RT-PCR the Applied Biosystems ViiA7 machine was used; samples were prepared in triplicates on 384 well plates. Primer mixes were prepared to a concentration of 100µM for each gene, and each reaction had a 6µM concentration of each primer. Primers are as follows: LOX forward, 5′-CGGCGGAGGAAAACTGTCT-3′ and reverse, 5′-TCGGCTGGGTAAGAAATCTGA?-3′; LOXL1 forward, 5′-GCCAGTGGATCGACATAACC-3′ and reverse, 5′-CCAAAACAATATACTTTGGGTTCA-3′; LOXL2 forward, 5′-TGACCTGCTGAACCTCAATG-3′ and reverse, 5′-TGGCACACTCGTAATTCTTCTG-3′; LOXL3 forward, 5′-CACATCACCTGCAAGAGGAC-3′ and reverse, 5′-TGCTGAGTGCAGCAACAGAT-3′; LOXL4 forward, 5′-TCGTGGCTACCTTTCTGAAAC-3′ and reverse, 5′-GTGGCCCTCATACTTCACCTC?-3′; PLOD1 forward, 5′-GAAGCTCTACCCCGGCTACT-3′ and reverse, 5′-CTTGTAGCGGACGACAAAGG-3′; PLOD2 forward, 5′-GGGAGTTCATTGCACCAGTT-3′ and reverse, 5′-GAGGACGAAGAGAACGC-3′; PLOD3 forward, 5′-GCTCTGCGGAGTTCTTCAAC-3′ and reverse, 5′-TAACCACCGGACCTTCTGTC-3′; Col1A1 forward, 5′-GCCTCAAGGTATTGCTGGAC-3′ and reverse, 5′-ACCTTGTTTGCCAGGTTCAC-3′; Col1A2 forward, 5′-TGGCTAGGAGAAACTATCAATGC-3′ and reverse, 5′-TGATGTTCTGAGAGGCATAGTTG-3′; Col3A1 forward, 5′-CCTCCAACTGCTCCTACTCG-3′ and reverse, 5′-GGGCATGATTCACAGATTCC-3′; FKBP10 forward, 5′-CTGGGCTATGGGAGCATC-3′ and reverse, 5′-GCACGGTGTCTTCCTTGTTC-3′; GLT25D1 forward, 5′-TGCTTCCTGAGCCACTACAA-3′ and reverse, 5′-CAAACACAAGCGATTTCTGC-3′; ACTA2 forward, 5′-CTGTTCCAGCCATCCTTCAT-3′ and reverse, 5′-TCATGATGCTGTTGTAGGTGGT-3′; YWHAZ forward, 5′-GATCCCCAATGCTTCACAAG-3′ and reverse, 5′-TGCTTGTTGTGACTGATCGAC-3′. Mastermix was made for each with 0.5µl of primer mix and 5µl of iTAQ SYBR Green (Roche) dye at 2x concentration. Each well contained 5µl of this mastermix and 5ul of 1ng/nl of cDNA sample. The plates were then placed in ViiA 7 Real-Time PCR System for the following Thermal Cycling Protocol: Enzyme activation at 95℃ for 10 minutes for 1 cycle; Denaturation at 95℃ for 15 seconds, Annealing at 60℃ for 30 seconds and Extension at 72℃ for 30 seconds for 40 cycles; and melting curve at an increasing temperature following the ViiA 7 melting protocol. The fold increase was calculated using the delta-delta ct method (2^-delta-delta ct).

Western Blotting. Protein lysates were generated using the BIO-RAD DC Protein Assay kit and absorption was measured at 750nm using a photospectrometer. Proteins (20µm) were loaded onto a 10% polyacrylamide gel (15 wells, 1.0mm)

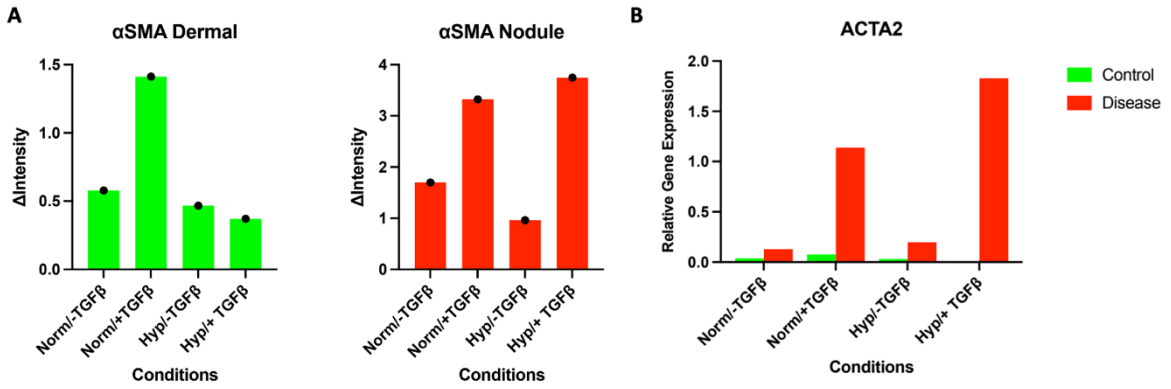

Fig. 1: Western blot and relative gene expression for α-SMA and ACTA2 Dermal and nodule cells were cultured in Deferoxamine (10mM) induced hypoxic condition and normoxic conditions with and without the addition of 5ng/ml TGFB. (A) Shows the Western Blot results of α-SMA protein concentration. (B) Shows relative Gene Expression of ACTA2.

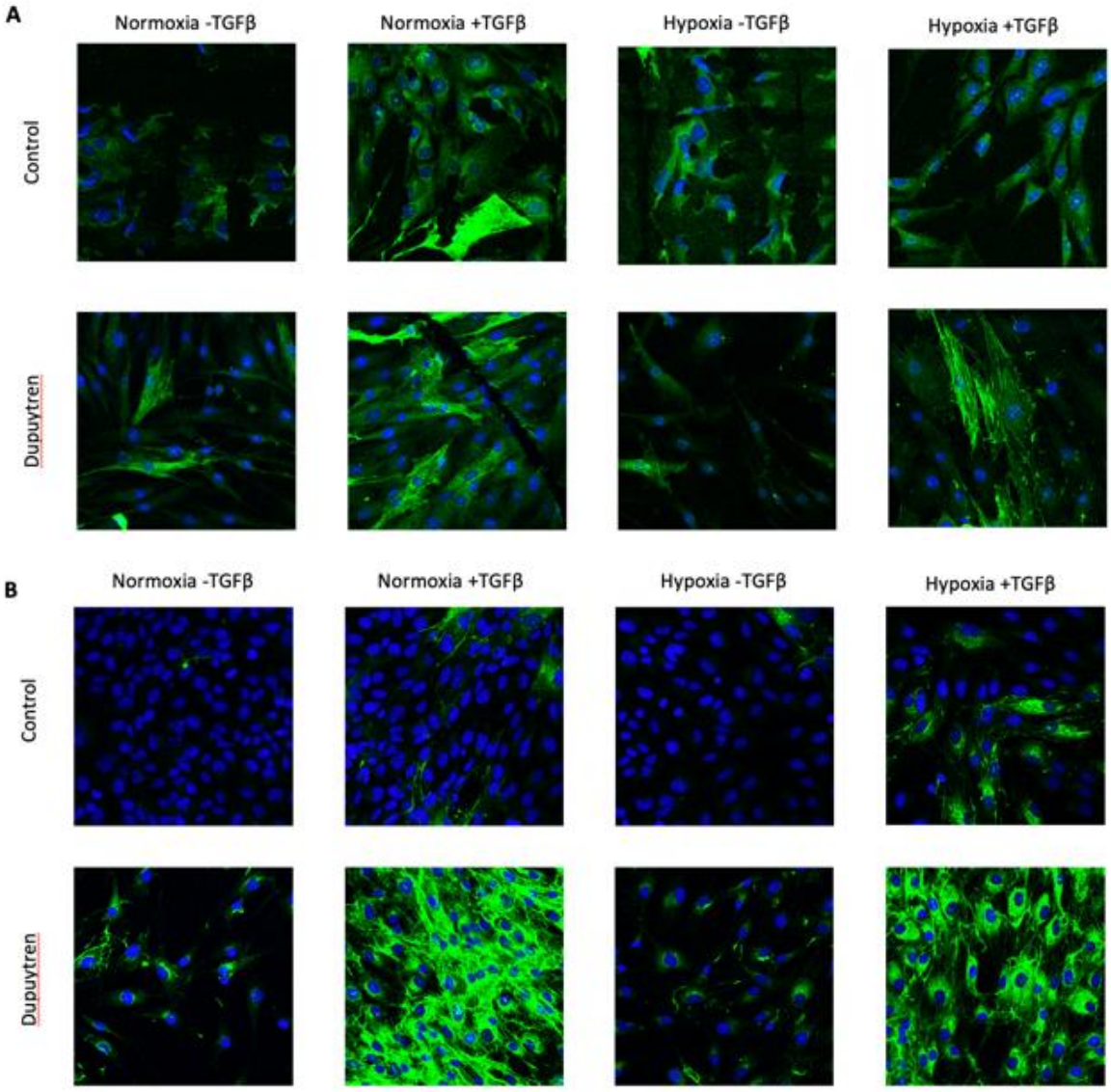

Fig. 2: Immunofluorescence staining for COL1A1 (Collagen type I) and α-SMA. Nuclei visualized with DAPI (blue), proteins in green. (A) Immunostaining for α-SMA. (B) Immunostaining for COL1A1.

along with the marker (PageRuler Plus Prestained Protein Ladder) and electrophoresis was done at 60V for 30min and at 1000V for one hour. Blotting was then done using the Trans-Blot® SD Semi-Dry Transfer Cell kit. Ponceau S was then used to detect proteins on nitrocellulose; blocking was done using 5% skim milk powder diluted with TBS and was left overnight at 4℃. The nitrocellulose sheets were incubated at room temperature for one hour with the primary antibodies suspended in 1% milk powder TBS-Tween (1:1000). The primary antibodies used were as follows: aSMA 1:250 DAKO M0851 (Mouse), YWHAZ 1:1000 ab51129 (Rabbit), PLOD2 1:300 SAB1400213 (Mouse), HiF1a 1:1000 Thermofisher 710059 (Rabbit) and Col1A1 1:1000 ab138492 (Rabbit). The membranes were then washed with TBS-Tween (1:1000) in 10 minute intervals for 30 minutes. The membranes were then incubated with the secondary antibodies for 40 minutes at room temperature. The secondary antibodies are as follows: RaM PO (1:5000) and GaR PO (1:5000). The membranes were then washed with TBS-Tween (1:1000) in 10 minute intervals for 30 minutes. Membranes were then incubated in the SuperSignal West Pico Chemiluminescent Substrate Kit. Chemiluminescent and colorimetric images were obtained with the BIO-RAD ChemiDoc imaging system.

Results

Dupuytren fibroblasts produce more α-SMA under hypoxic conditions.

We first determined the mRNA relative gene expression of ACTA2 in normal dermal fibroblasts and dupuytren fibroblasts. As is already known, α -SMA is indicative of myofibroblasts presence where it is in the cytoskeleton. The results from the western blot analysis (Fig.1a) and RT-PCR (Fig. 1b) indicate a higher level of α-SMA production in dupuytren cells compared to the controls. The addition of TGFβ increased the α-SMA protein levels and the production is even higher in hypoxic conditions. The immunohistochemistry results are consistent with the western blot and the mRNA relative gene expression (Fig. 2a). Therefore, these results point to higher fibroblasts differentiation under the effects of TGFβ and hypoxia.

Dupuytren fibroblasts produce more collage type I under hypoxic conditions.

We also determined the mRNA relative gene expression of COL1A1 and COL1A2 in the dermal and dupuytren fibroblasts and a western blot analysis of COL1A1. Collagen over-production is the hallmark of fibrosis and its deposition in the ECM contributes to the potentially devastating effects of fibrosis[3]. We observed an almost 10-fold increase of collagen protein in dupuytren cells under hypoxic conditions compared to normoxia; an overall increase of collagen protein with the addition of TGFβ1 can also be observed. However, the mRNA relative gene expression results indicated a lower expression of COL1A1 in hypoxic conditions (Fig. 3b) which was not consistent with the western blot results (Fig. 3a). This might be an indication of an underlying mechanism that could lead to such a discrepancy. As part of the immunohistochemistry, collagen deposition in the ECM can be observed under hypoxic conditions, especially with the addition of TGFβ1 (Fig. 2b). These results indicate an overall higher collagen production of collagen type I under hypoxic

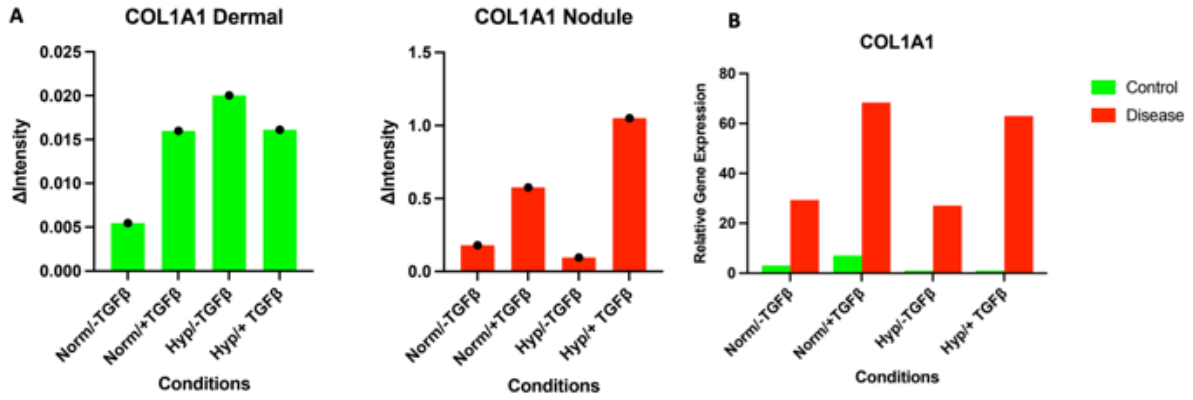

Fig. 3: Western blot and relative gene expression for COL1A1 Dermal and nodule cells were cultured in Deferoxamine (10mM) induced hypoxic condition and normoxic conditions with and without the addition of 5ng/ml TGFB. (A) Shows the Western Blot results of Collagen type I protein concentration. (B) Shows relative Gene Expression of COL1A1.

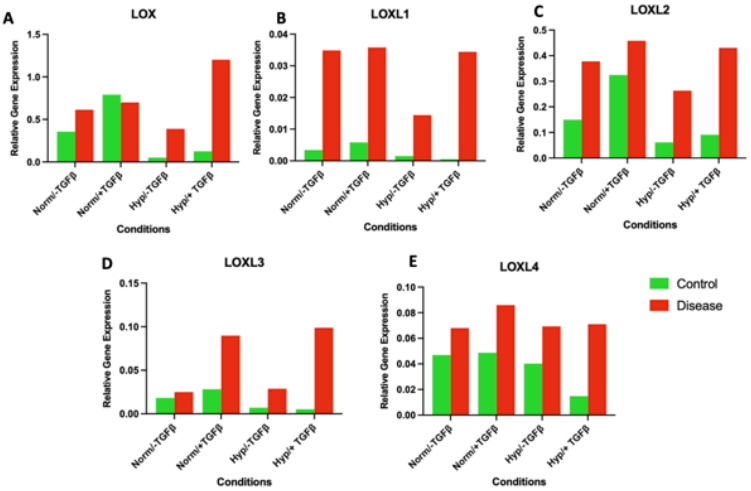

Fig. 4: Relative gene expression of (A) LOX (B) LOXL1 (C) LOXL2 (D) LOXL3 (E) LOXL4

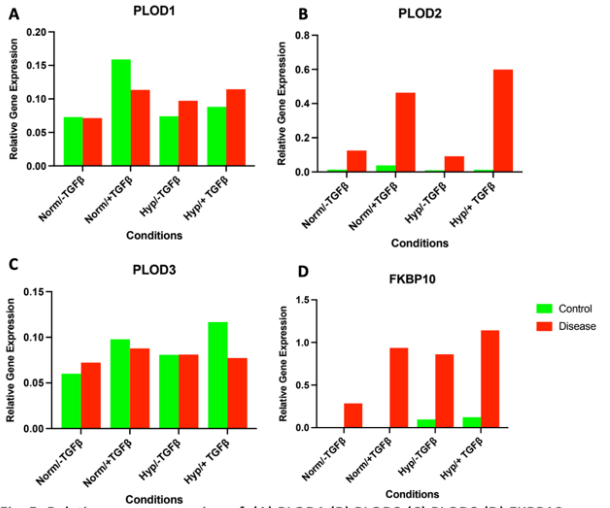

Fig. 5: Relative gene expression of (A) PLOD1 (B) PLOD2 (C) PLOD3 (D) FKBP10

conditions which are further elevated under the influence of TGFβ1.

Collagen produced by dupuytren cells under hypoxia is of different quality compared to that of normoxia.

The quality of collagen that is produced by the myofibroblast cell is determined by collagen modifying enzymes. Generally, there are higher mRNA levels of LOX (Fig. 4a) and LOXL1-4 (Fig. 4b-e) in dupuytren cells compared to normal dermal controls. Dermal fibroblasts show a higher level of LOX (Fig. 4a) and LOXL2(Fig. 4c) in the presence of TGFβ1. LOXL1,3,4 (Fig. 4b,d,e) show a decrease of mRNA in hypoxic conditions. The Dupuytren fibroblasts show an increase in LOXL3 mRNA levels in the presence of TGFβ1 (Fig. 4d); but generally, there is not much effect of hypoxia (both with and without TGFβ1).

A clear increase of PLOD2 in all conditions is seen in Dupuytren cells compared to the controls (Fig. 5b). The PLOD genes encode the enzyme lysyl hydroxylase which hydroxylase the lysine residues of preprocollagen[25]. The addition of TGFβ1 shows an increase in PLOD2 in both normoxic and hypoxic conditions for the Dupuytren fibroblasts, whereas this is only the case in normoxic conditions for the controls (Fig. 5b). The patterns are similar for its chaperone FKBP10 with the exception of a larger increase for the Dupuytren cells in hypoxia (Fig. 5d). PLOD1 and PLOD3 do not show much major differences between hypoxic and normoxic conditions. Generally, TGFβ1 did not have a great effect on PLOD1&3 (Fig. 5a,c).

GLT25D1 is responsible for the glycosylation of procollagen in the cytosol, aiding in its release into the ECM[10]. GLT25D1 mRNA levels observed are higher in Dupuytren cells in all conditions compared to the controls. Dupuytren fibroblasts show higher mRNA levels of GLT25D1 in the presence of TGFβ1 under normoxia; however, the addition of TGFβ1 does not increase the mRNA levels under hypoxia. In the controls, TGFβ1 causes an increase in mRNA under both normoxia and hypoxia, but hypoxia in itself does not elevate the levels (Fig. 6).

Overall, there are clear differences in mRNA levels in both controls and Dupuytren fibroblasts between normoxic and hypoxic conditions, as well as with the addition of TGFβ1. This indicates that the quality of collagen produced varies between the two cell types.

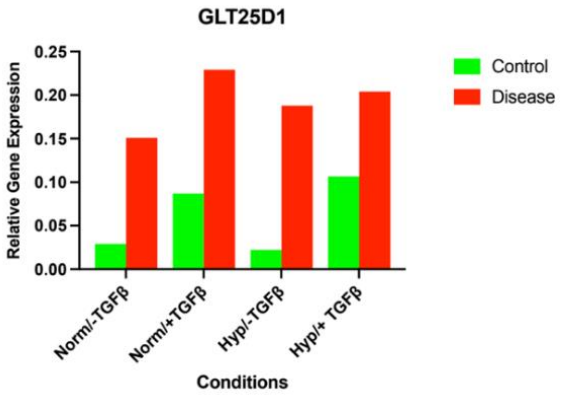

Fig. 6: Relative gene expression of GLT25D1

Discussion

Hypoxia has previously been shown to have an effect on fibroblast growth through the activation of HIF1-a[26]. HIF1-a helps the cell adapt to the stress of hypoxia by acting as a transcriptional regulator, which in turn suppresses or expresses certain genes[27]. Collagen modifying enzymes play a crucial role in determining the quality of collagen that is produced. A greater degree of hydroxylation, glycosylation and a greater amount of cross-links all contribute to the tensile strength of the collagen and the degree as to which the collagen can be degraded[7,9,10], therefore directly affecting the severity of fibrosis of the given tissue. TGFβ is a cytokine which activates fibroblasts and causes their differentiation into myofibroblasts which produce collagen and deposit it into the ECM[28]; adding TGFβ1 in the culture medium replicates the fibrotic conditions *in vitro*.

In this study, we aimed to investigate the differences between DD cells and healthy controls in order to observe gene expression changes and COL1A1 and α-SMA production levels using RT-PCR and Western Blot analysis. The aim was to uncover these differences in order to study what changes culturing under hypoxic conditions and the addition of TGFβ1 will result in. The data obtained suggests that gene expression definitely varies under hypoxic compared to normoxic conditions. The addition of TGFβ1 also brings about varying gene expression. Generally, the genes we studied were more expressed under hypoxic conditions and further expressed with the addition of TGFβ1, with, of course, some exceptions.

As was earlier reported, hypoxia is an aggravating factor contributing to fibrosis and inflammation resulting in a myofibroblast-like phenotype of cells, resulting in greater collagen production[29–31]. Our results align closely with previous suggestions by previous research. We show that collagen modifying enzyme-coding genes such as LOX and LOXL1-4, GLT25D1 and PLOD1-3 and FKBP10 are (mostly) expressed at varying levels in different conditions; indicating that the collagen produced in hypoxic conditions is more hydroxylated, glycosylated and has more cross-links compared to that of normoxic conditions. We also show that DD cells produce more collagen under hypoxic conditions especially with the

addition of TGFβ1; similar results were also obtained with α-SMA.

Taken together, we conclude that DD fibroblasts should be cultured under hypoxic conditions in order to be representative of the physiological environment. By studying cells in an environment closely similar to physiological, we believe more realistic and accurate conclusions can be made in further research. We also describe the differences we observed in the amount and quality of collagen as well as the amount of α-SMA produced.

Our results, although relevant, rely on only a single sample of cells obtained from one DD patient, as well as a single batch of control cells. In order to formulate more concrete and accurate conclusions more biological replicates would need to be obtained; this would allow for statistical significance to be determined, and hence a more representative study. It would also be beneficial to employ different techniques to induce hypoxic conditions, which would allow for comparison and verification of the obtained results.

For future research, we suggest a looking into the PLOD2 and FKBP10 genes. We observed a large increase in gene expression of both the genes in DD cells especially under hypoxia and we believe that inhibiting PLOD2 or its chaperone could provide a potential treatment of DD.

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