Effects of Oxygen Tension and TGF-β1 on Dupuytren's Disease Fibroblasts

By Tony Yoo (S3916545)
Research Project Biomedical Sciences
Supervisors:
Prof. Dr. Ruud Bank
Theo Borghuis

Abstract

Dupuytren's disease is a myofibroblastic disease, affecting up to 42% of the population in certain areas, most prevalent in older men. This is an example of a fibroproliferative disease, which different cases contribute to up to 45% of deaths in the western world. In this paper we aim identify differences in gene expressions and protein expressions by fibroblasts in different oxygen levels.

Introduction

Fibrosis is defined as an excessive deposition of extracellular matrix (ECM), which leads to scarring and consequently resulting in organ failure [1]. This is associated with the proliferation, differentiation and activation of fibroblasts and myofibroblasts, which lead to an increase in ECM components such as collagen[2] . While around 45% of deaths in developed countries around the world are associated with fibrosis[3], the progressivity and lack of effective treatments of fibrosis leave room for further understanding of the dynamics of fibrosis[4].

In most vertebrates, type I collagen is the most abundant structural protein, but synthesized at a relatively slow rate due to its long half-life of about 60 to 70 days. Yet often in cases of fibrosis, this rate shows an increase of up to several hundredfold[5].

Type I collagen is mainly found as a heterotrimer consisting of two α1(I) chains and one α2(I) chain, and is synthesized as a procollagen precursor consisting of N-terminal propeptide, a main collagen domain and C-terminal propeptide[6]. These preprocollagen polypeptides are co-translationally translocated into the endoplasmic reticulum (ER), where their proline and lysine are hydroxylased and these hydroxyl-lysines are then glycosylated. These polypeptides then form a triple helix before being secreted into the ECM and forming fibrils[5].

In fibroproliferative diseases, not only is collagen overexpressed, it also undergoes post translational modifications that stabilise collagen fibrils by formation of intermolecular cross-links which makes it even more difficult to target for degradation. Such modifications are mediated by several collagen modifying enzymes that play a role in at different stages of the biosynthesis of collagen. Examples of such enzymes include: lysyl hydroxylase, encoded by three isoforms of procollagen-lysine, 2-oxoglutarate and 5-dioxygenase (PLOD1 to 3); collagen galactosyltransferase, which mediate the glycosylation of the hydroxylysine residues, and in the case of type I collagen is encoded by the glycosyltransferase 25 domain 1 (GLT25D1) gene; lysyl oxidase (LOX), which mediate oxidative deamination of lysine and hydroxylysine to respective aldehydes, which undergo condensation reactions to form crosslinks within and between collagen molecules. In type I collagen, LOX (encoded by LOX gene) catalyzes reactions involving amine groups derived from telopeptidyl lysine and hydroxylysine, but studies have shown the presence of 4 LOX-like (LOXL) proteins, each encoded by LOXL1-4 genes, which although highly variable, show highly similar biological functions[7].

An example of such fibroproliferative disease is Dupuytren's disease (DD), which is reported to be prevalent in up to 42% of the population. Dupuytren's disease is an example of fibrosis, involving abnormal myofibroblast activation leading to and excess collagen (type I initially, followed by type III) deposition and subsequent decrease in hand mobility and grip strength. The onset of these symptoms start with the formation of palpable nodules in the palm, mainly consisting of myofibroblasts, which when progressed extend into cords that extend the palm and result in contracture[8].

Due to the persistent nature of fibrosis, especially in fibroproliferative diseases, research continues to look potentials for treatment in the pathology of fibrosis, in

hopes that a better understanding of the dynamics of fibrosis would uncover a potential therapeutic target. As such, an important factor that is considered is hypoxia, as studies have shown that hypoxic conditions have significant effect on fibroblast proliferation as well as collagen production[9] . Hypoxia refers to a state of insufficient oxygen level, and in fibrosis plays an important role through the transcription factor hypoxia-inducible factor-1 (HIF-1), associated with the cell mechanism that senses oxygen levels[10]. HIF-1 is also associated with an upregulation of transforming growth factor (TGF)-β1, which pushes the transition of fibroblasts into myofibroblasts, consequently leading to an increase in expression of α-smooth muscle actin (α-SMA) and type I and III collagen[11,12] .
In this experiment we aim to determine whether hypoxic conditions play a role in gene expressions of type I and type III collagen, as well as gene expressions of enzymes that play a role in post-translational collagen modification, including the gene FKBP10, which encodes for immunophilin FKBP65, which studies have shown that inhibition of FKBP65 result in consequent inhibition of lysyl hydroxylase 2 (LH2, encoded by PLOD2 gene) activity. We look into protein production of α-SMA as well as type I collagen, as studies have shown that these are some of the predominant proteins produced in myofibroblasts and fibrosis [5,12,13]. We induce fibrotic conditions in healthy dermal fibroblasts with TGF-β1 to compare the differences in gene expressions and subsequent protein production between healthy dermal fibroblasts and cells obtained from nodules of a DD patient.

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 CO2 incubator with 5% CO2. 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 CoCl2 100mM stock solution 1000x diluted with sterile water and HCl (end concentration of stock solution is 100uM), a separate culture was treated with Deferoxamine 10mM solution, 1000x diluted with sterile PBS (end concentration of stock solution is 10uM), all with/without 5ng/ml TGF-β1 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 α-SMA 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 α-SMA 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 -20C. 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 25C, 60min at 42C,5min 70C). 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 100uM for each gene, and each reaction had a 6uM 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 0.5ul of primer mix and 5ul of iTAQ SYBR Green (Roche) dye at 2x concentration. Each well contained 5ul 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 95C for 10 minutes for 1 cycle; Denaturation at 95C for 15 seconds, Annealing at 60C for 30 seconds and Extension at 72C 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 (20um) were loaded onto a 10% polyacrylamide gel (15 wells, 1.0mm) 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 4C. 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: α-SMA 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

Nodule fibroblasts produce more α-SMA and react differently towards TGF-β1 compared to dermal fibroblasts.

A clear indicator of myofibroblast presence in cell culture is the presence of α-SMA[14], this was also observed in this experiment. The immunohistochemistry images in figure 1 show clear presence of α-SMA, especially when fibroblasts were cultured in the presence of TGF-β1. This is replicated in the western blotting analysis (figure 2) of α-SMA production, where nodule fibroblasts have a clear increase in

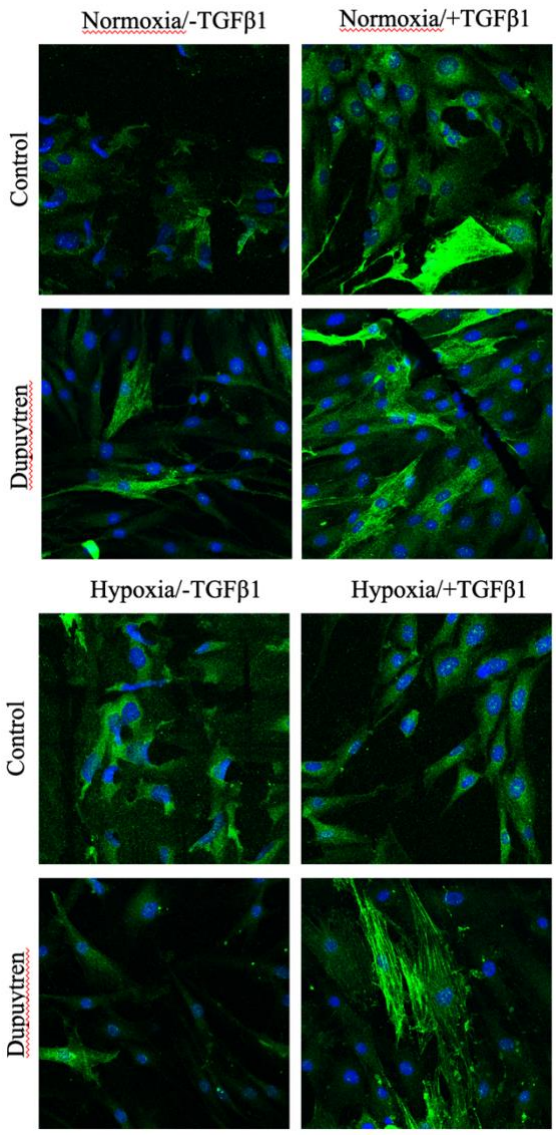


Figure 1: **Immunofluorescence staining for α-SMA, depicting dermal fibroblasts and nodule fibroblasts after culturing, and stimulated in different conditions.** Clear presence of α-SMA seen in the presence of TGF-β1 as well as myofibroblast differentiation and proliferation. Cell nucleus stained in blue.

α-SMA levels compared to dermal fibroblasts. Another difference is the reaction of these fibroblasts to addition of TGF-β1 in hypoxic conditions. Where the nodule fibroblasts have a clear drastic increase in α-SMA proteins levels with the addition of TGF-β1, dermal fibroblasts show a little decrease. This pattern is also seen in the relative gene expression data obtained from the PCR analysis (figure 3), where absolute gene expression of α-SMA is higher in the nodule fibroblast sample

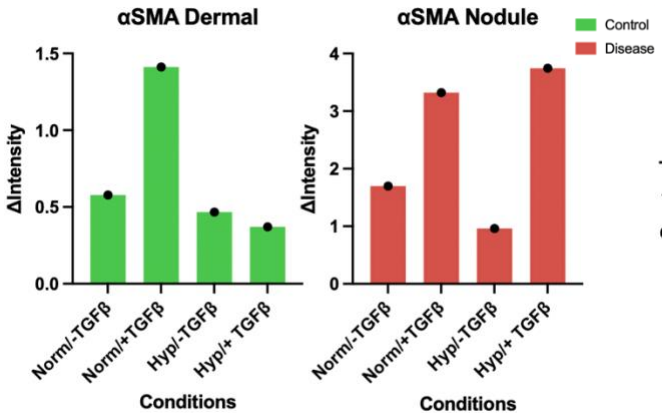


Figure 2:**Western Blot analysis of production of α-SMA protein.** ΔIntensity of each sample calculated relative to intensity of YWHAZ protein of each respective sample. Data shows increased baseline of α-SMA protein in nodule samples compared to dermal samples, and a similar pattern of increase with the addition of TGF-β1within same samples but varying between nodule and dermal.

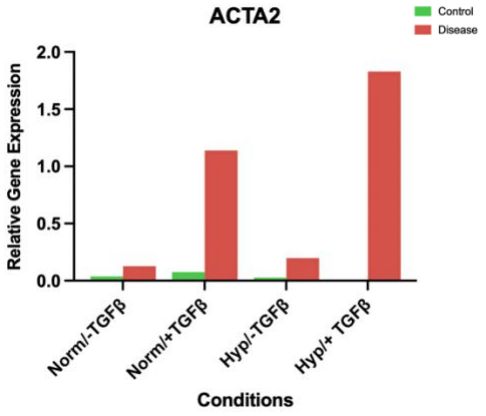


Figure 3: **Relative gene expression of ACTA2 gene (encoding for α-SMA) plotted with the different stimulation conditions.** ΔCt of each sample calculated relative to expression of YWHAZ gene of each respective sample. Data shows increased baseline of ACTA2 gene expression in nodule samples in all conditions compared to dermal samples.

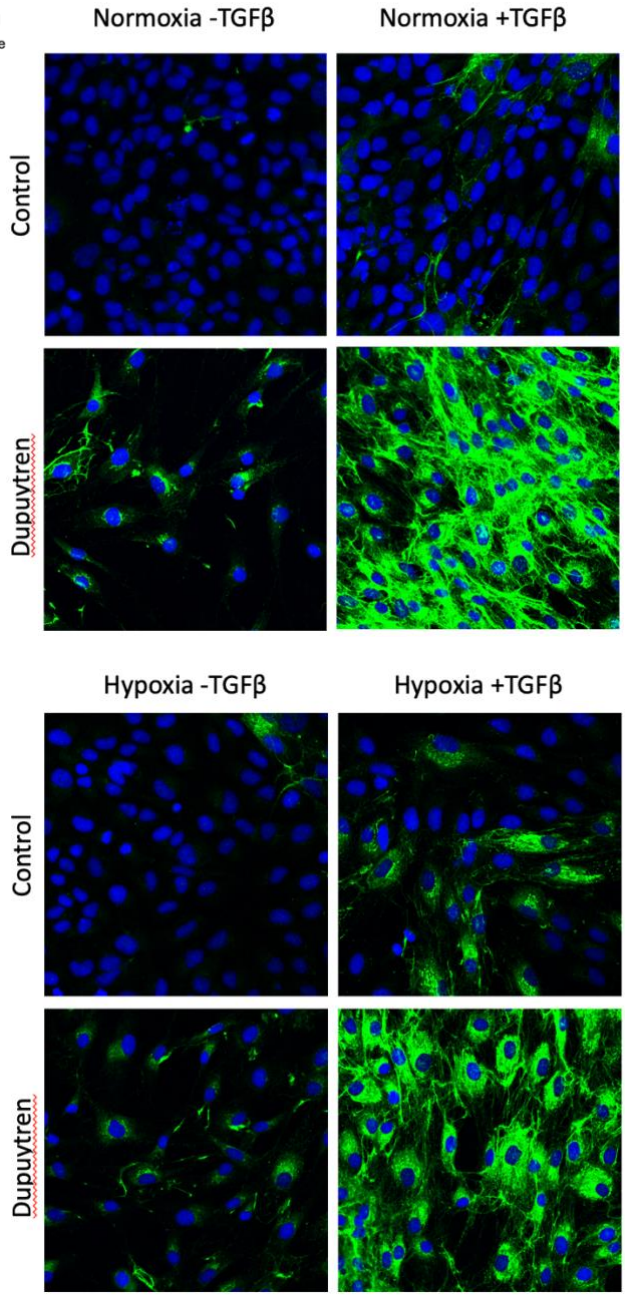


Figure 4: **Immunofluorescence staining for type I collagen, depicting dermal fibroblasts and nodule fibroblasts after culturing, and stimulated in different conditions.** Clear presence of collagen seen in the presence of TGF-β1 as well as in hypoxic conditions. Collagen deposition into ECM also observed in nodule samples. Cell nucleus stained in blue.

compared to the dermal fibroblasts. This is consistent with studies that show increase in myofibroblast activation in hypoxic conditions[11]. Hypoxic conditions resulted in an higher production α-SMA in nodule samples with TGF-β1 but the opposite is seen in dermal samples. Dermal samples show less production of α-SMA in hypoxic conditions, regardless of the addition of TGF-β1. In the absence of TGF-β1, nodule samples produced less α-SMA protein, yet this pattern was not observed in the gene expression of α-SMA, where there was a slight increase in expression in hypoxic conditions without the presence of TGF-β1.

Nodule fibroblasts produce more Collagen and react differently towards TGF-β1 compared to dermal fibroblasts

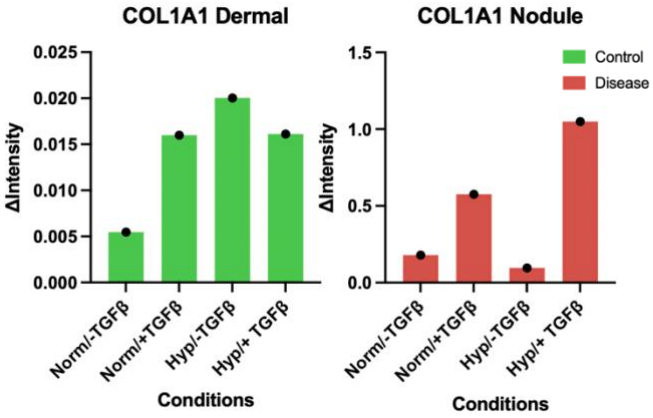
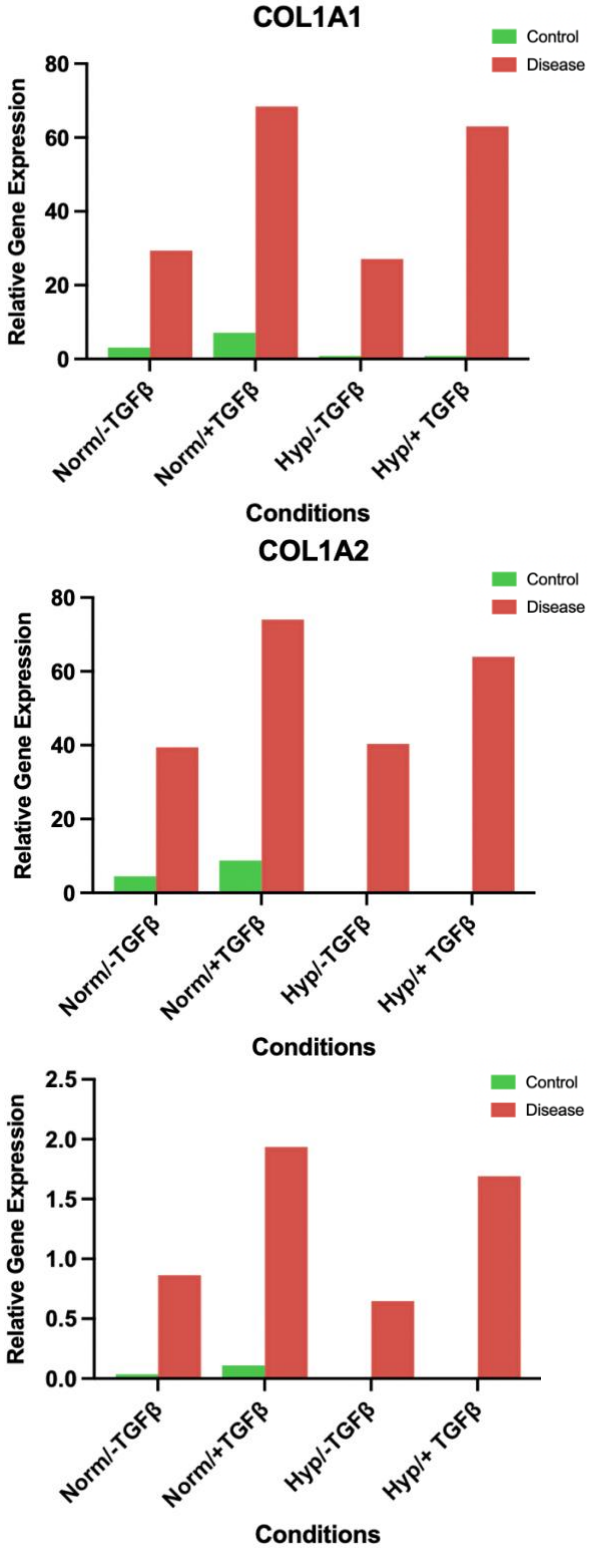


Figure 5: **Western Blot analysis of production of type I collagen.** ΔIntensity of each sample calculated relative to intensity of YWHAZ protein of each respective sample. Data shows increased baseline of type 1 collagen in nodule samples compared to dermal samples similar pattern of increase with the addition of TGF-β1within same samples but varying between nodule and dermal.

A similar pattern to α-SMA production is seen in type 1 collagen gene expression and subsequent protein production in nodule fibroblasts compared to dermal fibroblasts. Figure 4 shows clear distinction of collagen production and deposition into the ECM between dermal fibroblasts and nodule fibroblasts. Nodule fibroblasts show clear increase in collagen production and deposition, in both the presence of TGF-β1 and hypoxic conditions, whereas dermal fibroblasts show little collagen production. These results are more clearly seen quantified in figure 5 of the western blot analysis of type I collagen. Baseline levels of collagen produced is seen at an almost tenfold increase in the nodule fibroblasts compared to dermal fibroblasts. Nodule fibroblasts show a consistent increase with the addition of TGF-β1, in both normoxic and hypoxic conditions, but dermal fibroblasts show a different pattern. Where in normoxic conditions addition of TGF-β1 results in a drastic increase in collagen production, in hypoxic conditions this is actually seen to decrease. These results are also consistent with the relative gene expression graphs of COL1A1, COL1A2, and COL3A1. COL1A1 and COL1A2 genes encode for type I collagen and



represent expression of type 1 collagen production, whereas COL3A1 gene encodes for the alpha chain of type III collagen and indicates type III collagen expression.

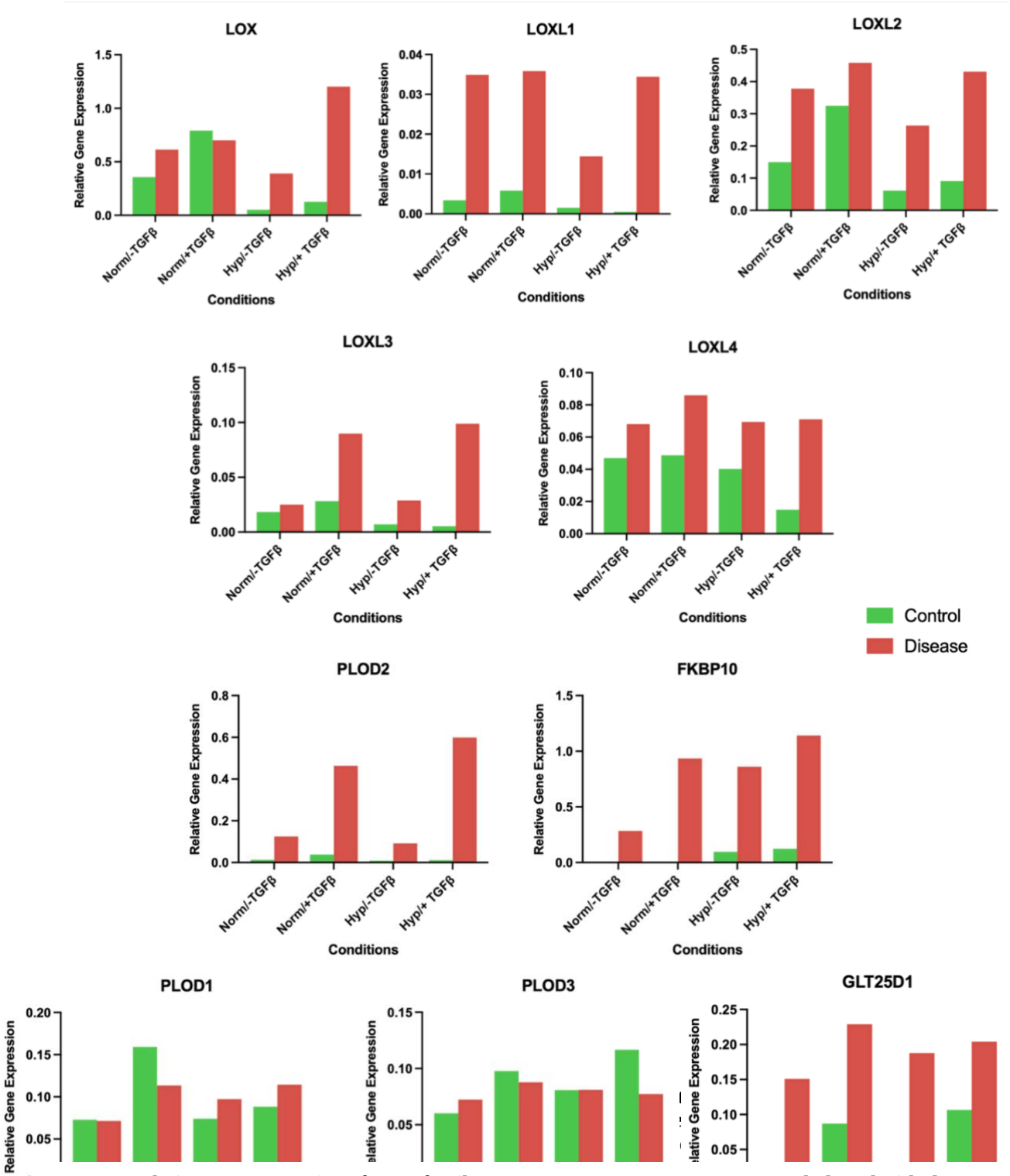


Figure 7: **Relative gene expression of LOX family genes, PLOD 1-3 genes, FKBP10 and plotted with the different stimulation conditions.** ΔCt of each sample calculated relative to expression of YWHAZ gene of each respective sample. Generally, expression of all LOX family genes, PLOD2 and FKBP10, and GLT25D1 is higher in nodule fibroblasts compared to dermal fibroblasts. PLOD1 and PLOD3 gene expressions were indifferent.

Nodule fibroblasts show higher mRNA relative LOX family,PLOD2 & FKBP10, and GLT25D1 gene expression

Figure 7 shows a clear distinction in gene expression of genes associated with collagen modification between nodule fibroblasts and dermal fibroblasts. While certain genes such as PLOD1 and PLOD3 were similarly expressed, nodule fibroblasts showed drastic increase in collagen modifying enzyme gene expressions. Differences were also clearly present between normoxic and hypoxic conditions. Dermal fibroblasts expressed higher levels of LOX family genes in normoxic conditions compared to hypoxic conditions, whereas nodule fibroblasts expressed similar levels of LOX family genes between normoxic and hypoxic conditions. Gene expression patterns of PLOD2, FKBP10 and GLT25D1 were similar, but absolute gene expression values were always higher in nodule fibroblasts for these genes.

Discussions

Fibrosis pathology is a dynamic process, with variations occurring between different tissues or organs, and affected by various signalling pathways and factors[2,15–17]. Through this study we have found that while healthy dermal fibroblasts do express certain genes similarly in normoxic and hypoxic conditions, clear discrepancies in absolute values and expression patterns are present. This suggests that for further future *in vitro* studies of fibrotic models, inducing hypoxic conditions may yield results that similarly replicate what may happen *in vivo.* This is also the case for the nodule fibroblasts, as clear differences were present between normoxic and hypoxic conditions. This study was however limited due to time constraints to a single concentration of deferoxamine and further studies into effects of different concentrations of deferoxamine may also yield different results, however it is indeed clear, as other studies also suggest, that hypoxia does indeed play a key role in fibrosis and is a factor that cannot be ignored[9–12,18,19] .

This study further clarifies how different genes play a role in fibrosis specific to DD. As discrepancies were definitely present between LOX family genes as well as different PLOD genes. It is clear that PLOD2 and FKBP10 do indeed play a significant role in fibrosis, as well as DD, and fibrotic conditions do result in a collagen production that present different modifications to collagen produced by healthy fibroblasts[14,20–24] .

While this study lacks in statistically significant data due to a small sample size, it is clear that replicating hypoxic conditions play a paramount role in fibrosis research.

While the dynamic process of fibrosis has yet to be fully understood and much is yet to be discovered of antifibrotic targets which could lead to potential therapeutics[3], we suggest that targeting signalling pathways or inhibiting PLOD2 or FKBP10 may be a potential target for treatments.

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