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Jackson College of Graduate Studies

Effect of Telomerase on Proliferation and Differentiation of a Cell Line Derived from Dupuytren's Contracture.

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By

Dima Sawalha

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Effect of Telomerase on Proliferation and Differentiation of a Cell Line Derived from Dupuytren's Contracture

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Thesis Title

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A THESIS APPROVED FOR

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I want to express my love and gratitude to my family; my husband, my daughters, my father, my mother, my brother, and my sisters, who gave me support and encouragement to complete my project.

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Abstract of Thesis

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Abstract:

Dupuytren's disease is a fibroproliferative disorder that affects the hand's palm and eventually leads to loss of hand function. It is characterized by shortening of the palmar fascia and an increased myofibroblast presence in diseased tissue excised from Dupuytren's contracture surgery. Transforming growth factor-beta enhances the formation of structural elements and elevated expression of alpha-smooth muscle actin in myofibroblasts. The objective of this study was to determine the ability of the immortalized myofibroblast to differentiate as

expected in response to transforming growth factor-beta to provide an unlimited supply of cells to investigate further the causes and treatments for Dupuytren's disease.

A 3-dimensional collagen lattice model was used to study myofibroblast tension generation and contraction using an immortalized cell line derived from Dupuytren's disease (DP147+hTERT). In addition, DP147+hTERT was compared to its parental cell line derived from Dupuytren's contracture (DP147). We determined tension generation and contraction for both cell line types by measuring the decrease in the surface area of the collagen lattice over time. It was followed by lattice immunostaining to calculate the percentage of myofibroblasts and proliferation. In addition, cells were plated on coverslips (under maximum tension) then immunostained to determine differences in differentiation and proliferation between DP147 and DP147+hTERT.

The results were encouraging for using the immortalized cell line for future studies on Dupuytren's disease; both cell lines showed increased myofibroblast differentiation in response to TGF-beta. Still, further investigations are required to understand differences in proliferation between DP147 and DP147+hTERT and its effect on the reliability of the immortalized cell line.

Introduction:

Dupuytren's Disease (DD) is characterized by the appearance of fibrotic lesions leading to flexion contractures of the fingers, followed by the loss of hand function (Tomasek 1999, Krause et al. 2011, Tripoli et al. 2016). In addition, DD is associated with thickening of the tissue underneath the skin (Tomasek 1999, Tse et al. 2004). This thickening occurs in the palm and extends to the fingers (Gabbiani et al. 1972, Tomasek 1999). DD treatment historically consists of surgical removal of the contracted tissues. High recurrence rates are associated with this type of surgery (Krause et al. 2011). At present percutaneous needle fasciotomy and collagenase injections in the cords are available alternatives to hand surgery (Hurst et al. 2009, van Rijssen et al. 2012, Tripoli et al. 2016); however, these procedures are not 100% effective and come with a high recurrence rate as well (Strömberg et al. 20016, Scherman et al. 2018). Researchers are working to maximize treatment strategies by investigating the causes. Several studies showed increased myofibroblast percentages in the tissue excised from Dupuytren's contracture (DC) surgery (Tse et al. 2004, Krause et al. 2011).

The myofibroblast is a specialized fibroblast responsible for generating contractile force associated with DD and characterized by α-smooth muscle actin (α-sma) within its contractile stress fibers (Desmouliere 1995, Vaughan et al. 2000, Tomasek et al. 2002, Chen et al. 2007). With their stress fibers and α-sma,

myofibroblasts cause wound contraction (Grinnell et al. 1999, Gabbiani 2003). During wound contraction, fibroblasts are responsible for the biosynthesis of new tissue matrix and differentiate to myofibroblasts (D'Urso and Kurniawan 2020).

The cytokine transforming growth factor-beta (TGF-β) plays an essential role in tissue repair and wound healing (Border and Ruoslahti 1992, Desmouliere et al.1993, Midgley et al. 2013). TGF-β levels are found elevated after wound healing events, proving its importance to the wound healing process (Masur et al. 1996, Chipev et al. 2000, Razdan et al. 2018). TGF-β affects the conversion of fibroblasts to myofibroblasts; it increases myofibroblast differentiation by the elevated expression of α-sma and contractile force generation (Desmouliere 1995, Badalamente et al. 1996, Vaughan et al. 2000, Tomasek et al. 2002). In mammals, three isoforms, TGF-β1, –2, and -3, have been found (Yang et al. 1999). TGF-β stimulates extracellular matrix deposition and fibroblast proliferation that characterizes DD (Kloen et al. 1995, D'Urso and Kurniawan 2020). Macrophages, fibroblasts, and several cell types may activate TGF-β during wound healing. In addition, TGF-β plays an essential role in other fibrotic diseases and cancer (Yang et al.1999).

Telomeres are the ends of linear chromosomes; they contain long repetitive DNA sequences composed of TTAGGG, bound to specific proteins (Blackburn 1991, McChesney et al. 2000, Wright and Shay 2002). With each cell cycle, the telomeres get shorter due to the inability of the DNA lagging strand to replicate the

far 3' end of the chromosome (Blackburn 1991, Brayan et al. 2000, McChesney et al. 2000, Wright and Shay 2002, Masutomi and Hahn 2003, Ramirez et al. 2003, Aubert and Lansdrop 2008). When telomeres become sufficiently short, the cell enters what is called senescence, which is irreversible growth arrest (Sager 1991, Brayan et al. 2000, Wright and Shay 2002). Telomerase helps stabilize telomere length in human reproductive cells, stem cells, and cancer cells by adding TTAGGG repeats onto the telomeres (Herbert et al. 1999, Zhang et al. 1999, Shay et al. 2001, Yatabe et al. 2002, Masutomi and Hahn 2003). Telomerase is the reverse transcriptase enzyme composed of two subunits, the protein catalytic subunit (TERT) and the template RNA subunit (TR) (Yatabe et al. 2002, Masutomi and Hahn 2003, Shay and Wright 2007, Hornsby 2007). Elevated telomerase activity has been discovered in immortalized, cancerous cells and in injured tissue and normal hematopoietic progenitor cells, endometrial cells, and the basal cells of skin and cervical keratinocytes (Hiyama et al. 1995, Funk et al. 2000, Shay et al. 2001, Razdan et al. 2018).

Telomeres have an essential role in stabilizing the ends of the chromosomes, but they do not contain active genes. Instead, they contain long repetitive sequences and specific binding proteins, which form the unique T-loop structure at the end of the chromosome. Therefore, any changes in the length of the telomeres should not affect gene expression (O'Connor 2008).

Telomere shortening during every cell cycle makes the life span of somatic cells limited. Human somatic cells have a limited proliferation capacity, after which the

cells enter a growth arrest state, which is known as replicative senescence (Aubert and Lansdrop 2008). Telomerase can reverse telomere shortening by the addition of the telomeric repeats. Telomeres and telomerase work together to maintain the ability of the cell to undergo cell division. Immortalization of human somatic cells by telomerase does not cause any growth transformation. However, telomerase extends the life span of cells without any alteration in functions. Telomerase is sufficient for fibroblast immortalization but not enough to cause malignant transformation (Morales et al. 1999). Jester et al. (2003) showed that TGF-β induces myofibroblast differentiation in hTERT-immortalized human corneal fibroblast cell lines similar to their normal counterpart. According to these studies, we may predict that DP147+hTERT would differentiate similar to its parental cell line, DP147, in the presence or absence of TGF-β because hTERT should not change the fibroblast phenotype; it just allows cell division to continue.

Several studies showed that human TERT (hTERT) is expressed in different normal cells and tissues, including many epithelial cells, hematopoietic precursors, and spermatogonia (Masutomi and Hahn 2003). In addition, it is expressed more in fibroblasts versus myofibroblasts (Liu et al. 2006). Kalson et al. (2018) showed a significant association between telomere length and fibrotic condition and that telomere repair defects contribute to joint fibrosis. In lung injury and fibrosis, telomerase activity was induced in fibroblasts versus myofibroblasts. This induced activity can be inhibited with IL-4 and TGF-β (Liu et al. 2006, Razdan et al. 2018). In these studies, reduced telomerase expression came with increased

expression of α–smooth muscle actin (α-sma), which indicated that there was myofibroblast differentiation (Liu et al. 2006, Tomasek et al. 2002). Based on these studies, we may predict that immortalized DP147+hTERT would not differentiate similar to DP147, its parental cell line, in the presence or absence of TGF-β.

Therefore based on previous studies there are two conflicting hypotheses; telomerase inhibits myofibroblast differentiation (Liu et al. 2006), or telomerase does not inhibit myofibroblast differentiation (Morales et al. 1999, Jester et al. 2003). Our goal is to test these hypotheses using a DD-derived cell line and determine whether telomerase has no effect on the myofibroblast phenotype or alters the phenotype.

The Objective of the Study:

The purpose of this study was to determine if hTERT-immortalized fibroblasts from DC would differentiate into myofibroblasts with and without TGF-β. Several studies showed that the myofibroblast is responsible for tissue contraction in DD (Gabbiani and Majno 1972, Tomasek and Rayan 1995, Tomasek et al.1999). We conducted *in vitro* studies to determine the ability of the immortal line of Dupuytren's cells to differentiate similar to their parental, non-immortalized cell line. Telomerase-immortalized cell lines have been used to study several diseases (Lee et al. 2004) with unlimited potential for future studies. This study used telomerase-immortalized fibroblasts derived from DD (DP147+hTERT) to determine their

ability to proliferate and differentiate using a coverslip culture assay, then study their ability to generate tension using collagen lattices. Coverslips are a reliable assay for studying the morphology of contractile cells (Tomasek and Rayan 1995). In addition, collagen lattices provide opportunities to understand the interaction between cells and their surroundings by providing a tissue-like environment (Bell et al. 1979, Tomasek et al. 1992, Vaughan et al. 2000, Grinnell 2003). We used the stress-relaxed collagen lattice to allow tension generation to produce the myofibroblast phenotype (Grinnell 1994, Vaughan et al. 2000).

Materials and Methods:

The study was conducted at Dr. Vaughan's Lab (HOH 253) in the biology department at the University of Central Oklahoma. The study was approved by UCO IRB (08077, 09027).

Cell cultures:

Two sets of cells were examined. One set was obtained from patients undergoing Dupuytren's contracture excision surgery (DP147), and another set was the hTERT immortalized cells from the same line (DP147+hTERT). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% Fetal Bovine Serum (FBS) and penicillin, streptomycin, and amphotericin mixture (ABAM, Sigma). Cells were incubated at 37^{o}C at 5% CO_2 with high humidity. Cells

were sub-cultured at least every seven days. The cells were determined immortal if they had a population-doubling rate (PD) beyond the non-immortalized cell. In addition, DNA fragment sequencing was performed by James Creecy (Dept. Biology, UCO) to determine whether DP147 and DP147+hTERT were derived from the same individual, thereby assuring that the immortalized cell is not a minor contaminant of a non-immortalized population (American Type Culture Collection Standards Development Organization Workgroup ASN-0002, 2010).

Coverslips:

Cells were plated on glass coverslips (12mm round, Fisher Scientific) placed into wells in a 24 well spot plate (Falcon Multiwell). Cells at a concentration of $0.3x10^5$ were placed onto each coverslip. DP147 cells and DP147+hTERT were plated with and without TGF-β. Cell proliferation was determined by treating cells with 10 μM Edu 24 hours after incubation (5-ethynyl-2'-deoxyuridine) per 2 ml media for four hours before fixing (Vaughan et al. 2014). The Edu is a modified nucleotide that will be incorporated into replicating DNA. Detection of the incorporated nucleotide was done by reaction of the ethyl group of Edu with the fluorescent azide group of the click-chemistry. Edu staining is an effective method to measure cells during S-phase. Unlike BrdU (5-bromo-2'deoxyuridine) labeling, Edu does not require harsh DNA treatment (Buck et al. 2008, Li et al. 2010, Cavanagh et al. 2011). At the

conclusion of cell culture, coverslips were washed with 1x PBS (Phosphate Buffered Saline). Then, coverslips were covered with a fixative (4% paraformaldehyde) for 20 minutes at room temperature in the fume hood. Next, coverslips were washed with 0.1 M phosphate buffer for 5 minutes, and this step was repeated two more times. Finally, coverslips were placed in 0.05 M Tris buffer for at least 30 minutes. Coverslips were stored in the refrigerator with PBS-Azide.

Cells were stained using the inverted staining on the parafilm method (Tomasek and Rayan, 1995). Cells were washed with PBS for 5 minutes then stained for α–sma using the standard procedure. Cells were blocked with 1:10 goat serum for 10 minutes. The primary antibody was 1:500 mouse anti-human α–sma (clone 1A4, Sigma). Then we used Edu stain (Click-IT) for 30 minutes. Cells were washed three times with PBS. The primary antibody was labeled using the secondary antibody Goat anti-mouse rhodamine (Santa Cruz) at 1:200 dilution for 15 min in the dark. Dapi stain was added for an extra 30 minutes in the dark to stain DNA in cells. Cells were washed three times with PBS and then mounted with 80% glycerol/PBS (Vaughan et al. 2014).

The stained coverslips were photographed using an inverted Olympus IX71 microscope with a digital camera (DP72; Olympus) and software program (CelSens). A computerized image analysis program (ImageJ) was used to determine the positive immunostaining of the cells. The number of cells in the field was determined based on DAPI-positive nuclei (Vaughan et al. 2014). Cells were quantified into four cell types: non-proliferative fibroblasts (NPF), proliferative

fibroblasts (PF), non-proliferative myofibroblasts (NPM), and proliferative myofibroblasts (PM) (Figure 1). At least 200 cells per set were counted, and each experiment was repeated three times. Two-way repeated-measures ANOVA (analysis of variance) was used to compare variation in myofibroblast differentiation between DP147 cells and DP147+hTERT cells and determine the effect of TGF-β on the two sets of cells. Repeated measures ANOVA was done to determine the variation in the percentage of myofibroblast proliferation and the percentage of fibroblast proliferation between DP147 cells and DP147+hTERT cells and between cells treated with TGF-β and cells that were not treated with TGF-β.

Stress-relaxed collagen lattice model:

We used fibroblast populated collagen lattice (FPCL) to determine the differences in TGF-β induced contraction changes between DP147 and DP147+hTERT (Herwig and Vaughan 2014). Cells were sub-cultured, and the final cell concentration in the FPCL was 1.25×10^5 cells/ml. The collagen mixture included rat-tail collagen type I (Shelton and Summers Rada 2009), 0.1N NaOH, 10X EMEM (Eagle's Minimum Essential Medium), and 10X $NaHCO_3$. Prepared collagen solution was mixed with the counted cell solution. The solution was mixed thoroughly and placed on ice. A 150µl collagen drop was plated in the center of a pre-warmed 35 mm TC plate. Twenty-four lattices contained DP147 fibroblasts,

and another 24 lattices contained DP147+hTERT fibroblasts. Plates were gently placed in a 37°C incubator for one hour. Then 2 ml of media with or without TGF-β was added to each plate. Collagen lattices were incubated for five days at 37°C to reach the maximum tension generation and then either released using a needle tip or pulsed with Edu for staining similar to the coverslip procedure. Digital images of the contracting cultures were captured using an inverted microscope (Olympus) with an attached camera (SPOT) (Herwig and Vaughan 2014). Images were captured before releasing the lattices and at 1, 2, 10, 30, and 60 minutes after the lattice was released from the bottom of the dish (Figure 2). A computerized image analysis program (ImageJ) was used to measure the FPCL area (mm^2). Repeated measures ANOVA was run to compare the means of the four tested groups: DP147 with and without TGF-β and DP147+hTERT with and without TGF-β. Tukey's multiple comparisons followed the ANOVA.

Collagen Lattice Staining:

Replicate lattices that were not released and measured were prepared for staining similar to coverslips. Each lattice was covered with 4% paraformaldehyde for 20 minutes at room temperature in the fume hood to preserve the protein structure and prepare the lattice for staining. Next, the collagen lattice was washed with 0.1 M phosphate buffer for 5 minutes, and this step was repeated two more times. Then collagen lattice was cut into four pieces for staining. Collagen lattice staining was conducted to identify proliferation and differentiation similar to coverslips and quantified in the same way.

Results:

Collagen lattice contraction:

The contraction of the lattices and reduction in diameter over time provide an indirect measurement of tension generation (Tomasek et al. 1992). The repeated measures (ANOVA) was performed to determine the mean contraction and tension generation differences between collagen lattices with DP147 or DP147+hTERT. It was then followed with Tukey's test, which was completed and indicated no significant difference in the contraction of the collagen lattices containing DP147 cells or the collagen lattice containing DP147+hTERT ($F(1,5) = 0.454$, $p = 0.506$). However, collagen lattices treated with TGF-β revealed more contraction in the collagen lattices with DP147 cells ($F(1,5) = 23.413$, $p<0.0001$) and the collagen lattices with DP147+hTERT cells ($F(1,5) = 68.695$, $p<0.0001$). The increase in the mean of contraction was statistically significant (Figure 3).

Collagen lattice staining:

Collagen lattice staining provides a direct correlation of structure to function, unlike coverslips. Proliferation and differentiation are associated with increased contraction/tension generation (Vaughan 2000). Therefore, we stained lattices to determine how proliferation and differentiation were correlated to contraction (Vaughan 2000). Repeated measures ANOVA was performed to determine the effect of TGF-β on the percentage of myofibroblasts for DP147 cells and DP147+hTERT cells. A boxplot was created to show median, interquartile range,

and outliers (Figure 4). The results showed an increase in myofibroblast percentage in both DP147 cells ($F(1,34) = 3.833$, $p = 0.058$) and DP147+hTERT cells ($F(1,34) = 0.055$, $p = 0.815$) after TGF-β treatment, but that increase was not statistically significant ($P > 0.05$). Also, the results show that percentage of myofibroblasts was not significantly different between DP147 cells and DP147+hTERT cells ($F(1,34) = 3.319$, $p = 0.077$) (Figure 4, Figure 5).

There was a significant increase in the percentage of myofibroblast proliferation in DP147+hTERT, comparing it with DP147 ($F(1,34) = 4.547$, $p = 0.040$). Treating the cells with TGF-β did not affect the percentage of myofibroblast proliferation in DP147 ($F(1,34) = 0.201$, $p = 0.656$) and DP147+hTERT ($F(1,34) = 0.292$, $p = 0.59$) (Figure 6, Figure 7). There was no significant difference in the percentage of fibroblast proliferation in DP147 and DP147+hTERT. Also, treating cells with TGF-β showed a significant increase in the percentage of fibroblast proliferation in DP147+hTERT ($F(1,34) = 6.624$, $p = 0.014$)) but not in DP147 ($F(1,34) = 0.139$, $p = 0.710$) (Figure 8, Figure 9).

Coverslips staining:

DP147+hTERT cells showed a significant increase in the percentage of myofibroblasts compared to DP147 cells. The repeated measures ANOVA was performed, and the results were significantly different between DP147+hTERT and DP147 ($F(1,28) = 37.291$, $p<0.0001$). Treating both cell types with TGF-β showed

a significant increase in myofibroblast percentage ($p < 0.05$) (Figure 10 and Figure 11).

The percentage of myofibroblast proliferation was determined, and results showed no difference between DP147 and DP147+hTERT ($F(1,28) = 1.020$, $p = 0.321$). Also, treating both cell types with TGF-β did not significantly affect the percentage of myofibroblast proliferation (Figure 12). Treating DP147 with TGF-β increased the percentage of fibroblast proliferation, but this increase was not statistically significant ($F(1,28) = 1.563$, $p = 0.221$) (Figure 13).

Discussion:

TGF-β, in addition to the mechanically stressed environment, is essential for the differentiation of fibroblasts into myofibroblasts *in vitro* (Tomasek et al. 2002). The focus of this research was to investigate the ability of hTERT-immortalized fibroblasts from DC to differentiate as expected in the presence of TGF-β, with the potential of conducting unlimited further studies and investigations. First, we used the fibroblast populated collagen lattice (FPCL) model to determine the TGF-β induced contraction differences between DP147 and DP147+hTERT (Herwig and Vaughan 2014). The collagen lattice model closely resembles what occurs *in vivo*; it provides a tissue-like environment to measure contraction at the tissue level (Vaughan et al. 2000, Griffith et al. 2006).

We measured the contraction of the FPCL with DP147 cells and compared it to the results of DP147+hTERT (Figure 3). The lattice contraction and reduction of diameter provides a measurement of tension generation. The results showed no significant differences in the contraction in DP147 and DP147+hTERT lattices. In addition, both FPCL showed a substantial increase in contraction when treated with TGF-β (Figure 2, Figure 3). Therefore, we predicted that DP147 collagen lattices would increase myofibroblasts with TGF-β treatment, but we expected that DP147+hTERT, when treated with TGF-β, to show no increase in myofibroblast. Liu et al. (2006) showed that telomerase inhibits myofibroblast differentiation, and the loss of telomerase activity increases myofibroblast differentiation. However, our results showed that FPCL with DP147+hTERT increased contraction and myofibroblast differentiation when treated with TGF-β, same as the results of FPCL with DP147. Thus, our collagen lattice results support the null hypothesis, indicating no difference between DP147 and DP147+hTERT in differentiation and collagen lattice contraction and agree with Jester et al. (2003).

The collagen lattice immunostaining with anti-smooth muscle alpha-actin is essential in determining the percentage of the differentiated myofibroblast. Collagen lattice immunostaining results showed that TGF-β increased the percentage of the myofibroblasts in both cell types, but the increase in myofibroblasts was not significantly different ($P>0.05$) (Figure 5). Thus, the collagen lattice stain results support the FPCL results. Furthermore, the increase of the percentage of myofibroblasts with TGF-β treatment means an increased

expression of the α-sma, which increases the contractile force as shown in the FPCL (Vaughan et al. 2000).

In addition, the coverslip results confirm that treating both types of cells (DP147 and DP147+hTERT) increases the percentage of myofibroblast, the same as the FPLC results. Vaughan et al.'s 2000 results showed that using FPCL resembles what occurs *in vivo*; while cells on coverslips grow under maximum tension, cells in the collagen lattices grow under tension but not maximum tension. Due to these differences, the coverslips staining results could show differences when compared to collagen lattice results.

DD is a fibroproliferative tissue rich in myofibroblasts (Tomasek et al. 1999, Tripoli et al. 2016). The proliferation of myofibroblasts is crucial for developing DD (Rehman et al. 2011). Therefore, it is essential to understand myofibroblast proliferation and differentiation as part of studying the mechanisms of developing this kind of palmar fibromatosis. Our collagen lattice results showed a significant increase in the percentage of myofibroblast proliferation in DP147+hTERT compared to DP147. Treatment with TGF-β did not significantly affect the proliferation of the myofibroblast in collagen lattice or the coverslips. However, the coverslips staining showed an increase in the percentage of fibroblast proliferation in DP147+hTERT more than DP147, not in the myofibroblast as in the collagen lattices results. As we mentioned before, growing the cells under maximum tension on a coverslip would affect the proliferation and differentiation potential (Vaughan et al. 2000).

The increase in the myofibroblast proliferation of the DP147+hTERT in collagen lattices compared to DP147 could be due to higher levels of telomerase in DP147+hTERT, which cause more proliferation in cells as what happens in tumor cells (Wright and Shay 2002). However, the DP147 cells used in this research were early in population doubling (PD), meaning they still had proliferation potential. Studies showed that telomeres in normal cells are variable in length (Ramirez et al. 2003, Martin-Kuiz et al. 2004); some have long telomeres, and some have short ones. Therefore, the DP147 cultures we used may have been heterogeneous; indeed, some cells may have been growth-arrested, which may have caused this reduced proliferation.

The DP147+hTERT shows promising results based on the percentage of myofibroblasts and contraction. However, further investigations are required to determine the effectiveness of using this cell line and its resemblance to the cells extracted from DC to advance our knowledge about this disease and further investigate advancements in the treatment of this condition.

Conclusions and Further Studies:

Our investigations on the immortalized cell line of DD enhance the understanding of this disorder. We examined the effectiveness of using the immortalized cell line so that future studies are not limited to a finite number of cells available. This study has demonstrated that the immortalized cell line shows promising results when comparing cells extracted from DD (DP147) with the immortalized (DP147+hTERT) cells. Similarities in myofibroblast percentage and similarities with the effect of TGF-β on the increase in the myofibroblast percentage yielded encouraging results to use further the immortalized cell line in future investigations.

Further investigations and experiments are required to understand better the differences in proliferation between DP147 and DP147+hTERT and the effectiveness of using this cell line to advance our understanding of the palmar fascia in DD. The question here is whether the proliferation and contraction of myofibroblasts *in vitro* is the same as *in vivo*. Furthermore, investigations and experiments on this disorder may require an animal model to understand the disease better or even find a therapy for this condition.

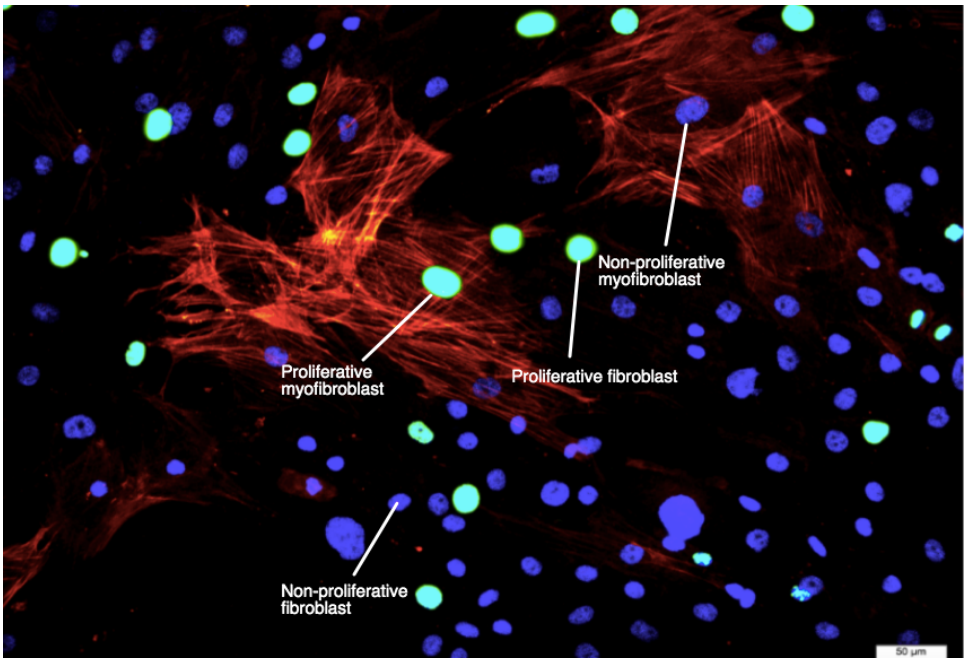

Figure 1: Coverslip immunostaining. Myofibroblast proliferation stain allows quantification of 4 cell types. For each field of view, images were collected using the blue (dapi-nuclei counterstain), the green (Edu-proliferation), and the red (α-sma-differentiation). Four cell types were identified when the images were combined: non-proliferative fibroblast, proliferative fibroblast, non-proliferative myofibroblast, and proliferative myofibroblast.

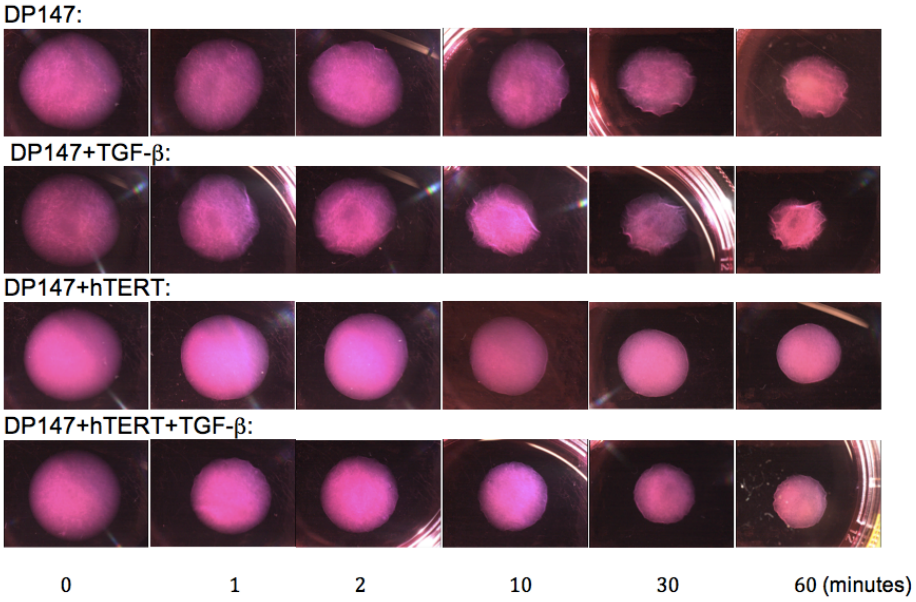

Figure 2: DP147 and DP147+hTERT contract similarly in the presence or absence of TGF-β. Images of collagen lattices showing the change caused by the fibroblast contraction at 0, 1, 2, 10, 30, and 60 minutes after release. Four series of images are collagen lattices with DP147, DP147+TGF-β, DP147+hTERT, and DP147+hTERT+TGF-β.

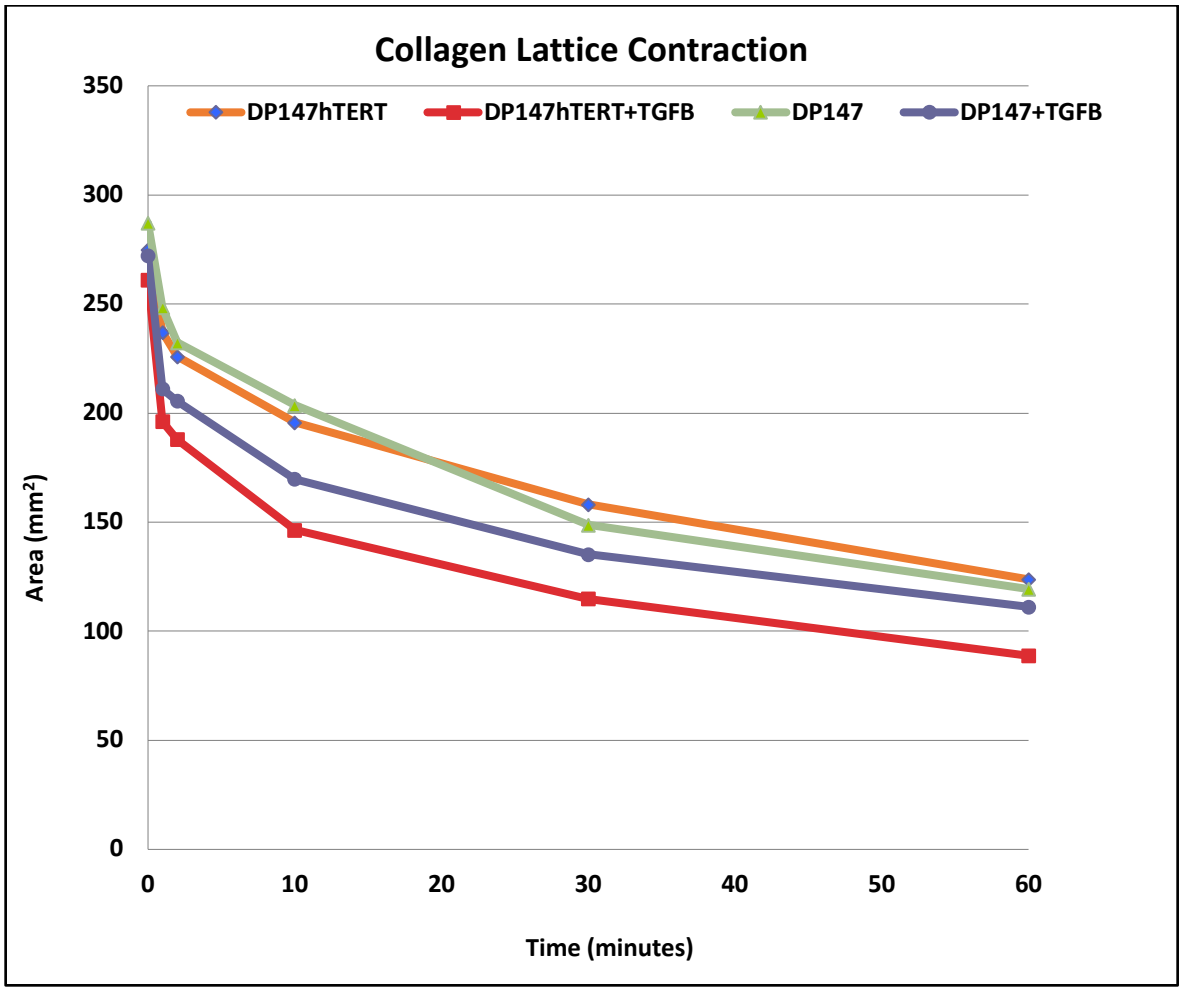

Figure 3: Mean contraction of collagen lattices in the presence or absence of TGF-β. Fibroblast plated collagen lattices (FPCL) were used to measure the reduction in diameter over time as a measurement of tension generation. Collagen lattices treated with TGF-β showed more contraction for both DP147 cells and DP147+hTERT cells.

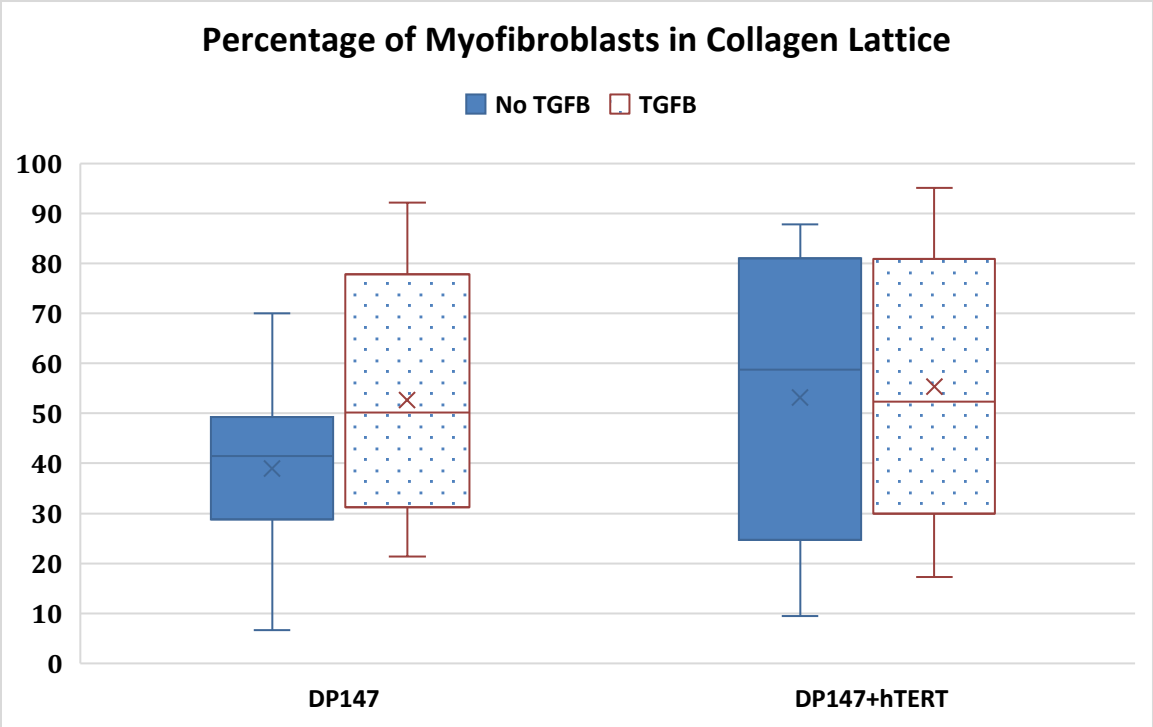

Figure 4: Percentage of myofibroblast in the FPCL with DP147 cells and DP147+hTERT cells. Results showed no significant difference between DP147 and DP147+hTERT in myofibroblast percentage ($F(1,34) = 3.319$, $p = 0.077$). Although, when treated with TGF-β, results showed an increase in the myofibroblast percentage in FPCL with DP147 and FPCL with DP147+hTERT, the increase was not statistically significant ($p>0.05$).

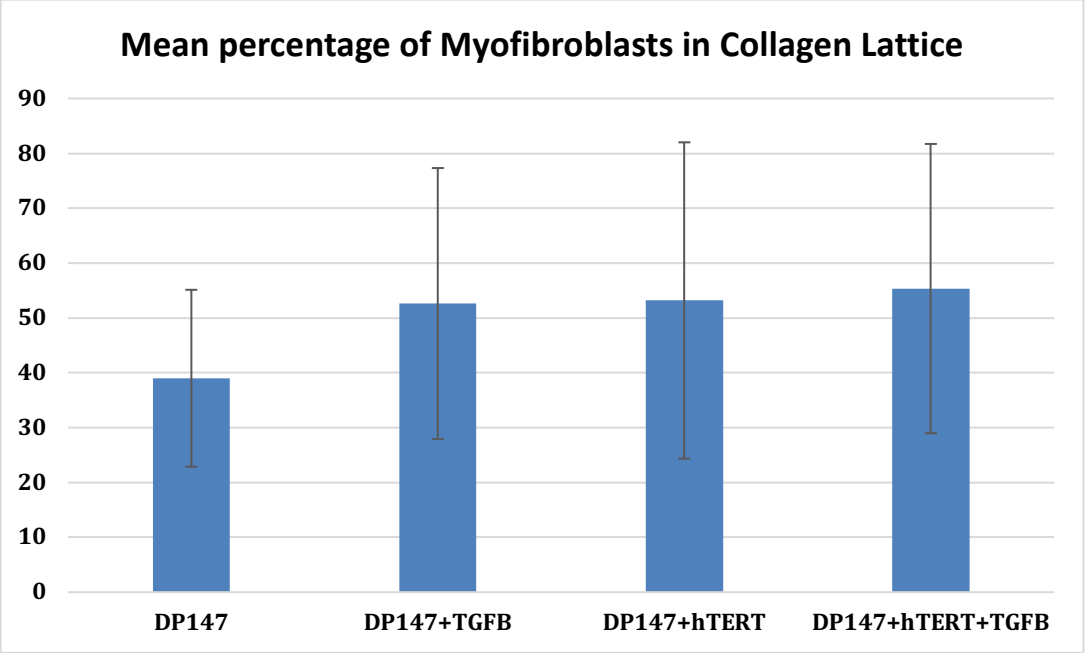

Figure 5: Mean percentage of myofibroblasts in FPCL with DP147 cells and DP147+hTERT cells, treated with TGF-β, and was not treated with TGF-β. Repeated measures ANOVA was performed, and results showed an increase in myofibroblast percentage with TGF-β treatment, but the increase was not significant ($p>0.05$).

Collagen lattice myofibroblast proliferation results:

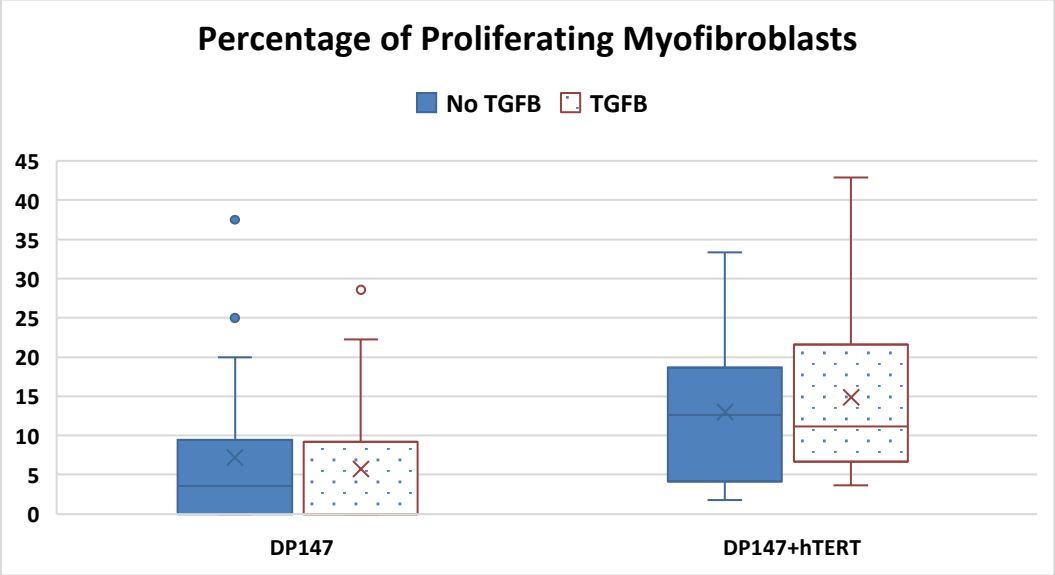

Figure 6: Percentage of proliferating myofibroblasts in collagen lattice. Results showed a significant increase in the percentage of myofibroblast proliferation in DP147+hTERT compared to DP147. Treatment with TGF-β showed an increase in myofibroblast proliferation, but the results were not statistically significant ($P>0.05$).

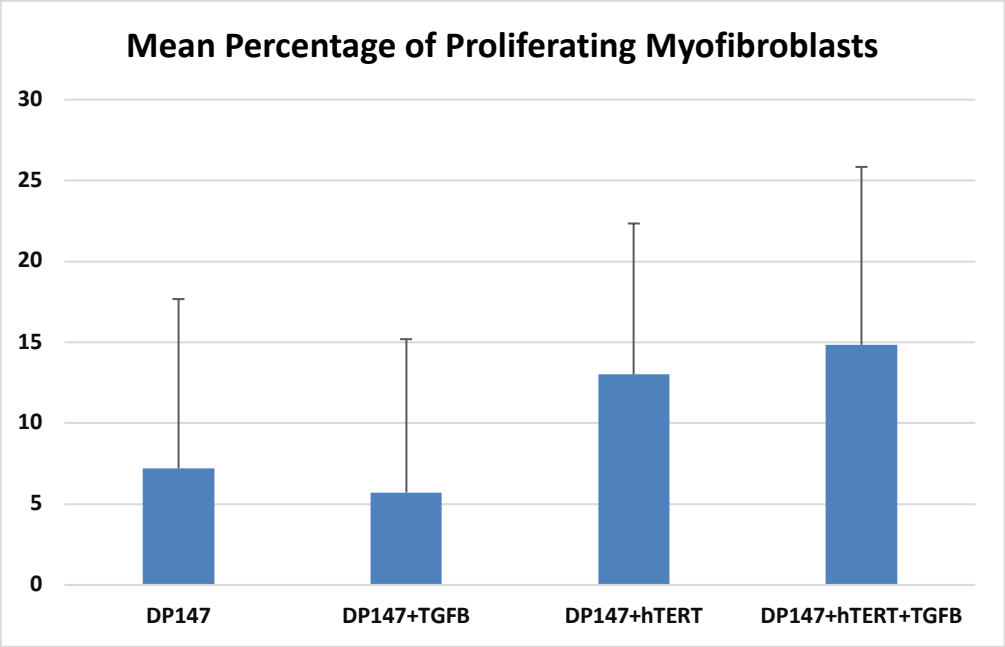

Figure 7: Mean percentage of proliferating myofibroblasts in collagen lattice. No significant increase in the percentage of proliferating myofibroblasts when treated with TGF-β ($P>0.05$) for both collagen lattices plated with DP147 and DP147+hTERT.

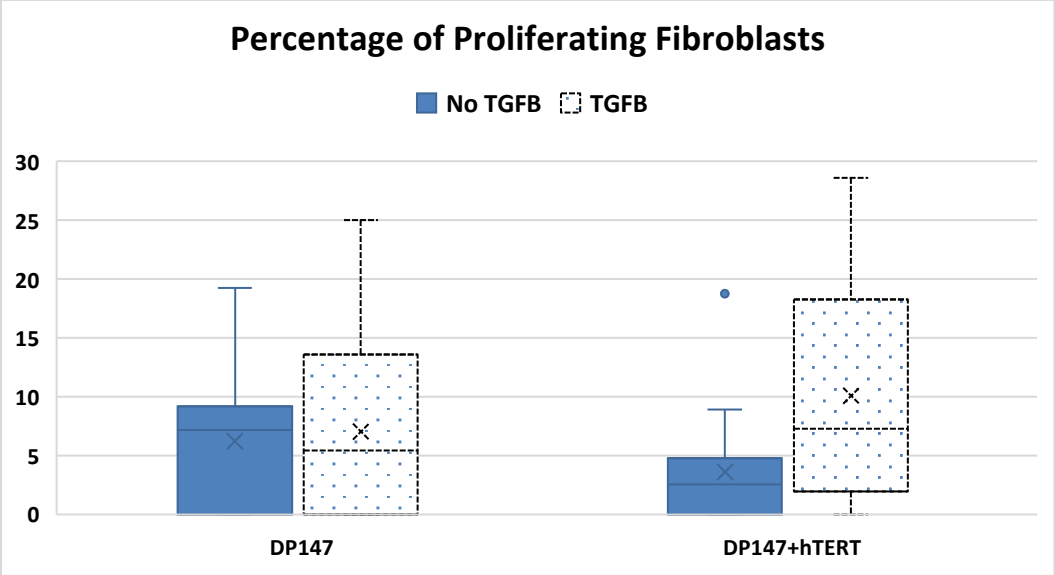

Figure 8: Percentage of proliferating fibroblasts in collagen lattice. Treating cells with TGF-β significantly affected fibroblast proliferation in DP147+hTERT ($F(1,34) = 6.624$, $p = 0.014$). Results show no difference in the percentage of fibroblast proliferation between DP147 and DP147+hTERT.

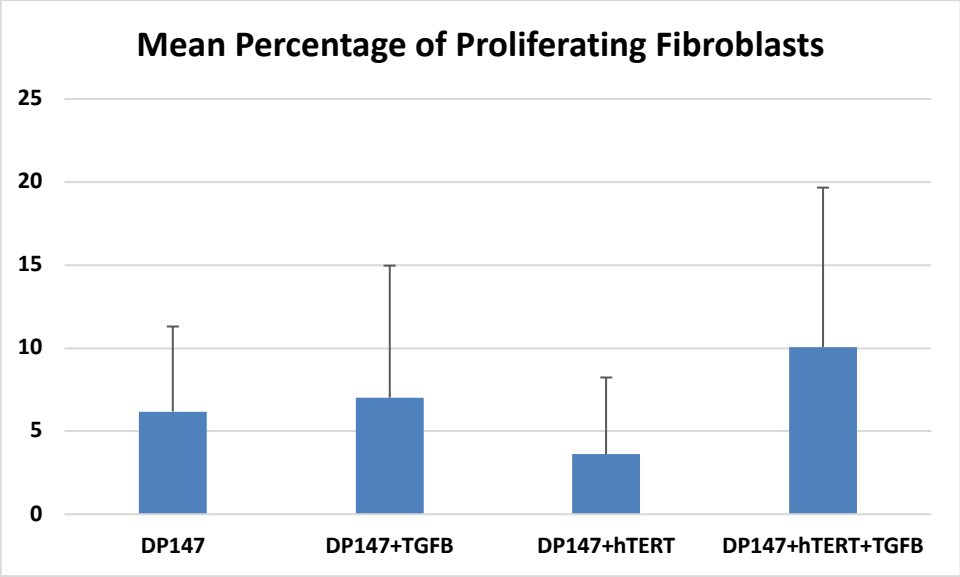

Figure 9: Mean percentage of proliferating fibroblasts in collagen lattices with DP147 and DP147+hTERT. Results showed a significant increase in the percentage of proliferating fibroblasts in collagen lattices with DP147+hTERT when treated with TGF-β.

Coverslips staining results:

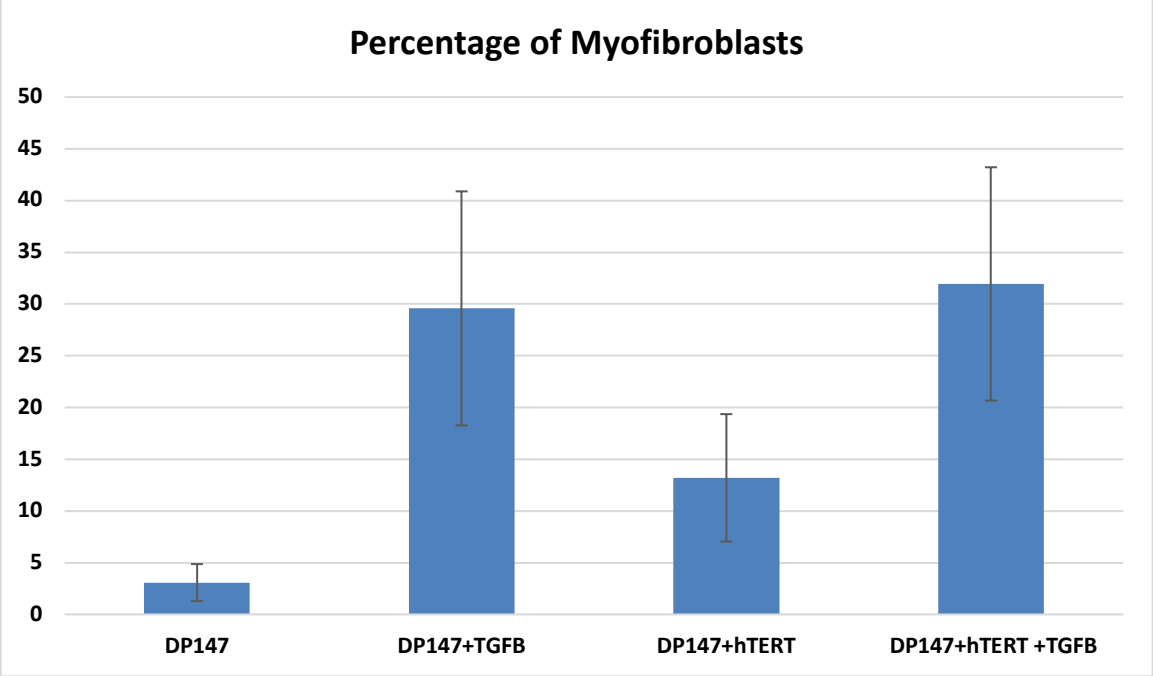

Figure 10: Mean percentage of myofibroblasts plated on the coverslips. A significant increase in the mean percentage of myofibroblasts when treated with TGF-β for DP147 and DP147+hTERT ($p<0.05$).

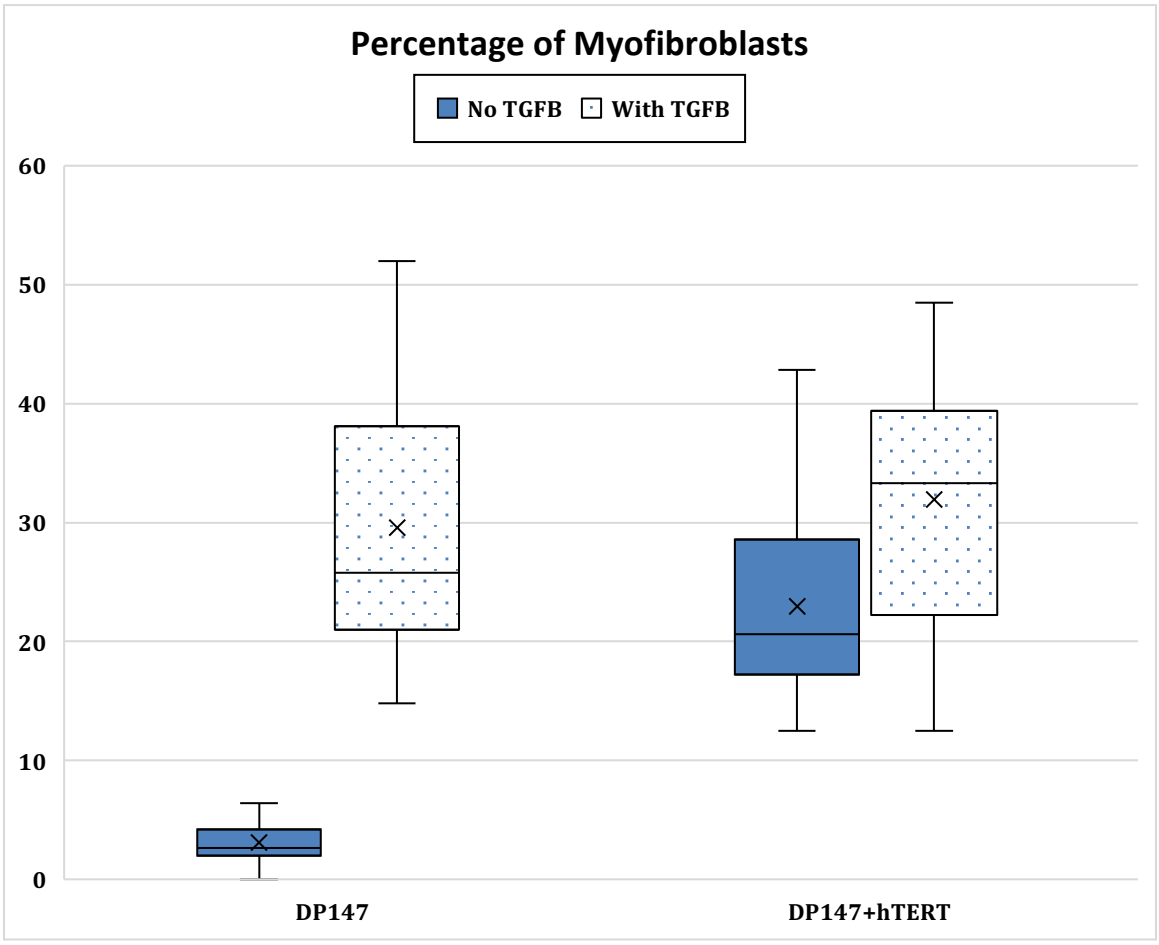

Figure 11: Percentage of myofibroblasts plated on the coverslips. Results showed a significant increase in the percentage of myofibroblasts when treated with TGF-β for both DP147 and DP147+hTERT ($p<0.05$).

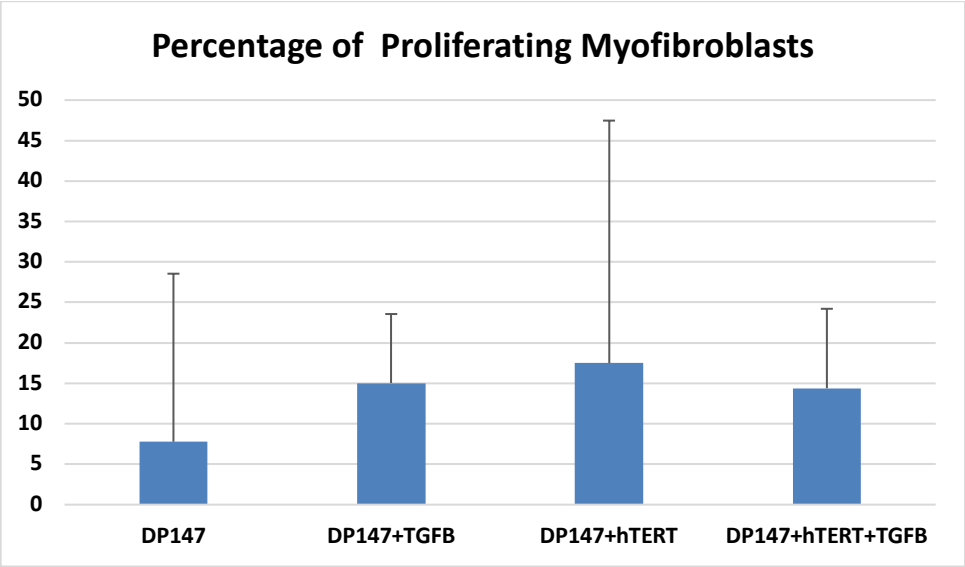

Figure 12: Mean percentage of proliferating myofibroblasts on the coverslips. Treating DP147 and DP147+hTERT with TGFβ did not statistically significantly affect the percentage of myofibroblast proliferation ($p > 0.05$).

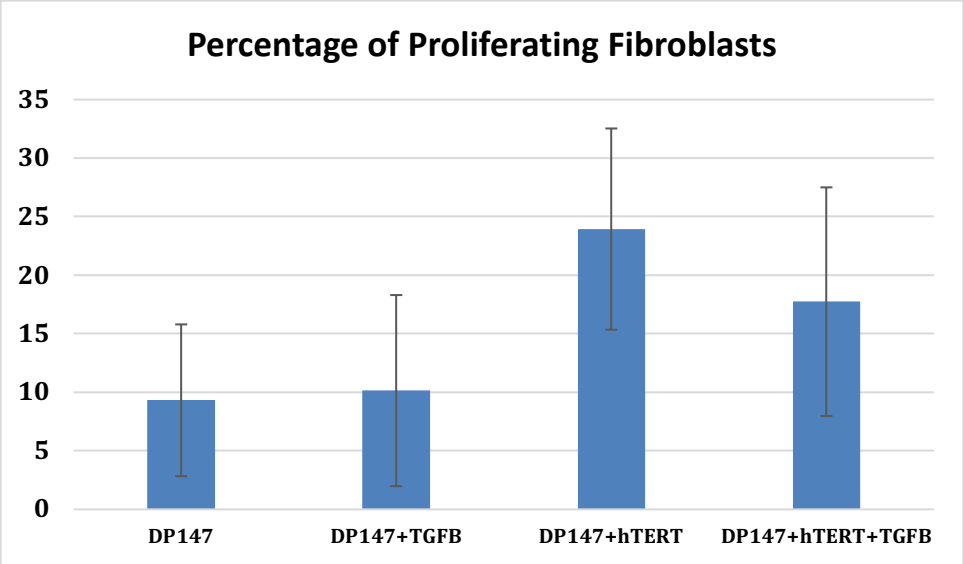

Figure 13: Mean percentage of fibroblast proliferation on the coverslips. Treating DP147 with TGF-β increased the percentage of fibroblast proliferation, but the increase was not significant ($F(1,28) = 3.407$, $p = 0.075$).

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