SRYAHWA PUBLICATIONS

Journal of Biotechnology and Bioengineering
ISSN: 2637-5362 | Volume 7, Issue 1, 2024
https://doi.org/10.22259/2637-5362.0701002

RESEARCH ARTICLE

The in Vitro Characterization of Primary Human Rectus Sheath Fibroblasts

Dr. Thomas Whitehead-Clarke, Dr. Alessandra Grillo, Prof. Vivek Mudera, Dr. Alvena Kureshi

The Centre for 3D models of Health and disease, Division of Surgery and Interventional Science, University College London.

Received: 18 August 2024 Accepted: 09 September 2024 Published: 27 September 2024

Corresponding Author: Dr. Thomas Whitehead-Clarke, The Centre for 3D models of Health and disease Division of Surgery and Interventional Science, University College London.

Abstract

Each day, thousands of patients undergo surgical procedures which require an incision through the abdominal fascia. Failure of this connective tissue layer to heal properly can result in uncomfortable and unsightly incisional hernias. Rectus sheath fibroblasts (RSFs) have a key role to play in tissue healing – specifically wound contraction and laying down new collagen. Fibroblasts from the human rectus sheath have not previously been studied in vitro, and little is known about their capacity to survive in vitro culture, their gene expression, or their ability to contract a collagen matrix. Our study isolated primary human fibroblasts from the rectus sheaths of 3 human volunteer patients and assessed them for a number of factors including their morphology, metabolic activity, intracellular markers and their contractility. Cells from all three donors underwent an identical characterization process and were compared directly to a well-characterised, immortalised cell line of human dermal fibroblasts. Our work demonstrates that human rectus sheath fibroblasts can be cultured successfully in vitro following cryopreservation – albeit with differing levels of proliferation. In 2D, these cells express typical intracellular fibroblast markers including α-Smooth Muscle Actin and Vimentin. An MTT assay revealed comparable levels of metabolic activity between cells from all donors with slightly greater activity in collective RSF populations. All RSF cells contracted collagen matrix in vitro, albeit less so than dermal fibroblasts. Our work demonstrates the possibility of working with this cell type in vitro and introduces the possible development of an in vitro rectus sheath tissue model.

Keywords: Fibroblast, Characterization, Rectus Sheath, Hernia, 3D Models.

1. Introduction

An abdominal wall hernia can be defined as an abnormal protrusion of visceral contents through abdominal wall fascia. They are associated with significant morbidity across the world and can be described as either “primary”- occurring spontaneously within virgin tissue or “incisional” - at the site of previous surgery. Incisional hernias occur secondary to poor quality healing of the abdominal wall fascia and can be caused by a number of factors including infection and obesity [1].

On a cellular level, healing is mediated by a number of cell types that play different roles throughout the healing process. After an initial ‘*inflammatory’* phase where immune cells release inflammatory mediators to increase blood flow and cell recruitment to the wound bed, a ‘*proliferative’* phase begins, in order to lay down new tissue and initiate wound contraction. This phase of healing is chiefly mediated by fibroblasts, and it is therefore *these* cells within the abdominal wall that are primarily responsible for healing the rectus sheath after surgery. Being able to study these cells *in vitro,* therefore, may help us better understand their characteristics and help future work to optimize patients’ fascial healing.

Whilst some groups have isolated and characterized rectus sheath fibroblasts (RSFs) from rats [2,3], we

Citation: Thomas Whitehead-Clarke, Alessandra Grillo, Vivek Mudera, et.al. The in Vitro Characterization of Primary Human Rectus Sheath Fibroblasts. Journal of Biotechnology and Bioengineering 2024;7(1): 7-16.

©The Author(s) 2024. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

believe our work is the first to do so with primary human cells. Although human fibroblast populations share several unifying characteristics, fibroblasts from different tissues are by no means identical. Recent work suggests that fibroblast populations can exist as sub-groups, which are then "in-printed" by their resident tissue [4]. It is our belief that the *in vitro* use of primary RSFs will improve understanding of this cell population and their ability to heal abdominal fascia. Studying these cells *in vitro* may also provide a better understanding of their interaction with medical devices such as suture material and hernia mesh, or more novel glue products that are used for mesh fixation [5].

Characterization of fibroblast populations can take a number of different forms. Classically, brightfield microscopy to examine cell morphology is a standard component of such work [6], with fibroblasts classically exhibiting an adherent elongated phenotype. This type of imaging is often coupled with immunofluorescence microscopy to assess the presence of typical cellular markers [7]. Such markers typically found in a fibroblast population include Vimentin and α-Smooth Muscle Actin [2]. Other factors helpful in characterizing a cell population include their functional characteristics – measurable through metabolic activity or proliferation. Another measurement of cellular function particularly relevant to fibroblast populations is their ability to contract collagen; a factor used in other studies to either compare cell populations [2,8] or the effect of mechanical strain upon cell function [3].

This work seeks to characterize a novel population of primary RSFs isolated from three different adult patients in terms of their metabolic activity, contractile capacity, morphology and presence of intracellular markers. Experiments aim to compare primary cell populations not only to each other but also a line of human dermal fibroblasts (HDFs) as an established cellular control.

2. Methods

2.1 Cell Populations Used

Throughout this work, experiments are conducted on four different fibroblast cell populations. Three primary human RSF cell populations are used (NH05,08 and 09) alongside one immortalised human dermal fibroblast (HDF) cell line (Human dermal fibroblasts – catalogue number C0135C, Gibco™, Thermo Fisher Scientific, Waltham MA, USA). From the moment of thawing post cryopreservation, processes, reagents and culture media remain consistent across all experimental cell populations.

2.2 Cell Isolation

In order to isolate the RSF cells, an established protocol was used across all cell populations. The detailed protocol for cell isolation, culture and cryopreservation has been previously published by our group [9]. In brief, ethical approval was obtained from the UK Health research authority to obtain human rectus sheath tissue from patients undergoing the formation of a stoma during surgery (IRAS ID: 254166). Tissue samples for this work were harvested from three individuals. The age and sex details of each patient can be found below:

- NH05 - Female, 46 Years
- NH08 – Male, 60 Years
- NH09 – Female, 34 Years

Tissue samples were immediately transported to the laboratory, minced into small pieces (pulp consistency) and digested overnight in a media/ collagenase solution. After digestion, cells and any persisting tissue were re-suspended in culture media and plated in cell culture flasks. Cell populations were expanded over 4-6 weeks until confluence at 2nd passage. Cells were cryopreserved in 1 ml cryovials containing at least 1 million cells and stored in liquid nitrogen until needed for experimentation.

For re-amination of cells, cryovials were thawed using a water bath (37°C) and then added to approximately 15mls of warmed cell culture solution- Dulbecco's modified eagles Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% Pencillin/ Streptomycin solution (P/S). Media and cell stock were then transferred into a 75cm^2 (T-75) cell culture flask and placed in an incubator (37°C 5% CO_2) overnight. A complete change of cell culture media was then undertaken the following day, with subsequent media changes occurring every 2 days, changing half of the media on each occasion. All primary RSFs in this work were used between 3rd and 4th passage. For each experiment, passage number was identical across RSF populations. HDFs were used within passage 8 and 9 (cell line consistency ensured until passage 12).

2.3 2D Characterization

2.3.1 Cell Morphology

Cells were thawed and plated as per the above description. After plating, cells were examined within

their culture flasks using a digital imaging microscope (EVOS M500, Invitrogen – Waltham MA, USA) at days one, three and five after initial plating. Cells were imaged at 10x and 4x magnification and their morphology was examined.

2.3.2 Immunofluorescence

Staining: All four cell populations underwent immunofluorescent staining for the presence of Vimentin and α-Smooth Muscle Actin. All steps took place at room temperature unless stated otherwise. Approximately 120,000 cells were seeded within the wells of a 24 well plate and then cultured until around 70-80% confluence. Each well plate was washed three times with Phosphate buffered saline (PBS) and fixed with 4% Paraformaldehyde (PFA) in PBS for 15 minutes. Cells were once again washed 3 times with PBS and then permeabilized with 0.5% Triton X in PBS for 15 minutes. After three further PBS washes, cells were then blocked with 1% BSA in PBS +0.1% Tween20 (PBST) (Bioworld Dublin OH, USA) for 30 minutes. After a further 3 washes, cells were incubated with primary antibodies overnight in the dark at 4°C. Details of the antibodies used are as follows:

- Conjugated Anti-Vimentin antibody – 200µl of 1:200 dilution - Alexa-Flour® 488 anti-Vimentin Antibody (Biolegend San Diego, CA, USA) in 1% BSA in PBST.
- Anti α-Smooth Muscle Actin antibodies - 200µl of 1:100 dilution - Rabbit anti α-smooth muscle actin recombinant monoclonal antibodies (Cambridge biosciences, Cambridge, UK).

After overnight incubation, cells stained with primary anti α-Smooth Muscle Actin antibodies were washed three times with PBS and then incubated in the dark at room temperature for 1 hour with a secondary antibody (Sheep Anti-rabbit IgG Heavy and Light chain Antibody Dylight 488 Conjugated- Cambridge Biosciences, Cambridge UK – used at 1:100 dilution). Finally, all cells were counter-stained with DAPI at room temperature for 15 minutes. Cells were once again washed in PBS three times and then stored in PBS at 4°C until ready for microscopy. Cells stained for Vimentin were compared to a single non-antibody control. Cells stained for α-Smooth Muscle Actin were compared to one non-antibody control and another without any *primary* antibody.

2.4 Microscopy

Before microscopy, excess PBS was removed from each well. Plates were then imaged at 20x magnification and 488nm using a Zeiss LSM 710 fluorescence microscope (Zeiss – Obercochen, Germany).

2.4.1 MTT Assay

In order to assess the proliferation/ metabolic activity of different cell populations, MTT assays were conducted across two time points of 48 and 72 hours (as per manufacturers protocol recommendations). For each assay, 5000 cells were seeded in each well (n=8 for each cell population, with n=8 acellular control samples) within a 96 well plate. Samples were cultured without media change for 48 and 72 hours respectively. After the culture period, media was removed from the wells, and 100µl of fresh culture media reintroduced. 10µl of MTT reagent (Biotium, San Francisco CA, USA) was added to the culture media, and plates incubated for four hours (37°C 5% CO_2). After incubation, 200µl of Dimethyl sulfoxide (DMSO Merck, Darmstadt, Germany) was added to each well plate. Each plate was then scanned for absorbance in a microplate reader (Infinite M Plex, Tecan – Mannedorf, Switzerland), with absorbance measured at 570nm, and background absorbance at 630 subtracted from it. Final results are denoted as 'net absorbance'.

2.5 3D Characterisation

Cells underwent two separate types of 3D characterisation; both of which involved cells seeded into collagen gels to form fibroblast populated collagen matrices (FPCMs). In the first study, FPCMs were immediately compressed and cultured for 5 days before being stained and imaged. In a separate experiment, cells were seeded within free-floating collagen gels for assessment of their contraction.

2.5.1 Compressed Gel 3D Characterisation

FPCM Production: FPCMs were produced as per the Lonza RAFT protocol [10]. Specifically, for each cell line, 1.2ml FPCMs were produced in triplicate within 24 well plates. FPCMS were produced by combining 80% type I rat tail collagen (2 mg/ml protein in 0.6% acetic acid: First Link UK Ltd, West Midlands, UK), 10% of 10x concentration of Minimum Essential Medium (10X MEM; Gibco™), 5.8% of neutralizing solution (HEPES Buffer with 1M Sodium Hydroxide) and finally 4.2% cell stock solution. Cells were seeded within the FPCM mixture at a concentration of 0.1×10^6 cells/ml. Gels then underwent plastic compression using RAFT absorbers (Lonza – Basel, Switzerland) for 5 minutes, after which 1 ml of culture media was added to each well. Gels were cultured for 5 days with media being exchanged every 2 days. After 5 days,

gels were washed 3 times with PBS then fixed for 30 minutes using 4% PFA. Gels were then stored at 4°C in PBS until staining.

2.5.2 Gel Staining and Imaging

All of the FPCMs (both compressed gels as well as contraction assays) were stained for both Vimentin and α-Smooth Muscle Actin as described in the 2-dimensional characterization. Identical reagents and antibodies were used throughout, aside from minor differences in timing; 3D samples were fixed in PFA for 30 minutes, permeabilised in 0.5% Triton X for 30 minutes, and blocked using 1% BSA for 60 minutes before staining. 3D samples were then mounted on microscopy slides using fluorescence mounting media (Fluorescent mounting media, KPL, Seracare Milford MA, USA) underneath a coverslip – then underwent microscopy as per 2-dimensional characterization. Representative single z-depth images were used for analysis.

2.5.3 3D Contraction Assay

FPCM Production

FPCMs were produced as per the above detailed protocol for compressed gels but were seeded with cells at a concentration of $0.5x10^6$ cells/ml. Once formed, gels were incubated for 15 minutes (37˚C and 5% CO_2) and allowed to set. Once set, an additional 1ml of culture media was added to each well plate and each gel was scored around its edge to separate it from the well – leading to free-floating gels within 1ml of culture media. FPCMS were cultured for 5 days in total – with culture media changed every 24 hours.

Analysis of Contraction

Each FPCM was photographed at 24-hour intervals using a digital camera (Cybershot; Sony Tokyo, Japan). FPCMs were photographed twice, and then each photograph was analysed twice using software package ImageJ™ (National Institutes of Health, Bethesda, MD, USA). The software was used to calculate the area of the upper surface of each FPCM compared to the area of the well plate. Measurements were used to calculate the percentage reduction in area of each FPCM throughout the experiment. Once the 5-day contraction assay was complete, all FPCMS were fixed in 4% PFA so they could undergo immunofluorescence staining.

Statistical Analysis

All statistical analysis was performed using SPSS (Version 29.0 IBM 2022). For both contraction and MTT assays, all four cell populations and acellular control were compared using a non-parametric analysis of variance (Kruskall-wallis) test with pair-wise comparisons.

For both assays, the combined results for the RSF population (NH05,08, and 09) were also treated as one population and compared to acellular and HDF cellular controls through two separate Mann-Whitney tests. Values were deemed statistically significant with a P value <0.05.

3. Results

3.1 Morphology

Images were taken on the first, third and fifth days after cells were plated post-thawing. All three RSF cell populations as well as HDFs attached to the culture flask well and began elongating at day 1 (figure 1a). All three primary RSF cell populations as well as the HDF control showed characteristic features of fibroblast cell lineage (adherent, elongated cells with spindle-like projections). Over the next 4 days, all cell populations multiplied well with HDFs and NH09 cells appearing more proliferative. Such was the case that at day five, NH09 cells became over confluent within culture (bottom right of figure 1b). These over confluent NH09 cultures were discarded and not used for further experimentation.

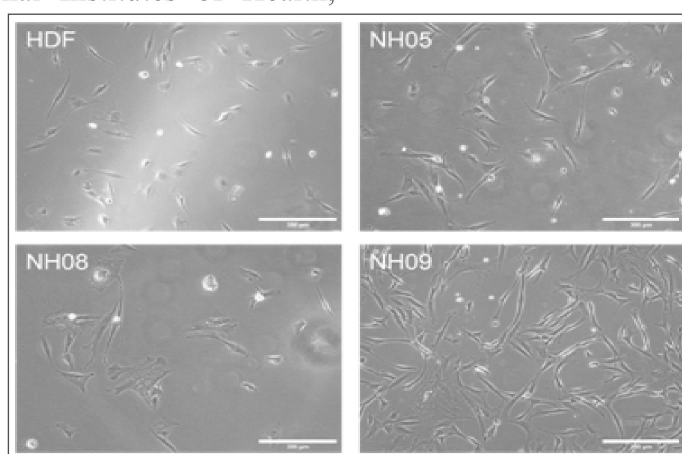

Figure 1a. *Day 1 of culture*

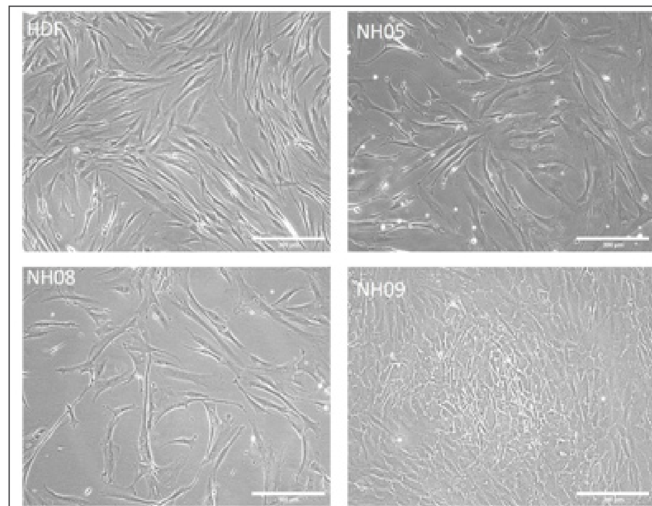

Figure 1b. *Day 5 of culture*

Figures 1a+b. *10x Magnification Microscopy images of HDFS and NH5,8, and 9 cells. 1a depicts all four cell populations 1 day after re-plating following thawing. 1b shows cells 5 days following replating following thawing. Scale Bar = 300µm.*

3.2 2D Immunofluorescence

When cultured on well plates, all three primary RSF cell populations as well as the HDF control stained positively for α-Smooth Muscle Actin as well as Vimentin. Positive staining in each cell type was confirmed by comparison with one or multiple negative controls. Figures 2 and 3 show 20x immunofluorescence images for each cell population stained for Vimentin and α-Smooth Muscle Actin respectively. The images provided for NH05 in both figures 2 and 3 have been previously published in our protocol article[9].

3.3 MTT Assay

Figure 4 shows the results of our MTT assay – a bar graph plotting the net absorbance levels provided by the plate reader after the 48 and 72 hour assays. A higher net absorbance represents higher the metabolic activity within the well plate. Each RSF cell population and HDF control showed significantly higher metabolic activity than the acellular control at 48 and 72 hours ($P<0.05$). The only exceptions were the HDF and NH05 cells which failed to reach statistical significance at 72 Hours ($p=0.16$ and 0.056 respectively). After 48 hours, each cell population showed a similar level of metabolic activity, with no significant difference between each population individually. At 72 Hours, Kruskal Wallis analysis identified a significantly higher metabolic activity in the NH09 population than both NH05 and HDF groups.

When RSF results were combined as one population, MTT results were significantly higher than acellular controls. When combined RSF populations were compared to the HDF population, there was a difference between populations that lacked significance after 48 hours ($P=0.064$) but became significant after 72 hours ($P=0.046$).

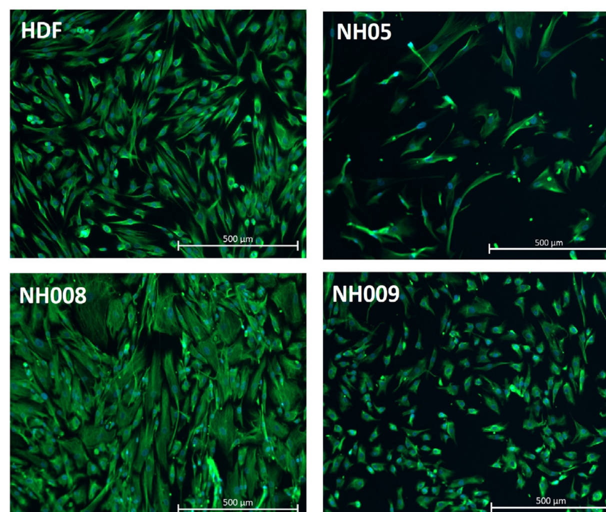

Figure 2. *20x immunofluorescence images of 3 RSF populations as well as an HDF control. Vimentin can be seen stained in green, whilst the cell nuclei can be been stained in blue (DAPI). Scale bars= 500µm.*

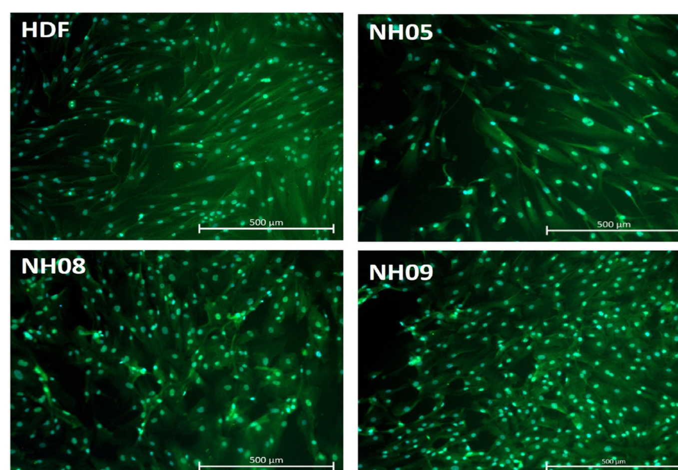

Figure 3. *20x images of each of the 3 RSF populations as well as an HDF control stained for α- Smooth Muscle Actin. Nuclei are stained in blue (DAPI), and α-Smooth Muscle Actin in green. Scale bars = 500µm.*

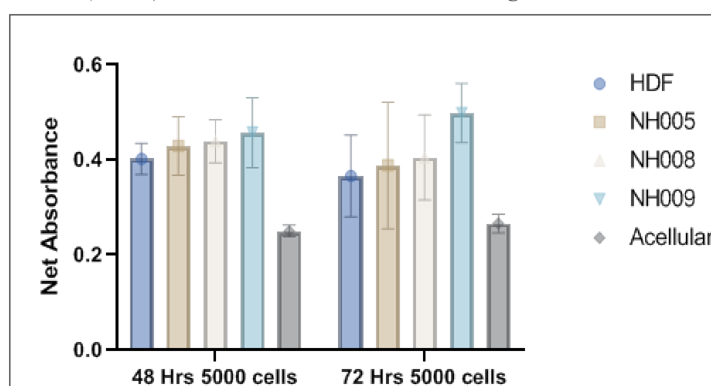

Figure 4. *A bar chart showing net absorbance levels from an MTT assay of each cell type. Each bar represents a mean +/-SD net absorbance value from n=8 wells of each cell population from a 96 well plate.*

3.4 3D Immunofluorescence

All RSF populations as well as HDF control stained positively for Vimentin. This was the case throughout both contraction assay FPCMs and compressed FPCMs. Figure 5 shows each cell population after staining for Vimentin, showing not only a positive result for staining, but also a consistent elongated cell morphology throughout all cell populations – matching our cellular control.

Staining for α-Smooth Muscle Actin revealed similar morphology, but a definitive positive staining result in 3D could not be confirmed due to significant autofluorescence. Further future work should look to optimize the 3D staining protocol or experiment with alternative antibodies.

3.5 3D Contraction Assay

All cellular FPCMs were seen to contract over the five days of the assay (see figures 6+7), whilst the

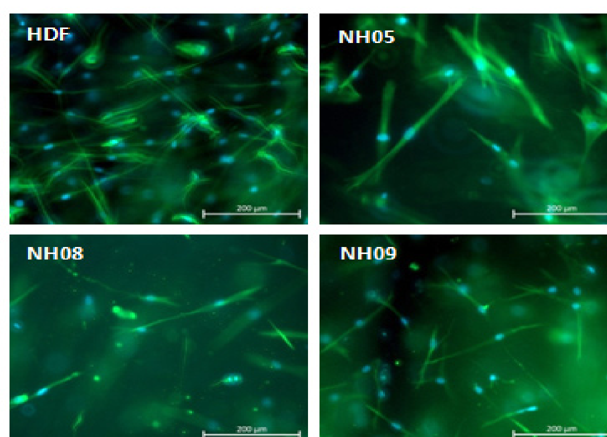

Figure 5. *20x single z-depth images of each of 4 cell populations within our compressed 3D collagen gel culture. Each sample is stained for Vimentin (green) with nuclei stained in blue (DAPI). Images show a consistent elongated cell morphology – expected from fibroblasts. Scale bar =200 µm.*

acellular collagen gels did not. Figure 7 shows the results of the assay, with each cell population showing an initial higher rate of contraction with a subsequent plateau – a pattern well documented within the literature [11]. All statistical analysis and group comparisons were undertaken on results of the contraction assay after the fifth day. Kruskall-wallis analysis revealed no significant differences between the three RSF lines when compared to each other. The analysis also showed a pair-wise significant difference between NH08 and HDF populations but no other inter- population differences. Only the HDF and NH05 populations showed significant difference at five days to the acellular gels, with the NH09 result being marginal (P=0.055). When RSF populations were combined as a whole, FPCMS were significantly less contractile over five days compared to FPCMs populated with HDF cells.

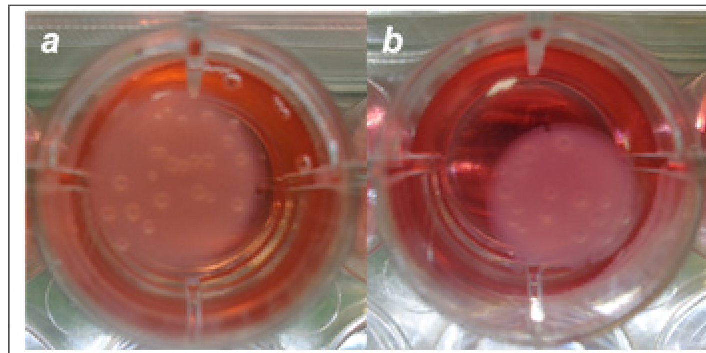

Figure 6a+b. *Photographs of a single free floating contraction assay made with NH09 cells. Image a shows the FPCM after 24 hours of culture, with b showing the same FPCM after 120 hours (5 days) of culture. Images show the clear reduction in size over the intervening 4 days.*

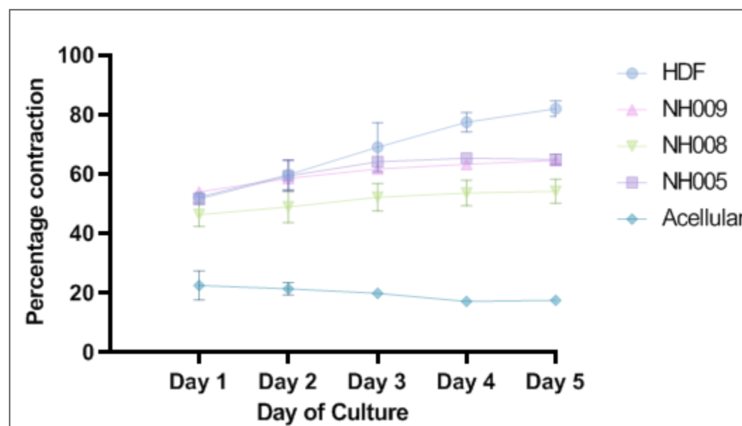

Figure 7. *A graph showing results of contraction assay across all 4 cell populations as well as an acellular control. Each point represents the mean contraction percentage across n=3 gels for that cell type with error bars representing standard deviation.*

4. Discussion

This work sought to undertake an *in vitro* characterization of primary human rectus sheath fibroblasts. The work was designed not only to analyse and compare cells isolated from three separate individuals, but also compare them to a control cell population of immortalised HDFs.

This work was crucial to deciphering the exact phenotype of newly isolated human rectus sheath fibroblasts and determining whether these maintained their phenotype in 2D and 3D *in vitro* culture. When culturing these RSF cells directly after cryopreservation, it was clear to see that all cell populations proliferated well, adherent to culture flasks. As per figures 1a+b, all cell populations exhibited a typical elongated fibroblast cellular morphology. Figure 1b shows the extent to which the NH09 cells were particularly proliferative over the first 5 days of culture, leading to initial over-confluence. This observation is in keeping with, and may explain, the findings of our MTT assay showing their elevated metabolic activity.

Immunofluorescence microscopy confirmed the positive presence of both Vimentin and α-Smooth Muscle Actin within each RSF cell population and HDF control. Whilst both these markers are indicative of a fibroblast lineage, neither is definitive. Vimentin is considered a reliable marker for any cells of mesenchymal origin[2], and α-Smooth Muscle Actin can play a role of mechano-transduction in a number of different cell types [12]. Whilst the presence of

α-Smooth Muscle Actin is considered to be more consistent with a myofibroblast than fibroblast phenotype, fibroblasts cultured on a rigid substrate in the presence of serum can exhibit a myofibroblast phenotype [13].

When undertaking immunofluorescence on RSF cell populations within our FPCMs, whilst Vimentin staining was successful, staining for α-Smooth Muscle Actin was not definitive, with cells staining no better than either of our negative controls. Future work should look to optimize this process with the use of either different antibody products or a more optimal 3D staining protocol.

With regards to the wider functional analysis (MTT and contraction assays) of our cell populations, several cautious conclusions can be reached. MTT assay results indicate that the metabolic activity of our RSF cells is similar to, if not slightly higher than, HDF cells. Whilst MTT assays are often used as a proxy for cell replication and validity, they are more specifically a measurement of cell metabolic activity. This metabolic activity can be influenced by other factors (e.g accumulation of intracellular biomass, and production of proteins and lipids [14]) and does not necessarily always correlate with proliferative activity. Our results, for example, show the HDF cell line to have a consistently (if not always statistically significant) lower activity than the RSF populations. This is despite our observations throughout that HDF cells were one of the more proliferative cell populations (evidenced by figures 1a+b and figure 5). We believe it is likely given the MTT assay results that the RSF populations are, even with the same cell number, more metabolically active than the HDF cell line. The reason behind this finding is unclear and more work should be undertaken to investigate potential causes. Aside from the tissue origin of the cells, the immortalization of the HDF cell line may play a key role. Immortalized cell lines are not completely reflective of human cells and may possess a number of genetic abnormalities or mutations which allow indefinite proliferation. Such mutations may result in cells with altered metabolism. A definitive way to investigate this confounder would be to directly compare primary cells from the skin Vs rectus sheath of the same individual.

Our contraction assay revealed that all cell populations exhibited contractile capacity that was sufficient to contract a collagen matrix. Contraction assays are a well-established tool to help characterize fibroblast cell populations [8,15]. Such an assay examines a principal function of fibroblast cells; their ability to contract extra-cellular matrix components. This cellular function enables fibroblasts to mediate wound contraction in the early stages of tissue healing. Direct comparison between RSF cell populations and HDFs suggests that HDFs were capable of contracting collagen at a greater rate. Like the MTT assay, there may be a number of factors that influence this – for example a greater rate of cell proliferation. Previous literature has described primary murine RSF populations as more contractile than murine dermal fibroblasts [2], however both of these cell populations were primary cells from the same murine source. An interesting addition to our work would be to compare the contractile capacity of primary human dermal and rectus sheath fibroblasts from the same patient to examine their function.

Now that these isolated cells have been established as a functional and viable cell population for experimentation, our group has begun work on using them to develop prototype 3D tissue models to test interactions with implantable medical devices such as hernia mesh. Such studies using cells *in vitro* to test hernia meshes have been conducted before [16-19], but not with the use of human RSF cells, and not within a 3D *in vitro* model. The emerging face of 3D model development is currently dominated by using 3D *in vitro* cancer models to examine factors such as invasion[20] or the effects of chemotherapeutic drugs [21]. It is reasonable to suggest that similar models could be used in the coming years to measure the effects of a great number of implantable medical devices upon human tissues.

Further experiments would help overcome some limitations of this work and could be used to gain a better understanding of these cell populations in the future. The contraction assay presented some technical issues as can be seen in figure 7. This shows an approximate 20% contraction of the acellular gels despite there being a known absence of any contraction. This 20% is an artefact of the FPCM gel floating in media, and therefore the 'true' contraction of the cellular gels in the same experiment is likely to be less than described. In addition, it is likely that the number of contracting FPCMs used for each cell population is not enough to sufficiently power the study. Figure 7 displays several clear differences between populations that did not reach the point of statistical significance (for example NH08 Vs acellular gels). It is very likely that such statistical significance would be reached with a greater number of experimental repeats; our work was, however, limited by the total

number of cells available and our intent to undertake a number of different experiments.

Whilst RSF cells were used up to their 4th passage, future work should look to establish the function and validity of these cells into their 5th passage and beyond. Further work could also look to establish quantitative data for levels of proliferation for each cell population by conducting cell counts at different passages – possibly with the addition of how different growth factors effect this.

5. Conclusion

This work is the first of its kind to isolate and culture human fibroblasts from posterior rectus sheath and confirms that human RSF cells isolated using our previously published protocol [9] can be cultured successfully for experimentation until their 4th passage. At this passage they display metabolic activity of a similar level to HDF cells, and successfully contract collagen *in vitro*. All cells displayed morphological and intracellular markers of fibroblast lineage.

The results confirm that primary human RSF cells possess promising potential for future *in vitro* experimentation in both 2D and 3D culture. Such future work may include the potential for these cells to investigate the effects of various medical implants such as hernia mesh.

Declaration of Interest

None of the authors have a financial conflict of interest to declare for this work.

Ethics Statement

The tissue used for this work was gathered from consenting volunteer patients. Ethical approval for this study was obtained from The UK National Health service, Health research authority – IRAS No. 254166.

Fundings

This work was partially funded by research grants from the British Hernia Society and the European Hernia society.

Acknowledgement

Authors would like to thank the British Hernia Society and the European Hernia society for providing funding for this work.

6. References

1. Walming S, Angenete E, Block M, Bock D, Gessler B, Haglind E (2017) Retrospective review of risk factors for surgical wound dehiscence and incisional hernia. BMC Surg 17 (1):19. doi:10.1186/s12893-017-0207-0
2. Dubay DA, Wang X, Kirk S, Adamson B, Robson MC, Franz MG (2004) Fascial fibroblast kinetic activity is increased during abdominal wall repair compared to dermal fibroblasts. Wound Repair Regen 12 (5):539-545. doi:10.1111/j.1067-1927.2004.012506.x
3. Culbertson EJ, Xing L, Wen Y, Franz MG (2011) Loss of mechanical strain impairs abdominal wall fibroblast proliferation, orientation, and collagen contraction function. Surgery 150 (3):410-417. doi:10.1016/j.surg.2011.06.011
4. Buechler MB, Pradhan RN, Krishnamurty AT, Cox C, Calviello AK, Wang AW, Yang YA, Tam L, Caothien R, Roose-Girma M, Modrusan Z, Arron JR, Bourgon R, Müller S, Turley SJ (2021) Cross-tissue organization of the fibroblast lineage. Nature 593 (7860):575-579. doi:10.1038/s41586-021-03549-5
5. Phoa S, Chan KS, Lim SH, Oo AM, Shelat VG (2022) Comparison of glue versus suture mesh fixation for primary open inguinal hernia mesh repair by Lichtenstein technique: a systematic review and meta-analysis. Hernia 26 (4):1105-1120. doi:10.1007/s10029-022-02571-4
6. Patil R, Kale AD, Mane DR, Patil D (2020) Isolation, culture and characterization of primary cell lines of human buccal mucosal fibroblasts: A combination of explant enzamytic technique. J Oral Maxillofac Pathol 24 (1):68-75. doi:10.4103/jomfp.JOMFP_282_19
7. Künzel SR, Rausch JSE, Schäffer C, Hoffmann M, Künzel K, Klapproth E, Kant T, Herzog N, Küpper JH, Lorenz K, Dudek S, Emig R, Ravens U, Rog-Zielinska EA, Peyronnet R, El-Armouche A (2020) Modeling atrial fibrosis in vitro-Generation and characterization of a novel human atrial fibroblast cell line. FEBS Open Bio 10 (7):1210-1218. doi:10.1002/2211-5463.12896
8. Haydont V, Neiveyans V, Perez P, Busson É, Lataillade J, Asselineau D, Fortunel NO (2020) Fibroblasts from the Human Skin Dermo-Hypodermal Junction are Distinct from Dermal Papillary and Reticular Fibroblasts and from Mesenchymal Stem Cells and Exhibit a Specific Molecular Profile Related to Extracellular Matrix Organization and Modeling. Cells 9 (2). doi:10.3390/cells9020368
9. Whitehead-Clarke T, Grillo A, Mudera V, Kureshi A (2023) The Isolation, Culture, and Cryopreservation of Human Rectus Sheath Fibroblasts. Curr Protoc 3 (5):e788. doi:10.1002/cpz1.788
10. Lonza Lonza RAFT 3D cell culture protocol - https://

bioscience.lonza.com/lonza_bs/CH/en/how-the-raft-system-works. Accessed 27/10/2022 2022

11. Mukhey D, Phillips JB, Daniels JT, Kureshi AK (2018) Controlling human corneal stromal stem cell contraction to mediate rapid cell and matrix organization of real architecture for 3-dimensional tissue equivalents. Acta Biomater 67:229-237. doi:10.1016/j.actbio.2017.11.047
12. Wang J, Zohar R, McCulloch CA (2006) Multiple roles of alpha-smooth muscle actin in mechanotransduction. Exp Cell Res 312 (3):205-214. doi:10.1016/j.yexcr.2005.11.004
13. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. Nature Reviews Molecular Cell Biology 3 (5):349-363. doi:10.1038/nrm809
14. Zhu J, Thompson CB (2019) Metabolic regulation of cell growth and proliferation. Nat Rev Mol Cell Biol 20 (7):436-450. doi:10.1038/s41580-019-0123-5
15. Haydont V, Neiveyans V, Zucchi H, Fortunel NO, Asselineau D (2019) Genome-wide profiling of adult human papillary and reticular fibroblasts identifies ACAN, Col XI α1, and PSG1 as general biomarkers of dermis ageing, and KANK4 as an exemplary effector of papillary fibroblast ageing, related to contractility. Mech Ageing Dev 177:157-181. doi:10.1016/j.mad.2018.06.003
16. Fehér D, Ferencz A, Szabó G, Juhos K, Csukás D, Voniatis C, Reininger L, Molnár K, Jedlovszky-Hajdú A, Wéber G (2021) Early and late effects of absorbable poly(vinyl alcohol) hernia mesh to tissue reconstruction. IET Nanobiotechnol 15 (6):565-574. doi:10.1049/nbt2.12015
17. Langer C, Schwartz P, Krause P, Mohammadi H, Kulle B, Schaper A, Füzesi L, Becker H (2005) [In-vitro study of the cellular response of human fibroblasts cultured on alloplastic hernia meshes. Influence of mesh material and structure]. Chirurg 76 (9):876-885. doi:10.1007/s00104-005-1036-8
18. Giuntoli G, Muzio G, Actis C, Ganora A, Calzone S, Bruno M, Ciardelli G, Carmagnola I, Tonda-Turo C (2020) In-vitro Characterization of a Hernia Mesh Featuring a Nanostructured Coating. Front Bioeng Biotechnol 8:589223. doi:10.3389/fbioe.2020.589223
19. Muzio G, Perero S, Miola M, Oraldi M, Ferraris S, Vernè E, Festa F, Canuto RA, Festa V, Ferraris M (2017) Biocompatibility versus peritoneal mesothelial cells of polypropylene prostheses for hernia repair, coated with a thin silica/silver layer. J Biomed Mater Res B Appl Biomater 105 (6):1586-1593. doi:10.1002/jbm.b.33697
20. Micalet A, Moeendarbary E, Cheema U (2021) 3D In Vitro Models for Investigating the Role of Stiffness in Cancer Invasion. ACS Biomater Sci Eng. doi:10.1021/acsbiomaterials.0c01530
21. Gupta P, Miller A, Olayanju A, Madhuri TK, Velliou E (2022) A Systematic Comparative Assessment of the Response of Ovarian Cancer Cells to the Chemotherapeutic Cisplatin in 3D Models of Various Structural and Biochemical Configurations-Does One Model Type Fit All? Cancers (Basel) 14 (5). doi:10.3390/cancers14051274