Tissue Engineering

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Journal:	*Tissue Engineering*
Manuscript ID	TEA-2017-0509.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	01-Mar-2018
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Keyword:	Tumor Models < Enabling Technologies in Tissue Engineering (DO NOT select this phrase; it is a header ONLY), 3-D Cell Culture < Enabling Technologies in Tissue Engineering (DO NOT select this phrase; it is a header ONLY), Adipose < Applications in Tissue Engineering (DO NOT select this phrase; it is a header ONLY), Mesenchymal Stem Cells < Fundamentals of Tissue Engineering (DO NOT select this phrase; it is a header ONLY), Cell Migration < Fundamentals of Tissue Engineering (DO NOT select this phrase; it is a header ONLY)
Manuscript Keywords (Search Terms):	Mesenchymal stem cells, Adipocytes, Breast cancer, Anisotropic, Collagen, Immortalisation
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	empty scaffold controls, demonstrating an adipocyte-mediated influence on breast cancer cell migration. Thus, this 3D in vitro model recapitulates the migratory effects of adipocytes observed on breast cancer cells and suggests that it could have utility with fresh breast tumour biopsies as an assay for cancer therapeutic efficacy in personalised medicine strategies.

An engineered human adipose/collagen model for *in vitro* breast cancer cell migration studies

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Abstract

Adipocytes are one of the major stromal cell components of the human breast. These cells play a key role in the development of the gland and are implicated in breast tumorigenesis. Frequently, directional stromal collagen I fibres are found surrounding aggressive breast tumours. These fibres enhance breast cancer cell migration and are associated with poor patient prognosis. We sought to recapitulate these stromal components *in vitro* to provide a 3D model comprising human adipose tissue and anisotropic collagen fibres. We developed a human mesenchymal stem cell (hMSC) cell line capable of undergoing differentiation into mature adipocytes by immortalising hMSCs, isolated from breast reduction mammoplasties, via retroviral transduction. These immortalised hMSCs were seeded in engineered collagen I scaffolds with directional internal architecture and adipogenesis was chemically induced, resulting in human adipose tissue being synthesised *in vitro* in an architectural structure associated with breast tumorigenesis. Subsequently, fluorescently labelled from an established breast cancer cell line were seeded into this model, co-cultured for 7 days and imaged using multiphoton microscopy. Enhanced breast cancer cell migration was observed in the adipose-containing model over empty scaffold controls, demonstrating an adipocyte-mediated influence on breast cancer cell migration. Thus, this 3D *in vitro* model recapitulates the migratory effects of adipocytes observed on breast cancer cells and suggests that it could have utility with fresh breast tumour biopsies as an assay for cancer therapeutic efficacy in personalised medicine strategies.

Introduction

The stromal extra-cellular matrix (ECM) at the periphery of breast tumours mainly comprises collagen I fibres that are organised in a number of so-called tumour-associated collagen signatures (TACS) [1]. One such signature, TACS-3, is characterised by aligned, or anisotropic, bundles of collagen fibres that are orientated perpendicular to the tumour boundary and this arrangement has been associated with poor patient prognosis [1,2]. We have developed a system that recapitulates the TACS-3 arrangement by synthesising 3D engineered collagen scaffolds with an anisotropic internal architecture [3].

During breast tumour progression, a variety of stromal cells contribute to the lethal metastatic spread of epithelial cancer cells to various organs [4]. One of the most abundant cellular components of the breast stroma is adipocytes and these cells have been implicated in tumour phenotype [5]. Through the synthesis and secretion of a variety of hormones and cytokines, including oestrogen, adipocytes communicate with breast epithelium [5]. It is therefore paramount that any model investigating breast cancer cell migration should incorporate adipocytes to provide a physiologically relevant setting.

Previous *in vitro* breast cancer models have frequently utilized the 3T3-L1 murine embryonic preadipocyte cell line to study adipocyte influences on cancer cell migration [6–8]. Although these models have provided important insight into cancer cell migratory potential, we sought to replace this murine cell line with a human alternative to more accurately recapitulate the human breast stroma. Human mesenchymal stem cells (hMSC) that have been isolated from donor tissue can be differentiated *in vitro* into mature adipocytes, making them an ideal candidate for this purpose [9]. Furthermore, hMSCs reside within the adult breast and can be

purified from breast reduction mammoplasty tissue, thus providing a more relevant cell type [10].

According to an NC3Rs review, it is estimated that >100 million rodents per year are culled worldwide for experimentation [11]. With a paradigm shift in opinions on animal testing alongside obvious financial incentives, scientists are being encouraged to minimise *in vivo* animal experimentation. Furthermore, there is a push to use 3D cell cultures as *in vitro* models as they more accurately resemble the *in vivo* environment, in comparison with their 2D counterparts [12]. Using this ideology, this study aimed to recapitulate breast cancer cell migration using 3D *in vitro* systems that only required cells from a human origin.

In this study, we initially set out to confirm if the previously described TACS-3 phenotype could be observed in a human breast tumour sample utilising histological, optical clearing and immunohistochemical approaches [1,2]. We then sought to immortalise hMSCs to provide a human breast-derived cell line capable of undergoing adipogenesis to replace the frequently used 3T3-L1 murine cell line and provide a humanised model. To generate adipose tissue in an collagen structure relevant to breast cancer, immortalised hMSC were then seeded into engineered collagen scaffolds with an anisotropic internal architecture [3] and differentiated with an adipogenic cocktail [9]. Successful differentiation was confirmed using immunolocalisation of mature adipocyte markers and specialised two-photon microscopy techniques. Following this, fluorescently tagged MDA-MB-231 breast cancer cells were seeded and their migration assessed. These data demonstrated a pro-migratory effect of human adipocytes on human breast cancer cells within our 3D model.

Materials and methods

Masson's Trichrome

Human ER+ breast tumour biopsies were paraffin embedded and sections were bathed in the following reagents for the following lengths of time: 100% Xylene: 6mins twice, 100% ethanol: 3mins, 95% methylated spirit: 2mins, 70% methylated spirit: 2mins, running tap water: 5mins, Weigert's haematoxylin: 15mins, running tap water: 5mins, 1% hydrochloric acid in 70% ethanol (acid alcohol): 5s, running tap water: 5mins, red mixture: 5mins, rinsed in 0.2% glacial acetic acid, filtered orange mixture: 5mins, rinsed in 0.2% glacial acetic acid, 0.5g aniline blue in 0.2% glacial acetic acid: 2mins, rinsed in 0.2% glacial acetic acid/70% methylated spirit: 40s, 95% methylated spirit: 1min, 100% ethanol: 3mins and 100% Xylene: 3mins. Sections were then mounted in Sub-x and left to dry.

Whole human tumour fragment immunostaining using Cleared Unobstructed Body Imaging Cocktails (CUBIC)

CUBIC was carried out according to a previously published protocol [13,14]. Human ER+ breast tumour biopsies were fixed overnight in 4% PFA at 4°C followed by immersion in Reagent 1a (modified from Reagent 1, unpublished, available at http://cubic.riken.jp/) at 37°C for three days changing into fresh Reagent 1a each day. Samples were blocked overnight in 0.5% Triton-X-10% NGS-PBS (blocking solution). Primary antibodies anti-K8 (DSHB, #TROMA-1, 1:150) and anti-ERα (Leica, #NCL-L-ER-6F11, 1:50) were diluted in blocking solution and agitated on a rocker for 5 days at 4°C. Samples were washed in PBS briefly followed by 3x1hour washes in fresh PBS. Secondary antibodies goat anti-rabbit Alexafluor647 (LifeTech, #A21245, 1:500) and goat anti-rat (LifeTech, #A21247, 1:500) were diluted in blocking solution and agitated on a rocker for 2days at 4°C. Samples were washed in PBS briefly followed by a 1hour wash in fresh PBS. Nuclei were marked with a

2hour wash in 10µM DAPI. Samples were washed in PBS briefly followed by a 1hour wash in fresh PBS and immersed in Reagent 2 at 37°C in a dry incubator for 24hours before imaging [13–15]. Two-photon microscopy techniques were carried out on a LaVision BioTec TriM Scope II upright 2-photon scanning fluorescence microscope.

The human breast biopsy material was provided by the Breast Cancer Now Tissue Bank held at Barts Cancer Institute, Queen Mary University of London, UK. This tissue bank is licensed by the Human Tissue Authority, according to UK ethical guidelines and approval, and biopsy material is obtained following informed consent. Anonymised data linked to each sample is provided. Applications to obtain material is assessed by the Tissue Access Committee, to ensure the research proposed is original and of high quality.

Cell Culture

All cell cultures were maintained in a humidified 5% CO_2 incubator at 37°C.

Human primary mesenchymal stem cells (MSC)

Maintenance

Human mesenchymal stem cells (hMSC) isolated from routine breast reduction mammoplasty surgery (passage 0) were provided by Professor Mohamed Bentires-Alj (Universität Basel, Switzerland). hMSCs were maintained in MSC MM (DMEM/F12 supplemented with 20% FCS, 15mM HEPES (Sigma #83264), 1nM 17-β-estradiol (Sigma #E2758), 100U/ml P/S, 50µg/ml gentamicin (Sigma #G1397), 10ng/ml EGF, 10ng/ml bFGF (Peprotech #100-18B). Cells were passaged at approximately 70% confluency, ensuring that 100% confluency was not reached during passage steps to avoid spontaneous differentiation.

Adipogenesis

hMSC grown to 100% confluency had their MSC MM removed and replenished with MSC adipogenic media (DMEM/F12 supplemented with 10% FCS, 15mM HEPES, 10nM 17-β-estradiol, 100U/ml P/S, 50µg/ml gentamicin, 5 µg/mL insulin, 1 µM dexamethasone, 0.5 mM IBMX, 60 µM indomethacin (Sigma #I7378)). Media was replaced with fresh MSC adipogenic media every 48 hours for 11 days.

Antibiotic titration

Primary hMSCs were treated with puromycin (Gibco #A11138) in the range 0-1 µg/ml or geneticin (Gibco #10131) in the range 0-600 µg/ml in MSC MM for 5 days to determine the minimum concentration of each antibiotic required to kill 100% of cells (Supplementary Table 1).

Human embryonic kidney 293T (HEK293T) cells

HEK293T cell maintenance media (HEK293T MM) comprised DMEM (Gibco #41965) supplemented with 10% FCS. Cells were passaged at 70-90% confluency. Cells were obtained from ATCC and were kept below passage 15.

MDA-MB-231 cells

MDA-MB-231 cells were maintained in DMEM supplemented with 10% FCS. Cells were passaged at 70-90% confluency. Cells were obtained from ATCC and were kept below passage 15. Cell lines in our laboratory are routinely tested for mycoplasma contamination.

Retroviral transduction of human mesenchymal stem cells (hMSC)

HEK293T cells were transfected using the PEI method for lentiviral transfection [16] with the exception of using 3 µg of viral DNA (retroviral plasmids pLXSN-neo-E6E7 and pBABE-puro-hTERT were gifted by Dr. Heike Laman (University of Cambridge, Department of Pathology)) and 2 µg of envelope vector pMD2.G.. Virus-containing media

was supplemented with MSC MM in a 1:1 ratio. Following transduction MSCs were passaged to remain sub-confluent. Transduced cells were selected by treatment with 600 µg/ml geneticin (pLXSN-neo-E6E7) or 1 µg/ml puromycin (pBABE-puro-hTERT) for 5 days (Supplementary Table 2). Surviving cells were then pooled into smaller wells to increase cell density and expanded for future use.

Senescence-associated β-Galactosidase staining

hMSC were seeded in 6 well culture plates and cultured until approximately 70% confluent. Media was removed and cells were washed with PBS before fixing with 4% PFA at room temperature for 15 mins. Cells were then washed twice with PBS and covered with β-galactosidase staining solution (β-Galactosidase staining kit, Cell Signalling #9680). Plates were then sealed with parafilm and incubated at 37°C in a dry incubator overnight. Cells were imaged under a light microscope. Senescent cells stain positively for β-galactosidase and appeared a blue/green colour.

Immunocytochemistry (ICC)

hMSC were cultured on glass coverslips and fixed in 4% PFA for 10mins. Permeabilization was carried out for 15mins in 0.5% Triton-X-PBS (VWR chemicals #28817.295) followed by 1hour blocking in normal goat serum (NGS). Primary antibodies anti-laminin (Abcam, #ab11575, 1:500), anti-collagen IV (Abcam, #ab6586, 1:500) or anti-Ki67 (Abcam, #ab15580, 1:100) were added and left overnight at 4°C. Secondary goat anti-rabbit AlexaFluor488 (LifeTech, #A11008, 1:500) was added to all samples for 1 hour. DNA was marked using DAPI and cells were visualized using epi-fluorescence.

Oil Red O

To prepare a stock Oil Red O solution, 0.5g Oil Red O (Sigma #O0625) was dissolved in 100ml isopropanol (5mg/ml), incubated at room temperature for 1hour, filtered through a 0.2-

mm filter and stored at room temperature. To prepare a working solution of Oil Red O, 6ml of stock solution was mixed with 4ml distilled water, incubated at room temperature for 1hour and filtered through a 0.2 mm filter. Undifferentiated hMSCs, or hMSCs following 11days adipogenesis, had their media removed and gently washed twice in PBS. Cells were fixed in 4% PFA at 4°C for 1hour followed by two more gentle washes in PBS. Oil Red O working solution was added, ensuring the entire cell surface was covered and incubated for 15mins at room temperature. Cells were then rinsed with distilled water approximately 5 times (until the solution was clear) and left in distilled water for light microscope imaging analysis.

SDS-PAGE and western blotting

Undifferentiated hMSCs, and hMSCs differentiated for 11 days, were collected in radioimmunoprecipitation assay (RIPA) buffer. A bicinchoninic acid assay (BCA) was then performed on an aliquot and 20 µg of protein was added per lane. Following electrophoresis and membrane transfer, blots were blocked in 5% bovine serum albumin (BSA) (Acros organics #240405000) in phosphate buffered saline-0.1% Tween-20 (PBST) for 1 hour. Primary antibody perilipin (Cell signalling #3470, 1:1000) and loading control anti-GAPDH (Abcam #ab9482, 1:10000) were diluted in 5% BSA-PBST, added to blots and left rocking overnight 4^{o}C. Blots were washed 3 times for 5 min in PBST, moved into 5% BSA-PBST containing the secondary antibody horseradish peroxidase (HRP)-anti-rabbit (Dako #PO448, 1:4000) and rocked at room temperature for 1 hour. Blots were then developed using enhanced chemiluminescence (ECL) (GE Healthcare #RPN2109) substrate and photographic film.

Anisotropic collagen scaffold synthesis

Anisotropic collagen scaffolds were prepared according to a modification of our previously published method [3]. Briefly, 1wt% collagen slurry was homogenised and aspirated into scaffold moulds containing copper pins. Moulds were placed into a pre-cooled (-40^{o}C) freeze- drier so that the copper pins were in direct contact with the metal shelf and thermally cycled. Following freeze-drying, scaffolds were removed and placed in cross-linking solution (70% ethanol, 33mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma #E6383) and 6mM *N*-hydroxysuccinimide (Sigma #130672)) for 30 mins with constant agitation on a rotating plate. Following cross-linking, the scaffolds were removed to fresh 70% ethanol and degassed under vacuum (approximately 10kPa) for 5mins. Samples were stored in 70% ethanol to ensure sterility until required.

Engineered human adipose model - E6/E7-hMSC seeding and differentiation

1x10^{6} E6/E7-hMSC cells were seeded into anisotropic collagen scaffolds, cultured for 7 days and differentiated using MSC adipogenic media for 11 days, replenishing MSC adipogenic media every other day.

Whole scaffold immunostaining

The human adipose model was fixed overnight in 4% PFA at 4°C followed by blocking overnight in 0.5% Triton-X-10% NGS-PBS (blocking solution). Primary antibodies anti-perilipin (1:50), anti-laminin (1:100) and anti-collagen IV (1:100) were diluted in blocking solution and agitated on a rocker for 5 days at 4°C. Samples were washed in PBS briefly followed by 3x1hour washes in fresh PBS. Secondary antibody goat anti-rabbit Alexafluor488 (1:500) were diluted in blocking solution and agitated on a rocker for 2days at

4°C. Samples were washed in PBS briefly followed by a 1hour wash in fresh PBS. Nuclei were marked with a 2hour wash in 10μM DAPI. Samples were washed in PBS briefly followed by a 1hour wash in fresh PBS before imaging. Two-photon microscopy techniques were carried out on a LaVision BioTec TriM Scope II upright 2-photon scanning fluorescence microscope.

Lentiviral transduction of MDA-MB-231 cells

5.84 μg pMD2.G envelope vector, 11.68 μg p8.91 packaging vector and 18.25 μg pCDH-EF1-MCS-T2A-tdTomato lentiviral transfer vector (cloned by modifying the pCDH-EF1-MCS-T2A-copGFP vector from System Biosciences (#CD521A-1)) were transfected into HEK293T cells using the previously published polyethylenimine (PEI) method [16]. Following this, virus-containing media from HEK293T cells was collected and added with 4μg/ml polybrene to MDA-MB-231 cells 48 hours after transfection. MDA-MB-231 cells were then spin transduced at 1800rpm for 45 mins at room temperature. This process of collecting viral-media and spin transducing was repeated again 24 hours later. Transduced MDA-MB-231 cells were then isolated by FACS and expanded in culture.

Fluorescent activated cell sorting (FACS)

tdTomato-expressing MDA-MB-231 cells were sorted using a MoJo cell sorter with the assistance of Nigel Miller (University of Cambridge) based on their fluorescence. Non-transduced MDA-MB-231 cells were used as a negative control.

tdTomato-expressing MDA-MB-231 cell / human adipose model migration assay

Migration assays were carried out according to a modified previously published protocol [3]. Briefly, empty anisotropic collagen scaffolds and adipose invested collagen scaffolds were placed into the upper wells of 6mm diameter Boyden chambers (0.4μm pore size, Costar

#3470) with upward facing seeding funnels. tdTomato^{+} MDA-MB-231 cells were trypsinized and seeded into scaffold seeding funnels at a concentration of 5×10^{6} cells/ml in a volume of 10µl. The bottom Boyden chamber was then filled with 750µl MSC MM and the scaffolds incubated for 4 hours. Once the cells had attached to the funnel, the upper chamber was filled with 250µl MSC MM. Samples were left to incubate for 7 days, with media changed every 48 hours. Following incubation, scaffolds were fixed for analysis in 4% PFA overnight at 4°C, washed in PBS and divided in half through the nucleation point using a scalpel blade. Cell nuclei were marked with SYTO16 (ThermoFisher #S7578) and samples were imaged using multi-photon microscopy.

Statistical analysis

Statistical analysis of data was carried out using an unpaired one-way ANOVA with a Tukey multiple comparisons test.

Results

Human oestrogen receptor alpha (ERα) positive breast tumour biopsy displays a tumour associated collagen signature-3 (TACS-3)

To investigate the TACS-3 signature associated with poor patient prognosis [1,2] at an increased level of detail, an ER^{+} breast tumour biopsy was analysed (Fig. 1). Following surgery, the biopsy was fixed and bisected, with one half embedded in paraffin for histological analyses and the other half processed for CUBIC optical clearing [14].

In order to visualise collagen, a Masson's Trichrome histological stain was employed (Fig. 1a). Directional anisotropic collagen fibres (Fig. 1a, arrows) were observed aligned perpendicular to the periphery of the tumour, projecting between the adipocytes constituting

the surrounding fat pad (Fig. 1a, asterisks). Epithelial cells were observed in the tumour bulk, on anisotropic collagen fibres at the tumour periphery and in the surrounding stroma (Fig. 1a), commensurate with a TACS-3 signature.

To investigate collagen structure in 3D, a CUBIC optical clearing protocol was utilised in conjunction with immunostaining, to permit deep 3D imaging [14]. Stereoscopic visualisation of the tumour following clearing revealed that CUBIC increased optical transparency considerably (Fig. 1b). Collagen can be detected specifically without the need for staining by using a non-liner imaging method called Second Harmonic Generation (SHG). Using second harmonic generation (SHG) and two-photon fluorescence (2pf) microscopy, anisotropic collagen fibres were observed running from right to left, between ERα^{+}/keratin-8^{+} (K8^{+}) epithelial cells, within the cleared tumour sample (Fig. 1c,d).

Human mesenchymal stem cells (hMSCs) can be immortalised by retroviral insertion of the E6/E7 genes but not the hTERT gene

Successive experiments focussed on immortalising hMSCs isolated from a breast reduction mammoplasty [10] via retroviral insertion of either human telomerase reverse transcriptase (hTERT) or the human papillomavirus oncogenes E6 and E7. Both hTERT and E6/E7 have been utilised previously to immortalise human primary cells for extended cell culture [17–21].

Following retroviral transduction, non-transduced hMSC (control, named hMSC hereafter), hTERT-hMSC and E6/E7-hMSC were investigated for the appearance of senescence, a phenomenon common to primary cells that are not immortalised, using a β-galactosidase staining protocol. These data showed a high proportion of hMSCs and hTERT-hMSC had

become senescent, whilst the majority of E6/E7-hMSC had escaped senescence (Fig. 2a, arrowheads).

As immortalisation can increase proliferative capacity [22], IHC analysis for the proliferation marker Ki67 was employed (Fig. 2b,c). Cell lines were analysed either at pre-confluency or post-confluency plus 11 days adipogenesis. At pre-confluency, significantly more proliferating E6/E7-hMSCs were observed compared to both hMSC and hTERT-hMSC (Fig. 2c). Since adipogenesis induces terminal differentiation, low proliferation rates were observed after 11 days in adipogenesis medium as expected (Fig. 2b,c) [23,24].

E6/E7-hMSCs successfully undergo adipogenesis in 2D culture

In order to confirm that the cell lines could undergo differentiation into adipocytes following 11 days culture in adipogenic medium, lipid droplets were detected using the fat soluble dye Oil Red O, which produces a red stain (Fig. 2d). Upon adipogenesis, both hMSC and E6/E7-hMSC generated multiple lipid droplets in the cytoplasm of the majority of cells (Fig. 2d). In contrast, hTERT-hMSC produced little or no detectable lipid droplets (Fig. 2d). To confirm differentiation status at the molecular level, cell lysates were compared by western blotting for the lipid vesicle membrane marker perilipin (Fig. 2e). In agreement with Fig. 2d, differentiated hMSC and E6/E7-hMSC cells both expressed perilipin, whilst hTERT-hMSC had undetectable levels (Fig. 2e).

Previously it has been reported that upon adipogenesis, both 3T3-L1 cells and MSCs synthesise the basement membrane proteins laminin and collagen IV [25–28]. Therefore, we next investigated their expression. Immunocytochemistry revealed a significantly increased deposition of laminin and collagen IV in differentiated hMSC and differentiated E6/E7-

hMSC compared to their undifferentiated states (Fig. 3). In contrast, although increased levels of laminin and collagen IV were present for differentiated hTERT-hMSC, these were not statistically significant (Fig. 3).

E6/E7-hMSC successfully undergo adipogenesis within anisotropic collagen scaffolds

Following successful immortalisation and adipogenesis of E6/E7-hMSC in 2D culture, this cell line was selected as the candidate to generate a human adipose model. Subsequent experiments investigated E6/E7-hMSC adipogenic capacity in 3D by seeding, culturing and differentiating E6/E7-hMSC in anisotropic collagen scaffolds and has been shown schematically in Fig 4a. Scaffolds were then fixed, bisected and 3D immunostained with anti-perilipin antibodies to mark lipid vesicles. Confocal microscopy tile scans revealed that differentiated E6/E7-hMSC with perlipin^{+} vesicles covered large areas of the scaffold's internal architecture, thus indicating the presence of mature adipocytes (Fig. 4b).

To further confirm the presence of lipids and therefore adipocytes, multi-photon microscopy and immunostaining techniques were employed (Fig. 4c). 2pf was used to image both DNA and anti-perilipin antibody staining, SHG to image collagen I and coherent anti-raman spectroscopy (CARS) to image lipids (Fig. 4c). CARS imaging is a revolutionary technique capable of imaging tissue without the use of labels or dyes. This technique can yield information about the distribution of specific cellular molecules by probing their vibrational properties. As the CARS input signal was specifically adjusted to the molecular vibration of lipids, the output signal therefore indicated the presence of lipids. These images showed that E6/E7-hMSC attached to the inner surface of the anisotropic collagen I pores with perilipin^{+}

vesicles containing an interior CARS signal (Fig. 4c). As a further confirmation of differentiation, immunostaining in 3D for laminin and collagen IV was employed. Both laminin and collagen IV expression were observed in all E6/E7-hMSC cells at a range of z-depths within the collagen I scaffold (Fig. 4d,e). Taken together, these data demonstrated that E6/E7-hMSC could successfully differentiate into mature adipocytes within anisotropic collagen I scaffolds.

E6/E7-hMSCs support the migration of MDA-MB-231 breast cancer cells

Subsequent experiments aimed to evaluate whether the model could recapitulate breast cancer cell migration in a TACS-3 setting. In previous work from our laboratory, we compared 3 different breast cancer cell lines (MDA-MB-231, MCF-7 and MDA-MB-468) for their migratory behaviour in empty collagen scaffolds [3]. We found that MDA-MB-231 cells were the most invasive. Thus, to provide us with sufficient migratory cells to compare scaffolds with and without the adipocyte fat pad, we chose this cell line and labelled them with the fluorescent protein tdTomato using lentiviral methods (Supplementary Fig. 1) to distinguish them from the E6/E7-hMSC. Anisotropic scaffolds invested with differentiated E6/E7-hMSC (human adipose model), alongside empty scaffold controls, were then seeded with tdTomato^{+} MDA-MB-231 cells into their scaffold funnels, cultured for 7 days and imaged using 2-photon microscopy (Fig. 5a) This methodology was according to a modified version of our previously published migration assay [3].

2pf and SHG revealed that after 7 days culture, the majority of tdTomato^{+} MDA-MB-231 cells in empty scaffolds were located within close proximity to the seeding funnel located at the top of the scaffold and also on the outer surface (Fig. 5b, red). In contrast, when co-

cultured with differentiated E6/E7-hMSC, tdTomato^{+} MDA-MB-231 cells were observed at all depths of the scaffold and were not confined to the funnel region (Fig. 5c). This demonstrated that MDA-MB-231 cells can survive in this environment and that adipocytes enhanced their migratory potential.

Discussion

In order to develop a 3D culture model of utility for analysis of breast tumour cell invasiveness and response to therapeutic drugs, we generated an immortal hMSC line from a breast reduction mammoplasty and demonstrated that these cells can be induced to differentiate into adipocytes in an anisotropic collagen scaffold. Furthermore, we have analysed the influence of the adipocyte microenvironment on the invasiveness of breast cancer cells.

It is interesting that the E6/E7 genes were more successful in immortalising hMSCs than hTERT. It is not clear why this should be, however, one possible explanation is that E6 and E7 interact with the master regulators of the cell cycle, p53 and pRB, respectively increasing the likelihood of immortalisation [29–31]. In contrast, although hTERT has been used successfully to immortalise a range of cell types, it was unable in our system to prevent telomere-controlled senescence [32].

The immortalised E6/E7-hMSC cell line is capable of *de novo* synthesis of lipids and extra-cellular matrix (ECM) proteins, such as laminin and collagen IV, in both 2D and within 3D anisotropic collagen scaffolds. Thus, our model comprises anisotropic collagen I, laminin, collagen IV and mature adipocytes; all major components of the stroma surrounding breast tumours with a poor prognosis [1,2].

Refractive index matching using reagents such as CUBIC, in conjunction with immunostaining and multiphoton imaging, allows direct imaging of collagen with SHG with the added advantage that the structural integrity of the tissue is relatively uncompromised. Hence, these techniques facilitated the detailed 3D visualisation of tumour cells associated with anisotropic collagen fibres.

Following 2D adipogenesis, E6/E7-hMSC synthesised the basement membrane proteins laminin and collagen IV, as previously reported in hMSCs and the murine preadipocyte 3T3-L1 cell line [25–28]. Although 2D adipogenesis was successful, it could not be presumed that scaffold culture would produce similar results since hMSC differentiation potential is affected by factors such as 3D culture, ECM substrate and surface topography [33–35]. Therefore, E6/E7-hMSC were differentiated within anisotropic collagen scaffolds to generate a human adipose model. Synthesis of lipids, laminin and collagen IV were all indicative of successful differentiation of E6/E7-hMSC to adipocytes and provide evidence that our model can recapitulate certain aspects of the breast stroma. It is worth noting that migration of MDA-MB-231 cells can be enhanced by uptake of lipid-derived fatty acids [8,36].

In conclusion, we have demonstrated the seeding and tracking of human breast cancer cells, from the aggressive MDA-MB-231 cell line, in a physiologically relevant 3D *in vitro* tissue engineered adipose model. This system successfully recapitulates multiple stromal elements of the breast tumour microenvironment and provides scientists with a novel tool to investigate cancer cell migration. Furthermore, these findings warrant further development to analyse additional breast cancer cell lines and to include breast cancer patient tumour

biopsies and the screening of a panel of cancer therapeutic drugs, to provide personalised treatments.

Acknowledgments

We thank Mohammed Bentires-Alj (Department of Biomedicine, University Hospital Basel, Universität Basel, Switzerland) for isolating and providing primary human mesenchymal stem cells from a reduction mammoplasty; Raza Ali (University of Cambridge, CRUK, UK) for examining the Masson's Trichrome sections and confirming the structures and cell types present; the Breast Cancer Now Biobank (Queen Mary University of London, UK) for supplying human breast tumour biopsies; and Dr. Heike Laman (University of Cambridge, Department of Pathology) for providing retroviral plasmids for hMSC immortalisation. This work is funded by the National Centre for the Refinement, Reduction and Replacement of animals in research (NC3Rs).

Author Disclosure Statement

No competing financial interests exist for all authors of this manuscript.

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Supplementary Table 1

Antibiotic	Concentration (µg/ml)	Dead MSC after 5 days (approx.)
Geneticin (G418)	0	1%
	150	75%
	300	90%
	400	98%
	500	99%
	600	100%
Puromycin	0	1%
	0.05	10%
	0.1	30%
	0.2	50%
	0.5	95%
	1	100%

Supplementary Table 1 - Kill curve to determine minimum antibiotic concentration required for the selection of immortalised hMSCs.
Non-transfected hMSC were treated with increasing concentrations of Geneticin/G418 and Puromycin. After 5 days treatment, cell death was quantified by the percentage of non-attached cells observed using light microscopy.

Supplementary Table 2

Plasmid name	Immortalising Gene	Antibiotic resistance	Concentration for selection
pLXSN-neo-E6E7	E6E7	Neomycin / G418 /Geneticin	600 µg/ml
pBABE-puro-hTERT	hTERT	Puromycin	1 µg/ml

Supplementary Table 2 - Optimal antibiotic concentrations for the selection of immortalised hMSCs.
The plasmids used for retroviral insertion of the E6E7 and hTERT genes for immortalisation and the corresponding antibiotic concentrations used for selection in hMSCs.

Figure legends

Figure 1 - The TACS-3 signature analysed in a human breast tumour biopsy using histological, optical clearing and 2-photon microscopy techniques.
A fresh human oestrogen receptor positive (ER^+) / human epidermal growth factor receptor negative ($HER2^-$) invasive ductal carcinoma (IDC) grade 2 was fixed following surgery and processed for either histological or CUBIC optical clearing. Grade, ER and HER2 status were determined by a trained medical pathologist (QMUL, Bart's Institute, UK). **a)** Paraffin embedded sections were stained with Masson's Trichrome; collagen (blue), nuclei (dark purple), cytoplasm (red). Columns of tumour cells can be seen invading between adipocytes (*) of the surrounding fat pad via anisotropic collagen fibres (arrows). Structures identified were confirmed by Dr. Raza Ali (University of Cambridge, CRUK), a trained medical pathologist. **b)** CUBIC optically cleared tumour fragment imaged using stereoscopic microscopy. **c)** Tumour fragment from b, imaged using two-photon fluorescence microscopy (2pf) and second harmonic generation (SHG) to visualise DAPI (round, white) and collagen (long fibrous, white), respectively. Directional anisotropic collagen fibres can be observed running right to left. A confocal z-stack is represented as individual z-slices. **d)** Tumour fragment and same z-sections from c, whole mount immunostained for keratin-8 (green) and ER (red) and imaged using 2pf. DAPI (blue) and collagen (blue) imaged with 2pf and SHG, respectively. Keratin-8^+ ER^+ epithelial cells can be observed, often in single file procession directionally aligned with anisotropic collagen fibres.

Figure 2 – Human mesenchymal stem cells (hMSCs) immortalised by insertion of E6/E7 genes escape senescence, have increased proliferative capacity and maintain successful adipogenic differentiation.
Human telomerase reverse transcriptase (hTERT) or E6/E7 genes were retrovirally inserted into hMSCs. These cell lines were named hMSC (non-transfected control), hTERT-hMSC and E6/E7-hMSC. **a)** Senescent cells were marked using β-galactosidase and produced a green stain (arrowheads). Due to low sensitivity of the assay, colour saturation and contrast alterations were applied equally all images (second row) to highlight differences between cell lines. Cells were at passage 12. **b)** Immunocytochemistry for proliferation marker Ki67 (green) on undifferentiated cell lines or after 11 days adipogenic induction. Nuclei marked with Hoechst (blue). **c)** The ratio of $Ki67^+$ nuclei to all cell nuclei was plotted for each cell line. Statistical significances were determined using an unpaired one-way ANOVA test (n=3, different passages). **p<0.01, ***p<0.001. **d)** Oil red O histological staining for intracellular lipids (red) to mark adipogenic differentiation. **e)** Western blotting of cell extracts, for the lipid vesicle marker perilipin (62 kDa) to mark differentiation. 'Adipo' samples are following 11 days adipogenic induction. GAPDH (37 kDa) and HEK293T cells were used as loading and negative controls, respectively.

Figure 3 – E6/E7-hMSC synthesise basement membrane proteins upon adipogenesis.
Immunocytochemistry of hMSC (non-transfected control), hTERT-hMSC and E6/E7-hMSC that are either undifferentiated or after 11 days adipogenic induction for basement membrane proteins **a)** laminin (green) and **b)** collagen IV (green). Nuclei were marked with Hoechst dye (blue). Image J analysis of detectable fluorescence for **c)** laminin and **d)** collagen IV. A.U. stands for arbitrary units. One way ANOVA statistical test was used to compare samples (n=3, different passages). *p<0.05, **p<0.01, ****p<0.0001.

Figure 4 - E6/E7-hMSC successfully undergo adipogenesis and synthesise basement membrane proteins in anisotropic collagen scaffolds to produce the human adipose model.

a) Following immortalisation of hMSC by retroviral insertion of E6 and E7 genes and antibiotic selection, E6/E7-hMSC were seeded in anisotropic collagen scaffolds, cultured for 7 days for proliferation and differentiated for 11 days with an adipogenic cocktail to produce the human adipose model. Scaffolds were then fixed, whole mount immunostained and imaged using 3D microscopy techniques. **b)** Immunostaining for the lipid vesicle and adipogenic marker perilipin (green). DNA marked with DAPI (blue). Tiled image z-stacks of undifferentiated E6/E7-hMSC and E6/E7-hMSC following adipogenesis, with zoomed in images (i,ii) and a digitally magnified image (ii, box insert). **c)** Scaffolds following adipogenesis from b, were imaged using non-linear excitation coherent anti-raman spectroscopy (CARS) to visualise lipids (red) directly. Perilipin (green) and DAPI (white) were imaged using 2-photon fluorescence excitation. Collagen was imaged directly using SHG microscopy (white). Image is shown as a maximum intensity projection of a z-stack. **d,e)** Immunostaining for basement membrane proteins laminin (d, green) and collagen IV (e, green). DNA is marked with DAPI (blue). Confocal images are shown as maximum intensity projections of DAPI, laminin/collagen IV and merged (white asterisk), or as a z-depth colour coded image of the laminin/collagen IV channel (white asterisk).

Supplementary Figure 1 - Lentiviral transduction of MDA-MB-231 cells with tdTomato expression construct and fluorescence activated cell sorting (FACS) enrichment of tdTomato-expressing cells.

MDA-MB-231 cells were lentivirally transduced to express the tdTomato fluorescent protein. **a)** FACS sorting of tdTomato MDA-MB-231 cells based on tdTomato fluorescence. **b)** Immunofluorescence of tdTomato MDA-MB-231 cells based on tdTomato fluorescence (red). DNA marked using Hoechst dye (blue). **a,b)** Non-transfected MDA-MB-231 cells were used as a negative control.

Figure 5 – The human adipose model promotes the migration of the MDA-MB-231 breast cancer cell line.

a) Empty anisotropic collagen I scaffolds and the human adipose model, were seeded with tdTomato-expressing MDA-MB-231 cells into the scaffold funnels and cultured for 7 days. Scaffolds were then fixed, bisected and DNA was marked. Scaffolds were then imaged using 2-photon microscopy. **b)** Empty scaffold control and **c)** human adipose model 2-photon microscopy images are shown. Collagen was imaged directly using SHG (blue). SYTO16 (green) was used to mark DNA and was imaged using 2pf. tdTomato-expressing MDA-MB-231 cells were imaged using 2pf.

Figure 1

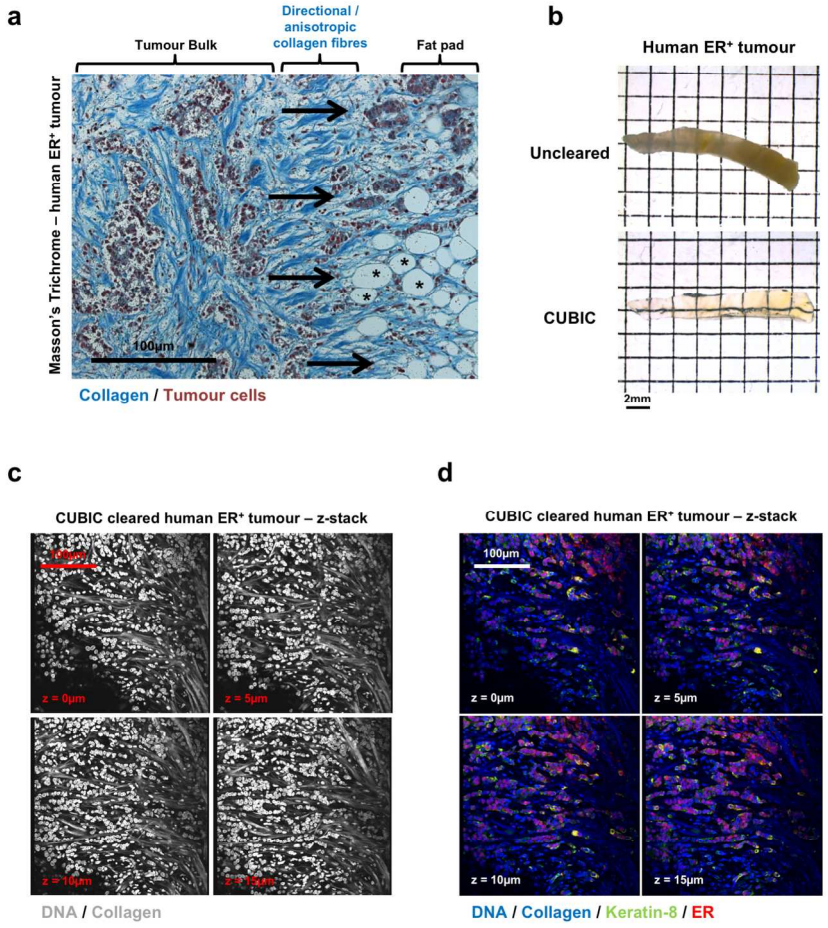

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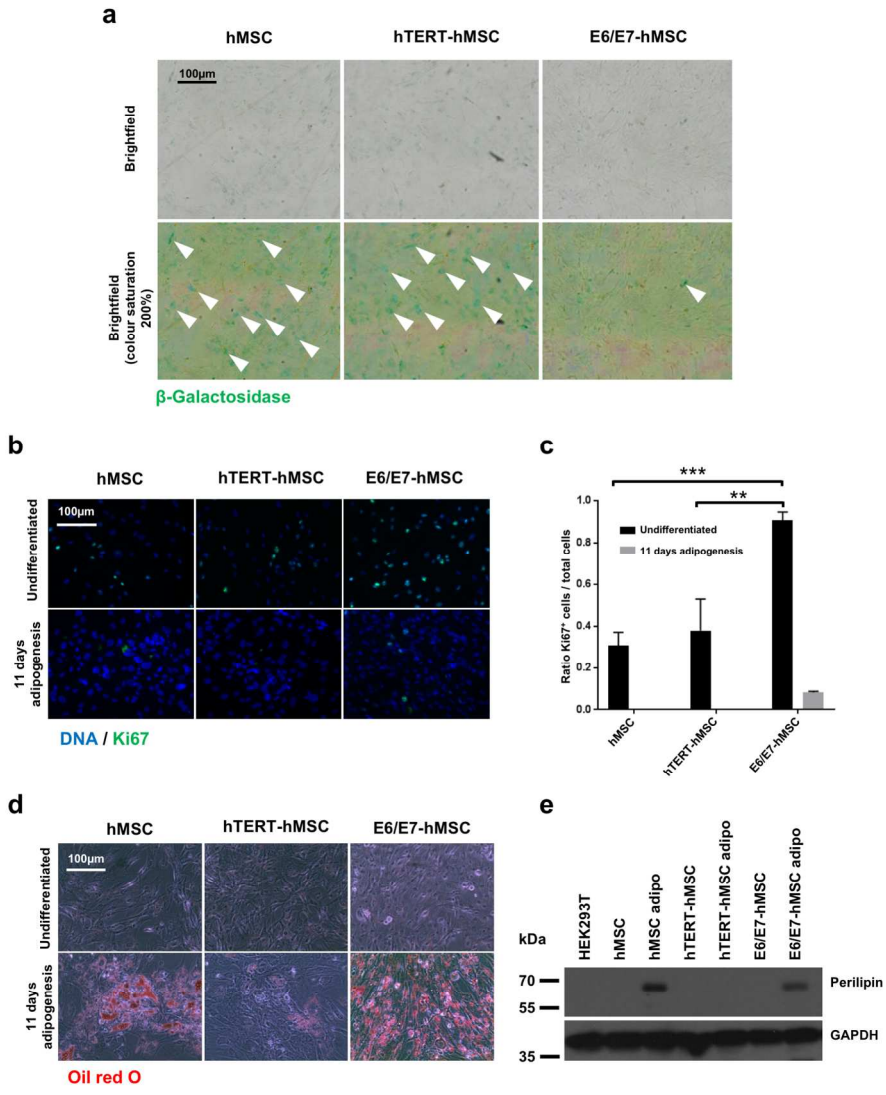
Figure 2



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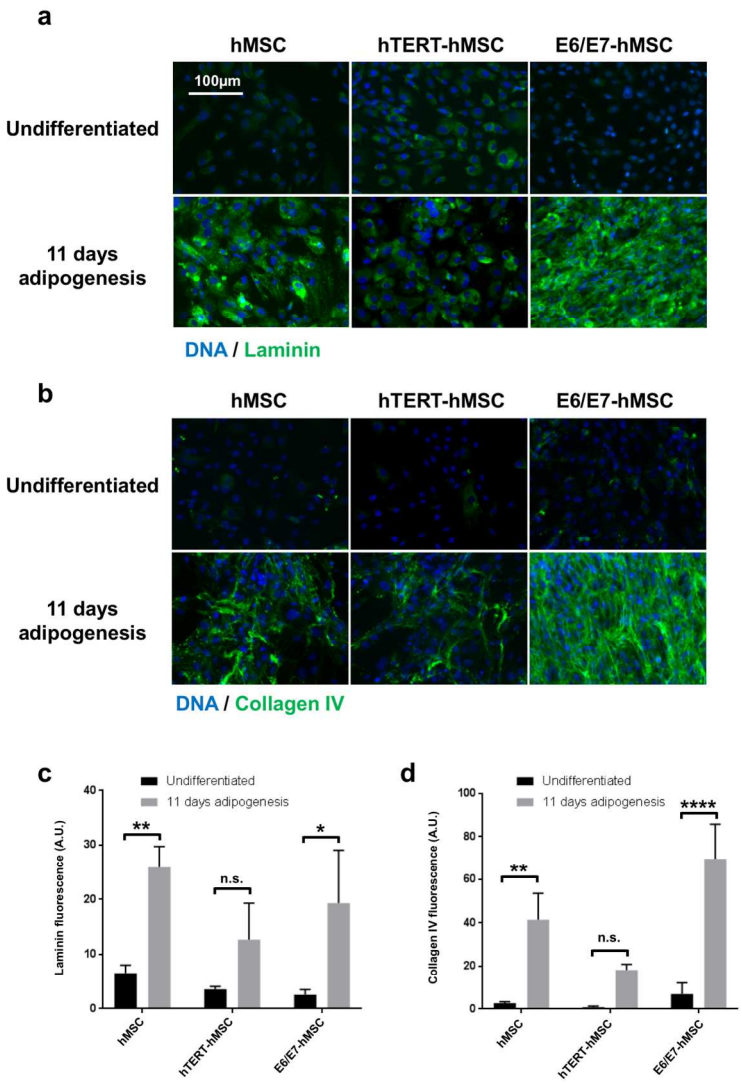

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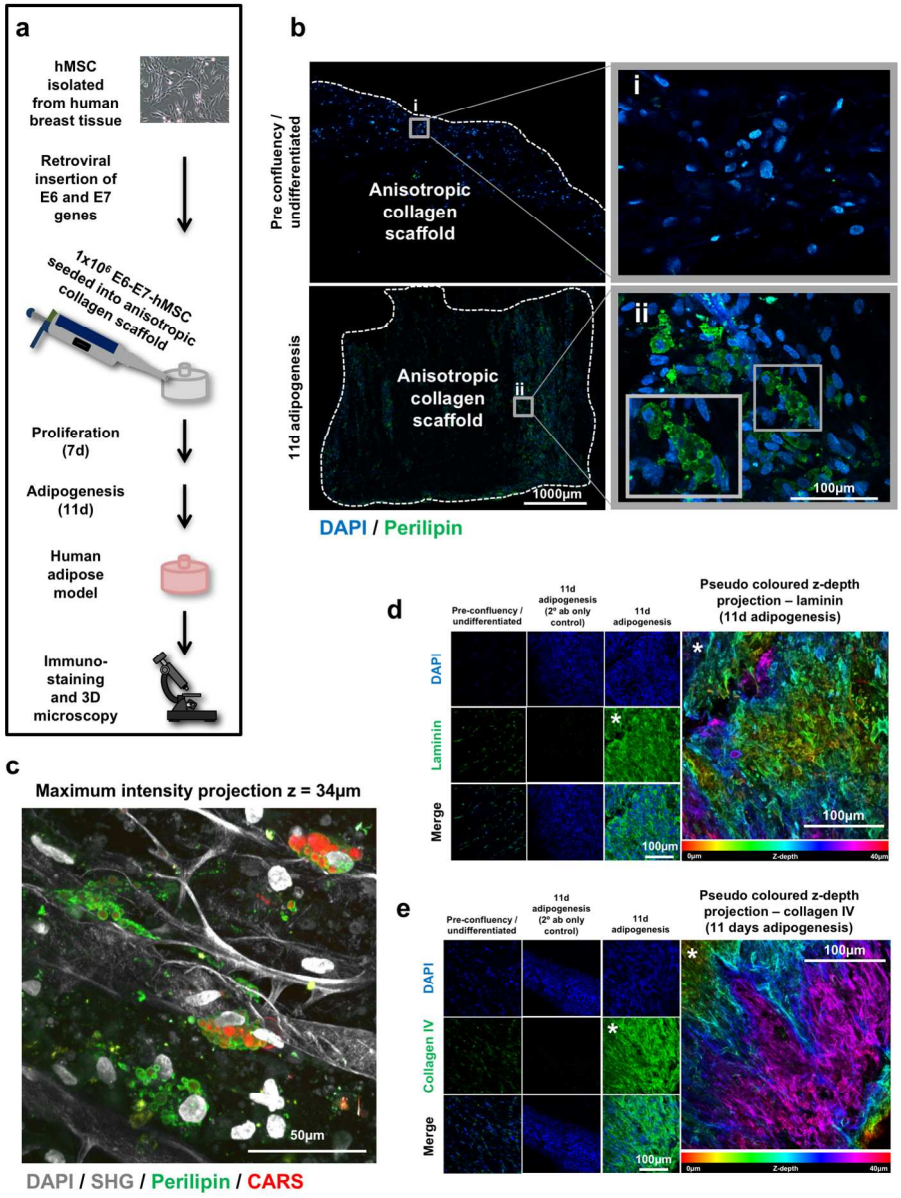

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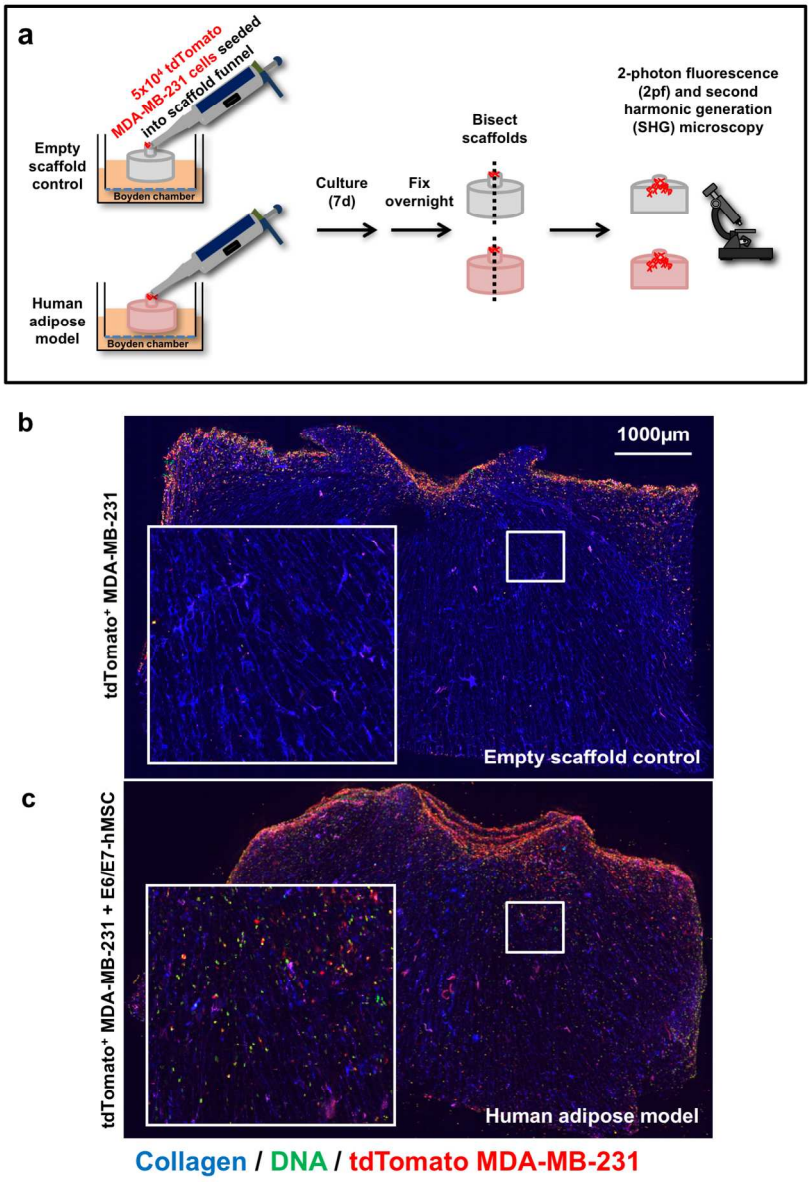

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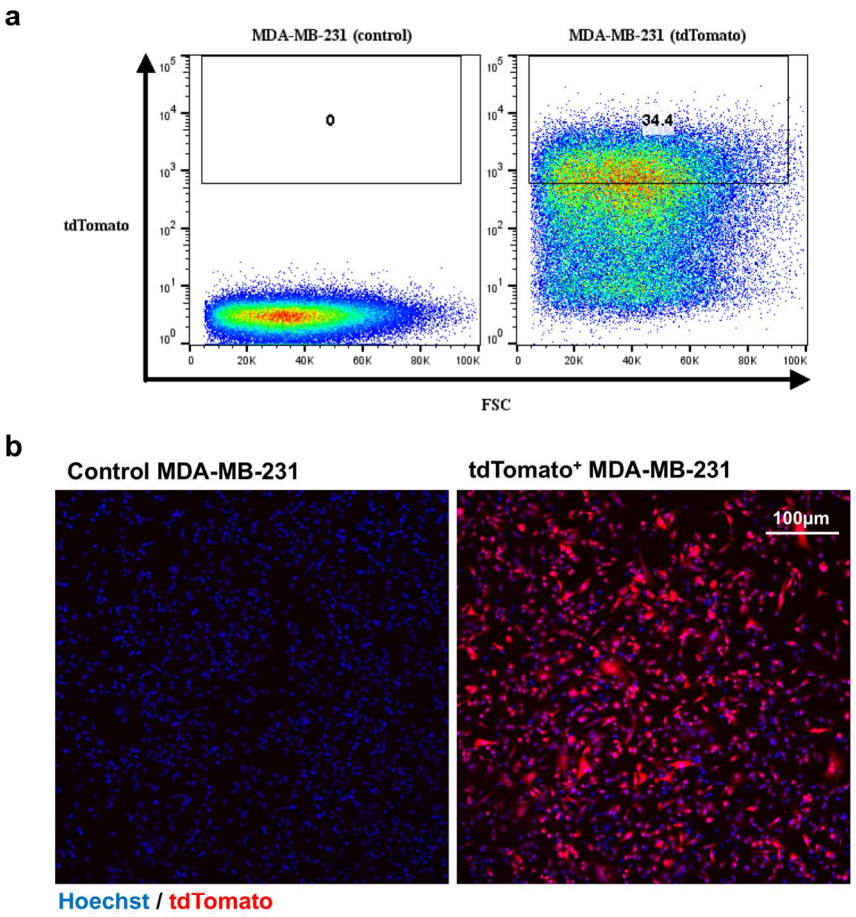

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