Human cardiac mesenchymal stem cell like cells, a novel cell population with therapeutic potential.

Rachel Oldershaw[1], W. Andrew Owens[2,4], Rachel Sutherland[2], Martin Linney[2], Rachel Liddle[2], Lissette Magana[2], Gendie E Lash[3], Jason H. Gill[5], Gavin Richardson[2] and Annette Meeson[2]

[1] Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease, Faculty of Health and Life Sciences, University of Liverpool, West Derby Street, Liverpool, L7 8TX.

[2]Institute of Genetic Medicine, Cardiovascular Research Centre, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK.

[3]Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Jinsui Road, Tianhe, Guangzhou, 510623, China.

[4]Department of Cardiothoracic Surgery, South Tees Hospitals NHS Foundation Trust, Middlesbrough TS4 3BW, UK

[5] School of Pharmacy, King George VI Building & Northern Institute for Cancer Research (NICR), Paul O'Gorman Building, The Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK.

Author details:

Author 1: Rachel Oldershaw: Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease, Faculty of Health and Life Sciences, University of Liverpool, West Derby Street, Liverpool, L7 8TX. Email: Rachel.Oldershaw@liverpool.ac.uk, Tel: +44 (0) 151 793 2322, FAX: +44 (0)151 7958420

Author 2: W. Andrew Owens: Institute of Genetic Medicine, Cardiovascular Research Centre, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK.
Email: a.owens@ncl.ac.uk, Tel: +44 0191 241 8859, Fax: +44 (0) 191-241-8666

Author 3: Rachel Sutherland: Institute of Genetic Medicine, Cardiovascular Research Centre, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK.

Email: rachel.sutherland2@ncl.ac.uk, Tel: +44 (0) 191-241-8856, Fax: +44 (0) 191-241-8666

Author 4: Martin Linney: Institute of Genetic Medicine, Cardiovascular Research Centre, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK. Email: martin_linney@hotmail.co.uk, Tel: +44 (0) 191-241-8856, Fax: +44 (0) 191-241-8666

Author 5: Rachel Liddle: Institute of Genetic Medicine, Cardiovascular Research Centre, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK. Email: rjll246@gmail.com, Tel: +44 (0) 191-241-8856, Fax: +44 (0) 191-241-8666

Author 7: Lissette Magana: Institute of Genetic Medicine, Cardiovascular Research Centre, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK. Email: Lmagana2@illinois.edu, Tel: +44 (0) 191-241-8856, Fax: +44 (0) 191-241-8666

Author 8: Gendie Elizabeth Lash: Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Jinsui Road, Tianhe, Guangzhou, 510623, China.

Email: Gendie.lash@hotmail.com, Tel: +86 18902268242, FAX +86 02038857698

Author 9: Jason Gill: School of Pharmacy, King George VI Building & Northern Institute for Cancer Research (NICR), Paul O'Gorman Building, The Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK.

Email: Jason.Gill@newcastle.ac.uk, Tel: +44 (0) 191-208-2346

Author 10: Gavin Richardson: Institute of Genetic Medicine, Cardiovascular Research Centre, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK.

Email: gavin.richardson@ncl.ac.uk, Tel: +44 (0)191 241 8615, Fax: +44 (0) 191-241-8666

Author 11 and Corresponding author: Annette Meeson, Institute of Genetic Medicine, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK

Email: Annette.Meeson@ncl.ac.uk, Tel: +44 (0) 191-241-8856, Fax: +44 (0) 191-241-8666

Running title: Novel cardiac stem cells for therapeutic use

Footnotes and abbreviations

Cardiac mesenchymal stem cell like stem cells (CMSCLC)

Abstract

Cardiac stem/progenitors are being used in the clinic to treat patients with a range of cardiac pathologies. However, improvements in heart function following treatment have been reported to be variable, with some showing no response. This discrepancy in response remains unresolved. MSCs have been highlighted as a regenerative tool as these cells display both immunomodulatory and pro-regenerative activity. The purpose of this study was to derive a cardiac MSC population to provide an alternative/support to current therapies. We derived human cardiac-mesenchymal-stem-cell-like-cells (CMSCLC) so named as they share some MSC characteristics. However, CMSCLC lack the MSC tri-lineage differentiation capacity, being capable of only rare adipogenic differentiation and demonstrating low/no osteogenic or chondrogenic potential, a phenotype that may have advantages following transplantation. Further, CMSCLC expressed low levels of p16, high levels of MHCI and low levels of MHCII. A lack of senescent cells would also be advantageous for cells to be used therapeutically, as would the ability to modulate the immune response. Crucially, CMSCLC display a transcriptional profile which includes genes associated with cardio-protective/cardio-beneficial effects. CMSCLC are also secretory and multipotent, giving rise to cardiomyocytes and endothelial cells. Our findings support CMSCLC as a novel cell population suitable for use for transplantation.

Introduction

Cellular strategies using bone marrow-derived cells or autologous cardiac derived cells have translated into the clinical setting as potential therapies for the treatment of patients with heart failure [1-3]. Although these trials have demonstrated some benefits of these therapies, they are also not without limitations, with reports of only modest improvements in cardiac function in some patients and no improvements in others. The mechanisms behind the improvements that have occurred remain unclear, it has been postulated that these transplanted cells either contribute to regeneration themselves giving rise to new cardiomyocytes or that they secrete paracrine factors to support native cardiac cells, spared by injury, allowing these cells to recover and promote some level of cardiac regeneration. Regardless of the mechanism, a better understanding of these cells may lead to improvement in cardiac regeneration. However, the choice of the most appropriate cell population for this application has as yet to be determined. Mesenchymal stem cells (MSCs) have been selected as a useful cell population for tissue regeneration as they have been reported to be safe, have immunomodulatory effects and to secrete pro-regenerative factors [4-8]. Human sub-endocardial MSCs have been derived, this cell population was capable of expansion *in vitro*, expressed markers normally expressed by MSCs and was capable of multi-lineage differentiation to osteoblasts, adipocytes and chondrocytes. Moreover, these cells express cardiac transcription factors while lacking expression of markers associated with mature cardiomyocytes [9].

In this study we aimed to determine if we could isolate a cardiac cell population using tissue that is normally discarded as surgical waste (and therefore not necessitating any additional invasive procedures) from which we could derive cells that were MSCs or had MSC-like qualities and might therefore make them a more optimal tool to study cardiac regeneration. We opted for specimens of right atrial appendage (RAA) which are excised during coronary artery bypass surgery. RAA has previously been described as having characteristics of both atrial and ventricular tissue [10] and has been used as a source of stem cells used in a clinical trial [1]. We isolated a stem cell population that we have termed cardiac-derived mesenchymal stem cell-like cells (CMSCLC) and compared these with

cardiosphere-cardiac-derived cells (CS-CDCs) (also RAA derived) and to bone marrow-derived MSCs. We demonstrate that while these CMSCLC are similar to other reported MSC cell populations e.g. have an MSC like morphology; they also differ as they are largely incapable of the tri-lineage differentiation normally displayed by MSCs. Some CMSCLC could be made to differentiate into cells of the adipogenic lineage, and while displaying the morphology of osteoblasts under osteogenic culture conditions, contributed to no or negligible matrix mineralisation, and under chondrogenic culture conditions did not undergo chondrogenesis. This was in contrast to CS-CDCs which differentiated readily to the osteogenic lineage, while also only displaying a low level of adipogenic lineage differentiation potential. However, both CMSCLC and CS-CDCs under *in vitro* cardiac differentiation conditions displayed morphological changes indicative of a more mature cardiac lineage cell, while the CMSCLC also showed early striation formation. In addition we show that some CMSCLC express the senescence marker p16. We also undertook transcriptional analysis of single CMSCLC cells from three individual human heart tissue donors and showed that they expressed at varying levels; genes associated with pluripotency, proliferation, migration, differentiation, endothelial cells, cardiogenesis and cardioprotective factors. This data provides further support for results obtained using a range of other techniques employed in this study. Taken together our data suggests that these CMSCLC cells differ from CS-CDCs. CMSCLC have a more limited ability to differentiate to non-cardiac cell lineages than CS-CDCs but can be expanded *in vitro* while retaining cardiac and some MSC characteristics.

Methods

Study Approval

All studies were performed according to the amended Declaration of Helsinki. All cardiac tissue samples were collected from consenting patients undergoing cardiac surgery at the James Cook University Hospital, Middlesbrough, UK. Approval for collection and use of tissue was given by Local ethics committee under REC number UKCRN ID: 20120092.

Derivation and culture of CMSCLC

Right atrial appendage (RAA) (from 5 patients, 3 males - ages 62 (patient number 1179), 66 (patient number 1201) and 73 (patient number 1201) and two females aged 66 (patient number 1114) and 78 (patient number 1132)) all undergoing coronary artery bypass surgery) was washed and chopped followed by digestion for 45 minutes in 0.01% (wt/vol) pronase (Calbiochem), during which the tissue suspension was rotated on a MACSrotator (Miltenyi) at 37^0C and then tissue was dissociated using a gentleMACS dissociator (Miltenyi). The resulting cell suspension was then filtered thought a 70 µm cell strainer (BD). Cardiac cell suspensions were made up to a total of 6ml with MSC medium (αMEM, 10% (vol/vol) FBS, 1% (vol/vol) Glutamax™, 5ng/ml FGF2, (Peprotech), seeded into T-25 culture flasks and cultured at 5% CO_2, 5% O_2. The culture medium was removed 72 hours post-seeding and non-adherent cells were removed by rinsing 3 times with DPBS. Fresh culture medium was added and further medium changes were performed every three days thereafter. Colony-forming unit fibroblasts (CFU-Fs) where defined as colonies if they contained 50 or more cells. CFUs were observed approximately 15 days post-seeding, these ranged from 8-11 CFUs per CMSCLC culture and 18 CFUs for bone marrow (Figure 1). All colonies from each individual donor were expanded as a pool until confluent. Upon confluence, cell cultures were expanded by passage at a ratio of 1:3 using TrypLE™ Express.

Cell population doublings of CMSCLC

Cell population doubling of CMSCLC per day were calculated over three cell passages, equation to calculate this is provided under the statistics heading and based on [11].

Derivation and culture of cardiac derived cells from cardiospheres

Cardiospheres were derived using methods described previously [12]. In brief, RAA (from 3 patients, 1 female aged 84 and 2 males aged 59 and 65 all undergoing coronary artery bypass surgery) were minced into small <1mm^3 fragments in trypsin and transferred to fibronectin coated plates in DMEM

with 20% serum containing 0.1 mmol/l 2-mercaptoethanol (GIBCO). Following culture, for 10 days, non-adherent phase bright cell were harvested and cultured on Poly-D-Lysine in cardiosphere growing media. Several days later, cells that remained adherent to the poly-D-lysine coated dishes were discarded, while detached cardiospheres were harvested and expanded as a monolayer of CDCs on fibronectin coated plates.

Derivation and culture of bone marrow-derived MSCs

Bone marrow mononuclear cells (BMMNCs) were purchased from Lonza (27 year old male donor). BMMNCs were resuspended in MSC medium, seeded at a density of 1.25 $x10^7$ cells/T-75 flask and cultured at 5% CO_2, 5% O_2. The culture medium was removed 72 hours post-seeding and non-adherent cells were removed by rinsing 3 times with DPBS. Fresh culture medium was added and further medium changes were performed every three days thereafter. Upon confluence, cell cultures were expanded by passage at a ratio of 1:3 using TrypLE™ Express.

Immunophenotyping of MSCs

Cardiac cells were detached from tissue culture plastic using TrypLE™ Express and resuspended in FACS buffer to a cell density of 1 x 10^6 cells/ml. Aliquots of 200µl were transferred to 1.5ml microfuge tubes and incubated at 4°C for 1 hour with 5µg/ml of primary antibody (CD44, CD90, CD105, CD106, CD146, CD166, CD19, CD45 or IgG isotype control). Primary antibodies and IgG control were taken from the Human Multipotent Mesenchymal Stromal Cell Marker Antibody Panel kit (R&D Systems). Cells were rinsed with PBS and then resuspended in FACS buffer with donkey anti-mouse secondary antibody conjugated to Alexa Flour® 488 diluted 1:250 (Invitrogen). Cells were labeled with directly conjugated primary antibodies raised against MHC class I and MHC class II antigens (HLA-ABC-FITC, 5µl of stock reagent in 200µl of cell suspension, Beckman Coulter; PE mouse antihuman HLA-DR 5µl of stock reagent in 200µl of cell suspension, BD) and c-kit (5µg/ml of PE conjugated primary antibody; BD) Controls where unstained CDC and CMSCLC from the same cell preparations. Analysis was done

using a FACS Canto II (BD) using a 488 laser and 530/30 emission filter and data analysis collated using FACS DiVa software.

Analysis of PDGFR-alpha expression by CMSCLC

Cells were prepared and analyzed as for immunophenotyping. Primary antibody used was 5µg/ml of anti-human PDGFR-alpha (R&D systems, MAB322) and secondary was donkey anti-mouse secondary antibody conjugated to Alexa Flour® 488 diluted 1:250 (Invitrogen).

Chondrogenic differentiation of cell populations

Chondrogenic differentiation of cell populations was performed as previously described [13], with slight modification. Briefly, cells were collected by enzymatic detachment from tissue culture plastic and centrifugation at 700 x g for 3 minutes. Cells were resuspended in chondrogenic medium at a cell density of 5 x 10^5 cells/ml. Aliquots of 1ml volume were dispensed into 15ml conical tubes and cell aggregates formed by centrifugation at 700 x g for 3 minutes. The caps were loosened to allow for gas exchange and the cultures incubated at 5% CO_2, 5% O_2 for 14 days with medium changes every 2 days.

Osteogenic differentiation of cell populations

Osteogenic differentiation of cell populations was performed as previously described [14]. Briefly, cells were seeded in MSC medium into 12 well tissue culture plates at a density of 2.5 x 10^3 cells/cm^2. Twenty-four hours post-seeding the medium was replaced with osteogenic medium. Cultures were maintained for 28 days at 5% CO_2, 5% O_2 with medium changes performed every 3-4 days.

Adipogenic differentiation of cell populations

Adipogenic differentiation of cell populations was performed using the StemPro® Adipogenesis Differentiation Kit (Gibco) as per manufacturer's instructions; cultures were maintained under standard oxygen conditions for a total of 21 days.

Histological evaluation of differentiated cell populations

Adipogenic cultures were evaluated by phase contrast microscopy and adipogenic cells identified as cells with prominent clusters of cytoplasmic lipid vesicles at 21 days for cardiac cells these were then stained with oil red O. Adipogenic cultures were incubated for 30 minutes at room temperature with oil red O (stock solution of 30% (vol/vol) oil red O in isopropanol diluted to 60% (vol/vol) in ddH_2O. Excess oil red O solution was removed and the cultures rinsed with ddH_2O. Osteogenic cultures were evaluated for matrix mineralisation by alizarin red staining. Osteogenic cultures were incubated for 2 hours at room temperature in 2% (wt/vol) alizarin red (pH 4.3 with 10% (vol/vol) ammonium hydroxide). Excess alizarin red solution was removed and the cultures rinsed extensively with DPBS to remove background staining. Chondrogenic cell aggregates were embedded in OCT cryopreservation medium and frozen on dry ice. Cryosections (7 µm) were cut onto slides for histological analysis of cartilage tissue formation. For Safranin O staining, cell pellet sections were stained with Harris' hematoxylin for 4 minutes, de-stained in acid alcohol (1% vol/vol HCl, 70% (vol/vol) for 10 seconds and rinsed in deionised water. Sections were counter-stained with 0.02% aqueous fast green FCF for 3 minutes, rinsed in 1% (vol/vol) acetic acid and then stained with 0.1% aqueous Safranin O for 5 minutes. The slides were rinsed, dehydrated and mounted using DePeX mounting medium.

Cardiac differentiation of cell populations

CS-CDCs and CMSCLC were seeded into 12 well tissue culture plates at a density of 2.5 x 10^3cells/cm^2 and placed under their respective culture conditions. After 3 days, the culture medium was replaced with cardiac differentiation medium (Cellutions) and this in turn was replaced every 4 days. After 7 days in cardiac differentiation medium the differentiating CMSCLC cultures were transferred to incubation at 5% CO_2, 22% O_2 for a further 14 days of culture.

Endothelial cell differentiation of CMSCLC – CMSCLC were derived as described above and then cultured in Endothelial Cell Growth Medium 2 (Promocell) for 9 days under standard oxygen conditions, with medium being replaced every 3 days.

Immunocytochemistry

Cardiac differentiated cells grown either on coverslips or in chamber slides were harvested after two or three weeks in cardiac differentiation media, rinsed with DPBS and fixed in cold methanol at -20°C for 20 minutes. Primary antibodies used were Cardiac troponin C 1:200 (Abcam, Ab30807), NXK2.5 1:200 (Abcam, Ab35842), alpha Tropomyosin 1:200 (GeneTex, GTX113857) and Cardiac Actin 1:200 (GeneTex, GTX101876). The secondary antibodies used were, donkey anti goat-AF488 (Invitrogen A-11055), donkey Anti-Rabbit AF594 (Invitrogen ab150076) and donkey anti rabbit-AF488 (Invitrogen A11008). Negative controls were sections incubated as for primary staining but without the inclusion of primary antibodies. As a positive control cells of the AC10 cell line (derived from adult human ventricular cardiomyocytes) [15] where also stained will the aforementioned antibodies. For Confocal Z-stack imaging a Nikon Eclipse Ti was used running NIS Elements AR 4.20.02 software.

Endothelial cell differentiation cells, were grown in chamber slides for 9 days, medium removed and cells fixed in ethanol for 10min. Cells were immunostained for CD34 (1:250; M7165; Dako Cytomation) using an avidin-biotin-peroxidase technique (Vectastain Elite ABC kit; Vector Laboratories) and the reaction developed for 1-2 min with 3,3**'**-diaminobenzidine (DAB; Sigma) containing 0.01% H_2O_2 to give a brown reaction product. Sections were lightly counterstained with Mayer's haematoxylin for 30 sec, dehydrated, cleared in xylene and mounted with DPX synthetic resin.

CMSCLC for senescence analysis at passage 3 where maintained under standard MSC culture conditions in chamber slides for 24 hours and then fixed. Primary antibody used was p16 1:50 (Santa Cruz, J0411), secondary was Donkey anti-Rabbit IgG (H+L), Alexa Fluor 488 (A-21206 Thermofisher) (1/1000). Under x20 magnification 5 distinct fields of cells were imaged using an Axio imager 2 microscope (Zeiss) and the percent of positive p16 expressing cells determined from these images. ICC

images to show p16 where obtained using a confocal Nikon Eclipse Ti using NIS Elements AR 4.20.02 software as mentioned above.

In vitro protein expression quantification.

Following staining with NKX2.5 and troponin C antibodies, cultures were imaged at random locations (n>5). Using ImageJ software (ImageJ; U.S. National Institutes of Health; http://rsbweb.nih.gov/ij/) total cell numbers were counted based on DAPI stained nuclei. Percentage positive cells for each protein was calculated based on number of cells labelled with the specific antibody as a percentage of the total cell numbers. All quantification were performed in a blinded manner.

Polymerase chain reaction (PCR)

PCR was performed as described previously [16], PCR was run for 25 and 35 cycles in order to ascertain that comparisons of expression were made at the exponential phase and not once cDNA amplification had plateaued, data is shown for 35 cycles. The primer sequences used were as follows: MEF2C forward: CGAGATACCCACAACACACG, reverse: CGCTTGACTGAGGGACTTTC, GATA4 forward: TCCCTCTTCCCTCCTCAAAT, reverse: TCAGCGTGTAAAGGCATCTG, β-actin forward: GCGGGAAATCGTGCGTGAC, reverse: GGAAGGAAGGCTGGAAGAG.

ELISA analysis

Cell culture supernatants (control was MSC media only) from CMSCLC at day 14 and day 17 of culture were analysed for IL-10, VEGF, FGF2 and TGFβ1 secretion by ELISA according to the manufacturer's instructions (IL-10, VEGF, FGF2, TGFβ1 Duosets; R&D).

Single cell capture

Single cells were captured and the loci of interest pre-amplified using the C1 system (Fluidigm, software version 2.2.1) following the manufacturers protocol. Cells were partitioned using a 10-17 µm

C1 Single-Cell Preamp integrated fluidic circuit (IFC) (Fluidigm PN 100-5479). IFC priming, cell loading and lysis, reverse transcription and pre-amplification was then carried out using reagents from the following kits; C1 Single-Cell Auto Prep Reagent Kit (Fluidigm PN 100-5139), Ambion Single Cell-to-CT qRT-PCR Kit (Thermo Fisher Scientific 4458237) and 20X TaqMan Gene Expression primers (Thermo Fisher Scientific). Amplicons were transferred from the IFC to a 96 well plate and stored at -20°C.

Single cell gene expression analysis

Single cell gene expression analysis was carried out using the Biomark HD system and IFC Controller HX (Fluidigm) as per manufacturer's instructions. Gene expression analysis was carried out using Dynamic Array Flex Six Gene Expression IFCs (Fluidigm). Assays were run using reagents from the following kits; Flex Six Gene Expression Reagent Kit (Fluidigm), 20X TaqMan Gene Expression primers (Thermo Fisher Scientific) and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). TaqMan primers used are listed in Supplemental Table 1.

Analysis of single cell data

CT values were calculated using the Fludigm Real-time Analysis software. The remainder of the analysis was carried out using Data analysis was carried out primarily in R (version 3.4.1) and the expression values extracted using SINGuLAR Analysis Toolset v3.6.2. Low quality cells were excluded from the analysis if total expression per cell was less than 250; or the number of detected genes per cell less than 10. The filtered data was normalised using the computeSumFactors from the Scran R package. The remainder of the analysis was carried out using R core functions.

Statistical analysis

Cell population doubling of CMSCLC per day were calculated over three cell passages using the following equation [26], population doublings per day = $\ln N / N_0 \times t^{-1}$. Where N_0 = number of cells seeded, N = number of cells counted at passage, t^{-1} = number of days to passage. Percentage of p16

expressing cells was determined using SPSS box plot analysis. Excel 2013 was used to generate bar chart for single cell data.

Results

Morphological, CFU forming potential and doubling time of CMSCLC

CMSCLC were examined to determine if they had MSC like characteristics when cultured under standard MSC culture conditions. At passage 3 bone marrow-derived MSC (BM-MSCs) were plastic adherent and displayed a distinct fibroblast like morphology (Fig. 1A), CMSCLC also displayed these characteristics at passage 3 (Fig. 1 B-D). The morphology of CS-CDC cells is also show as a comparison to the CMSCLC (1E). Colony forming potential is a recognised stem cell characteristic, an example of a CFU is shown (Fig. 1F). CMSCLC from 3 different patient derived cultures readily formed similar numbers of CFUs around day 15 post seeding into culture (Fig.1G) as a control BM-MSCs were also used and formed CFUs at day 7 post seeding *in vitro,* CFUs where counted at day 15 (Fig. 1G). Cell doubling rates of CMSCLC were also determined for all 3 patient derived cell cultures and no significant difference in doubling rates was observed (Supplemental Fig.1).

Phenotyping of CMSCLC

CDCs and CMSCLC were screened for expression of cell surface antigens known to be expressed by MSCs. Both cell populations expressed CD44, CD105 and CD166 but the CDCs express all these markers at much lower levels than the CMSCLC (Fig 2), while both expressed low levels of the hematopoietic lineages markers CD19 and CD45 (Fig. 2). Controls included IgG isotype control and unstained CDC and CMSCLC (Fig. 2). CMSCLC were also tested for the expression of MHC I and II. Representative images of the data generated are shown in (Fig. 3A) demonstrating the majority of cells expressed MHC I (98%) and lacked expression of MHC II (0.4%). Controls included IgG isotype control stained and unstained CMSCLC (data not shown). Three different patient-derived CMSCLC cultures were analyzed for MHC expression and the percentages of positive cells per culture are shown in (Fig. 3B). CMSCLC were also

examined for expression of c-kit, this analysis revealed the presence of c-kit expression in only a rare sub-population of cells (0.5% - 2.6%) (Fig 3C and Table). Controls were CMSCLC stained with IgG isotype control only and unstained cells (data not shown). In addition to immunophenotyping, the CMSCLC they were also immunostained (after nine days in culture) for the expression of CD34. Cells from the same donor derived CMSCLC line cultured under normal CMSCLC culture conditions showed no/low CD34 expression, while cells cultured under endothelial cell differentiation conditions showed increased expression of CD34 (Fig 3D). We also examined CMSCLC for expression of PDGFR-alpha and showed that some cells express this marker (Supplemental figure 2).

Secretion of IL-10, VEGF, FGF2 and TGFβ1 by CMSCLC

To determine if CMSCLC secreted IL-10, VEGF, FGF2 and TGFβ1 CMSCLC were cultured under normal CMSCLC conditions as described above and media was collected at day 14 and 17 of culture. In all cases there was an increase in fold expression when compared to control media. After 14 days culture there was a 1.3 fold increase in IL-10 secretion, but by day 17 there was a 4.5 fold increase in IL-10 secretion. For VEGF at day 14 there was a 13.5 fold increase in VEGF secretion which dropped to an 11.7 fold increase at day 17 compared to control, whereas for FGF2 at both day 14 and 17 there was an 8.9 fold increase compared to control. For TGFβ1 there was a 1.5 fold increase at both times points compared to control (Supplemental Fig. 3A). In all cases except for IL10 at day 14 the increase in expression compared to control was significant (Supplemental Fig. 3B).

Osteogenic, adipogenic and chondrogenic differentiation potential of CMSCLC

To determine the differentiation potential to the above-mentioned lineages CMSCLC, CDCs and BM-MSCs were cultured under the appropriate lineage differentiation conditions. Cultures were examined microscopically for morphological changes, both CDCs (Fig. 4A) and CMSCLC (Fig. 4B) underwent morphological changes when cultured for 28 days under osteogenic culture conditions but when stained for matrix mineralization using alizarin red only the CDCs cultures stained (Fig. 4C) whereas

the CMSCLC showed low/no positive staining (Fig. 4D). Adipogenic differentiation was determined by the presence of cells with prominent clusters of cytoplasmic lipid vesicles that stained positively with Oil red O after 21 days in culture (Fig. 4E and 4F). When cultured under chondrogenic culture conditions at 14 days the CDCs (Fig. 4G) and CMSCLC (Fig. 4H) formed aggregates but when evaluated histologically failed to stain with Safranin O, and had not undergone chondrogenic differentiation. BM-MSC cultured under the same tri-lineage differentiation culture conditions provided a control and successfully differentiated to; the osteogenic lineage and displayed matrix mineralization as indicated by alizarin red staining (4I) the adipogenic lineage as indicated by Oil red O staining of cells containing cytoplasmic lipid vesicles (4J) and to the chondrogenic lineage as shown by staining of aggregates with Safranin O (4K).

Expression of p16 by CMSCLC

In order to establish if the level of senescence was higher in some patients CMSCLC than others, we compared the level of senescence from CMSCLC at passage 3 from 3 different patients. The cultures were stained using p16 antisera. A representative image of p16 stained cells in a CMSCLC culture is shown (Fig. 5A). All cultures contained low percentages of p16 expressing cells (Fig. 5B).

Cardiac differentiation potential of CMSCLC

To determine the ability of CMSCLC to differentiate to a more mature cardiac lineage, cells were cultured under cardiac differentiation conditions, after two weeks the cells were stained for cardiac troponin I and NXK2.5 expression. CDCs have a previously demonstrated cardiogenic potential, and were used as a positive control being cultured under the same cardiac differentiation conditions for the same length of time. For both the CDCs (Fig. 6A) and CMSCLCs (Fig. 6B) for all cultures examined, troponin C expressing cells with a flattened, spread straight edged morphology were present. While in all of CDC cultures and one CMSCLC cultures some cells were present that expressed nuclear NKX2.5. Representative images of NKX2.5 positive cells are shown in CDC derived cultures (Fig. 6C) and from

the CMSCLC culture (Fig. 6D). The morphology of these cells differed from those in (Fig. 6A and 6B) as these cells had a more primitive rounded morphology. To further demonstrate cardiomyocyte differentiation, CMSCLC were differentiated for 3 weeks, and labelled for troponin C, cardiac actin or the striated muscle specific isoform of tropomyosin, α-tropomyosin, and imaged using Z-stack confocal microscopy. Maximum intensity z-stack projections demonstrated filamentous staining for all proteins (Fig 6 E-H). Additionally, maximum intensity volume projection of alpha-tropomyosin labelling clearly demonstrates the striated phenotype of these differentiating cells (Fig 6H, H*i*). Note the absence of staining in negative controls (Supplemental Fig. 4A-C) and staining with all antibodies of cells of the AC10 cell line (Supplemental Fig. 4D). Analysis for percentage of cells expressing NKX2.5 and Troponin C shows that the percentage of NKX2.5 expressing cells is lower than that of cells expressing Troponin C for both cell populations, with the percentages of cells expressing troponin C being higher in all cases for CMSCLC (98, 99 and 84%) compared with CDCs (86, 68, 63%) (Fig 6I)
Molecular analysis was used to examine the undifferentiated CMSCLC and after culture under cardiac differentiation conditions for expression of genes important for cardiogenesis both populations expressed MEF2C and GATA4 (Supplemental Fig. 5)

Single cell transcriptomics analysis

We examined CMSCLC derived from 3 patients, only cells that passed the quality control steps (see materials and methods) were included in the downstream analysis. The percentage of genes being expressed by cells in each culture was calculated (Fig. 7). B-actin was used as a housekeeping gene and was expressed by all cells. CD44 and tropomyosin were also expressed by all the cells analysed. GATA4 (percentage of cells expressing ranging from 82.5 -100%) VEGF (percentage of cells expressing ranging from 97.8-100%) TGF-β (percentage of cells expressing ranging from 96.5-100%) were also highly expressed. CDC73 was more variable, being expressed by 100% of the cells in one culture and by 95.6 and 79.3% of cells in the other two cultures. The expression of FGF-2 (100, 56.5 and 62%), HGF (18.5, 89.1, 41.3%), PCNA (98.5, 71.7, 55.1%), MEF2 (81.5, 86.9, 31%) and MCAM (58.4, 32.6, 27.5%)

also varied between the different donors cultures. c-Kit expression was also variable between the different donor derived cultures with percentage of cells expressing being 3 and 10.8% and with one culture having no detectable expression (Fig. 7). No expression was observed for NXK.25 in any of the cells of the 3 donor cultures analysed (data not shown).

Discussion

The International Society for Cell therapy position paper (2006) set the minimum requirements for cells to be considered MSCs; as the ability to adhere to plastic under standard culture conditions, to express a number of cell surface antigens, to lack expression of a number of markers including the hematopoietic lineage markers CD45 and CD19, and to differentiate to osteoblasts, adipocytes and chondroblasts in culture [17]. This paper included the caveat that these criteria would need modification as our knowledge of MSCs increased. MSCs also have distinct cell morphology *in vitro* displaying a fibroblast-like morphology with cells showing parallel alignment and tapering of the fibroblast-like cellular protrusions [18]. CMSCLC displayed some of these characteristics; being plastic adherent in MSC culture conditions, having an MSC-like morphology and expressing MSC markers CD105, CD166 and CD44 [19]. CMSCLC differed from CS-CDCs, displaying higher levels of expression of these antigens. Although controversial, human MSC have been reported as being MHC I positive and having low/no expression of MHC II [20]. CDCs also express MHC I and lack expression of MHC II [21] and CMSCLC express MHC I and have low expression of MHC II. MSCs have been reported to have immunomodulatory properties, in part due to their ability to secrete the anti-inflammatory cytokine IL10 which has been suggested to be cardio-beneficial. In a rat model of myocardial infarction transplanted MSCs help reduce the inflammatory response and this was in part due to increased levels of IL10 [22]. We examined CMSCLC for IL10 expression and observed increased expression when cells where cultured for 14-17 days. In contrast to CS-CDCs, CMSCLC were unable to differentiate to the osteogenic lineage as they exhibit little/no matrix mineralization as indicated by the low/absence of alizarin red staining, while all CS-CDCs stained strongly for alizarin red. However, both cell populations

exhibited low adipogenic differentiation and both failed to undergo chondrogenesis. This contrasts with BM-MSCs and sub-endocardial MSCs that have this tri-lineage differentiation potential [9]. These differences might be due to these being distinct cell populations, differences in tissue used for cell derivation (sub-endocardial MSC are derived from ventricle while CMSCLC are derived from atrial tissue), or due to differences in culture conditions used. CDCs and sub-endocardial MSC are grown out from tissue explants, whereas, CMSCLC are derived from single cell suspensions and CMSCLC are cultured in 5% CO_2, 5% O_2 whereas no information is provided on the oxygen conditions for the culture of sub-endocardial MSCs. We have reported on increased; derivation rates, osteogenic potential and cellular health of MSCs derived from haemarthrosis fluid cultured under low oxygen conditions versus those cultured under standard conditions [14], supporting the hypothesis that culture conditions may influence cell behavior or possibly support the preferential selection of a subset of cells within a heterogeneous cell population. Certain cardiac stem/progenitor cells have been shown to be preferentially located in distinct anatomical regions of the heart e.g. adult human cardiac side population cells have been reported to be confined to the left atrium and absent from the right [23]. In a molecular study of multiple mouse cardiac stem cell populations, a comparison was made between the mouse data and microarray data available in the public domain and CS-CDCs appeared to be closely related to BM-MSCs [24]. This might also account for the osteogenic response displayed by our CS-CDCs. In addition, sub-endocardial MSC were reported as being strongly positive for c-kit expression [9] and human CS-CDCs have been reported to express c-kit (about 18% of the total cell population) [25], while CMSCLC showed only low-level expression of c-kit. Identification of cardiac stem cells based on expression of c-kit alone has been brought into question. However, in a recent study it has been reported that within the $CD45^{neg}$c-kit^{pos} cells found in the heart a small subpopulation of between 1-2% have the characteristics of true multipotent cardiac stem cells [26]. Therefore, the small percentage of c-kit expressing cells observed in CMSCLC cultures could represent such a population. The CMSCLC containing the highest percentage of c-kit expressing cells (2.6%) was also the only culture containing NkX2.5 expressing cells. PDGFR-αlpha has been reported to be expressed

by progenitor cells in human fetal and diseased adult heart [27]. Therefore we also examined CMSCLC and observed that some expressed PDGFR-alpha.

CMSCLC displayed other stem cell characteristics including the ability to form CFUs and be expanded *in vitro*. All CMSCLC cultures had similar potential with regard to cell doubling rates, while rates for CS-CDCs have been reported to be similar for cells generated from both non-transplant heart failure and transplant heart patient's biopsy specimens [25]. Under cardiac differentiation culture conditions, both CS-CDC and CMSCLC had a phenotype previously reported for cardiomyocytes in culture [28]. Moreover, in these conditions all CS-CDC cultures but only one CMSCLC culture also contained a population of cells that expressed nuclear NXK2.5 and had an immature rounded morphology. These characteristics have previously been suggested to be indicative of a more primitive or early cardiac committed cell [29] and may suggest that the CS-CDC are more heterogeneous than the CMSCLC or that the CMSCLC are more mature. This is further support by our quantification of cells expressing troponin C that shows all CMSCLC containing higher numbers of Troponin C expressing cells than CS-CDCs. In addition, we also observed that undifferentiated and differentiated CMSCLC expressed genes important for myocardial lineage development including MEF2C and GATA4 [30, 31]. Atrial appendage derived cardiac stem cells also express markers important for myocardial development and even post-induction of differentiation to a more mature cardiac lineage they continue to express MEF2C and GATA4 [32]. It is recognised that for MSCs derived from patient tissue, patient to patient variability does exist [33], and this could be responsible for the difference in the presence/absence of the NXK2.5 expressing cells in the CMSCLC cultures.

If cardiac stem/progenitors are to be used as a cellular therapy or indicator of heart health the level of cellular senescence needs to be determined. We examined CMSCLC derived from 3 different donors at passage 3 and observed the presence of between 5 to 15% senescent cells. Whether the presence of senescent cells within CMSCLC would prove to be a problem in utilizing these as a cellular therapy would need to be further explored. CSC expanded over approximately 113 days have been shown to contain small percentages of senescent cells (however the authors of this study did not provide data

on % observed) and yet still deemed suitable for intracoronary perfusion in patients with heart failure [1].

Human cardiac atrial appendage stem cells (CASCs), like CMSCLC, also fail to differentiate towards the osteogenic lineage. However, unlike the CMSCLC they also failed to differentiate towards the adipogenic lineage [34]. CASCs also lack expression of c-kit and CD44 [34], while most of our CMSCLC cultures contain small numbers of c-kit+ cells and express CD44. It is interesting to note that both atrial appendages can be used as a source of stem cells. Why the atrial appendages are such good sources of stem cells is not entirely understood, but they have a distinct developmental origin to the atria and display many trabeculae similar to ventricles [10]. They are also a source of atrial natriuretic peptide factor (ANP). Nppa, the gene encoding ANP, has been shown to be important for heart development as well as being reactivated in adult disease [35]. It may be these developmental/fetal links that make the appendages such rich sources of stem cells. A study of mouse left atrial appendage showed it to be a source of more than one type of cardiac progenitor population [36].

As a cellular therapeutic, RAA-derived CMSCLC might have advantages over sub-endocardial derived MSCs (that readily differentiate to osteogenic, adipogenic and chondrogenic lineages, but remain primitive under cardiac differentiation conditions) and CS-CDCs or BM-MSCs as they lack the osteogenic potential of the former and the osteogenic and chondrogenic potential of the latter. This may have important implications for the use of CMSCLC as a potential cellular therapeutic as they could be less likely to contribute to cardiac calcification. The cause of this remains controversial, and while there is no evidence from clinical trials to date that cardiac derived cell transplantation causes calcification, there is evidence that cells with osteogenic and chondrogenic differentiation potential may be involved [37]. CMSCLC also differ to CS-CDCs as they do not require the rounds of harvesting/replating required to generate CS-CDCs [25].

We did not observe spontaneous beating of CMSCLC cells under cardiac differentiation conditions; this may be a limitation of the technique used. Human CS-CDC cells also failed to beat *in vitro* unless co-cultured with neonatal rat ventricular myocytes [25], this was also the case with human CASCs [34].

Our single cell analysis demonstrated that 100% of CMSCLC expressed CD44, a recognised marker of MSCs [19]. A large % of CMSCLC also expressed CDC73 which is associated with stem cell pluripotency [38]. Our single cell analysis also supported our standard PCR analysis showing that GATA4 and MEF2 were also detectable at the single cell level. GATA4 was expressed by all CMSCLC for all three donors (100%, 84.7%, and 82.5%), while MEF2C expression was more variable with two cultures having high numbers of MEF2C positive cells (86.9% and 81.5%) but in the third only 31%. 100% of CMSCLC expressed Tropomyosin and 98.5%, 71.7 and 55% of CMSCLC expressed PCNA and nearly all expressed VEGF. Lower numbers of CMSCLC expressed MCAM (also known as CD146) with percentages varying from the highest at 58.4% to the lowest at 27.5%. CD146 has been reported to be expressed by a subpopulation of human MSCs from a telomerised bone marrow derived stromal cell line [39]. While in the heart it has been reported to be a marker of perivascular mesenchymal precursor cells [40]. Together this data suggests that CMSCLC are; proliferative, pluripotent and have some multi-lineage differentiation potential to other cells important in the heart. It also supports our *in vitro* culture observation of some CMSCLC being capable of expressing the endothelial cell marker CD34 under EC differentiation conditions. CMSCLC for all donors also expressed TGF-β. The role of TGF-β in the heart remains controversial it has been reported to contribute to heart failure but has also been suggested to be important for suppression of inflammation following myocardial infarction and been reported to be cardio-protective [41, 42]. CMSCLC also expressed VEGF (97.8 to 100 %) FGF2 (56.6, 62.0 and 100%) and HGF (18.0, 41.3 and 89.1%), which have all been reported to be cardio-protective/cardio-beneficial [42-44]. We also demonstrated using CMSCLC conditioned media that CMSCLC secrete a number of potentially cardio-beneficial factors including IL10, VEGF, FGF2 and TGFβ1. The role of MSC paracrine secreted factors that have protective/regenerative effects has now been demonstrated in several different tissues. In a rat neonatal hyperoxic lung injury model, human umbilical cord blood MSCs that secrete VEGF improved the survival rates on rat lung epithelial cells treated with hydrogen peroxide, to confirm this protective effect these cells where then used in an *in vivo* model of hyperoxic injury where is was again shown that the VEGF secreting MSCs protected against hyperoxia [45]. In a

rat model of liver regeneration, BM-MSC alone or transfected with the VEGF gene were injected into the liver after major hepatic resection, both groups of cells engrafted into the liver where they secreted a number of paracrine factors including VEGF, FGF, TGFβ and HGF, and in both cases bile duct and liver hepatocyte proliferation occurred [46]. The paracrine activity of MSCs may be multi-modal as they have also been reported to be able to cause cardiomyocyte proliferation, be immunomodulatory and activate resident cardiac stem cells spared by cardiac injury [reviewed in 47]. Additionally, single cell analysis showed only a few CMSCLC expressed c-kit, data which supports the low percentages of c-kit expressing cells observed in the CMSCLC using FACS. Single cell profiling as a means of determining the paracrine activity of MSCs has previously been used successfully in a study on the role of MSCs in the infracted mouse heart, where the authors showed using a single cell approach that MSCs transplanted into injured heart expressed paracrine factors *in vivo* [48].

We have shown that CMSCLC cultured under cardiac differentiation conditions display the morphology and expressed some markers of more mature cardiac lineage-committed cells, even showing early striation formation. Similar changes have been observed in rat CDCs differentiated to a more mature cardiac lineage *in vitro* [49]. In addition CMSCLC secrete cardio-beneficial factors and have low/no adipogenic, chondrogenic or osteogenic differentiation potential, all qualities that should be advantageous for cells to be used therapeutically for cardiac regeneration.

Acknowledgements

We wish to thank Barbara Innes (Institute of Cellular Medicine, Newcastle University) for cutting of frozen chondrogenic sections. We wish to thank Lina Zaripova for technical assistance with bone marrow differentiation to osteogenic and adipogenic lineages. We acknowledge the Newcastle University Flow Cytometry Core Facility for assistance with the generation of Flow Cytometry data, the Newcastle University Single cell genomics facility for assistance with single cell transcriptomics analysis and the Bioinformatics Facility for assistance with analysis of single cell data.

This work was supported by the British Heart Foundation (grant number PG/15/85/31744) (www.BHF.org.uk) and by the Wellcome Trust Institutional Strategic Support Fund at Newcastle University and at the University of Liverpool, the latter as part of a Tenure-Track Fellowship awarded to Rachel Oldershaw.

Author Disclosure Statement: "No competing financial interests exist."

References

1. Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, Beache GM, Wagner SG, Leri A, Hosoda T, Elmore JB, Goihberg P, Cappetta D, Solankhi NK, Fahsah I, Rokosh DG, Slaughter MS, Kajstura J, Anversa P. (2011). Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet 378:1847-1857.

2. Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marbán L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G, Marbán E. (2012). Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet 379:895-904.

3. Mathiasen AB, Jørgensen E, Qayyum AA, Haack-Sørensen M, Ekblond A, Kastrup J. (2012). Rationale and design of the first randomized, double-blind, placebo-controlled trial of intramyocardial injection of autologous bone-marrow derived Mesenchymal Stromal Cells in chronic ischemic Heart Failure (MSC-HF Trial). AHJ 164: 285-291.

4. Akiyama K, Chen C, Wang D, Xu X, Qu C, Yamaza T, Cai T, Chen W, Sun L, Shi S. (2012). Mesenchymal-Stem-Cell-Induced Immunoregulation Involves FAS-Ligand-/FAS-Mediated T Cell Apoptosis. Cell Stem Cell 10:544–555.

5. Caplan AJ, Correa D. The MSC: An Injury Drugstore. (2011). Cell Stem Cell 8:11-15.

6. Cutler AJ, Limbani V, Girdlestone J, Navarrete CV. (2010). Umbilical Cord-Derived Mesenchymal Stromal Cells Modulate Monocyte Function to Suppress T Cell Proliferation. J Immunol 185:6617-6623.

7. Nauta AJ, Fibbe WE. (2007). Immunomodulatory properties of mesenchymal stromal cells. Blood 110:3499-3506.

8. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. (2008). Mesenchymal Stem Cell-Mediated Immunosuppression Occurs via Concerted Action of Chemokines and Nitric Oxide. Cell Stem Cell 2:141-150.

9. Anzalone R, Corrao S, Lo Iacono M, Loria T, Corsello T, Cappello F, Di Stefano A, Giannuzzi P, Zummo G, Farina F, La Rocca G. (2013). Isolation and Characterization of CD276 + /HLA-E + Human Subendocardial Mesenchymal Stem Cells from Chronic Heart Failure Patients: Analysis of Differentiative Potential and Immunomodulatory Markers Expression. Stem Cells and Dev 22:1-17.

10. Moorman A, Webb S, Brown NA, Lamers W, Anderson RH. (2003). Development of the heart: (1) formation of the cardiac chambers and arterial trunks. Heart 89:806–814.

11. Kinner B, Zaleskas JM, Spector M. (2002). Regulation of Smooth Muscle Actin Expression and Contraction in Adult Human Mesenchymal Stem Cells. Exp Cell Res 278:72-83.

12. Davis DR, Kizana E, Terrovitis J, Barth AS, Zhang Y, Smith RR, Miake J, Marbán E. (2010). Isolation and expansion of functionally-competent cardiac progenitor cells directly from heart biopsies**.** J Mol Cell Cardiol 49: 312-21.

13. Oldershaw RA, Tew SR, Russell AM, Meade K, Hawkins R, McKay TR, Hardingham TE. (2008). Notch signaling through jagged-1 is necessary to initiate chondrogenesis in human bone marrow stromal cells but must be switched off to complete chondrogenesis*.* Stem Cells **26:** 666-674.

14. Knuth CA, Clark ME, Meeson AP, Khan SK, Dowen DJ, Deehan DJ, Oldershaw RA. (2013). Low Oxygen Tension is Critical for the Culture of Human Mesenchymal Stem Cells with Strong Osteogenic Potential from Haemarthrosis Fluid. Stem Cell Rev and Rep 9:599-608.

15. Davidson MM, Nesti C, Palenzuela L, Walker W, Hernandez E, Protas L, Hirano M, N.D. I (2005) Novel cell lines derived from adult human ventricular cardiomyocytes. J Mol Cell Cardiol 39:133-47

16. Shaharuddin B, Harvey I, Ahmad S, Ali S, Meeson A. (2014). Characterisation of Human Limbal Side Population Cells Isolated Using an Optimised Protocol From an Immortalised Epithelial Cell Line and Primary Limbal Cultures. Stem Cell Rev and Rep 10:240–250.

17. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop, Horwitz E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315-317.

18. Mauney JR, Kaplan DL, Volloch V. (2004). Matrix-mediated retention of osteogenic differentiation potential by human adult bone marrow stromal cells during ex vivo expansion. Biomaterials 25:3233-3243.

19. Mareschi K, Ferrero I, Rustichelli D, Aschero S, Gammaitoni L, Aglietta M, Madon E, Fagioli F. (2006). Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow. Journal of Cell Biochem 97:744-754.

20. Ryan JM, Barry FP, Murphy JM, Mahon BP. (2005). Mesenchymal stem cells avoid allogeneic rejection. J Inflamm 2:1-1.

21. Malliaras K, Li TS, Luthringer D, Terrovitis J, Cheng K, Chakravarty T, Galang G, Zhang Y, Schoenhoff F, Van Eyk J, Marbán L, Marbán E. (2012). Safety and Efficacy of Allogeneic Cell Therapy in Infarcted Rats Transplanted With Mismatched Cardiosphere-Derived Cells. Circ 125:100-112.

22. Du YY, Zhou SH, Zhou T, Su H, Pan HW, Du WH, Ziu B, Liu QM. (2008). Immuno-inflammatory regulation effect of mesenchymal stem cell transplantation in a rat model of myocardial infarction. Cytotherapy 10:469-78.

23. Sandstedt J, Jonsson M, Kajic K, Sandstedt M, Lindahl A, Dellgren G, Jeppsson A, Asp J. (2012). Left atrium of the human adult heart contains a population of side population cells. Basic Res Cardiol 107:255, 1-10.

24. Dey D, Han L, Bauer M, Sanada F, Oikonomopoulos A, Hosoda T, Unno K, De Almeida P, Leri A, Wu JC. (2013). Dissecting the Molecular Relationship Among Various Cardiogenic Progenitor Cells Novelty and Significance. Circ Res 112:1253-1262.

25. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marbán E. (2007). Regenerative Potential of Cardiosphere-Derived Cells Expanded From Percutaneous Endomyocardial Biopsy Specimens. Circ 115:896-908.

26. Vicinanza C, Aquila I, Scalise M, Cristiano F, Marino F, Cianflone E, Mancuso T, Marotta P, Sacco W, Lewis FC, Couch L, Shone V, Gritti G, Torella A, Smith AJ, Terracciano CMN, Britti D, Veltri P, Indolfi C, Nadal-Ginard B, Ellison-Hughes GM, Torella D. (2017). Adult cardiac stem cells are multipotent and robustly myogenic: c-kit expression is necessary but not sufficient for their identification. Cell Death and Differentiation. 24:2101–2116

27. Chong JJ, Reinecke H, Iwata M, Torok-Storb B, Stempien-Otero A, Murry CE. Progenitor cells identified by PDGFR-alpha expression in the developing and diseased human heart. (20130 *Stem Cells Dev*. 22(13):1932-43.

28. Bird SD, Doevendans PA, van Rooijen MA, Brutel de la Riviere A, Hassink RJ, Passier R, Mummery CL. (2003). The human adult cardiomyocyte phenotype. Cardiovasc Res 58:423–434.

29. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa, P. (2003). Adult Cardiac Stem Cells Are Multipotent and Support Myocardial Regeneration. Cell 114:763–776.

30. Buckingham M, Meilhac S, Zaffran S. (2005). Building the mammalian heart from two sources of myocardial cells. Nature Reviews 6:826-835.

31. Evans SM, Yelon D, Conlon FL, Kirby ML. (2010). Myocardial Lineage Development. Circ Res. 107:1428-1444.

32. He JQ, Vu DM, Hunt G, Chugh A, Bhatnagar A, Bolli R. (2011). Human Cardiac Stem Cells Isolated from Atrial Appendages Stably Express c-kit . PLoS ONE **6**:e27719.

33. Wegmeyer H, Bröske AM, Leddin M, Kuentzer K, Nisslbeck AK, Hupfeld J, Wiechmann K, Kuhlen J, von Schwerin C, Stein C, Knothe S, Funk J, Huss R, Neubauer M. (2013). Mesenchymal Stromal Cell Characteristics Vary Depending on Their Origin**.** Stem Cells and Dev 22(19):2606-2618.

34. Koninckx R, Daniëls A, Windmolders S, Mees U, Macianskiene R, Mubagwa K, Steels P, Jamaer L, Dubois J, Robic B, Hendrikx M, Rummens JL, Hensen K. (2013). The cardiac atrial appendage stem cell: a new and promising candidate for myocardial repair. Cardiovasc Res 97:413–423.

35. Houweling AC, van Borren MM, Moorman AF, Christoffels VM. (2005). Expression and regulation of the atrial natriuretic factor encoding gene Nppa during development and disease. Cardiovasc Res 67:583–593.

36. Leinonen JV, Emanuelov AK, Platt Y, Helman Y, Feinberg Y, Lotan C, Beeri R. (2013). Left Atrial Appendages from Adult Hearts Contain a Reservoir of Diverse Cardiac Progenitor Cells. PLOS One 8(3):e59228.

37. Yoon YS, Park JS, Tkebuchava T, Luedeman C, Losordo DW. (2004). Unexpected severe calcification after transplantation of bone marrow cells in acute myocardial infarction. Circ 109:3154-3157.

38. Ding J, Xu H, Faiola F, Ma'ayan A, Wang J. (2012). Oct4 links multiple epigenetic pathways to the pluripotency network. Cell Res 22:155-167.

39. Harkness L, Zaher W, Ditzel N, Isa A, Kassem M. (2016). CD146/MCAM defines functionality of human bone marrow stromal stem cell populations. Stem Cell Res Ther 7:4:1-13

40. Chen WCW, Baily JE, Corselli M, Diaz M, Sun B, Xiang G, Gray GA, Huard J, Peault. (2016). Human Myocardial Pericytes: Multipotent Mesodermal Precursors Exhibiting Cardiac Specificity. Stem Cells 33:2: 557-573.

41. Bujak M, Frangogiannis NG. (2007). The role of TGF-β Signaling in Myocardial Infarction and Cardiac Remodeling. Cardiovasc Res 74:2:184-195.

42. Hausenloy DJ, Yellon DM. (2009). Cardioprotective growth factors. Cardiovasc Res 83:179–194.

43. Sadat S, Gehmert S, Song YH, Yen Y, Bai X, Gaiser S, Klein H, Alt E. (2007). The cardioprotective effect of mesenchymal stem cells is mediated by IGF-I and VEGF. Biochem Biophys Res Commun 363:3:674-9.

44. Ueda H, Nakamura , Matsumoto K, Sawa Y, Matsuda H, Nakamura T. (2001). A potential cardioprotective role of hepatocyte growth factor in myocardial infarction in rats. Cardiovasc Res. 51:41-50.

45. Chang YS, Ahn SY, Jeon HB, Sung DY, Kin ES, Sung SI, Yoo HS, Choi SJ, Oh WI, Park WS. (2014). Critical Role of Vascular Endothelial Growth Factor Secreted by Mesenchymal Stem Cells in Hyperoxic Lung Injury. American Journal of Respiratory Cell and Molecular Biology 51:3:391-399.

46. Adas G, Koc B, Adas M, Duruksu G, Subasi C, Kemik O, Kemik A, Sakiz D, Kalayci M, Purisa S, Unal S, Karaoz E. (2016). Effects of mesenchymal stem cells and VEGF on liver regeneration following major resection. Langenbecks Arch Surg 401:725–740.

47. Hodgkinson CP, Bareja A, Gomez JA, Dzau VJ. (2016). Emerging Concepts in Paracrine Mechanisms in Regenerative Cardiovascular Medicine and Biology. Circ Res 118:1: 95–107.

48. Yao Y, Huang J, Geng Y, Qian H, Wang F, Liu X, Shang M, Nie S, Liu N, Du X, Dong J, Ma C. (2015). Paracrine Action of Mesenchymal Stem Cells Revealed by Single Cell Gene Profiling in Infarcted Murine Hearts. PLOS ONE doi: 10.1371/journal.pone.0129164

49. Carr CA, Stuckey DJ, Tan JJ, Tan SC, Gomes RS, Camelliti P, Messina E, Giacomello A, Ellison GM, Clarke K. (2011). Cardiosphere-Derived Cells Improve Function in the Infarcted Rat Heart for at Least 16 Weeks – an MRI Study. PLoS ONE 6(10):e25669.

Fig. 1 Morphology of different primary cells in culture. Representative brightfield images of cells *in vitro* at passage 3**.** (A) BM-derived MSCs, (B –D) show images from 3 different patients derived cultures of CMSCLC. Brightfield image showing morphology of human CDCs (E) Brightfield image of CMSCLC CFU (F). All scale bars = 100µm. CMSCLC cultures derived from 3 different patient tissue samples were assessed for their ability to form CFUs under standard MSC culture conditions, BM was also assessed for CFU formation ability (G). The number of CFUs formed per culture at passage 1 are represented graphically.

Fig. 2 Immunophenotyping of CS-CDC and CMSCLC. Representative images of FACS histograms showing results of immunophenotyping of CDC (n=3) and CMSCLC (n=3) using cell surface antigens of both hematopoietic lineage committed cells (CD45 and CD19) and those known to be expressed by MSCs (CD44, CD105, CD166). Controls are isotype control and unstained cells for both cell populations. All cells cultures were immunophenotyped at passage 3

Fig. 3 FACS analysis for expression of MHCs and c-kit, and IHC analysis for CD34 expression, by CMSCLC**.** (A) Representative images of FACS histograms showing expression levels of MHC I and MHC II by CMSCLC (B) Table showing percentage of cells expressing MHCI and MHCII in 3 different patient derived CMSCLC cultures. (C) Representative image of FACS histogram showing expression level of c-kit in CMSCLC and table showing percentage of cells expressing c-kit in 3 different patient derived CMSCLC cultures. (D) Cells from the same patient-derived CMSCLC cell line where cultured for 9 days under normal CMSCLC culture conditions (Control) and under Endothelial cells culture conditions (EC differentiation media) and were analyzed for the expression of CD34 (CD34 brown, cell nuclei blue). Note lack of CD34 in cells cultured under normal CMSCLC culture conditions (magnification x400). Scale bars = 100µm. All cultures were examined at passage 3

Fig. 4 Characterization of tri-lineage differentiation potential of CDC and CMSCLC at passage 3. Representative brightfield images from 3 different patients tissue samples of (A) CDC and (B) CMSCLC after 28 days in osteogenic culture conditions and prior to alizarin red staining demonstrates cellular proliferation and matrix deposition. Images of cells from the same samples but after being stained with alizarin red, staining reveals mineralisation of osteoid matrix within osteogenic cultures differentiated from CDCs (C) whereas (D) there is no mineralisation of culture matrix when CMSCLC were differentiated. Images of CDC (E) and CMSCLC (F) after 21 days under adipogenic culture conditions stained with Oil red O. Note the presence of red stained cells in both cultures. Images of cryosections of cell aggregates formed under pro-chondrogenic culture conditions after 14 days and after staining with Safranin O (counter stained with haematoxylin and fast green) for CDC (G) and CMSCLC (H). Note that while cell aggregates formed for both CDCs and CMSCLC chondrogenesis did not occur as evidenced by the absence of safranin O staining for sulphated glycosaminoglycans. Histological evaluation of cell aggregates shows lack of extracellular matrix deposition between cells and hence weak tissue structure. All scale bars = 100µm. BM-MSCs are shown after culture using the same differentiation protocols as for CDCs and CMSCLC and stained with alizarin red (I), Oil red O (J) and safranin O (K). Note BM-MSCs can differentiate to all three lineages. Scale bars for (I and J) = 100µm. Scale bar for (k) = 400 µm.

Fig. 5 Quantification of CMSCLC p16 immunostaining**.** Representative image of p16 ICC showing results of staining of CMSCLC from patient 1179, p16 (green), all nuclei stained with DAPI (blue) scale bar = 100µm **(A)** CMSCLC cultures derived from 3 different patient tissue samples were immunostained for the cell senescence marker p16 and counterstained with DAPI. The percentage of p16 positive cells within each culture was calculated by counting five independent fields within

each culture. Box-plot analysis (SPSS) showed no significant difference in p16 expression between n= 3 patient cultures (B).

Fig 6 Characterization of cardiac differentiation potential of CDC and CMSCLC. Representative images of CDC (n=3 donors) and CMSCLC (n=4) donors at 14 days under cardiac differentiation culture conditions show that some of both the CDC (A) and (B) CMSCLC differentiated cells have a more mature cardiac morphology and express troponin C (green-Alexa fluor 488). While other cells from the same patient samples cultured under the same conditions but stained for expression of NKX2.5 have a more primitive rounded morphology and express nuclear NKX2.5 (NKX2.5 green-Alexa fluor 488) in differentiated CDC (C) and differentiated CMSCLC culture (D). White arrows in (C) and (D) indicate cells shown in inserts of NKX2.5 staining without the DAPI overlay to shown the nuclear localization of NKX2.5. (E –H) Representative images of CMSCLC (n=4 donors) differentiated for 3 weeks and analyzed by confocal microscopy. (E) Maximum intensity projections of differentiated CMSCLC expressing Troponin C (green- Alexa fluor 488). (E*i*) Higher magnification image demonstrates early striations, indicated by yellow arrow. (F) Maximum intensity volume projection of differentiated CMSCLC labelled with Cardiac Actin (red- Alexa fluor 594). (G) Maximum intensity volume projection of differentiated CMSCLC labelled with Alpha-tropomyosin (red- Alexa fluor 594). (H and H*i*) Maximum intensity volume projection demonstrates striated pattern of Alpha-Tropomyosin expression, indicated by yellow arrows. For all images nuclei labelled with DAPI-Blue. Scale bars were either 20 or 50 µm as indicated. (I) Bar chart showing percentage of cells expressing NXK2.5 or troponin C, n= 3 for both CDC and CMSCLC cultures analyzed.

Fig 7 Graphical representation of Single cell data representing percentage of CMSCLC expressing genes of interest for n=3 donors. The percentage of cells expressing each gene is shown.

Supplemental Fig. 1 Analysis of doubling time of CMSCLC cultures. Cell population doublings per day were calculated for 3 patient derived cultures, with values presented as the average rate of population doubling over three cell culture passages. No significant differences in cell doubling rates were observed between the 3 different cultures

Supplemental Fig. 2 FACS analysis for expression of PDGFR-alpha by CMSCLC. Representative images of FACS histograms showing unstained CMSCLC (A) and cells stained for expression of PDGFR-alpha (B).

Supplemental Fig. 3 Bar chart showing ELISA detected fold changes in IL10, VEGF, FGF2 and TGFβ1 expression levels from CMSCLC cultured under standard CMSCLC conditions over time compared with control media (A). Note increased expression for all factors at day 14 and 17 compared with control. Note increases in fold change are significant for all factors at both time point with the exception of Il10 and 14 days which was not significantly (NS) different (B).

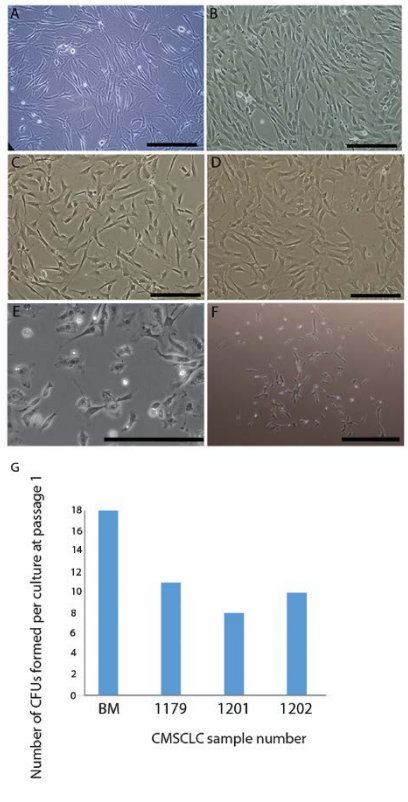
Supplemental Fig. 4 ICC controls for staining of CMSCLC.

ICC analysis showing results of CMSCLC stained using secondary antibodies only (no primary) for Troponin C, NKX2.5 and tropomyosin (A). All nuclei are stained with DAPI (Blue) A and B, Scale bars = 20µm; C, scale bar = 50µm. ICC analysis showing results of staining of the AC10 cell line (D) for expression of Troponin C (green), Alpha-tropomyosin (red), NXK 2.5 (green) and Cardiac Actin (red). All nuclei are stained with DAPI (Blue). Scale bars = 25 µm.

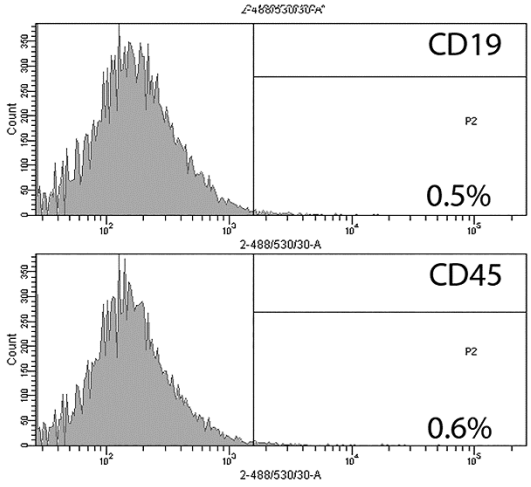
Supplemental Fig. 5 Transcriptional expression of MEF2C and GATA4 in undifferentiated and differentiated CMSCLC. Results of RT-PCR analysis of CMSCLC derived from 3 different patients. Note that expression of mRNA from both genes can be detected in both undifferentiated CMSCLC

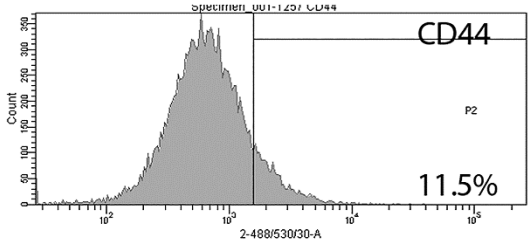
and cells from the same cultures after differentiation under cardiomyocyte differentiation conditions. β-actin was used as a loading control. Abbreviations: UD=undifferentiated, D=differentiated

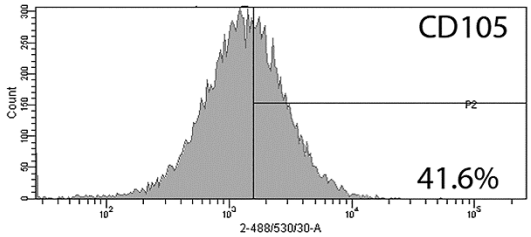
Supplemental Table 1 TaqMan® primers used in single-cell qPCR

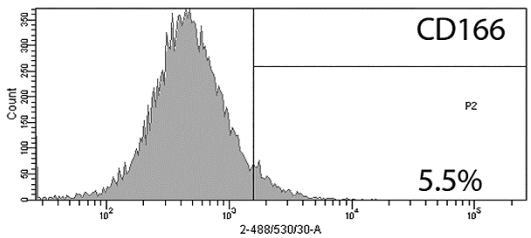

A
B
C
D
E
F
G
Number of CFUs formed per culture at passage 1
18
16
14
12
10
8
6
4
2
0
BM
1179
1201
1202
CMSCLC sample number

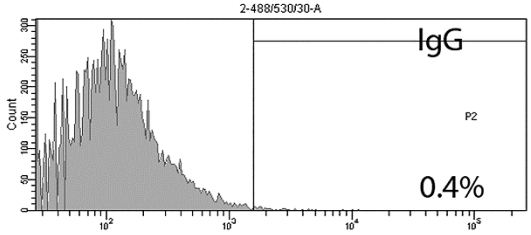
CS-CDC (1257)

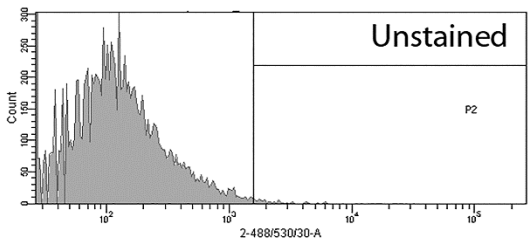




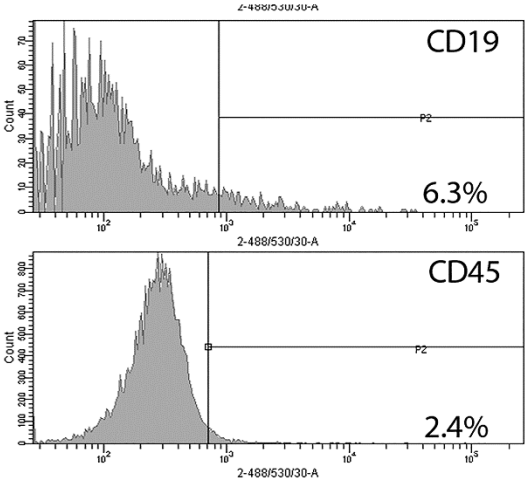


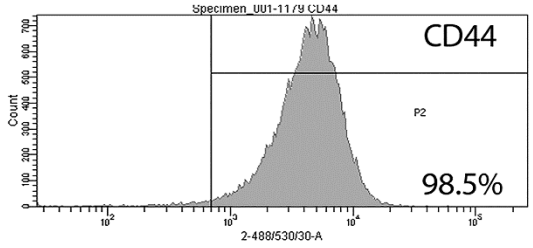


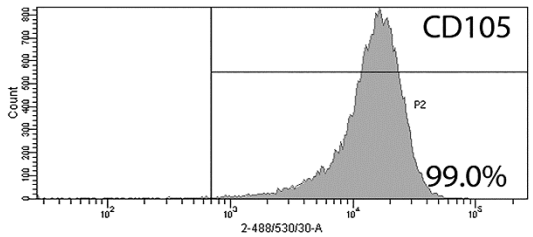


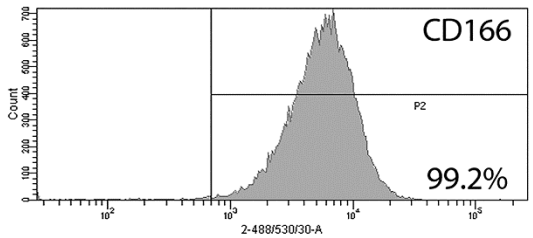


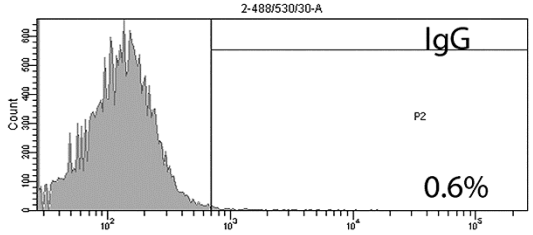
CMSCLC (1179)

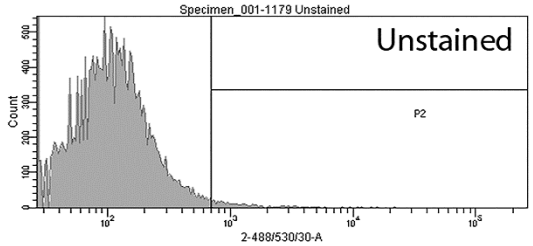




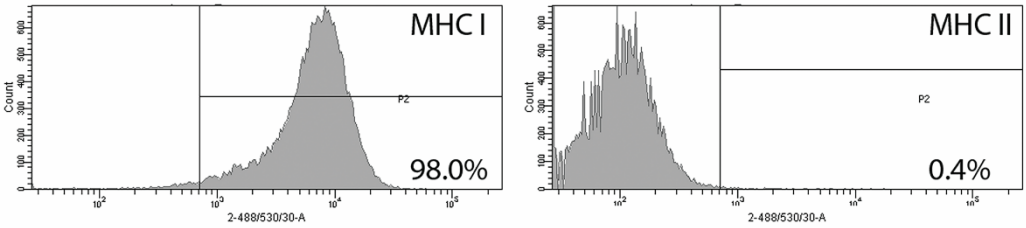








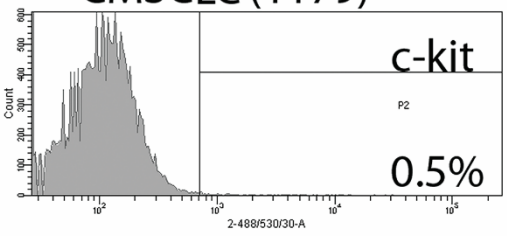
A



B

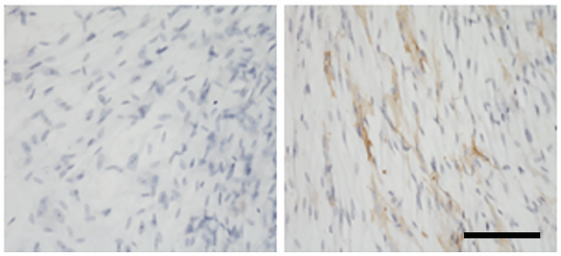
	CMSCLC sample number		
	1179	1201	1202
MHC I	98.0	96.9	59.6
MHC II	0.4	0.7	1.2

C



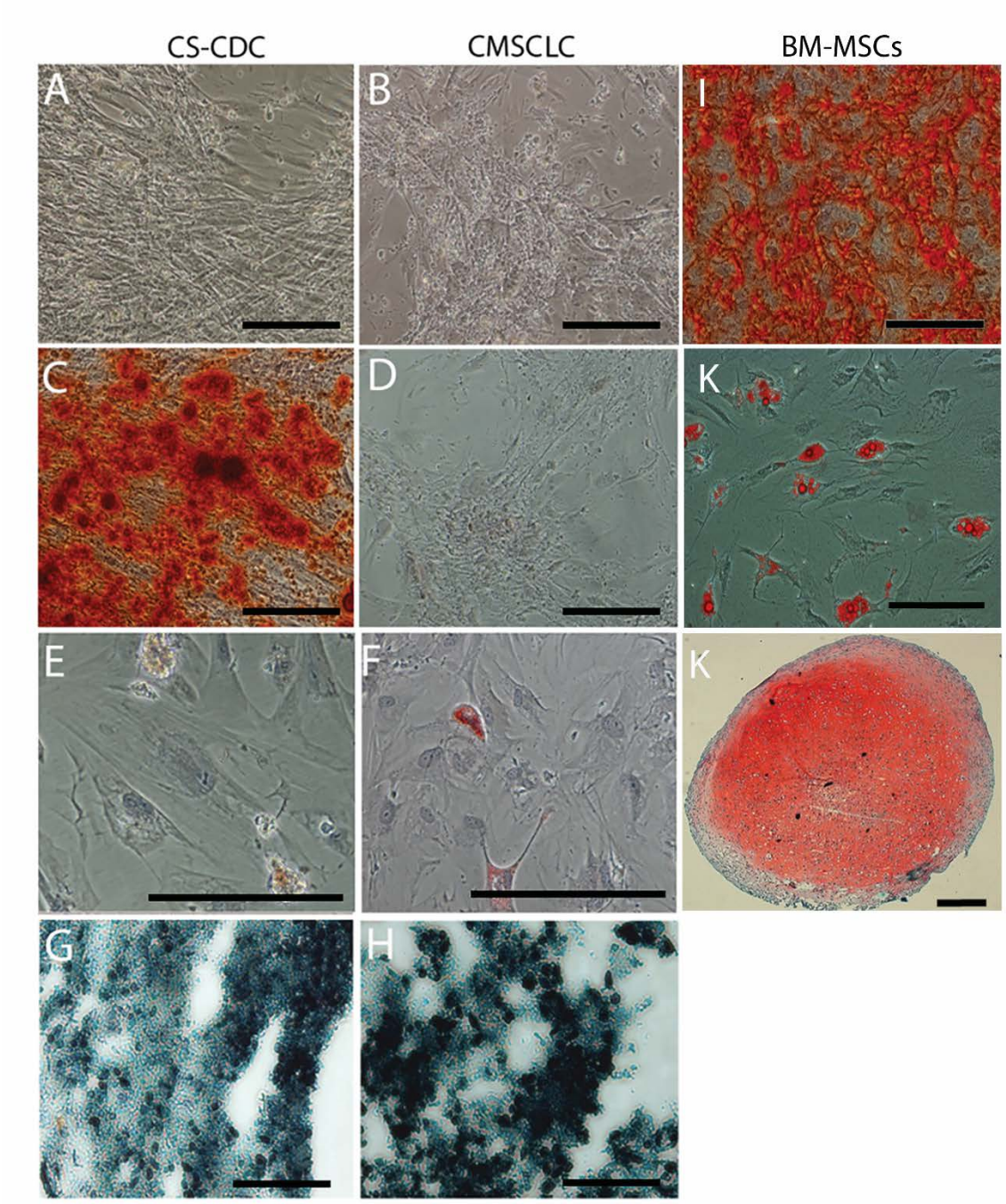
	CMSCLC sample number		
	1179	1201	1202
% c-kit positive	0.5	1.9	2.6

D

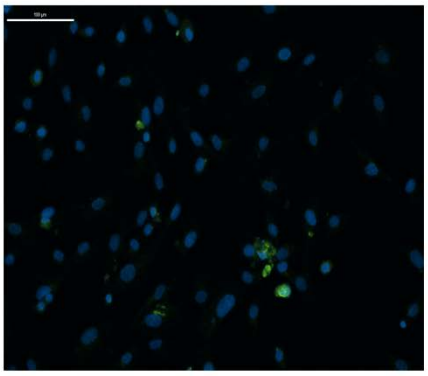


Control media

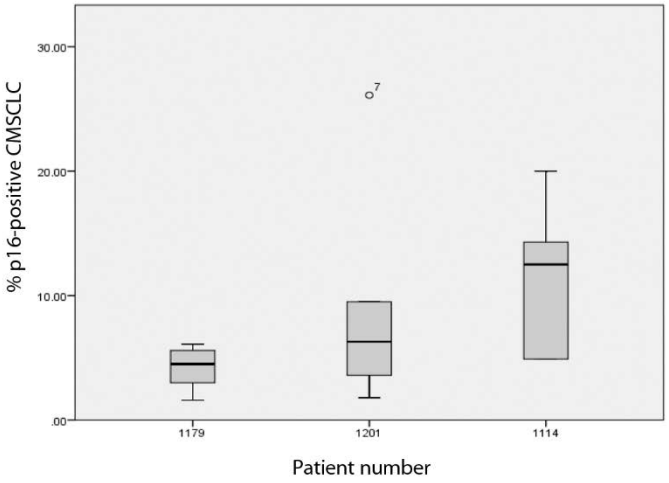
EC differentiation media

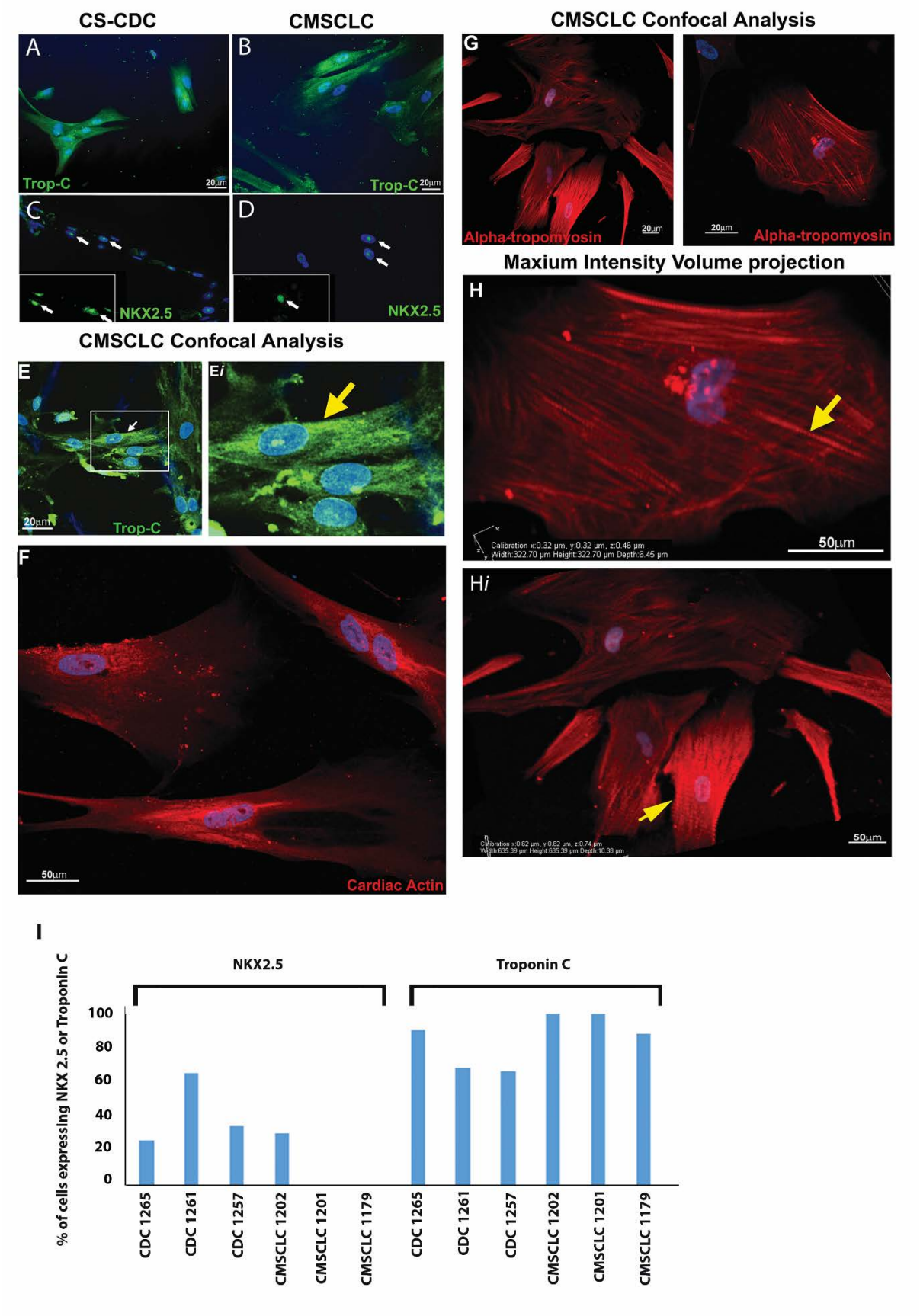

CS-CDC
CMSCLC
BM-MSCs
A
B
I
C
D
K
E
F
K
G
H

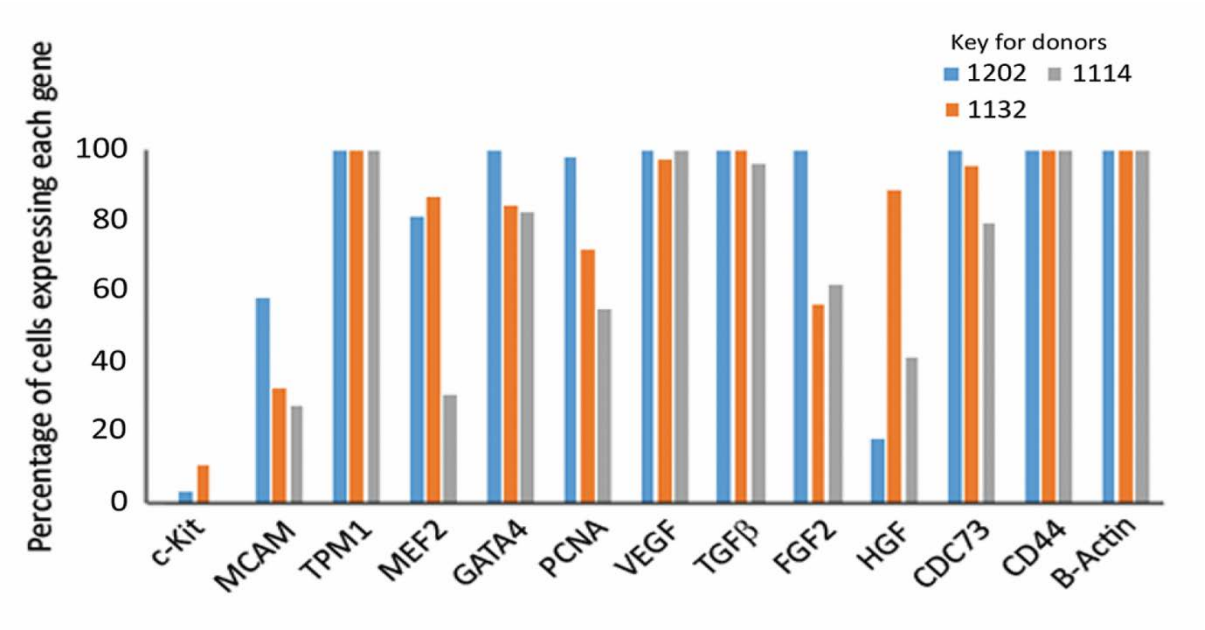
A

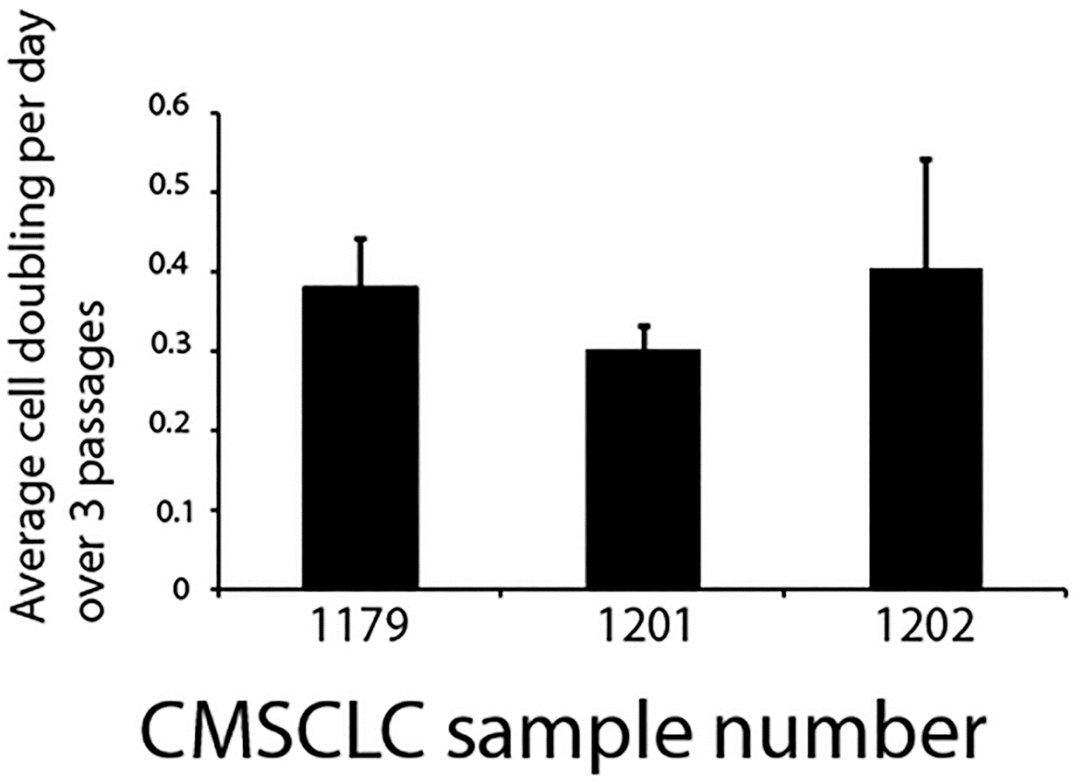


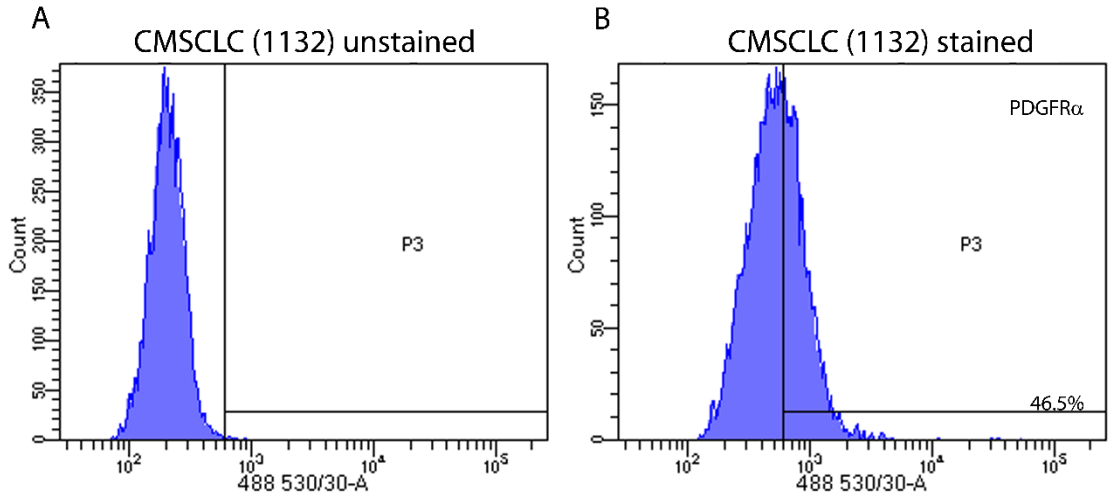
B

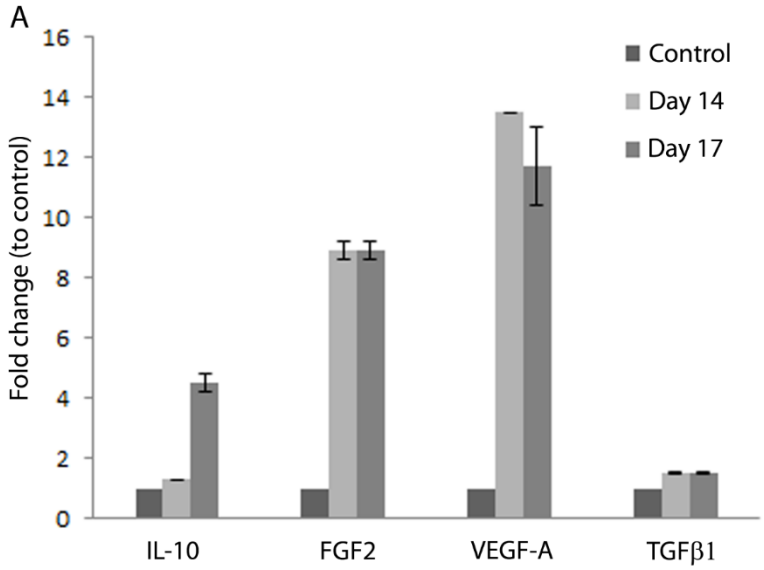

30.00
20.00
10.00
.00
% p16-positive CMSCLC
7
1179
1201
1114
Patient number


CS-CDC
CMSCLC
A
B
Trop-C
20µm
Trop-C
20µm
C
D
NKX2.5
NKX2.5
CMSCLC Confocal Analysis
E
Ei
20µm
Trop-C
F
50µm
Cardiac Actin
CMSCLC Confocal Analysis
G
Alpha-tropomyosin
20µm
20µm
Alpha-tropomyosin
Maxium Intensity Volume projection
H
Calibration x:0.32 µm, y:0.32 µm, z:0.46 µm
Width:322.70 µm Height:322.70 µm Depth:6.45 µm
50µm
Hi
50µm
I
NKX2.5
Troponin C
% of cells expressing NKX 2.5 or Troponin C
100
80
60
40
20
0
CDC 1265
CDC 1261
CDC 1257
CMSCLC 1202
CMSCLC 1201
CMSCLC 1179
CDC 1265
CDC 1261
CDC 1257
CMSCLC 1202
CMSCLC 1201
CMSCLC 1179


Key for donors
1202
1114
1132
Percentage of cells expressing each gene
100
80
60
40
20
0
c-Kit
MCAM
TPM1
MEF2
GATA4
PCNA
VEGF
TGFβ
FGF2
HGF
CDC73
CD44
B-Actin

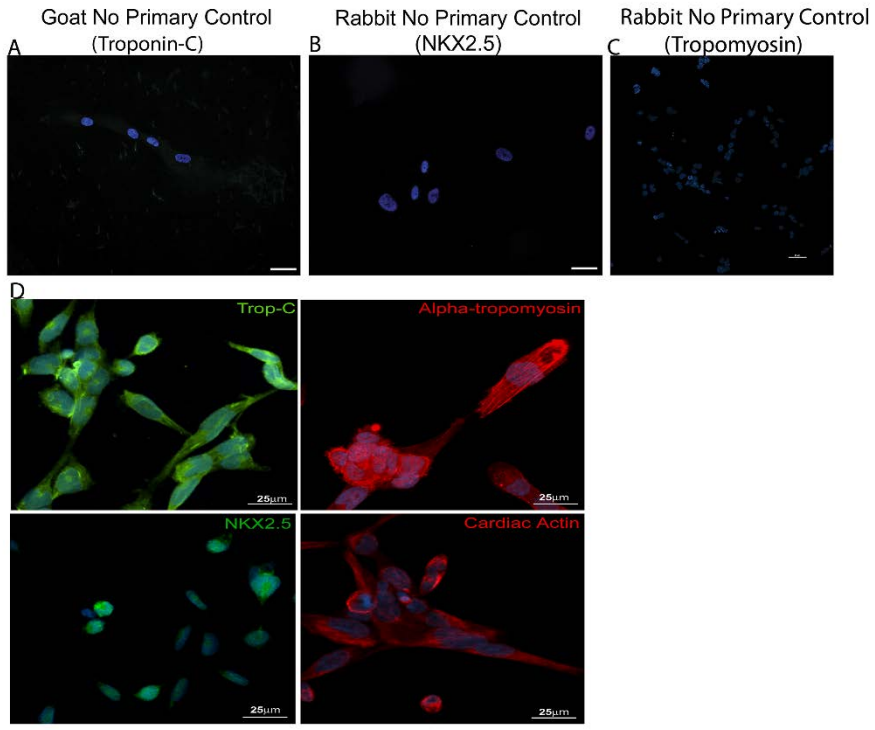

Average cell doubling per day over 3 passages
0.6
0.5
0.4
0.3
0.2
0.1
0
1179
1201
1202
CMSCLC sample number

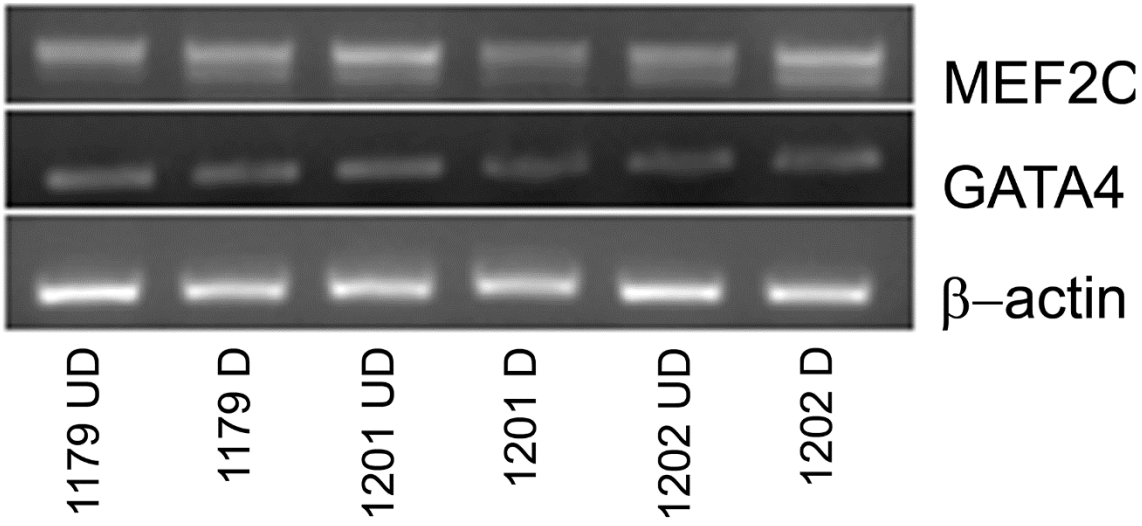




B

	IL-10	FGF2	VEGF-A	TGFβ1
Control - Day 14	NS	<0.0001	0.002	<0.0001
Control - Day 17	0.0005	<0.0001	0.001	<0.0001


Goat No Primary Control
(Troponin-C)
A
Rabbit No Primary Control
(NKX2.5)
B
Rabbit No Primary Control
(Tropomyosin)
C
D
Trop-C
Alpha-tropomyosin
NKX2.5
Cardiac Actin
25µm
25µm
25µm
25µm


MEF2C
GATA4
β–actin
1179 UD
1179 D
1201 UD
1201 D
1202 UD
1202 D

Gene	Cat.# of TaqMan Primer
NKX2.5	Hs00231763_m1
c-KIT	Hs00174029_m1
MCAM	Hs00174838_m1
TPM1	Hs04398572_m1
MEF2C	Hs00231149_m1
GATA4	Hs00171403_m1
PCNA	Hs00427214_g1
VEGF	Hs00900055_m1
TGFβ	Hs00998133_m1
FGF-2	Hs00266645_m1
HGF	Hs00300159_m1
CDC73	Hs00363810_m1
CD44	Hs01075864_m1
B-actin	Hs01060665_g1