Deformation-induced transitional myofibroblasts contribute to compensatory lung growth

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Deformation-induced transitional myofibroblasts remodel the lung during compensatory growth

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Abbreviations: SMA, α-smooth muscle actin; PCA, principal component analysis; qPCR, quantitative polymerase chain reaction
Abstract

In many mammals, including humans, removal of one lung (pneumonectomy) results in the compensatory growth of the remaining lung. Compensatory growth involves not only an increase in lung size, but also an increase in the number of alveoli in the peripheral lung; however, the process of compensatory neoalveolarization remains poorly understood. Here, we show that the expression of α-smooth muscle actin (SMA)—a cytoplasmic protein characteristic of myofibroblasts—is induced in the pleura following pneumonectomy. SMA induction appears to be dependent on pleural deformation (stretch) as induction is prevented by plombage or phrenic nerve transection (p<.001). Within 3 days of pneumonectomy, the frequency of SMA⁺ cells in subpleural alveolar ducts was significantly increased (p<.01). To determine the functional activity of these SMA⁺ cells, we isolated regenerating alveolar ducts by laser microdissection and analyzed individual cells using microfluidic single-cell quantitative PCR. Single cells expressing the SMA (Acta2) gene demonstrated significantly greater transcriptional activity than endothelial cells or other discrete cell populations in the alveolar duct (p<.05). The transcriptional activity of the Acta2⁺ cells, including expression of TGF signaling as well as repair-related genes, suggests a principal role for these myofibroblast-like cells in regulating septal remodeling and lung regeneration.
Introduction

In most mammals, including humans (Butler et al., 2012), removal of one lung (pneumonectomy) results in the compensatory growth of the remaining lung (Hsia et al., 2004). After murine pneumonectomy, the remaining lung demonstrates an increase in the volume of all 4 lobes—with particular increase in the volume of the cardiac lobe (Filipovic et al., 2014). Coincident with the increase in lung volume, there is an increase in weight and cell number (Chamoto et al., 2012; Konerding et al., 2012). Moreover, the process of post-pneumonectomy lung growth is rapid with compensatory growth occurring within weeks; most of the new alveoli (74%) are detectable within 7 days of surgery (Fehrenbach et al., 2008). The mechanism of compensatory growth is unknown.

There are few mechanical or morphologic clues suggesting the mechanism of neoalveolarization. In development, the importance of mechanical forces in lung growth has been suggested by several in vivo observations. Limited mechanical stretch—associated with congenital diaphragmatic hernia (Kitagawa et al., 1971), oligohydramnios (Adzick et al., 1984), and phrenic nerve dysfunction (Harding and Hooper, 1996)—has been associated with underdeveloped alveolar septa. Excessive mechanical stretch, commonly associated with neonatal mechanical ventilation, has been associated with disordered alveolar septa (Pierce et al., 1997).

In compensatory lung growth, histology of the post-pneumonectomy lung demonstrates no dominant cellular aggregates and only a modest increase in septal thickness (Voswinckel et al., 2004; Ysasi et al., 2015). Within days of pneumonectomy, approximately 20-30% of alveolar ducts are dilated as a result of septal retraction. Ysasi et al. have suggested that new alveoli are formed by repartitioning of the alveolar septa within the dilated ducts (Ysasi et al., 2015).

A cell type potentially involved in the re-partitioning of the alveolar duct is the myofibroblast. Myofibroblasts have been implicated in the alveolarization stage of lung
development—a process involving the “lifting” of alveolar septa (Mund et al., 2008).

Characterized by the cytoplasmic expression of α-smooth muscle actin (SMA) and the production of extracellular matrix components, myofibroblasts have been spatially associated with the development of alveolar septa during lung development (Dickie et al., 2008; Yamada et al., 2002). In adult lungs, myofibroblasts have been associated with a variety of diseases including pulmonary fibrosis (Noble et al., 2012) and pulmonary hypertension (Kapanci et al., 1990).

In this report, we manipulated the in vivo forces applied to the remaining lung after pneumonectomy—namely, static and cyclic stretch—to show that post-pneumonectomy deformation induces SMA+ cells in the pleura. We tracked the apparent migration of the SMA+ cells into subpleural alveolar ducts. The functional activity of the SMA+ cells in these regenerative “hot spots” was investigated using single-cell qPCR.
Methods

**Mice.** Male mice, eight- to ten-week old wild type C57BL/6 (Jackson Laboratory, Bar Harbor, ME, USA) were used for all experiments. The mice were anesthetized as previously described (Gibney et al., 2011). The care of the animals was consistent with guidelines of the American Association for Accreditation of Laboratory Animal Care (Bethesda, MD, USA) and approved by our Institutional Animal Care and Use Committee.

**Pneumonectomy.** Each animal undergoing pneumonectomy was ventilated on a Flexivent (SciReq, Montreal, QC Canada) at ventilator settings of 200/min, 10 ml/kg, and PEEP of 2 cmH$_2$O with a pressure limited constant flow profile (Gibney et al., 2011). A left fifth intercostal space thoracotomy provided exposure for hilar ligation and left pneumonectomy. In some mice, sham thoracotomy (N=4), plombage (N=4) and phrenic nerve transection (N=5) was performed as previously described (Ysasi et al., 2013). Postoperatively, the animal was weaned from mechanical ventilation and maintained on supplemental oxygen until normal spontaneous ventilation was observed.

**Immunohistochemistry.** Cryostat sections were obtained from lung specimens perfused with O.C.T. compound and snap frozen. After warming the slide to 27°C, the sections were fixed for 10 minutes (2% paraformaldehyde and PBS at pH 7.43). The slides were washed with buffer (PBS, 5% sheep serum, 0.1% azide, 1mM MgCl$_2$, 1mM CaCl$_2$) and blocked with 20% sheep serum, 20% goat serum, 0.1% azide in PBS. The slides were treated with anti-SMA polyclonal antibody (primary rabbit ab5694, Abcam; secondary goat anti-rabbit Texas Red; Life Technologies, Carlsbad, CA, USA) and second antibody control. The slides were incubated for one hour at 27°C, washed 3 times and mounted with either DAPI-containing medium (Vector Laboratories. Burlingame, CA, USA) or Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA).

**Fluorescence microscopy.** The tissue sections were imaged with a Nikon Eclipse TE2000 inverted epifluorescence microscope using Nikon objectives of 10, 20, and 40 linear
magnification with infinity correction. An X-Cite™ (Exfo, Vanier, QC, Canada) 120 W metal halide light source and a liquid light guide were used to illuminate the tissue samples. The tri-color excitation and emission filters (Chroma Technology, Bellows Falls, VT, USA) were controlled by a MAC5000 controller (Ludl, Hawthorne, NY, USA) and MetaMorph® software 7.8 (Molecular Devices, Downingtown, PA, USA). The fluorescence microscopy 14-bit fluorescent images were digitally recorded on a C9100-02 camera (Hamamatsu, Japan), digitally recombined and pseudocolored based on recording wavelength.

**Image segmentation and cytometry.** Automated 16-bit image acquisition and montage creation was performed using MetaMorph 7.8 (Molecular Devices) and the MAC5000 controller (Ludl). Fine adjustments of the montage were performed manually. The montage image segmentation was performed using custom routines created using CellProfiler (Broad Institute, Cambridge, MA, USA). Image parsing was used to manage memory overruns. Image cytometry of the segmented image was performed using the FCS Express 6 (beta) software (De Novo Software, Los Angeles, CA, USA).

**Precision-cut lung slices.** Agarose at 3% (w/v) or Alginate (1% w/v) and Gelatin (5% w/v) was thoroughly mixed and warmed to 37°C. The trachea was cannulated and the warm embedding medium infused through the trachea using the lowest pressure necessary to inflate the peripheral lung. At total lung capacity, the trachea was clamped and the lung block placed in 34 mM calcium chloride solution (in deionized water reconstituted to isotonicity with NaCl) at 4°C for 30 minutes to allow for gelation. Sectioning was performed with the Leica VT1000 S vibrating blade microtome (Leica Biosystems, Nussloch, Germany) using stainless steel razor blades (Gillette, Boston, MA). The microtome was operated at the following adjustable settings: knife angle, 5-7°; sectioning speed, 0.05-0.2 mm/sec; oscillation frequency, 80-100 Hz; and oscillation amplitude, 0.6 mm. Sections 200-300 µm thick were mounted on a polyethylene naphthalene (PEN) membrane frame slide (Life Technologies) for laser microdissection.
Laser microdissection. The Arcturus XT LCM System (Life Technologies) was used for all ultraviolet (UV) laser dissection. The UV laser was a specially adapted beta-test laser for wet tissue applications. The Arcturus XT software was used to target tissue for UV dissection. Peripheral alveolar ductal units were optically targeted and dissected free from surrounding tissue using the UV laser (Bennett et al., 2014). Dissected tissue was then placed into collagenase solution in a single well of a 96-well plate for enzymatic tissue dissociation.

Enzymatic digestion. Enzymatic digestion of the lung reflected a previously published protocol (Bennett et al., 2014). Briefly, 1mg/mL collagenase Type IV (Worthington, Lakewood, NJ, USA) and 0.01mg/mL DNase I (Fisher Scientific, Pittsburgh, PA, USA) in Dulbecco’s Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA, USA) was used to dissociate the tissue. Dissociation was performed at 37ºC under constant agitation for 30-45 minutes. The digest was filtered through 35µm nylon mesh, and remaining debris was removed by centrifugation at 1200 RPM for 3 minutes. The process of microfiltration and centrifugation was then repeated once more in preparation for microfluidic analysis.

Viability assessment. A small aliquot of cell suspension (7.5-10µL) was used to assess cell concentration and viability by trypan blue exclusion. Trypan blue (Sigma-Aldrich) was added in 1:1 ratio and cell concentration determined using a standard microscope hemacytometer. Each cell counted was determined to be live or dead based on trypan blue exclusion and viability calculated as a percentage of total cells.

C1 Specific Target Amplification (STA). Single mouse lung cell capture and STA were carried out using the Fluidigm C1 Single-Cell Auto Prep System and Single-Cell Auto Prep Array Integrated Fluidic Circuits (IFCs) (Fluidigm, South San Francisco, CA). For these experiments, medium sized (10-17µm cell diameter) STA IFCs were used. Chip-priming, cell-loading, lysis, reverse transcription, and pre-amplification were performed in accordance with Fluidigm’s recommended protocol using lysis and pre-amplification reagents from the Single Cell-to-CT Kit (Ambion/Life Technologies) and pooled pre-amplification primers custom
designed to enrich for 96 loci of interest (outer primer sequences provided in the Supplementary Table). Cells were loaded onto the chip at concentrations ranging from 120 to 370 cells/µL and captured cells were imaged using an Olympus IX71 microscope to assess cell number and quality. Fluidigm’s standard STA script was modified using C1™ Script Builder™ (Fluidigm) so that the total capture volume was increased four-fold.

**Single Cell Multiplexed Quantitative PCR.** Preamplified cDNA samples from single cells were analyzed by qPCR using 96.96 Dynamic Array™ IFCs and the Biomark™ HD System from Fluidigm. Processing of the IFCs and operation of the instruments were performed according to the manufacturer’s procedures. A Master Mix was prepared consisting of 420 µL SsoFast™ EvaGreen Supermix with Low ROX (BioRad 172-5211), 42 µL 20× DNA Binding Dye Sample Loading Reagent (Fluidigm 100-5360), plus 18 µL H₂O, and 4 µL of this mix was dispensed to each well of a 96-well assay plate. Three µL of preamplified cDNA sample was added to each well and the plate was briefly vortexed and centrifuged. Following priming of the IFC in the IFC Controller HX, 5 µL of the cDNA sample and Master Mix were dispensed to each Sample Inlet of the 96.96 IFC. Then, 4.5 µL of each 10× Assay (5 µL each primer, inner primer sequences (Supplementary Information) was dispensed to each Detector Inlet of the 96.96 IFC. After loading the assays and samples into the IFC in the IFC Controller HX, the IFC was transferred to the Biomark HD system and PCR was performed using the thermal protocol GE Fast 96x96 PCR+Melt v2.pcl. This protocol consists of a Thermal Mix of 70°C, 40 min; 60°C, 30 sec, Hot Start at 95°C, 1 min, PCR Cycle of 30 cycles of (96°C, 5 sec; 60°C, 20 sec), and Melting using a ramp from 60°C to 95°C at 1°C/3 sec.

**Data Analysis and Graphical Display.** Data was analyzed using Fluidigm Real-Time PCR Analysis software using the Linear (Derivative) Baseline Correction Method and the Auto (Global) Ct Threshold Method. The C_q values determined were exported to Excel for further processing using Singular™ Analysis Toolset (Fluidigm) in the R software environment. Principle component analysis and hierarchical clustering by Euclidean distance were performed.
for all cells. qPCR experiments were performed for control (4 biological replicates), day 1 (3 biological replicates), day 3 (5 biological replicates) and day 7 (4 biological replicates) time points. The single-cell data was converted into an FCS file standard (Dean et al., 1990) using available conversion software (GenePattern, Broad Institute). The FCS file was subsequently analyzed using hierarchical and combination gates using the FCS Express 6 (beta) software (De Novo). Statistical analyses were based on measurements in at least three different mice. The unpaired Student’s t test for samples of unequal variances was used to calculate statistical significance. The data was expressed as mean ± one standard deviation. The significance level for the sample distribution was defined as P< 0.05.
Results

**α-Smooth muscle actin (SMA) expression in the lung.** To identify resident myofibroblasts, we performed SMA immunohistochemistry on non-surgical murine cardiac lobes. The immunostained lobes were imaged and whole-lobe montage image stacks were created (Fig 1A). Control tissue sections, treated with secondary antibody alone, were used to define SMA positivity (Fig 1B). As expected, SMA staining was prominent in the central lobar regions reflecting perivascular and peribronchial smooth muscle cells as well as a smaller population of myofibroblasts. Gating in the subpleural regions, where septal remodeling occurs, demonstrated that less than 2% of the cells were SMA⁺; less than 1% of the SMA⁺ cells were localized to alveolar ducts (Fig 1C, D).

**Influence of mechanical forces on SMA expression.** To investigate the influence of in vivo lung deformation on SMA expression, we examined the cardiac lobe for cells expressing SMA after pneumonectomy (Fig 2A), sham thoracotomy (N=5), pneumonectomy plus phrenic nerve transection (inhibition of cyclic stretch; N=7), and pneumonectomy plus plombage (inhibition of static stretch; N=7). Systematic whole-lobe immunohistochemical staining and image cytometry demonstrated a significant rise in SMA expression within the first 3 days after pneumonectomy (Fig 2B). The sham thoracotomy, phrenic nerve transection and plombage conditions were indistinguishable from nonsurgical controls; all three control conditions were significantly different from pneumonectomy alone (p<.001). In the pneumonectomy condition, image cytometry permitted the localization of the enhanced SMA expression to the pleural and subpleural regions of the lung (Fig 2C). There was little change in the density of central SMA⁺ cells. A frequency analysis of SMA⁺ cell location demonstrated a progressive increase in SMA⁺ cells in the subpleural regions of the lung (Fig 3).

**Transcriptional profile of subpleural cells.** The functional role of the SMA⁺ cells appearing in the subpleural region after pneumonectomy was explored using single-cell
analysis. Laser microdissection of subpleural alveolar ducts facilitated single cell-isolation by microfluidics (C1 System, Fluidigm)(Fig 4A). Of the 2211 cells analyzed by microfluidics and qPCR, 1107 passed rigorous criteria for single cell analysis Consistent with image cytometry, 4% of control samples were positive for the SMA gene Acta2; 6.9% of the cells on post-pneumonectomy days 1,3 and 7 were Acta2+. Because fewer than 3% of the cells in the phrenic nerve and plombage controls were positive for Acta2, these conditions were excluded from further analysis.

The post-pneumonectomy Acta2+ cells demonstrated two distinctive expression profiles by principal component analysis (PCA) and hierarchical clustering. Population 1 (designated P1, Fig 4C) was relatively less transcriptionally active but demonstrated a similar profile to Population 2 (P2, Fig 4C). There was no statistical difference in transcriptional activity of the two Acta2+ cell populations between POD 1,3, 7 (p>.05). The distinctive transcriptional profile of P1 and P2 was demonstrated by a comparison with the Acta2- cells in Population 3 (P3)(Fig 5).

Most Acta2+ cells were also Itgav+ and Itgb1+ (61%). Gating hierarchies demonstrated that of the Itgav+ cells, 100% were Itgav+/Itgb1+, 95% were Itgav+/Itgb1+/Itgb2+, and 84% were Itgav+/Itgb1+/Itgb2+/Itgb5+. The broad gene co-expression of the integrin beta subunits was unexpected; particularly, the β2 subunit commonly associated with leukocyte expression. As expected, the Acta2+ myofibroblasts expressed Tgfb1 (as well as intracellular signaling molecules Smad4 and Smad2), Pdga and Pdgfrb. The most notable gene expression pathway was the mitogen-activated protein kinase (Mapk) expression in P2. Mapk expression effectively discriminated P1 and P2; similarly, the Acta2- cells (P3) demonstrated a much lower number of cells with detectable Mapk expression. Mapk1, Mapk3, Mapk8, Mapk14 and Map2k1 were co-expressed in 35% of Acta2+ cells.

Remodeling profile of the Acta2+ cells. Since the Acta2+ cells were harvested from regenerative "hot spots," we examined the cells for gene expression related to lung growth (Fig 6). Nearly all Acta2+ cells co-expressed notable levels of the genes associated with lung
remodeling: elastin (80%, *Eln*), the elastin scaffold protein fibrillin-1 (55%, *Fbn1*), collagen (83%, *Col18a1*), and fibronectin (64%, *Fn1*). The cross-linking enzyme lysyl oxidase (*Lox*) was prominently expressed in both *Acta2*+ and *Acta2*− cells.

More specialized genes associated with lung remodeling were predominantly expressed in the *Acta2*+ cells. Fibulins (*Fbln*) and latent transforming growth factor beta binding proteins (*Ltbp*) were prominently expressed in *Acta2*+ cells. Of note, 23% of the *Acta2*+ cells co-expressed *Fbln1*, *Fbln5*, *Ltbp2* and *Ltbp4*. Other lung remodeling genes, such as those involved in angiogenesis (e.g. *Vegfa*, *Epha2*, *Ephb4* and *Eng*), demonstrated significantly higher expression in the *Acta2*+ population than *Acta2*− cells (p<.001). Finally, the fibroblast growth factor (*Fgf*) gene family demonstrated variable expression; no clear pattern of *Fgf* co-expression was found (Fig 6).
Discussion

In this report, an in vivo analysis of myofibroblast population dynamics—defined by SMA$^+$ protein expression or Acta2$^+$ gene expression—produced 4 principal findings. 1) SMA$^+$ cells were induced in the pleura by post-pneumonectomy deformation. 2) The induction of SMA$^+$ pleural cells preceded the accumulation of SMA$^+$ cells in the subpleural alveolar ducts. 3) Cells in the subpleural alveolar ducts expressing the SMA gene (Acta2) demonstrated a broad and highly active transcriptional profile. 4) Cells expressing the SMA gene (Acta2) also demonstrated a transcriptional profile specific for lung repair and regeneration. We conclude that SMA$^+/Acta2^+$ cells induced in the pleural mesothelium play a pivotal regenerative role in post-pneumonectomy lung growth.

A major contribution of this work is the in vivo characterization of a low frequency and spatially distributed cell-type during the process of lung remodeling and regeneration. Myofibroblasts, commonly defined as demonstrating fibroblast morphology, expressing cytoplasmic α-smooth muscle actin, and producing matrix components (Hinz et al., 2007), are rare in tissues. The low frequency and "enigmatic" phenotype of myofibroblasts (Orenstein, 2014), has led to acknowledged controversy regarding myofibroblast function (Hinz et al., 2012). Here, we empirically defined our target cell population as expressing the SMA protein or the Acta2 gene. Optical tissue imaging and quantitative cytometry of SMA$^+$ cells demonstrated the dynamic transitions of this cell-type—from the induction of SMA expression in pleural cells to their apparent migration into regenerating alveolar ducts. In addition to inferring function from anatomic positioning, single-cell isolation and qPCR provided useful insights into the profound regenerative capabilities of these SMA$^+/Acta2^+$ cells.

After murine pneumonectomy, the cardiac lobe is displaced into the empty hemithorax (Gibney et al., 2012). Displacement of the cardiac lobe is associated with "static" deformation (Filipovic et al., 2014) and "cyclic" stretch (Filipovic et al., 2013). An intriguing observation in a
growing number of studies is that preventing cardiac lobe displacement (plombage) (Cowan and
Crystal, 1975; Hoffman et al., 2010) or inhibiting cyclic stretch (phrenic nerve transection) (Ysasi
et al., 2013) blocks lung growth (Ysasi et al., 2013). Despite the common element of
deformation or “stretch,” a mechanistic explanation for these observations has remained
elusive. Here, we show that pneumonectomy is associated with rapid (24 hours) transition of the
pleural mesothelium into SMA⁺ cells. Consistent with contemporary understanding of epithelial-
mesenchymal transition (EMT)(Thiery et al., 2009)(alternatively, mesothelial-mesenchymal
transition), the transition of mesothelial cells into SMA⁺ cells required both static and cyclic
stretch since both plombage and phrenic nerve transection blocked this transition. Also
consistent with EMT, the SMA⁺ cells appeared to acquire migratory behavior (Nieto, 2013).
Within 3 days, SMA⁺ cells were detected in subpleural alveolar ducts.

The induction of pleural SMA⁺ cells and their apparent migration into the subpleural
alveolar ducts provides an explanation for two puzzling post-pneumonectomy observations: 1)
compensatory lung growth is not associated with histologic evidence of cellular aggregates or
classic wound healing (Chamoto et al., 2013); and 2) compensatory lung growth in mice is not
associated with compromised gas exchange (Gibney et al., 2011). A plausible explanation for
these observations is that the increase in the frequency of SMA⁺ cells (4 to 7%) was modest
and undetectable without specific SMA staining. In addition, the spatial distribution of the SMA⁺
cells in the pleura allowed direct access of the migratory SMA⁺ cells to regenerating ducts--
without the need for large-scale cellular proliferation and without functionally compromising gas
exchange.

An important observation of the single-cell qPCR analysis was the broad transcriptional
activity of the Acta2⁺ cells. In addition to the production of matrix components commonly
associated with myofibroblasts (e.g. elastin and collagen), the Acta2⁺ cells transcribed a variety
of growth factors including numerous genes associated with angiogenesis (e.g. Vegfa). Given
these findings, we have revised our conception of "myofibroblast" to include a broader role in regeneration and repair.

The most convincing evidence that Acta2+ cells contribute to lung remodeling was the transcription of elastin (Eln) and the scaffold protein fibrillin-1 (Fbn1). Elastin is not only a fundamental structural protein in the lung parenchyma, but elastin, collagen and fibrillin microfibrils constitute the core of the cable line element that supports the lung (Wagner et al., 2015). In development, the cable line element "lifts" the septa which form alveolar walls. Analogously, post-pneumonectomy neovalveolarization is associated with remodeling of the cable line element (Hsia et al., 2004; Weibel, 2008; Ysasi et al., 2015). The transcriptional profiles associated with the Acta2+ cells suggest that these cells play a central role in both septal and line element remodeling.

Single-cell qPCR allowed us to avoid “Simpson's paradox” (Simpson, 1951); that is, the statistical observation that findings attributable to a particular subset (e.g. myofibroblasts) may diminish or even reverse when subset data is combined (e.g. bulk transcriptional analysis). Single-cell qPCR also allowed us to determine gene co-expression. When analyzed by combinatorial gating, our single-cell data demonstrated the co-expression of multiple integrin β subunits and Mapk genes. Gene co-expression suggests that these co-expressed genes are both functionally related and controlled by the same transcriptional regulatory program. In conventional gene co-expression networks, co-expression is inferred by correlation or dependency (Stuart et al., 2003). Gene co-expression networks depend upon large datasets—datasets in which low frequency cell populations may be under-represented. Complimenting the “big data” approach, the single-cell approach uses actual gene co-expression within individual cells, harvested within a defined functional context, to infer a common transcriptional regulatory program. We anticipate that this approach will be usefully applied to other low frequency populations in future studies.
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Figure 1. SMA⁺ cell distribution in the murine lung. Tissue sections of the murine lung were stained with a nuclear intercalation dye (Hoechst or DAPI) as well as primary anti-SMA rabbit polyclonal antibody followed by fluorescent anti-rabbit detection antibody. Whole-lobe montages were created of the cardiac lobe for subsequent segmentation and image cytometry (A). Paired tissue samples, stained with the detection antibody alone, served as negative controls (B). The SMA⁺ cells were gated and the corresponding cells localized in the tissue image. Most of the SMA⁺ cells were localized to the central regions of the lung corresponding to large airways and blood vessels. Less than 5% localized to the pleura and less than 1% to subpleural regions of the lung (D). Areas of atelectasis or gross inflammation were excluded from the analysis. N=7 mice; mean number of cells per montage = 1.53 x10⁵ cells.
Figure 2. α-SMA expression in the cardiac lobe after pneumonectomy. A) A left pneumonectomy was performed and the remaining cardiac lobe was examined on postoperative days (POD) 1, 3, 7 and 14. B) A representative sequence of SMA expression in the pleura and subpleural region is shown. A significant increase in SMA⁺ cells was seen on POD 1 and 3 with subsequent decline on POD 7 and 14. Statistics in the upper quadrants reflects the percentage of SMA⁺ cells; the right upper quadrant reflects cells with enhanced SMA expression and increased DNA content. C) Using image cytometry, SMA⁺ cells were gated and localized to the corresponding segmented image. The initial rise in SMA⁺ cells was identified in the pleura with subsequent increase in SMA⁺ cells within the subpleural regions. To illustrate the variability and trends of SMA expression, the percent of SMA⁺ cells for 7 animals is shown for the pleura (c1), subpleura (c2) and central regions (c3) of the cardiac lobe (bar=aggregate mean). The difference between pleura and subpleural SMA⁺ cells was highly significant on postoperative days 1, 3, and 7 (p<.001).
Figure 3. Whole-lobe montages, segmented and analyzed by image cytometry, are shown for the cardiac lobe (A). Representative regions of the pleura and subpleural regions are magnified (B). C) For each cardiac lobe, the frequency of SMA⁺ cells was plotted as a function of the distance from the pleura; the modal distribution of N=5 animals is shown for each time point. Pleural SMA⁺ cells increased on day 3 with progressive increase in SMA⁺ cells in the subpleural and central regions. Note the increase in the size of the cardiac lobe, reflecting compensatory growth, on day 14. Bar = 200um.
Figure 4. Single-cell qPCR analysis of Acta2⁺ cells after pneumonectomy. A) Laser microdissection isolated subpleural "hot spots" associated with SMA staining and lung regeneration (Bennett et al., 2014). A) After laser microdissection, single cells were isolated by microfluidics and qPCR transcriptional analysis was performed (see Methods). B) Cells were identified by cell surface staining with anti-CD31 mab or the transcription profile; 4% of total cells were Acta2⁺. Principal component analysis (PCA)(C) and hierarchical clustering (D) identified two major populations of Acta2⁺ cells; a lower transcriptional activity population (designated P1) and a higher transcriptional activity population (designated P2).
Figure 5. Tiered gene expression histograms of the transcriptional profile of cells isolated from alveolar ducts. The high transcriptional activity population (designated P2 and colored pink) is bracketed by the low transcriptional activity population (P1) and all Acta2− cells (P3). The majority of the Acta2+ cells in P2 co-expressed Itgav, Itgb1 and Itgb2. As expected, the P2 cells expressed high levels of Tgfβ signaling molecules. Notably, Mapk expression was significantly higher in P2 than P1.
Figure 6. Gene expression histograms of the transcriptional profile of cells isolated from alveolar ducts. All Acta2⁺ cells (P1⁺, P2⁺, pink) were compared to all Acta2⁻ cells (P3, white). The Acta2⁺ cells consistently demonstrated expression of lung-related matrix genes (Eln, Fbn1, Col18a1, Lox, Fn1, Fbln1, Ltbp2 and Ltbp4). The Fgf gene family, commonly associated with lung development, demonstrated variable expression.