Video Article

Isolation of Pulmonary Artery Smooth Muscle Cells from Neonatal Mice

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Abstract

Pulmonary hypertension is a significant cause of morbidity and mortality in infants. Historically, there has been significant study of the signaling pathways involved in vascular smooth muscle contraction in PASMC from fetal sheep. While sheep make an excellent model of term pulmonary hypertension, they are very expensive and lack the advantage of genetic manipulation found in mice. Conversely, the inability to isolate PASMC from mice was a significant limitation of that system. Here we described the isolation of primary cultures of mouse PASMC from P7, P14, and P21 mice using a variation of the previously described technique of Marshall *et al.*[26] that was previously used to isolate rat PASMC. These murine PASMC represent a novel tool for the study of signaling pathways in the neonatal period. Briefly, a slurry of 0.5% (w/v) agarose + 0.5% iron particles in M199 media is infused into the pulmonary vascular bed via the right ventricle (RV). The iron particles are 0.2 μM in diameter and cannot pass through the pulmonary capillary bed. Thus, the iron lodges in the small pulmonary arteries (PA). The lungs are inflated with agarose, removed and dissociated. The iron-containing vessels are pulled down with a magnet. After collagenase (80 U/ml) treatment and further dissociation, the vessels are put into a tissue culture dish in M199 media containing 20% fetal bovine serum (FBS), and antibiotics (M199 complete media) to allow cell migration onto the culture dish. This initial plate of cells is a 50-50 mixture of fibroblasts and PASMC. Thus, the pull down procedure is repeated multiple times to achieve a more pure PASMC population and remove any residual iron. Smooth muscle cell identity is confirmed by immunostaining for smooth muscle myosin and desmin.

Video Link

The video component of this article can be found at https://www.jove.com/video/50889/

Introduction

Pulmonary hypertension is normal during intrauterine life since the placenta serves as the major organ of gas exchange and only 10% of the cardiac output is circulated through the pulmonary vascular bed. *In utero*, pulmonary pressures are similar to systemic pressures due to elevated pulmonary vascular resistance. As gestation progresses, there is rapid growth of the small PA within the lung, preparing the fetus for the dramatic increase in pulmonary blood flow that occurs at birth[1]. When the normal perinatal transition fails in near-term and full term infants, the result is persistent pulmonary hypertension of the newborn (PPHN). PPHN is a clinical syndrome caused by many different underlying pathologies. However, all of these infants share common pathophysiologic features such as elevated pulmonary vascular resistance, hypoxemia, and right-to-left shunting of blood flow across persistent fetal connections such as the ductus arteriosus or foramen ovale. PPHN affects 2-6 per 1,000 live births and conveys an 8-10% risk of mortality as well as significant short-term and long-term morbidity[2]. Additionally, very low birth weight premature infants may develop pulmonary hypertension as a result of their underlying lung disease. The most common underlying lung disease of premature infants is bronchopulmonary dysplasia (BPD). While the overall risk of BPD correlates with gestational age and birth weight, it remains unclear why a subset of these infants develops significant pulmonary hypertension and how to appropriately treat these infants. Poor outcomes, including prolonged hospital stays and increased mortality, are common[3-6].

Historically, ovine fetal PASMC or porcine fetal PASMC from healthy animals have been used to study the signaling pathways involved in the normal pulmonary vascular transition after birth. These are typically isolated from fifth generation resistance PA of an ovine or porcine fetus that is delivered and euthanized prior to any spontaneous respiration[7-9]. Additionally, some investigators have isolated and utilized PASMC from slightly older and spontaneously breathing lambs and piglets at 3 days, 2 weeks, and 4 weeks[10-12]. More recently, some groups have successfully isolated and utilized PASMC isolated from lambs with PPHN to examine the derangements in signaling pathways in the disease state[13-17]. These cells have proved to be a valuable tool to examine which signaling pathways are crucial in both the normal and diseased near-term and term pulmonary vasculature. However, they do not give insight into the signaling pathways impacted in premature infants with pulmonary hypertension. Nor do they allow the possibilities of genetic manipulation seen in mouse models of disease.

Rat and mouse models have long been used to model BPD and more recently are being used to model pulmonary hypertension resulting from BPD[18-22]. Neonatal rats are enticing to work with due to their larger size, but they also suffer from lack of potential for genetic modification. Genetically modified animals have been extensively used to investigate the effects of specific gene targets on whole animal physiology in neonatal mice, but to date no one has previously successfully isolated PASMC from these small mice. By isolating PASMC, greater information can be obtained about how pathways change in response to environmental stimuli and/or genetic modification specifically in the pulmonary

artery smooth muscle. Additionally, live PASMC can be imaged in real time to examine rapid changes in key signaling molecules such as calcium and reactive oxygen species[23-25]. We recently described the successful isolation of PASMC from adult mice using a variation of the technique of Marshall *et al.*[26] used to isolate rat PASMC[23,25,26]. We now have adapted and extended this technique to small mice 7-21 days of age (P7, P14, and P21). The primary limitation to this new PASMC isolation technique is that it requires multiple mice to generate sufficient cells for experiments and that the cells grow very slowly, which is characteristic of primary smooth muscle cells. Despite these limitations, we believe this technique to isolate neonatal mouse PASMC will allow for the enhanced investigation of key signaling pathways involved in the development of pulmonary hypertension and represents a significant advance in this field.

Protocol

The Institutional Animal Care and Use Committee at Northwestern University approved this protocol.

1. Pulmonary Artery Isolation from Neonatal Mice - Day One

1. Prepare the **Complete M199 media** - Mix 400 ml M199 with 100 ml (final concentration = 20%) heat inactivated FBS and 5 ml (final concentration = 1%) Penicillin/Streptomycin.
2. Prepare the **Serum Free M199 Media -** Mix 500 ml M199 with 5 ml (final concentration = 1%) Penicillin/Streptomycin.
3. Prepare the **PA Agarose** - Mix 0.05 g agarose plus 0.05 g iron particles in 10 ml sterile, serum-free M199 media.
4. Prepare the **Lung Agarose** - Mix 0.1 g agarose in 10 ml sterile, serum-free M199 media.
5. Prepare the **Collagenase** - Mix collagenase into sterile, serum-free M199 media at a final concentration of 80 U/ml and then sterile filter this mixture.
6. Set up all of the equipment ahead of time before euthanizing the mouse.
7. Set PA agarose and lung agarose to boil right before euthanizing the mouse.
8. Euthanize the mouse in the anesthetic jar with isoflurane overdose and be careful not to allow the mouse pup to come in contact with the isoflurane. The best cell yield is obtained when the cells are harvested immediately after death in order to avoid micro-clots that form in the circulation after death.
9. Remove the mouse from the anesthetic jar, secure the mouse to the operating board, and sterilize the mouse, instruments, and gloves with alcohol.
10. Load the scalpel with the blade and incise the mouse from the neck to the inferior abdomen.
11. Open the abdomen on the mouse's left side, expose the kidney, and cut the renal artery to allow the mouse to exsanguinate, thereby avoiding clots in the small vessels of the lungs.
12. Use scissors to cut along the mouse's left side of the sternum to open the thorax.
13. Carefully use scalpel to cut the diaphragm on both sides and fully open the chest cavity. Use forceps or pins to hold the chest cavity open.
14. Stick the RV with a 27 G ½ inch needle directed towards the PA and perfuse the RV/PA/lungs with sterile PBS until the lungs appear white (all blood flushed out of the pulmonary circulation). This will take approximately 3-5 ml of PBS depending on the size of the mouse. It is important to flush first to prevent clot formation in the small lung vessels.
15. Carefully use blunt dissection to expose the trachea and thread one suture underneath.
16. Put the smallest forceps underneath the trachea for support and place the 24 G angiocatheter into the trachea. Place the angiocatheter as if placing an IV.
17. Secure the angiocatheter into the trachea with the suture threaded in step 1.16.
18. Take boiling PA agarose out of boiling water, and invert to mix well. Cool the agarose to approximately body temperature by swirling on ice. The agarose needs to remain in solution but not be so hot that it will damage the cells of the PA.
19. Stick the RV with a 27 G ½ inch needle and perfuse the RV/PA/lungs with PA agarose until the lungs appear grey. The iron particles in the agarose mixture will make the lungs appear grey in color. This will take approximately 3-5 ml of PA agarose depending on the size of the mouse. It is also important to go slowly as injecting too fast might cause the iron to leak into the airways.
20. Take the lung agarose out of boiling water and invert to mix well. Then cool the agarose to approximately body temperature by swirling on ice. The agarose needs to remain in solution but not be so hot that it will damage the cells of the lung.
21. Draw up the lung agarose and attach the syringe to the 24 G angiocatheter in the trachea. Infuse the agarose through the trachea to fully inflate the lungs. The lungs will be easier to mince (see later steps) if the lungs are appropriately inflated.
22. Remove the heart-lung bloc, and submerge it into 25 ml of ice cold PBS to solidify the agarose (~5 min). Use this time to clean up the surgical area.
23. For neonatal mice (P7-P21), the best PASMC yield comes from combining 2-3 pups into one batch of cells. PASMC prefer to be in contact with other cells for optimal cell health and growth. If one mouse is used per isolation, then the cells are very sparse and do not grow well. When combining animals, harvest and chill the heart-lung blocs together and then move to mincing once all heart-lung blocs have been removed from the animals.
24. Move to the cell culture hood for sterility from this point onward.
25. Place the chilled lungs into the lid of the 10 cm tissue culture dish and carefully remove the heart, thymus, trachea, and any connective tissue from the lungs.
26. Move the lungs to the bottom of the 10 cm tissue culture dish and add approximately 1 ml ice-cold sterile PBS.
27. Mince the lungs into very small pieces (1-2 mm) with 2 scalpels.
28. Break the tip off of a sterile 5 ml serological pipette. Pipette minced lung slurry into sterile 50 ml conical tube. Use additional sterile ice-cold PBS to wash the plate to insure that all of the lung tissue gets into the 50 ml conical tube.
29. Place the 50 ml conical tube onto the magnet. Wait for the iron-containing tissue to move to side of the tube next to the magnet. Depending on the ratio of iron-containing vessel to lung tissue, some of the vessels may still be floating at this point. Aspirate off the PBS slowly and carefully trying not to aspirate floating lung tissue since it is unclear if it contains vessels.
30. Wash the lung pellet 3x with 5 ml sterile PBS, again trying to minimize the amount of lung tissue that is aspirated.
31. Resuspend the lung pellet in 3 ml collagenase and pour into 60 mm tissue culture dish. Do not try to pipette this; the lung chunks will stick to the interior of your pipette.

32. Use an additional 3 ml to rinse the tube after pouring, and pour this into 60 mm tissue culture dish as well.
33. Place into 37 °C tissue culture incubator for 1 hr.
34. Pipette tissue+collagenase slurry up and down with a 15 G blunt needle on a 5 ml syringe until all the large lung pieces are disrupted. The tissue+collagenase slurry will be cloudy at this point with no visible tissue chunks.
35. Pour into a new sterile 50 ml conical tube and attach to the magnet.
36. Wash the tissue culture dish with approximately 5 ml of complete M199 media to insure that all of the tissue has been collected, and pour this into the conical tube.
37. Aspirate off the collagenase+media supernatant. This time there will be very few floaters, and a compact iron-containing pellet will be on the side of the conical tube next to the magnet.
38. Wash with 5 ml complete media 3x. This is required to inactivate the collagenase.
39. Add 3 ml of complete media, and mix up and down a few times to resuspend the iron-containing pellet.
40. Pour the resuspended pellet into a 35 mm tissue culture dish. Using a microscope, there will be small vessel fragments floating around with iron particles contained in the vessel lumen.
41. Place the tissue culture dish (labeled plate zero) in the tissue culture incubator overnight. The first cells to migrate off onto plate zero are approximately 50% fibroblasts and 50% PASMC, based on staining for smooth muscle markers. This plate is called plated zero and ultimately discarded after the subsequent enrichment steps.

2. Pulmonary Artery Isolation from Neonatal Mice - Day Two

1. Pour the media supernatant from plate zero into a clean 50 ml conical tube.
2. Aspirate off the media supernatant. This time there will be almost no floaters, and a compact iron-containing pellet will form on the side of the conical tube next to the magnet.
3. Wash with 5 ml complete media 3x.
4. Add 3 ml of complete media, and mix up and down a few times to resuspend the iron-containing pellet.
5. Pour into a 35 mm tissue culture dish. Using a microscope, small vessel fragments floating around with iron particles contained in the vessel lumen are seen. This will be plate one (**Figure 1A**).
6. Place the tissue culture dish in the tissue culture incubator.

3. Pulmonary Artery Isolation from Neonatal Mice - Day Six

1. Pour the media supernatant from plate one into a clean 50 ml conical tube. Repeating this step multiple times insures that all of the smooth muscle cells resident in the wall of the vessel have an opportunity to migrate off onto a cell culture plate. There is a diminishing return with each step *i.e.* each plate will have fewer and fewer cells adhere.
2. Refeed plate one with complete M199 media, and place back into the tissue culture incubator.
3. Aspirate off the media supernatant. This time there will be almost no floaters, and a compact iron-containing pellet will form on the side of the conical tube next to the magnet.
4. Wash with 5 ml complete media 3x.
5. Add 3 ml of complete media, and mix up and down a few times to resuspend the iron-containing pellet.
6. Pour into a 35 mm tissue culture dish. Using a microscope, small vessel fragments floating around with iron particles contained in the vessel lumen are seen. This will be plate two.
7. Place the tissue culture dish in the tissue culture incubator.

4. Pulmonary Artery Isolation from Neonatal Mice - Day Nine

1. Pour the media supernatant from the tissue culture dish plate two into a clean 50 ml conical tube.
2. Refeed plate two with complete M199 media, and place back into the tissue culture incubator.
3. Aspirate off the media supernatant. This time there will be almost no floaters, and a compact iron-containing pellet will form on the side of the conical tube next to the magnet.
4. Wash with 5 ml complete media 3x.
5. Add 3 ml of complete media and mix up and down a few times to resuspend the iron-containing pellet.
6. Pour into a 35 mm tissue culture dish. Using a microscope, small vessel fragments floating around with iron particles contained in the vessel lumen are seen. This will be plate three.
7. Place the tissue culture dish in the tissue culture incubator.

5. Pulmonary Artery Isolation from Neonatal Mice - Day Thirteen

1. Aspirate the media off of plates 1-3.
2. Gently wash the cells with warm, sterile PBS.
3. Add a thin film of trypsin (~0.25-0.5 ml) trypsin to each plate to trypsinize off cells. Cells are very adherent and will need to be in trypsin in the tissue culture incubator for approximately 10 min to lift off the plate.
4. Harvest cells off the plates into 5 ml complete media in a 50 ml conical tube attached to the magnet to pull out any residual iron particles.
5. Wash each of the plates with an additional 0.5 ml of complete media and add that media to the 50 ml conical tube.
6. This time, take the media supernatant (do not aspirate), and discard the iron particle pellet.
7. Plate the cells in 5 ml complete M199 media in a 60 mm dish to have confluent cells in a few days. Alternatively, plate the cells in 10 ml complete M199 media in a 10 cm plate, and PASMC will take approximately another 1-2 weeks to reach confluence.

6. Pulmonary Artery Isolation from Neonatal Mice - Day Fourteen

1. Change the media to fresh complete M199 media to remove any residual trypsin. There will be a thin population of PASMC on the tissue culture dish (**Figure 1B**).

7. Routine Care

1. Change the media to fresh complete M199 media approximately 2x per week.
2. When splitting these cells, approximately 10 min in the incubator in a film of trypsin (0.25-0.5 ml) is required for the cells to lift off the plate. Using the trypsin film allows replating of the PASMC without spinning down the cells. If too much trypsin is used, then the PASMC must be centrifuged to spin out the trypsin, or the cells will not readhere to the plate. Sometimes, after spinning down the cells, it is hard to get the PASMC resuspended into complete M199 media.
3. In observations, the isolated PASMC behave like smooth muscle cells reliably for 4-5 passages, but the isolated cells will stain for smooth muscle cell markers up to passage 7. Count the first pooled 60 mm or 10 cm plate on day thirteen as passage 2 (plating after first exposure to trypsin).

Representative Results

During and after isolation, PASMC are examined both by light microscopy and by immunostaining for smooth muscle cell markers. By light microscopy early in the protocol, PASMC are seen migrating onto the tissue culture dish from the small iron containing vessels (**Figure 1A**). After pooling plates one through three on day thirteen, then iron particles are no longer seen as those have been pulled out in the final pooling step. Instead, a population of PASMC is seen on the tissue culture dish (**Figure 1B**).

Based on immunostaining, the initial cells that migrate off the iron-containing vessels are approximately 50% fibroblasts and 50% PASMC (data not shown). Since fibroblasts have a significant growth advantage, the fibroblasts will over time overgrow the PASMC on plate zero. For this reason, plate zero is discarded once there are clearly isolated PASMC on the subsequent plates. After pooling, the population of PASMC is >90% PASMC which stain positive for both α-smooth muscle myosin and desmin (**Figure 2**). When imaged at 20X, multiple spindle-shaped cells are seen consistent with a smooth muscle cell phenotype (**Figure 2A**). When imaged at higher magnification (40X), the lamellopodia of the leading edge of single cells are visualized as they migrate and grow towards other smooth muscle cells on the dish (**Figure 2B**).

Phosphodiesterase 5 (PDE5) is expressed in PASMC and hydrolyzes cGMP, a key mediator of vascular tone, into inactive GMP. Decrease in PDE5 plays a critical role in the normal pulmonary vascular transition after birth. In large animal models, PDE5 is developmentally regulated across gestation, and its expression and activity fall dramatically after birth[27]. In order to confirm that these isolated PASMC retain a phenotype consistent with their developmental stage, we examined PDE5 enzyme activity in mouse PASMC isolated from P7, P14, and P21 mice, as well as adult mice. We see the highest levels of PDE5 activity in the PASMC isolated from P7 mice. These levels fall in the PASMC isolated from P14 and P21 mice. The lowest levels of PDE5 activity are noted in the PASMC isolated from adult mice (**Figure 3**).

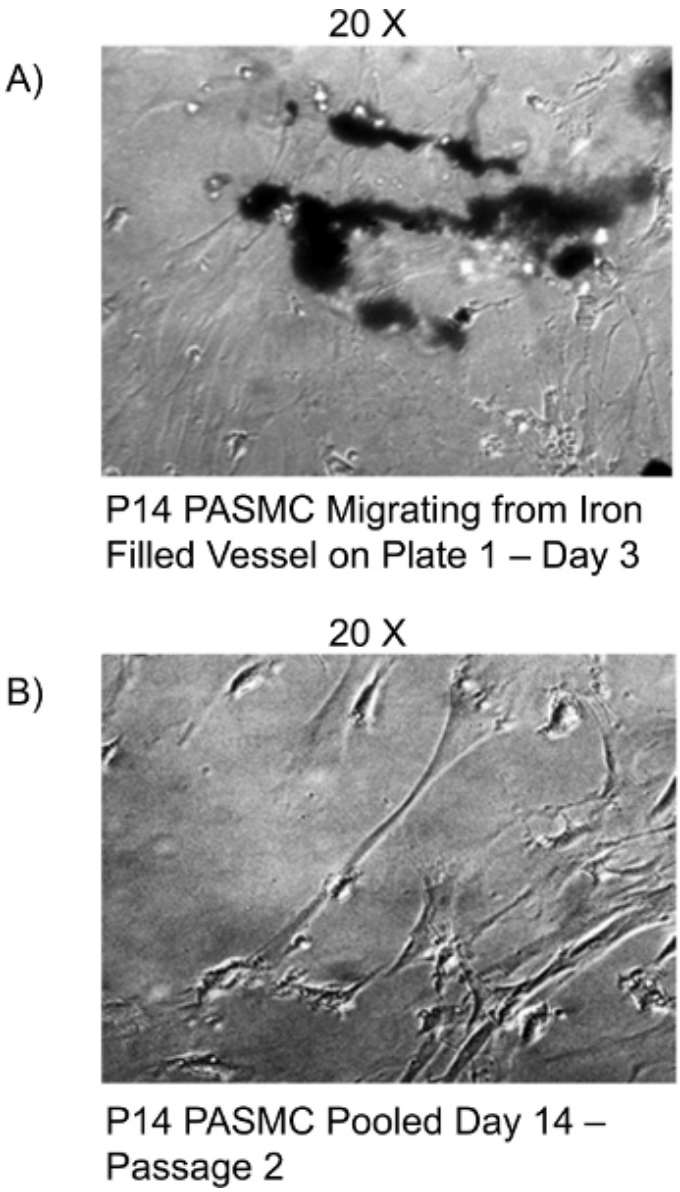

Figure 1. Neonatal PASMC Visualized By Light Microscopy. PASMC are visualized using a light microscope at 20X. **A)** On day three of the protocol, spindle-shaped PASMC can be seen migrating from the black iron-filled small PA onto the tissue culture dish. **B)** On day fourteen after pooling, a population of PASMC can be seen on the tissue culture dish.

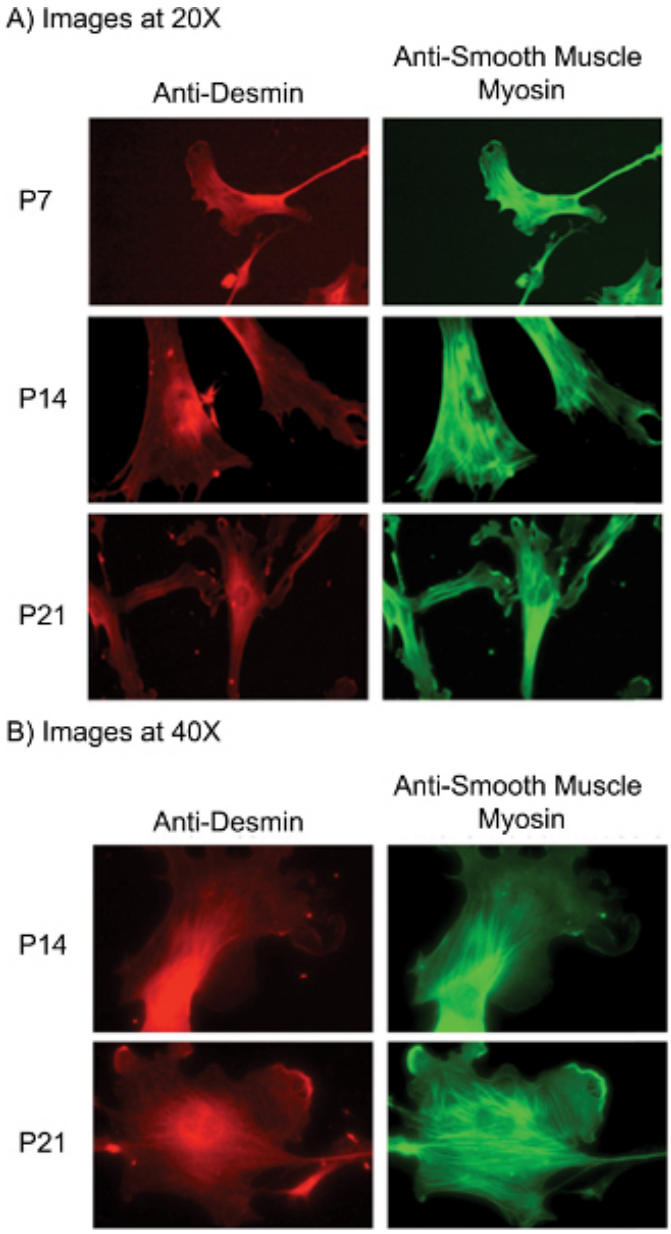

Figure 2. Neonatal PASMC Stain Positively for Smooth Muscle Markers. PASMC were plated onto collagen-treated glass coverslips, fixed in 4% formaldehyde, and permeabilized with 0.2% Triton-X as previously described[7,23]. **A)** PASMC from P7, P14, and P21 mice were stained with anti-desmin (1:200 dilution) or anti-smooth muscle myosin (1:2,000 dilution) in 5% BSA, followed by rhodamine-red anti-rabbit secondary at a 1:200 dilution. Fluorescence was visualized with an epifluorescence microscope at 20X. **B)** PASMC from P14, and P21 mice were stained with anti-smooth muscle myosin or desmin as above, and fluorescence was visualized as above at 40X.

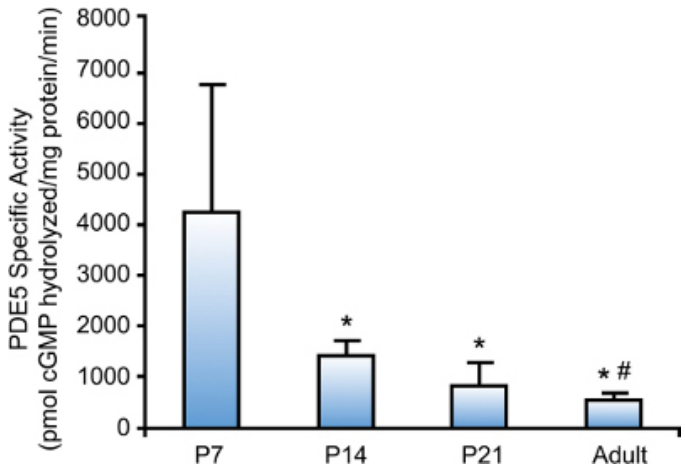

Figure 3. Phosphodiesterase 5 (PDE5) Activity is Developmentally Regulated in PASMC. PASMC were harvested for total protein and assayed for PDE5 enzymatic activity as previously described using a commercially available colorimetric cyclic nucleotide phosphodiesterase assay kit[7]. Each sample was read ± sildenafil (100 nM). The difference between the pmol cGMP hydrolyzed per mg total protein per minute ± sildenafil represents the PDE5-specific cGMP-hydrolytic activity. N=4 for P7 PASMC, n=10 for P14 PASMC, n=4 for P21 PASMC, and n=8 for adult PASMC. * $p<0.05$ versus P7 PASMC, # $p<0.05$ versus P14 PASMC.

Discussion

In this manuscript, we describe for the first time the isolation of PASMC from mice at P7, P14, and P21. In order to accomplish this, a slurry of agarose and 0.2 μM iron particles are infused through the RV into the PA. Due to the small size of the iron particles, they cannot pass through the pulmonary capillary bed and are thus deposited in the small PA. The lungs are inflated, removed and dissociated. The iron-containing vessels are pulled out of solution using a magnet. Ultimately, the vessels are plated into a tissue culture dish, and cells migrate off the vessels and onto the culture plate. The initial plate of cells is a mixture of fibroblasts and PASMC, and the pull down procedure is repeated multiple times to derive an enriched PASMC population. With prolonged exposure at high levels, there is a theoretical risk that residual iron may impact the redox balance within the isolated cells. Thus, care is taken in the last step to remove all residual iron prior to pooling and propagating the cells. Immunostaining for α-smooth muscle myosin and desmin is done to confirm that the enriched population is >90% PASMC. These cells have PDE5 enzymatic activity, consistent with a vascular smooth muscle cell phenotype. Interestingly, the PDE5 enzyme activity is different in PASMC isolated from mice of different ages, suggesting continued developmental regulation within the PASMC after isolation.

While this technique is intellectually very straightforward, a common problem is low yield of PASMC. There can be multiple reasons for this problem, even when 2-3 mice are pooled together. First, the best cell yield is achieved if the isolation procedure is initiated immediately after the mouse succumbs to the isoflurane overdose. Once death has occurred, micro-clots form throughout the vascular bed. These clots in the pulmonary vasculature will prevent the iron particles from getting into as many small PA, thereby decreasing the yield. Also, the viability of the isolated PASMC becomes of greater concern the longer the mouse is dead prior to isolation. The absolute key step that impacts PASMC yield is the iron infusion. If the lungs are not noticeably grey after the iron infusion, the possible reasons are: 1) the iron is going retrograde into the right atrium and liver, 2) there is hole or tear in the RV, or 3) there are micro-clots in the pulmonary circulation somewhere preventing good iron infusion. Lastly, we have noticed that PASMC isolated from different genetically modified strains of mice exhibit strikingly different growth rates in culture after isolation. Therefore, when first attempting this technique, it is best to use a wild-type strain of mice.

Obviously, contamination is a concern any time one is isolating cells from a whole animal. All instruments are sterilized and wiped with alcohol prior to the procedure, and then once the heart-lung bloc is removed, all of the subsequent steps are done in a tissue culture hood with sterile technique. Using these precautions together with antibiotic-containing media has virtually eliminated any contamination.

With the isolation of any primary cell line, there is a concern about how long a cell will maintain its phenotype in culture. Ovine FPASMC isolated from control and PPHN lambs maintain their smooth muscle phenotype for 8 and 5 passages, respectively[7,14]. For the mouse PASMC, the cells begin to lose signaling responses by passage 5 and smooth muscle markers by passage 7 (data not shown). This highlights one of the limitations of this technique. Multiple mice are required to achieve small amounts of cells, and the time from isolation to enough cells for experiments is long; 2-4 weeks depending on the number of cells needed. Finally, once cells are isolated, one only has three passages to work with them before they lose their smooth muscle cell signaling responses. One potential area for improvement would be to develop a method whereby these cells could be frozen and recovered successfully from liquid nitrogen. This would allow a lab to regularly make and freeze cells as mice are available, and then investigators could thaw and utilize the cells for experiments as needed.

Despite the technical challenge of performing the isolation and the long timeline for isolation, these PASMC represent a novel and invaluable tool for the study of signaling pathways in the developing murine pulmonary vasculature. We believe this technique to isolate neonatal mouse PASMC will allow for the enhanced investigation of key signaling pathways involved in the development of pulmonary hypertension, which will lead to better understanding of the disease pathogenesis and allow for development of new treatments.

Disclosures

The authors declare that they have no competing financial interests.

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