University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme
Summer Studentship
Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student

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2. Supervisor:

Surname: Johnstone Forename: Scott

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3. Research Project Report

3.1 Project Title (maximum 20 words):

Pannexin 1 Expression in Vascular Tissue in Cardiovascular Diseases

3.2 Project Lay Summary (copied from application):

Atherosclerosis is a major underlying condition causing cardiovascular diseases such as myocardial infarction. It is known that altered expression of connexin 43 (Cx43) is associated with increased atherosclerosis risk. Links have now been shown between Cx43 and Pannexin 1 (Panx1) leading to ATP release, cell apoptosis and inflammation. Thus balance of protein expression may be important in atherogenesis. In this project, it is hypothesized that Cx43 and Panx1 expression are inversely related and may control vascular inflammation. In this project, changes in protein expression in vascular tissue are detected- aorta & carotids.

3.3 Start Date: 09 Jun 2014 Finish Date: 01 Aug 2014

3.4 Original project aims and objectives (100 words max):

Based on preliminary data, we hypothesized that expression of Cx43 and Panx1 are inversely related and may regulate vascular inflammation. To investigate this effect Panx1 expression in mice with normal (wild type) and reduced (heterozygous) Cx43 expression are compared along with diseased vessels (ligated carotids).

3.5 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

Samples: All tissues samples from 12-18 month old Cx43wt (C57BL/6) and $Cx43^{+/-}$ mice were harvested in advance of the project start date as paraffin embedded for imaging or in lysis buffer for protein analysis. An n=3 samples for each genotype and experiment was provided

Immunofluorescence: Tissues in paraffin blocks were sectioned, de-paraffined and proteins were detected with primary and secondary antibodies which were then visualized on a Zeiss 510 confocal microscope. The images generated were used to report on levels of expression and cellular localization of the proteins in vascular tissues (carotid and aorta).

Western Blotting: To investigate the levels of protein expression in vascular tissues, Western blotting of tissue lysate samples was carried. Following samples preparation, gel separation, transfer to nitrocellulose membranes, the expression of Panx1 and Cx43 was detected using beta-tubulin as a loading control. Protein concentration in each of the samples was determined and quantified using Licor Odyssey near infrared scanner and software.

H&E Staining: To identify the structure of of each of the vessel walls and check for gross morphological changes, H&E staining was done using well-established protocols. Images of each of the tissues were taken and compared.
Reporting: Data were quantitated and statistical significance was determined between samples

3.6 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

Samples: Aorta samples were embedded in paraffin blocks before start of project. Carotids were provided in paraformaldehyde, I dehydrated these and paraffin embedded the carotids. Using a microtome, I performed tissue sectioning for each of the samples, producing slides with 5μm samples on each. For the tissue samples I cut, dounced then sonicated to break down the tissues and cells. Then samples were spun to remove large contaminants and proteins quantified for each for Western blotting.

Immunofluorescence: After some troubleshooting, I eventually optimized the protocol such that the immunofluorescence produced a good signal, showing specific Cx43 staining in the aortas and carotids (Cx43 found in the tunica media layer) as shown in **Appendix 1-2**. Following this, I tested Panx1 staining which I identified in the endothelial layers as shown in **Appendix 3**. To represent a disease state (neointimal formation), I performed immunofluorescence on ligated carotids for Cx43 and Panx1 as shown in **Appendix 4.**

H&E Staining: H&E staining shows fairly similar vascular structure of wild type and heterozygous aorta & carotids, we saw no apparent gross morphological changes between the two tissue types (**Appendix 5**)

Western Blotting: Similar to immunofluorescence staining, there were issues with the blots developed due to formation of dimer proteins. Our internal control protein (β-tubulin) exists as a monomer at 50kDa, yet I identified it mainly at 100kDa suggesting a dimer form. As a result of this quantification of Cx43 or Panx1 expression in these tissue samples was not possible. Due to time constraint, we turned to cell culture lysates in order to check Cx43 expression in vascular smooth muscle cells and quantify the proteins (**Appendix 6**).

3.7 Discussion (500 words max):

Immunofluorescent staining for Cx43 in the carotids and aortas appeared to show reduced levels of Cx43 staining in the heterozygous mice smooth muscle layers. This is in keeping with several publications suggesting that heterozygous deletion of Cx43 reduces the overall signal of the proteins. After obtaining images for specific staining for the proteins, I attempted to measure the fluorescence of the images with Image J. However, due to the different background of the control, I was not unable to determine the actual fluorescence of the images as the software showed similar fluorescence of both due to the different background of the tissue. The issue of background staining reduced the reproducibility of my results. In future studies I would aim to reduce this background autofluorescence by pretreating the sections with Sudan Black which is a known protocol for reducing auto fluorescence.

Another problem I encountered during quantification was the apparent aggregated expression of proteins in several regions of the tissue which made measurements skewed towards higher protein expression. The primary reason for this appeared to be the thickness of the tissue, compounded by taking confocal images. The confocal measures signal in a precise location on the tissue meaning that staining above or below the plane of view is not seen. Our tissues appear to be thicker than expected and were found to be on multiple focal planes. In future studies I would aim to take thinner sections and analyse the images using a compiled Z-stack of images.

Staining for Panx1 which has previously been identified in the endothelium layer was seen in my samples. However there were no apparent differences (as expected) between the wild type and knockdown mice. In addition it was difficult to quantify changes in the endothelium as it forms a very thin layer which is difficult demarcate with the software.
Some of the complicating factors may also have been compounded by the use of older mice. Mice were 12-18 months old and we identified several abnormalities in their tissues e.g. weakened vessel walls. Additionally comparisons should have been made with more diseased animals such as the ApoE-/- mice, but given time constraints we never investigated these.

Initial use of tissue lysate from aorta and carotids sample showed dimerization of proteins. We tried to break down the dimers by heating the samples at 95^{o}C for 1 minute or adding dithiothreitol (DTT) to the loading dye. Despite our attempts we were not able to produce sufficient quality Western blots for analysis. In order to practice detection and measurement of proteins we used cell culture lysates from human coronary artery smooth muscle cells. Using these samples I was able to identify Cx43 and B-tubulin at the correct molecular weights. The samples demonstrated a general decrease in Cx43 expression as a

result of increasing passage number and decreasing Foetal Bovine Serum (FBS) concentration in the media.

4. Reflection by the student on the experience and value of the studentship (300 words max):
Through this studentship, I was exposed to the different components of research work- including wet laboratory experiments, reading of scientific journals, data analysis, attending talks as well as presentation skills. This studentship definitely provides me with a better concept of what a career in research work is like. I was given the opportunity to talk to several post-doctor fellows, PhD and master students who shared their experience with me and provided advice which will help in my future education decision.

Given the unforeseen difficulties and obstacles met, the number of sections stained and gel ran were more than expected. It took me 6 weeks to get the immunofluorescence protocol right which was time-consuming but at the same time served as an opportunity to practice skills and coordination required for the procedures but also gave me the chance to familiarise myself with the procedure, read up on reagents used, previous papers published and to troubleshoot and question each and every step of the protocol. It was only with these misfortunes that I sense fulfilment when my final usable results were obtained at the end of the studentship.

Apart from the hands-on experience, I also learnt about data analysis and presentation that are the basis for writing up reports and doing presentation slides.

Within the short span of 8 weeks, not only was I able to look at wild type and heterozygous mice, I also had to opportunity to explore and look into diseased mice (ligated carotids, POVPC-treated carotids and PDGF-treated carotids).

5. Dissemination: (note any presentations/publications submitted/planned from the work):

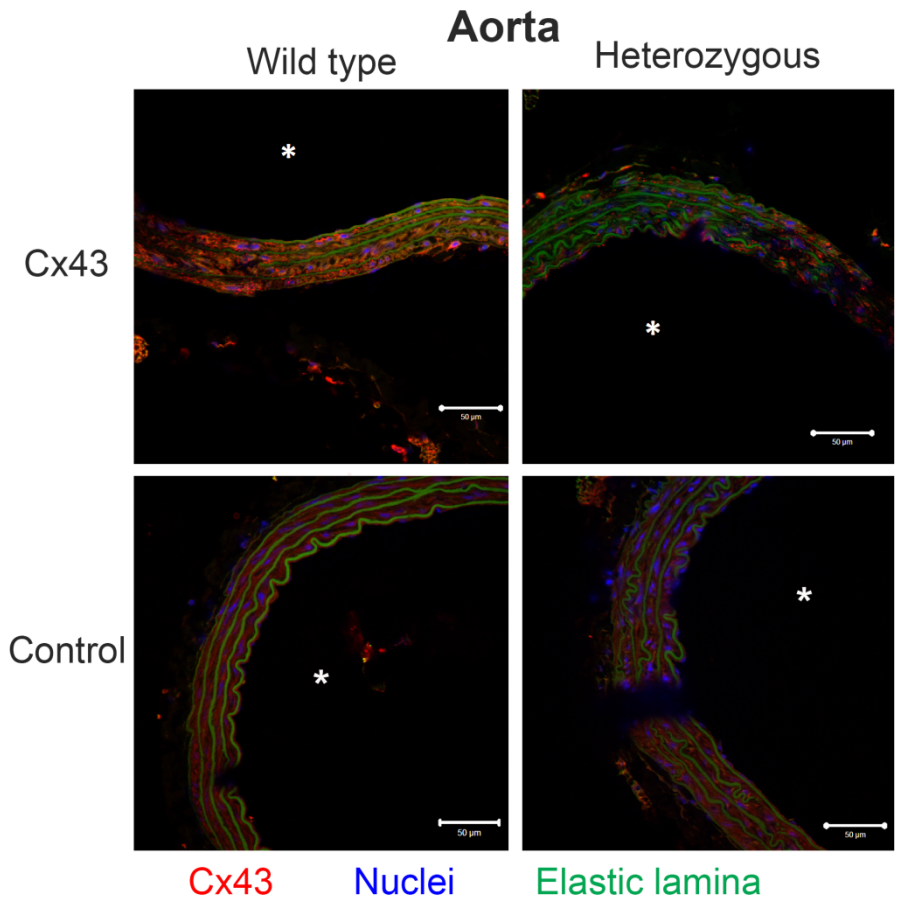
I presented my findings at the Young Investigator Network meeting, however further dissemination of my data is not anticipated.

6. Signatures:

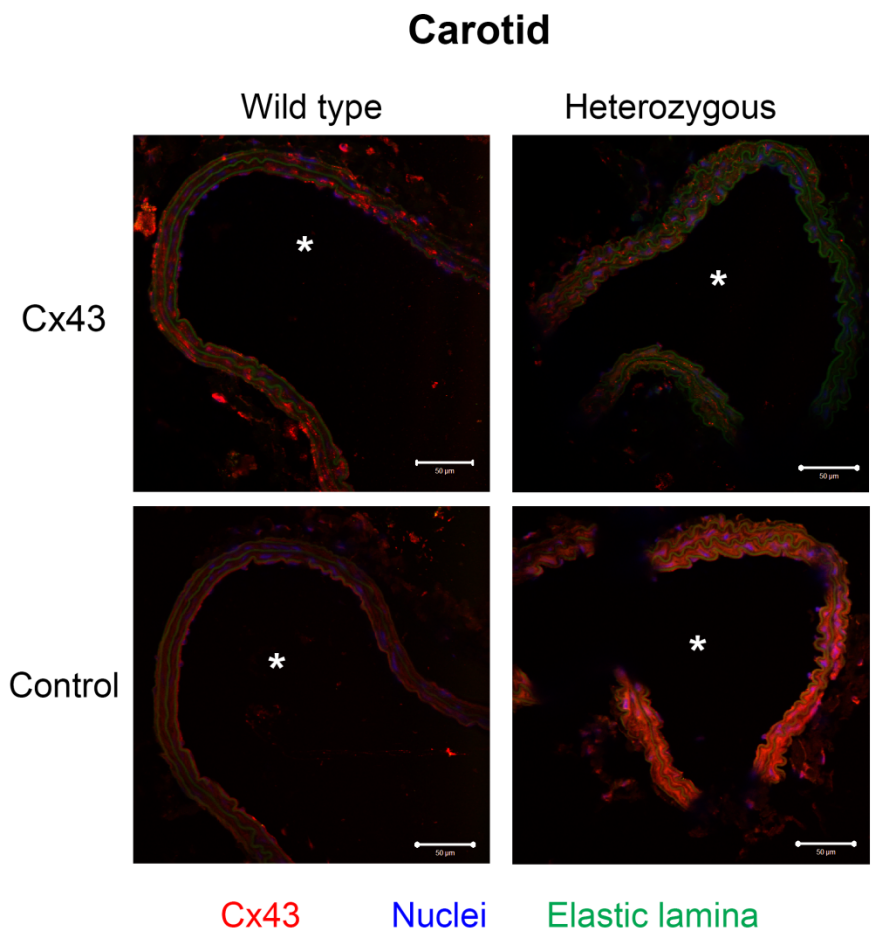
Supervisor S. R. Wheatcroft Date: 01/08/2014

Student Date: 01/08/2014

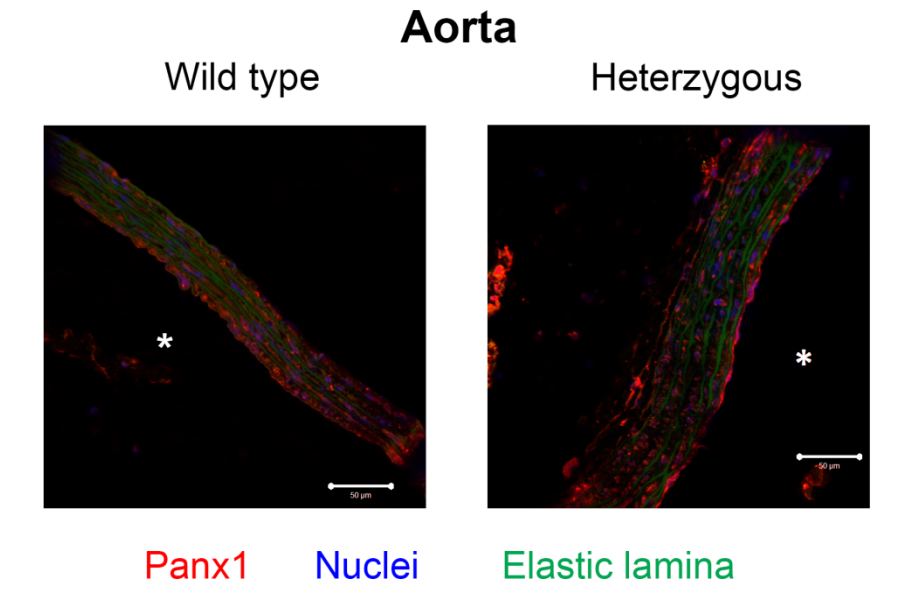
Appendix

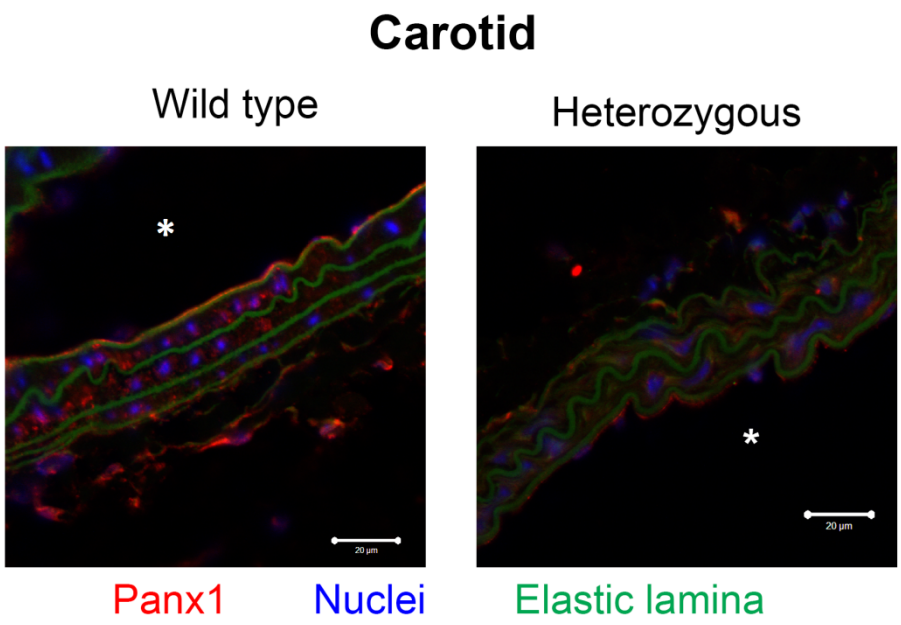


Appendix 1: Cx43 staining in the mouse aortic wall. Staining of Cx43 was performed in wt (top left) and heterozygous mice (top right). No-primary controls were used for each sample (bottom left and right). Scale bar is 50um, Cx43 (Red), elastic lamina (green) nuclei (blue) are shown in each image. * represents the luminal side of the vessel.

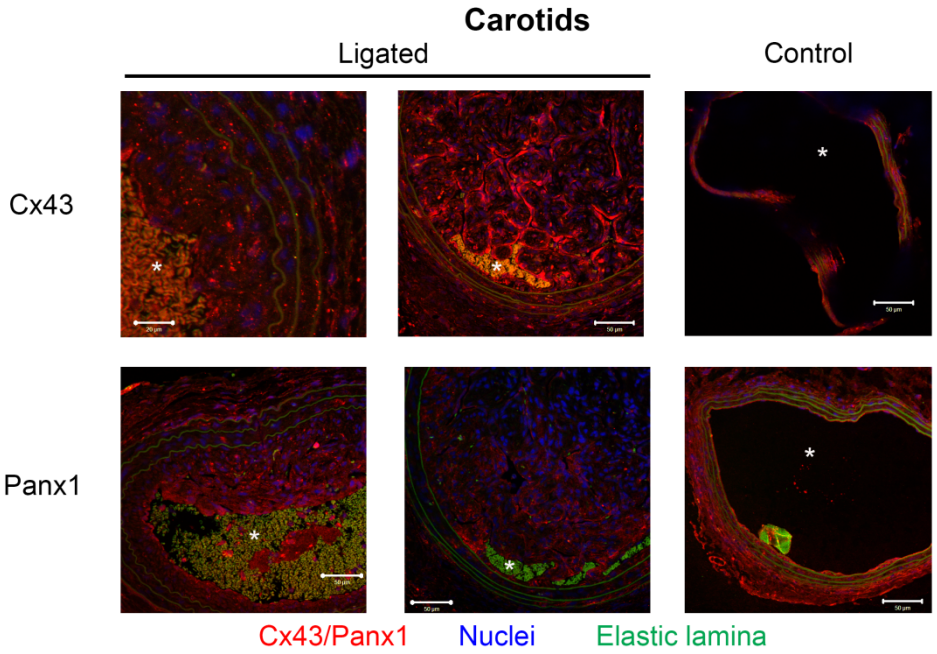


Appendix 2: Cx43 staining in the mouse carotid. Staining of Cx43 was performed in wt (top left) and heterozygous mice (top right). No-primary controls were used for each sample (bottom left and right). Scale bar is 50um, Cx43 (Red), elastic lamina (green) nuclei (blue) are shown in each image. * represents the luminal side of the vessel.

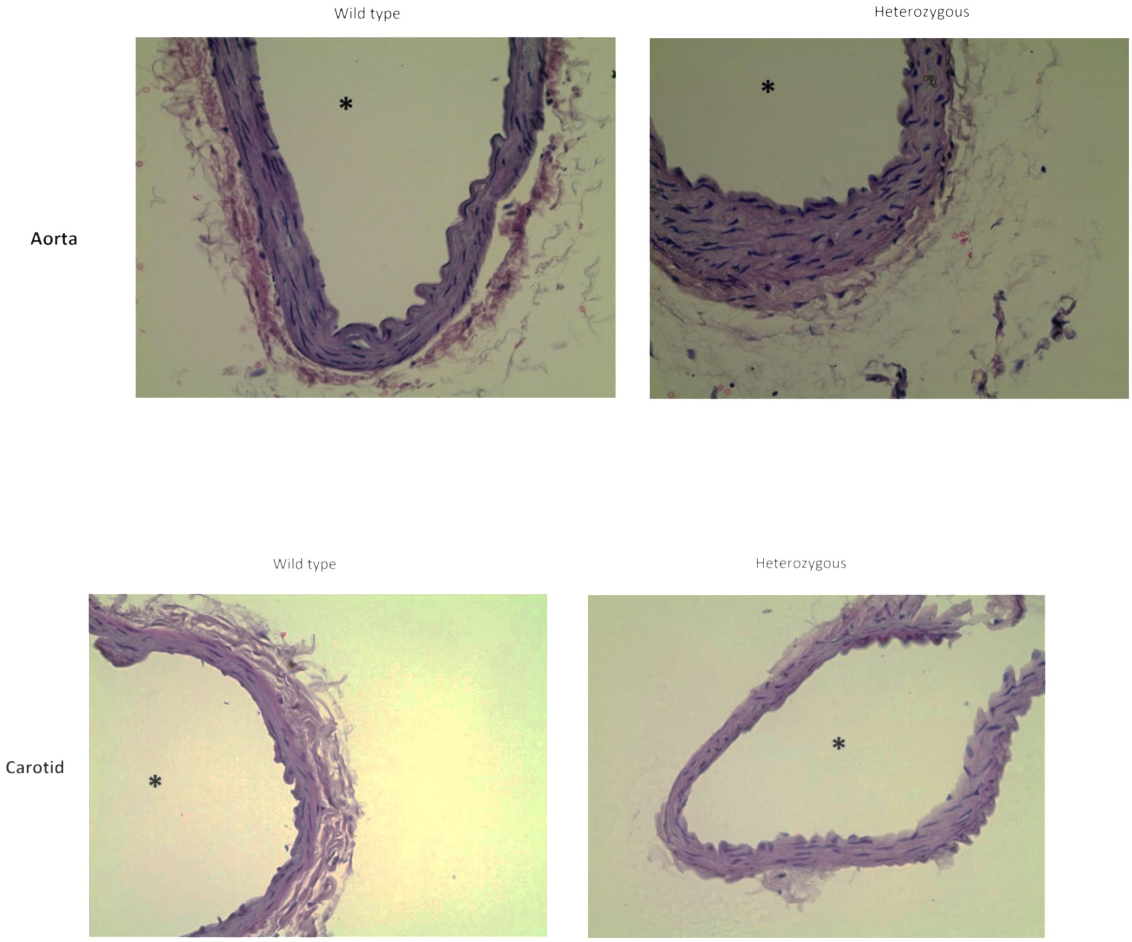




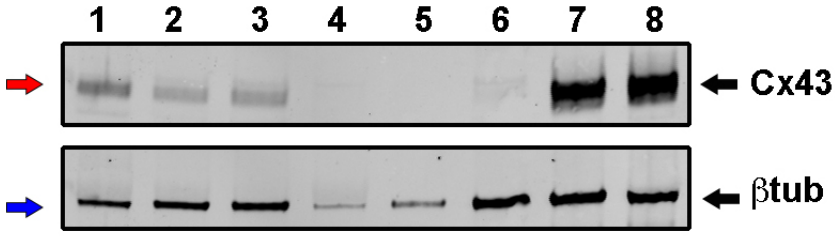
Appendix 3: Panx1 staining in the mouse aorta and carotid walls. Staining of Panx1 and Cx43 was performed in wt and heterozygous mice. No-primary controls were used for each sample (not shown). Scale bar is 50um, Cx43 (Red), elastic lamina (green) nuclei (blue) are shown in each image. * represents the luminal side of the vessel. Panx 1 expression can be clearly seen in the endothelium (cells lining the lumen). Significant changes in Panx1 staining were not identified.



Appendix 4: Cx43 staining in the mouse ligated carotids. Staining of Cx43 and Panx1 was performed. No-primary controls were used for each sample (right panels). Scale bar is 50um, Cx43 (Red), elastic lamina (green) nuclei (blue) are shown in each image. * represents the luminal side of the vessel. Pannexin 1 Expression was detected in each sample, but quantification was impaired by background autofluorescence.



Appendix 5: H&E staining of Cx43 wt and Heterozygous mouse aorta (top) and carotids (lower). No apparent morphological changes were detected. * represents the vessel lumen.



1 - Passage 3
2- Passage 4
3- 15% Serum
4- 10% serum
5 - 1% Serum
6 - 0.5% Serum
7 - 0.1% SErum
8 - PDGF

Appendix 6: Western Blotting of Cx43 in vascular smooth muscle cells. Lysates of vascular smooth muscle cells at different passage numbers and using different concentrations of serum in the media were tested for expression of Cx43. Molecular weights are shown Red arrow is 37kDa and Blue arrow is 50kDa. The results show an apparent decrease in Cx43 expression with increase passage number and reduced serum levels.