Project 1: Investigating the role of FAM198b in angiogenesis, tumourgenesis and wound repair

by

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Contents

Tables Page Number

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Introduction

Family with sequence similarity 198, member B (FAM198b), which is also known as C4orf18, ENED, AD021, AD036, TCPD2512, DKFZp434L142 and FLJ38155, has been identified as being expressed in human umbilical cord endothelial cells (HUVECs) (unpublished data). Additionally microarray data published by Sun *et al*. (2005) identified the expression of FAM198b as being increased in human microvascular endothelial cells in a 3-D microcarrier bead based angiogenesis system. Currently the structure and function of FAM198b is not known, but its presence in endothelial cells and upregulation in bead based angiogenesis assays (sprouting, branching, and capillary network formation) could indicate a potential role in angiogenesis.

Currently, little is known about the structure or function of FAM198b. However some databases have compiled information from whole genome microarray studies, from which the expression of FAM198b within different tissues can be explored (figure 1). Many of these studies have been carried out in cancer cell lines or cells from mammalian animal models, but can shed some light on the expression of the human equivalent of FAM198b. For example FAM198b is consistently shown to be under expressed in tissues associated with the liver.

Microarray data sets can be compiled and analysed to produce genes maps, which can predict interactions between genes. One such gene map analysed by Hecker *et al*. (2009)

predicted that FAM198b could be under the transcriptional control of the TATA-binding protein; Transcription Factor II D and the Hepatocyte Nuclear Factor 1 homeobox A (HNF-1A). Whilst these interactions have not been validated it is interesting that the up-regulation of HNF-1A has been linked to the down-regulation of FAM198b after the onset of anti-rheumatic therapy. HNF-1A is known to be predominantly expressed in the liver, which would be consistent with observation of FAM198b being down-regulated in liver. With respect to angiogenesis it is also interesting that FAM198b was down-regulated after the onset of anti-rheumatic therapy. As angiogenesis can be stimulated by inflammation, this data could indicate that FAM198b is a pro-angiogenic factor. The microarray data does not however address the fact that FAM198b has been predicted to have three splice variants, which would produce two different proteins (figure 2). Additionally the microarray data does not differentiate between the different expressions of different splice variants. It is possible that the expression of the FAM198b splice variants could be preferentially expressed in different tissues.

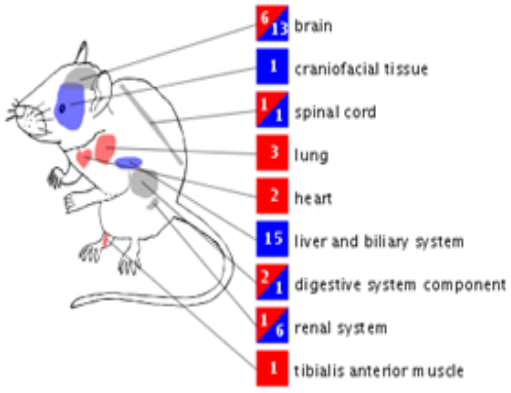

Figure 1: Compilation of microarray studies where FAM198b expression is modified

The number of published studies utilising microarray data, which have found FAM198b to be over expressed has been depicted in red. The studies which suggest that FAM198b is under expressed have been depicted in blue (Diagram obtained from European Bioinformatics Institute, 2011).

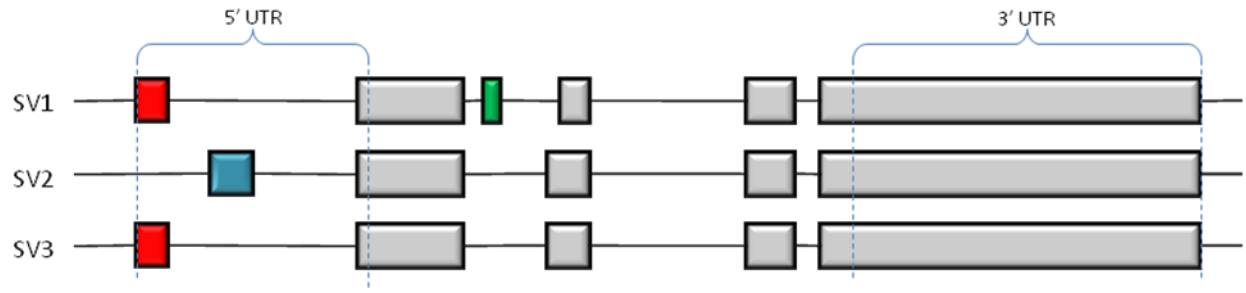

Figure 2: A diagrammatic representation of the three splice variants predicted to be expressed by FAM198b.

The regions of the FAM198b gene thought to be exons are depicted by boxes, whereas the introns are depicted by black lines. The exons shared by the three variants are shown as grey, whereas the differing regions are coloured.

Splice Variant 1 (SV1) - SV1 shares an alternative exon in the five prime untranslated region (5′ UTR) with the third splice variant. SV1 also contains a unique exon, which is not expressed in the second or third variants. The forth exon in SV1 also thought to be a shortened version of the third exon thought to be present in the other two variants.

Splice Variant 2 (SV2) - SV2 is thought to contain a unique exon in the three prime region of its transcript.

Splice Variant 3 (SV3) - SV3 would encode a protein identical to SV2 despite sharing a 5′ UTR with SV1.

(Original Diagram).

It is currently unknown whether the first and second isoforms of the FAM198b protein have similar or different functions. But it is possible to determine the degree to which FAM198b has been conserved over time. This is possible by aligning the protein sequences of isoform 1 (SV1) and isoform 2 (SV2 and 3) to their homologous proteins in different species. This information can give insights into the importance of the protein within the cell. A protein which is essential to the viability of an organism would have a highly conserved sequence, whereas a redundant protein might not be highly conserved. Groups of amino acid sequences which are more highly conserved than others within a protein can also indicate which regions of a protein are more important to its function. This is particularly

true for angiogenesis, largely due to the high level of conservation among vertebrates of signalling factors involved in angiogenesis (Adams and Eichmann, 2010).

FAM198b-like proteins are present in a wide variety of organisms and are relatively highly conserved when compared to the sequence of FAM198b in man. The key difference between FAM198b isoform 1 and 2 stems from the alternative exon in SV1, the protein sequences for this region in both isoform 1 and 2 are shown in figure 3. The unique sequence encoded in isoform 1 has only been found in the transcripts of higher primates such as the chimpanzee. Whereas the alternative sequence found in isoform 2 has been conserved in many different phylogenies. The knowledge that FAM198b isoform 1 is present in chimpanzee provides evidence that the protein is probably expressed. However it is likely that isoform 2 could have a more significant role in the viability of an organism, as it is conserved in many phylogenies. This does not exclude the possibility that isoform 1 could play an important role in higher primates and man.

The analysis of the protein sequence can also provide information into the function of FAM198b using bioinformatics. Clark *et al*. (2003) listed FAM198b in their supplementary information as being a possible transmembrane protein in the golgi apparatus. This was predicting using several approaches including the prediction of signal proteins, hydrophobic properties of signal sequences and searching for sequence similarities to known receptors and ligands.

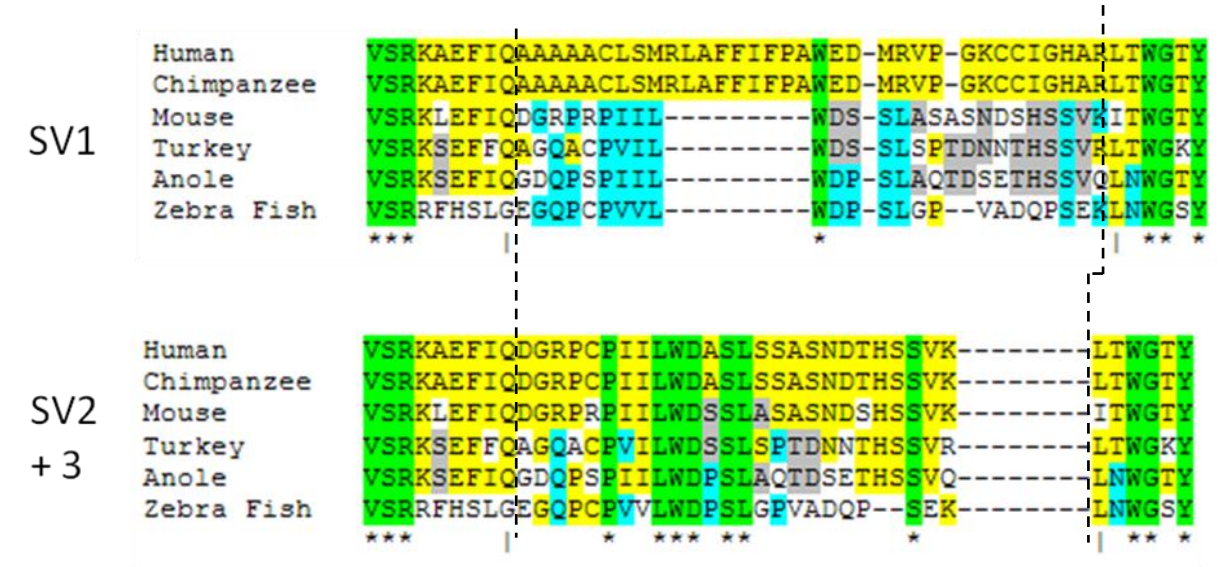

Figure 3: Protein sequence alignments for FAM198b and FAM198b-like proteins.

The amino acids which are conserved in all the above species are represented in green. The amino acids conserved in multiple species except zebra fish are represented in yellow, whereas the amino acids which were conserved but not humans are represented in blue (Original diagram).

Angiogenesis

Angiogenesis is the process of developing new blood vessels from existing vessels. The process of developing new vessels has an essential role in wound healing and normal development. However angiogenesis can also be a precipitating factor in the etiology of many diseases such as cancer. Endothelial cells are a significant contributor to these processes. Despite their low turn over, endothelial cells retain the potential for high levels of proliferation in the adult, which when activated can contribute to regenerative or pathological processes. Due to the connection between the vasculature and cancer, blood vessels in the tumour have been the target of anti-cancer therapies. These strategies are not only designed to inhibit tumour growth, but also cancer cell dissemination (Riasu, 1997; Carmeliet 2003; Ferrara and Kerbel 2005; Martin and Murray, 2009; Arroyo and Iruela-Arispe, 2010).

Angiogenesis is complicated process involving many mechanisms. So far four types of guidance molecules have been shown to be involved in vascular development. It is interesting to note that these molecules are also involved in neuronal guidance. The Netrins, Ephrins, Semaphorins and Slits act to attract or repel migrating endothelial cells. Different combinations of attractive and repulsive signals are expressed in the surrounding tissue, which aids in the navigation of developing vessels through “tissue corridors” (Martin and Murray, 2009; Arroyo and Iruela-Arispe, 2010).

In adults capillary sprouting is one of the two known mechanisms of forming new blood vessels. The vascular endothelial growth factor (VEGF) pathway is one of the most significant regulators of capillary formation. Many of the biological functions in the VEGF pathway are induced by VEGF and are transduced by vascular endothelial growth factor receptor (VEGFR) 1 and 2. Gradients of soluble VEGFR1 are able to modulate the activation of VEGFR2. This enables the fine tuning of the events which guide endothelial cells during capillary sprouting (Martin and Murray, 2009; Arroyo and Iruela-Arispe, 2010).

The endothelial cells responsible for the initial stages of vascular development and capillary sprouting differentiate into tip cells once stimulated (figure 4). Tip cell formation and differentiation is controlled by the Notch pathway. These specialised endothelial cells respond to gradients of VEGF and other molecules, at which point they proceed to invade the extracellular matrix (ECM). Tip cells break through the basement membrane, interstitial matrix, and the fibrin matrix by using proteases such as matrix metalloproteinase’s. Tip cells are capable of extending long filopodial protrusions, which control the guidance of migration and react to external stimuli. Only a small proportion of endothelial cells acquire the characteristics associated with tip cells. The majority of the endothelial cells remain in place to maintain the functional integrity of the vessel. The endothelial cells that remain behind

the tip cell proliferate, elongate and form a lumen as the tip cell migrates towards attractive signals. Once the capillary sprout has formed a lumen and fuses with a blood vessel to allow blood flow, the endothelial cells return to their quiescent state (Adams and Eichmann, 2010; Arroyo and Iruela-Arispe, 2010; Carmeliet and Jian, 2011).

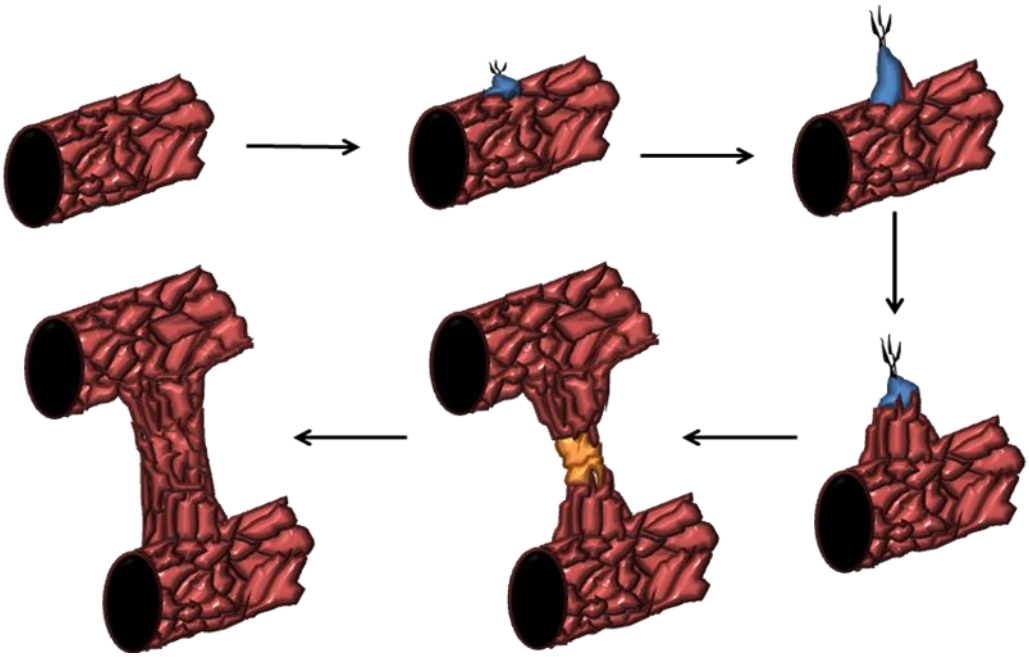

Figure 4: Tip cell formation, angiogenic sprouting and blood vessel growth.

When endothelial cells in mature blood vessels are activated in response to angiogenic stimuli they form tip cells. The tip cell will start to migrate once has broken through the basement membrane and has lost contact with surrounding mural cells. These tip cells are followed by proliferating endothelial cells, which assemble to form capillary-like sprouts. The new sprouts will eventually fuse with themselves or existing blood vessels to establish blood flow. (Original diagram compiled using information from Carmeliet, 2003; Adams and Eichmann, 2010).

Capillary sprouting is a significant contributor to angiogenesis, especially in areas like the brain foetal brain and areas not containing angioblasts. However Intussusceptive (non-sprouting) angiogenesis is the predominant mechanism in the lung, where endogenous endothelial precursors contribute to angiogenesis. Intussusception can be caused by the

proliferation of endothelial cells within a vessel to produce a wide lumen. This enlarged lumen can be split by fusion of capillaries and transcapillary pillars (the development formation of columns by thickened endothelial cells) (Riasu, 1997; Adams and Eichmann, 2010).

Anti-angiogenic agents and cancer therapy

Knowledge of whether FAM198b contributes to angiogenesis is interesting with reference to basic science, but it also could aid in the discovery or refinement of agents that target the tumour vasculature. Blocking the development and advancement of established tumour vasculature has been explored as a method to complement, or even replace current anti-cancer therapies. This option is particularly attractive as endothelial cells have intimate contact with the blood. Additionally anti-cancer therapies could be targeted to the tumour vasculature exclusively, as current anti-cancer therapeutics have a wide array of side effects. It has been known for some time that the endothelium differs in tumours from that of normal tissues. Tumour endothelium proliferates at a high rate and is a substantially contributes to angiogenesis. Whilst a small number of molecular differences have recently been characterised, many remain undefined (Neri and Bicknell, 2005).

The VEGF signalling pathway was considered to be a promising target for anti-cancer therapeutics. This was largely due to the fundamental role VEGF plays in promoting angiogenesis and tumour growth. Currently two methods have been employed to target the VEGF signalling pathway. Bevacizumab (avastin) was the first angiogenesis inhibitor to be made available commercially for the treatment of breast, colorectal renal and lung cancers in combination with standard chemotherapy. Bevacizumab is a monoclonal antibody which binds to circulating VEGF-A. This tactic reduces angiogenesis by limiting the binding of VEGF-

A to cell surface receptors (Chung *et al*., 2010; Kazazi-Hyseni *et al*., 2010). The second approach is based upon the specific targeting of the VEGF receptor complex. The tyrosine kinase inhibitors sorafenib (nexavar) and sunitinib (stutent) are capable of reducing angiogenesis. These molecules inhibit the phosphorylation of the VEGF receptors and therefore stop the activation of the angiogenesis cascade (Gupta and Zhang, 2005).

The use of the anti-angiogenic drugs mentioned above has been effective with regard to improving the survival rates of cancer patients. However it is important to discover novel targets for anti-angiogenic drugs. This would allow for possibility of combination therapies being developed, which would allow for the efficiency of angiogenesis inhibition to be maximised. These developments are important because many cancer patients do not respond to the anti-angiogenic therapeutics or experience disease reoccurrence. The development of tumour resistance against anti-VEGF treatment could be caused by many factors. This resistance could be caused by the exploitation of alterative pro-angiogenic pathways or vastly upregulated levels of VEGF/VEGFR molecules (Chung *et al*., 2010; Nussenbaum and Herman, 2010).

Models of angiogenesis

The aim of this project is to determine whether FAM198b plays a role in angiogenesis tumourgenesis and wound repair. This will be carried out by utilising HUVECs as *in vitro* models following the knockdown of FAM198b with small interfering RNA (siRNA) molecules. siRNA has been recognised as a natural gene silencing mechanism in plants, vertebrates and invertebrates. *In vitro* siRNA mediated knockdown of gene expression has been achieved by transfecting mammalian cells with synthetic double stranded RNA molecules. The suppression of gene expression is mediated through highly regulated enzymatic reactions.

The RNA interference (RNAi) pathway requires the RNA-induced silencing complex (RISC) to bind to the anti-sense strand of the siRNA molecule. Once the siRNA has bound to the RISC and pathway has been activated, mRNA targets which are complementary to the siRNA molecule can be recognised and cleaved (Xia *et al*., 2002; Reynolds *et al*., 2004)

In vitro models of angiogenesis are an indispensable prelimary tool for studying the molecular mechanisms involved in angiogenesis. The *in vitro* use of HUVECs allows for information to be gathered by imitating each of the three main phases involved in angiogenesis, migration, proliferation and tube formation. To study these three phases, this preliminary study of FAM198b utilised the scratch wound healing, matrigel tube formation and proliferation assays. These assays have many limitations and are extremely simplistic when compared to the processed involved in angiogenesis. But these assays can provide invaluable information for the preliminary phases of investigating and validating potential therapeutic targets (Staton *et al*., 2006).

Materials and Methods

Cell Culture

Materials	Source
Human umbilical vein endothelial cells (HUVEC)	Birmingham Women's Hospital, Birmingham
Media 199	Cancer research UK and Sigma, UK
Bovine brain extracts	Pel-Freez, USA. and prepared as described by Maciag *et al*. (1979)
Fetal calf serum	PAA, The Cell Culture Co., UK
L-glutamine	Sigma, UK
Porcine skin gelatine	Sigma, UK
Trypsin-EDTA	Sigma, UK
FastRead Haemocytometer	Immune Systems, UK
Tryphan Blue	Sigma, UK
Leica DM IL inverted microscope	Leica Microsystems, USA

Method:

Informed consent was obtained prior to obtaining human umbilical cords from Birmingham women's health care NHS trust. Collagenase type 1a was used to isolate human umbilical vein endothelial cells (HUVECs) from umbilical cords using a standard protocol. Media 199 containing 10% (v/v) foetal calf serum (FCS), 90 µg/ml heparin, 4mM L-glutamine was supplemented with bovine brain extract and was used to culture the HUVECs to confluence. The cells were cultured in sterile plastic culture dishes coated with 0.1% (w/v) gelatine and maintained at 37°C in a humidified atmosphere with 5%CO_2.

The HUVECs were split and re-plated at a ratio of 1:3 once confluent. The HUVECs were detached from the culture dish by washing with 1x phosphate-buffered saline (PBS) and incubated in 1xtrypsin-EDTA solution for 5 minutes in the conditions stated above. Once detached supplemented Media 199 was added to the cells before being centrifuged at 250 RCF for 5 minutes, the pellet was

resuspended in fresh supplemented Media 199 and the cells were plated for further cultivation. The HUVECs were only used for the following assays between passages 1 and 6.

RNA analyses

RNA extraction

Materials	Source
QIAShredder	Qiagen, UK
RNeasy mini kit	Qiagen, UK
2-mercaptoethanol	Sigma-Aldrich, UK
70% ethanol (in RNase-free water)	n/a

Method:

The HUVECs were lysed using the QIAShredder columns as according to the manufacturer's instructions. RNA was purified from the lysate using the RNeasy mini kit (including the optional DNase step) according to the manufacturer's instructions.

Generation of cDNA

Materials	Source
SuperScript III First-Strand Synthesis System	Invitrogen, UK

Method:

SuperScript III First-Strand Synthesis System kit was used to generate total complementary cDNA from the isolated RNA according to the manufacturer's instructions.

Real Time Quantitative Polymerase Chain Reaction

Materials	Source
Sensimix™	Quantace, UK
Universal Probe Library set, Human	Roche Applied Science, UK
PCR Primers	Eurogenetec, UK
Rotor-Gene RG-3000 qPCR machine	Corbett Research Ltd, Australia
Rotor-Gene 6 software	Corbett Research Ltd, Australia

Method:

The Exiqon universal probe system was used to perform real time quantitative polymerase chain reaction (qPCR). A web based tool produced by Roche Applied Science was used to design the forward and reverse primers shown below. These primers amplify a region containing a sequence where a probe could bind and to amplify a product which crossed an exon boundary where possible (table 1).

A reaction mix totalling 25µL was used:

- 12.5µL Sensimix™
- 1µL forward primer (10 µM)
- 1µL reverse primer (10 µM)
- 0.25µL of the appropriate probe
- 0.25µL deionised water
- 10µL of cDNA

A Rotor-gene RG-3000 was used to conduct the qPCR using the 36 well rotors and the following program:

- 95°C for 10 minutes
- 95°C for 15 seconds
- 60°C for 45 seconds

40 Cycles

Primers	Sequence	Probe
Splice variant 1 (SV1)	Forward - 5’ AAGCAGAGTTCATCCAAGCAG 3’	#64
	Reverse - 5’ TGCTGATAAGTTCCCAGGTG 3’	
Splice variant 2 (SV2)	Forward - 5’ ACAATTCTTCCTGGAGCCAAGC 3’	#33
	Reverse - 5’ AGAGGTGGAAGGAAAGGGTTG 3’	
FAM198b Universal Splice Variant	Forward - 5’ GAAAAAGCATGCAGTGGCATC 3’	#37
	Reverse - 5’ TACAGCATCAGCTTCCCTTGC 3’	
Splice variant 1+3 (SV1+3)	Forward - 5’ CTTCTAGGCCTTCTCTTTCCAG 3’	#31
	Reverse - 5’ CAAGGAGAAATGCGGCTTG 3’	
β-actin	Forward - 5’ GCACCCAGCACAATGAAGA 3’	#63
	Reverse - 5’ CGATCCACACGGAGTACTTG 3’	

Table 1: PCR Primer sequences.

The forward and reverse primer sequences are contained in this table. Additionally the specific probe number from the Universal Probe Library set for each amplicon has been included.

The expression levels of FAM198b were determined by using known dilutions of cDNA (1 in 10, 1 in 100 and 1 in 1000) to generate standard curves using Rotor-Gene 6 software. The normalisation of expression to β-actin was used to accurately quantify gene expression and compare between different samples.

siRNA Transfection and Knockdown of FAM198b

Materials	Source
OptiMEM	Invitrogen, UK
Lipofectamine RNAi MAX	Invitrogen, UK
FAM198b specific duplexes	Eurogentec, UK
Negative control duplex	Eurogentec, UK

Method:

Two siRNA duplexes (table 2) were designed to target the coding region of FAM198b according to the criteria proposed by Reynolds *et al*. (2004). A third siRNA duplex was obtained from Eurogenetec (UK) which is not complementary to any known sequence. The Duplexes were diluted to working stocks of 20µM in RNase-free water and aliquoted to avoid repeated freeze-thaw cycling.

SiRNA Duplex	Sequence
FAM198b Duplex 1 (D1)	5'-GAUAAAGUGUAUUGGGAAA55
FAM198b Duplex 2 (D2)	5'-GAAUCGGGUUGUACUGAAA55

Table 2: The siRNA duplex sequences for the FAM198b knockdown.

The two duplexes were designed to specifically target the 3' UTR of FAM198b. It is important to note that the 3' end of the duplexes were tagged with two thymine nucleotides (represented by the number 55). This modification creates an overhang at the 3' end of the duplex and aids in the preferential destruction of the siRNA sense strand and binding of the anti-sense strand to the proteins in the RISC pathway.

HUVECs were seeded onto gelatine coated dishes the day before the cells were transfected with siRNA. The size of dish and number of cells varied depending on the assay being conducted (table 3). For transfection of HUVECs in single 10cm culture dish D1 and D2 or the negative duplex was diluted in 680µL of opti-MEM to give the appropriate final concentration. 10% (v/v) lipofectamine RNAi MAX was also diluted in opti-MEM to obtain a total volume of 120µL. Both

mixtures were incubated for 10 minutes at room temperature after gentle mixing. The two mixtures were combined, flicked and incubated for a further 10 minutes. After washing twice with PBS, 3.2mL of opti-MEM was added the cells. The 800 µL transfection mix was added to the cells to give a total volume of 4mL and mixed by tilting. The HUVECs were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 4 hours; the transfection mix was then replaced with fresh media 199 without antibiotics. The volumes required for the transfection mix was scaled down to accommodate transfection in a 6 well plate for the scratch wound healing assay.

Assay	Culture dish	Number of cells	siRNA Concentration
Cell Growth	10cm plate	1x10^6	25nM D1 and 25nM D2 or 50nM Negative Duplex
Wound healing	6-well dish	3x10^5	
Tube formation	10cm plate	1x10^6	

Table 3: The cell numbers and dish size required for each assay

Angiogenesis Assays

Cell Growth - Proliferation Assay

Materials	Source
FastRead Haemocytometer	Immune Systems, UK
Tryphan Blue	Sigma, UK
Leica DM IL inverted microscope	Leica Microsystems, USA

Method:

Three gelatine coated 6 well plates per condition were prepared for each condition. HUVECs were seeded onto the 6 well plates at a density of $9x10^4$ cells four hours after the siRNA transfection. The number of cells per well were counted 1-3 days post seeding using a haemocytometer and a Leica DM IL light microscope.

Cell Migration - Scratch Wound Healing Assay

Materials	Source
Mitomycin C	Sigma, UK
Leica DM IL inverted microscope	Leica Microsystems, USA
USB 2.0 2M Xli camera	Leica Microsystems, USA
Image J Software	National Institutes of Health

Method:

48 hours after the HUVECs were transfected with the siRNA (as described above) vertical scratches were made with sterile 200µL pipette tips. Cell debris from the scratch process was washed of with PBS before adding fresh media containing 2.5µg/mL mitomycin C and incubating at 37°C in a humidified atmosphere with 5%CO_2 for 8 hours. The amount of cell migration was observed by taking pictures at 0, 4 and 8 hours from the same location using a USB 2M Xli camera and a Leica DM IL microscope. The pictures were analysed using Image J software to quantify the wound healing by measuring the area of wound remaining open. The percentage of the scratch wound remaining open was calculated by dividing the area of the wounds at 4 or 8 hours by the area measured at 0 hours, wounds were considered to be 100% open at the 0 hour time point. The degree of wound closure could then be compared between the knockdown of FAM198b and the negative duplex at each time point.

Tube formation - Matrigel ™ Tube Formation Assay

Materials	Source
BD Matrigel™, basement membrane matrix	VWR, Canada
Leica DM IL inverted microscope	Leica Microsystems, USA
USB 2.0 2M Xli camera	Leica Microsystems, USA

Method:

HUVECs were used 24 hours after transfection with siRNA for the tube formation assay. Matrigel™ was thawed overnight was thawed overnight at 4°C and kept on ice before being added to 12 well plates. The 12 well plates were washed with PBS before adding 70µL of Matrigel™, which was allowed to solidify at 37°C for 30 minutes. The transfected cells were seeded on top of the Matrigel™ at a density of $1.4x10^5$ in 1mL of complete Media 199. The HUVECs were incubated for 10 hours at 37°C in a humidified atmosphere with 5%CO_2 and tube formation was observed by taking pictures of 5 random fields of vision at 10 hours. The degree of tube formation for each condition was quantified by counting the number of nodes with two, three or four or more branches per field of vision.

Results

FAM198b Expression

Real time quantitative PCR (qPCR) was conducted to assess whether FAM198b was truly expressed in HUVECs (Figure 5). It was determined that splice variant 2 was definitely expressed. However the first splice variant could not be amplified, this could be because the splice variant was not expressed in HUVECs or because of primer inefficiency. It is important to note that the 5' UTR shared by the first and third variants was amplified and therefore at least one of those splice variants exists.

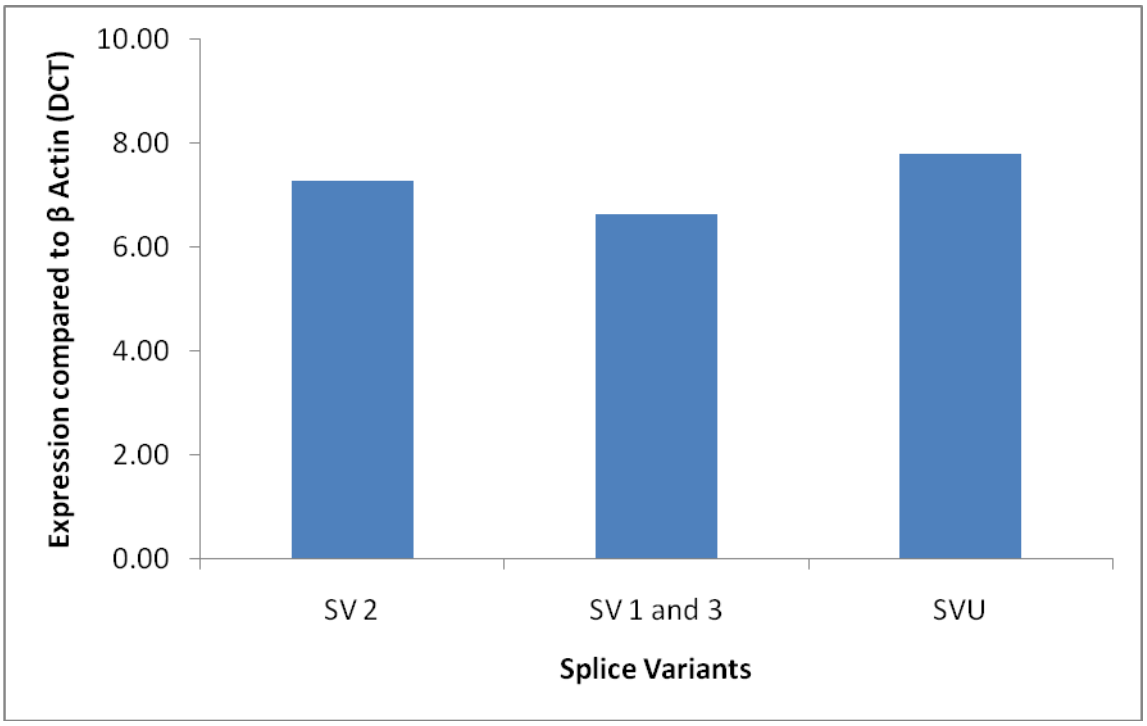

Figure 5: qPCR expression data to confirm the expression of FAM198b

The expression levels of the splice variants above were determined by deducting the cycle threshold of the house keeping gene, β-actin, from the cycle threshold of the FAM198b signal to obtain the Delta cycle threshold values (DCT).

Confirmation of knockdown

In the absence of specific antibodies for FAM198b qPCR was the only method by which the expression of FAM198b could be monitored post knockdown (Figure 6). The qPCR results demonstrate that duplex 1 and 2 successfully knocked down FAM198b by roughly 90%.

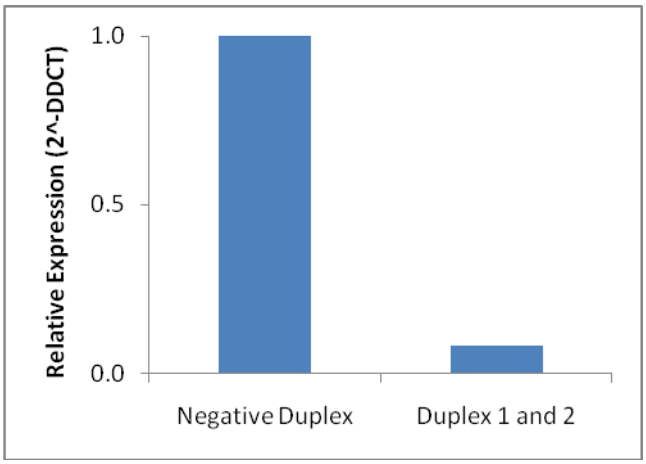

Figure 6: qPCR data to confirm the efficiency of the siRNA knockdown

The FAM198b expression levels for both the double knock down and negative control were compared to an internal β-actin control, which allowed for the calculation of 2^{-DDCT}.

Angiogenesis Assays

Cell Growth - Proliferation Assay

The proliferation assay shows that for the first two days after transfection there is not a difference in cell growth between the cells that have been transfected with duplexes to knockdown FAM198b and the negative control duplex. But by the third day there was a difference between the cells that had been knocked down and the negative control, fewer HUVECs were counted for the group which had been knocked down (figure 7). However whilst the means differed by over 150,000 cells and the error bars did not overlap, the difference was determined to not be statistically significant when the p value was calculated using the 'Kruskal–Wallis one-way analysis of variance'.

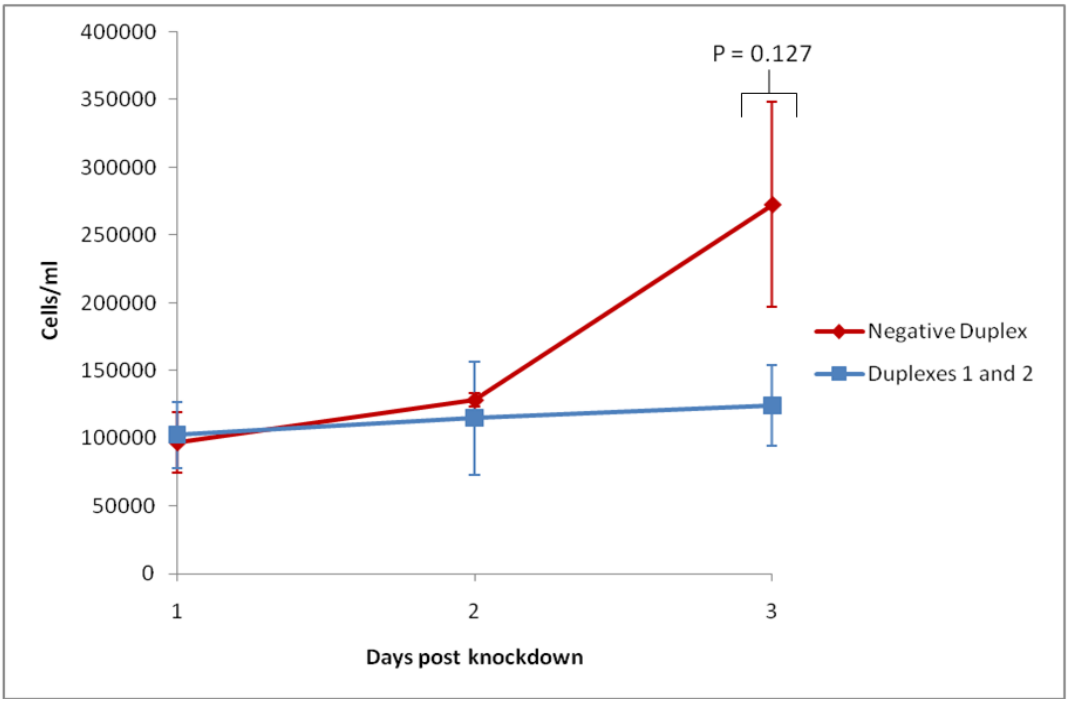

Figure 7: The effect a FAM198b knockdown has on cell growth

Cell counting using a haemocytometer was used to quantify the difference of cell growth between the cells transfected with D1 and 2 and the negative duplex. The error bars represent the standard error of the mean number of cells at each time point per condition. The P value was calculated using the Kruskal–Wallis one-way analysis of variance statistical test, which compared the variance between the raw data sets for each condition.

Cell Migration - Scratch Wound Healing Assay

The scratch wound healing assay shows that there was a small increase in migration when the HUVECs had been knocked down with siRNA targeting FAM198b (figure 8). The increased speed of was quantified to be just over 10% faster when FAM198b was knocked down, furthermore this difference was determined to be statistically significant at both 4 and 8 hours.

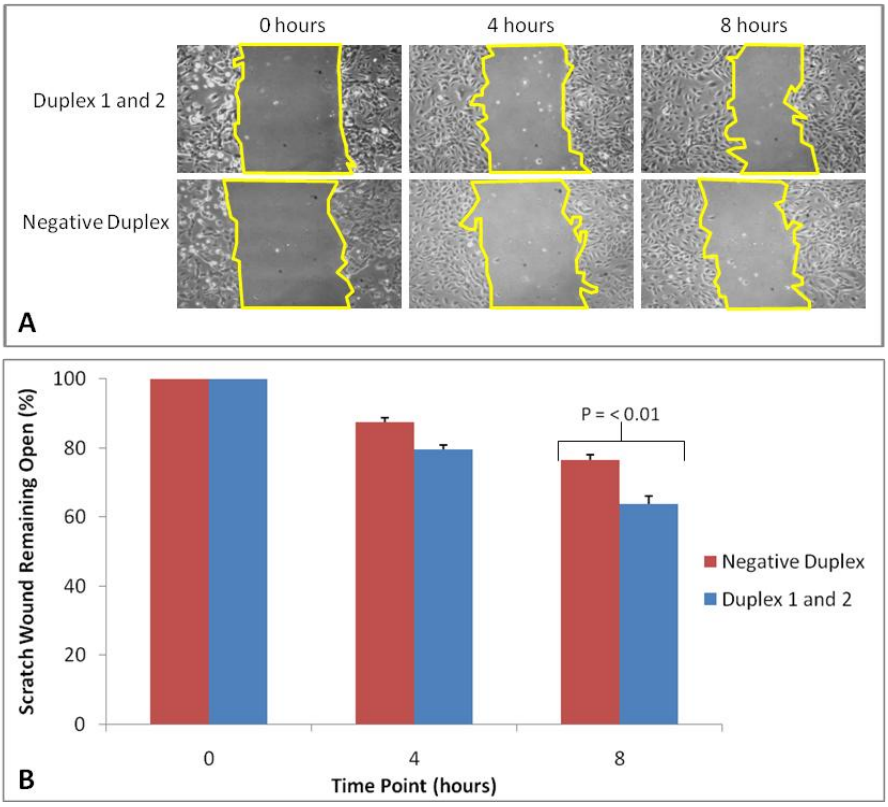

Figure 8: The effect a FAM198b knockdown has on scratch wound healing

A) The pictures of the scratch wounds were taken at the same location for each time point using a USB 2M Xli camera and Leica DM IL microscope.

B) Six independent scratch wounds per condition were analysed using Image J software, which allowed for the quantification of scratch wound healing. The scratch wounds at 0 hours were considered to be 100% open, whereas the percentage wound healing at 4 and 8 hours was determined by comparing the area at these time points to the scratch at 0 hours. The error bars represent the standard error of the mean amount of scratch wound remaining open. The P value was calculated using the Kruskal–Wallis one-way analysis of variance statistical test.

Tube formation - Matrigel™ Tube Formation Assay

The two dimensional Matrigel™ tube formation assay demonstrated that when FAM198b has been knocked down there is an effect on tube formation (figure 9). The number of nodes forming two or three branches is statistically lower when FAM198b has been knocked down. However the number of nodes with four or more branches was similar for both conditions, the nodes formed by HUVECs that had been knocked down appeared to be larger.

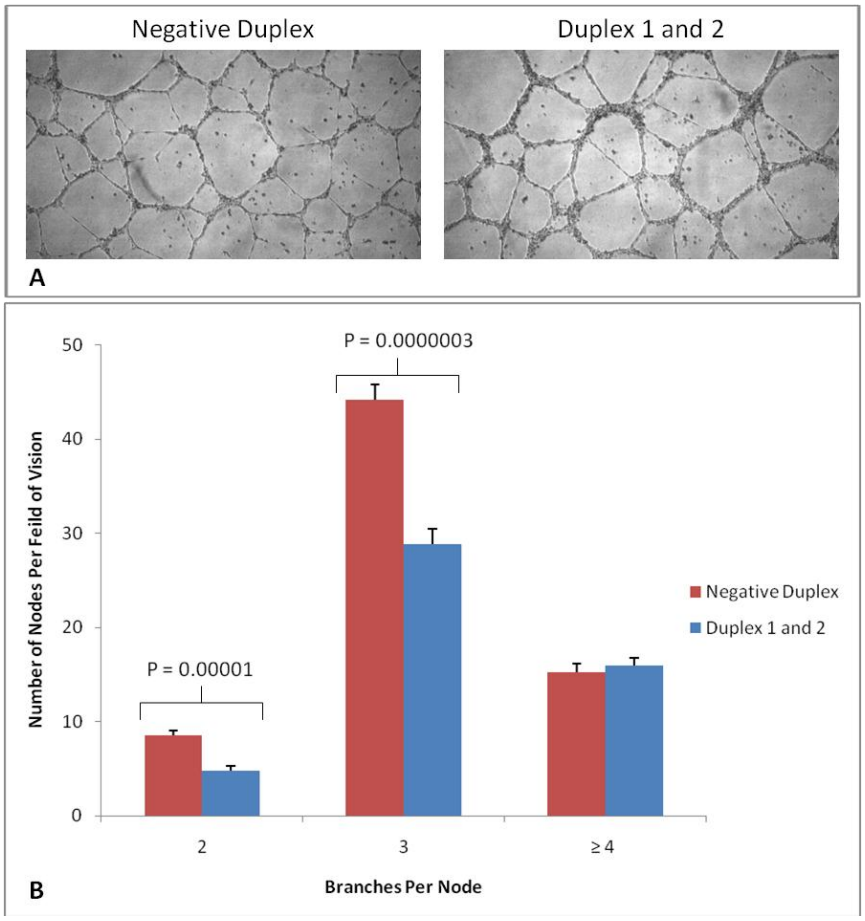

Figure 9: The effect a FAM198b knockdown has on tube formation

A) The pictures obtained from the USB 2M Xli camera and Leica DM IL microscope demonstrate that the level of tube formation by HUVECs with the FAM198b knockdown compared to cells transfected with the negative duplex.

B) The degree of tube formation was quantified by counting the number of nodes with 2, 3 or 4 or more branches. The error bars represent the standard error of the mean number of nodes per field of vision. The P values were calculated using the Kruskal–Wallis one-way analysis of variance statistical test.

Discussion

Real time quantitative PCR has been a valuable tool in this project. It was initially useful for confirming that HUVECs expressed the second splice variant and the alternate exon in the 5' prime region of the first and third splice variant. As there were no specific antibodies available for the detection of FAM198b, qPCR provided a simple way to detect and quantify the expression of FAM198b following knockdown with siRNA. A double knockdown of FAM198 with duplex 1 and 2 was used to transfect the HUVECs. This strategy provided a 90% knockdown of FAM198b compared to β actin. The double knockdown was employed because a greater degree of knockdown could be obtained. Additionally by employing a double knockdown the required concentration of each siRNA duplex could be halved. Not only could the high concentrations of siRNA provide a prolonged knockdown, but also there was less chance of off-target silencing because a lower concentration of each siRNA molecule was used.

The use of qPCR for confirming the knockdown of FAM198b relies upon the assumption that protein encoded by FAM198b has a short life-span. qPCR can be used to determine the effectiveness of the duplexes at the mRNA level. But it is not possible to know whether this relates to an equivalent reduction of FAM198b protein. However qPCR was the only viable option available to confirm the knockdown due to the short duration of the project. Additionally this limitation is not significant if an effect is observed after the knockdown, because for this difference to occur there must have been a significant protein deficiency.

Angiogenesis assays

The greatest difference in cell growth was observed on the third day following the FAM198b knock down. This result could indicate that without the expression of FAM198b HUVECs are not able to proliferate as efficiently. But the effect of the FAM198b knockdown was determined to not be statistically significant when comparing the raw data sets using the Kruskal–Wallis one-way analysis of variance. There is a large difference between the means of the data and the error bars do not overlap, which indicates that FAM198b could have a real effect on cell growth. More replicates over a greater time period would be required to explore this possibility. Should a significant effect be seen, additional assays would have to be carried out to determine whether FAM198b has a direct effect on proliferation and which stage of the cell cycle FAM198b effects. But also the possibility that the absence of FAM198b in HUVECs induces apoptosis or whether duplexes 1 and 2 induce tumour necrosis factor-α (TNFα) induced apoptosis.

The knockdown of FAM198b significantly increases the rate of scratch wound healing. This effect was solely due to the migration of HUVECs, as mitocycin C was used to ensure that the HUVECs could not proliferate. An increase in the rate of migration indicates that FAM198b could be either an adhesion factor or involved in the signalling, stability, production or trafficking of adhesion factors (Sanabria *et al*., 2011).

The scratch assay was conducted 48 hours following knockdown. It is possible that a greater difference in migration could have been observed if the scratch wound assay was conducted at a later time point. A greater length of time between the knockdown and the assay could have resulted in a further reduction of residual FAM198b, providing the knockdown remained effective.

The effect of FAM198b on migration could also be explored using Dunn and Boyden chambers. These assays could be more useful than the scratch assay if FAM198b had a function relating to adhesion factors of cytoskeletal dynamics. Dunn and Boyden chambers assess the ability of cells react to chemokinetic and chemotactic factors in addition to their ability to migrate and squeeze through pores in filters. The ability to move through the filters is heavy influenced by the activity of adhesion molecules and cytoskeletal remodelling (Staton, 2006). It would also be interesting to carryout further scratch wound assays to determine whether HUVECs over expressing FAM198b migrated at a slower rate.

The matrigel™ tube formation assay demonstrated that FAM198b depleted cells were able to align themselves into primitive tube-like structures. Connolly *et al*. (2002) has described the changes that contact with Matrigel™ induces in HUVECs. These changes include the cellular protrusions and microtubule dynamics required to align HUVECs into short tube-like structures. FAM198b depleted HUVECs appear to be capable of making these changes. However quantitative analysis revealed that the knockdown of FAM198b appeared to cause an increase in node size. Additionally there was a statistically significant decrease in the number of nodes being formed with fewer than four branches compared to the control HUVECs. This indicates a reduced ability for FAM198b deficient cells to effectively differentiate, migrate and form the complex tubule networks.

It is important to note that the results from this assay coincide with the microarray results published by Sun *et al.* (2005), which were highlighted in the introduction. FAM198b appeared to be upregulated in endothelial cells, indicating a possible involvement during the later stages of tubule formation.

The results from these *in vitro* assays indicate that FAM198b could play a significant role in angiogenesis. But it is important to note that this study does have some limitations. Firstly the HUVECs for these assays were derived from only one umbilical cord and therefore only one patient. It is possible that the knockdown of FAM198b would not have had significant results if the cells were derived from another patient, due to the genetic variability between individuals. Additionally, the use of HUVECs has some associated limitations, despite being a widely used model for *in vitro* angiogenesis assays. The results from the FAM198b knockdown observed in HUVECs might not relate accurately the role of FAM198b in an adult, as HUVECs are derived from foetal material.

Further research into the role of FAM198b could be justified based on the results presented in this study. But this research would have to demonstrate that the effects reported in this paper occurred in HUVECs from multiple patients. Additionally more *in vitro* assays could be employed, such as those discussed earlier. In the longer term some *in vivo* assays could be employed to verify the effect of FAM198b in a more complex environment. For example zebrafish could be used to analyse the knockdown and over expression of FAM198b. This model had many advantages because zebrafish embryos are transparent and embryogenesis occurs rapidly outside the mother's body, which allows for easy observation of vascular development, which can be carried out in real-time (Staton, 2006).

FAM198b is predicted to be a golgi membrane protein, which presents challenges for the development of therapies. It would not be possible to use relatively simple therapies which would target proteins on the cell membrane, such as monoclonal antibodies. Currently no anti-angiogenic specific therapies are in clinical use which target proteins contained within endothelial cells. But it could be possible to target FAM198b using small

organic molecules. This would require the large scale screening of compounds using nuclear magnetic resonance imaging to identify compounds with sufficient structure-activity relationships to FAM198b (Neri and Bicknell, 2005).

A significant disadvantage of FAM198b as a therapeutic target is that the microarray data discussed earlier in this paper suggests that FAM198b could be expressed in many tissues. Ideally it would be best for a prospective target to only be present in tumour vasculature. But as the object of these therapies is to inhibit angiogenesis FAM198b could still be an appropriate therapeutic target. Further experimental work would be required to determine that FAM198b is not essential to the viability of non-cancerous tissues. If FAM198b is not essential to the viability of those tissues, it could be an appropriate target for use in a combination therapy along side bevacizumab, sorafenib or sunitinib.

Conclusion

Many genes are involved with the regulation of angiogenesis. The *in vitro* assays detailed in this paper indicate that FAM198b plays a role in allowing HUVECs to migration and form tubules. These results support the unverified microarray data published by Sun *et al*. (2005), which indicated that FAM198b was upregulated during endothelial sprouting, branching, and capillary network formation. Angiogenesis is a natural process involved in wound healing, which can be hijacked and contribute to tumourgenesis. If FAM198b was determined to significantly affect angiogenesis and endothelial cell viability *in vivo*, FAM198b could be utilised as a novel target for ant-angiogenic therapeutics. Anti-angiogenic therapies could be a significant tool to selectively kill or limit the potential for tumour cells to rapidly proliferate. This option is preferential to conventional cancer therapies because chemotherapy is non-specific and can cause significant toxicity to proliferating non-tumour cells.

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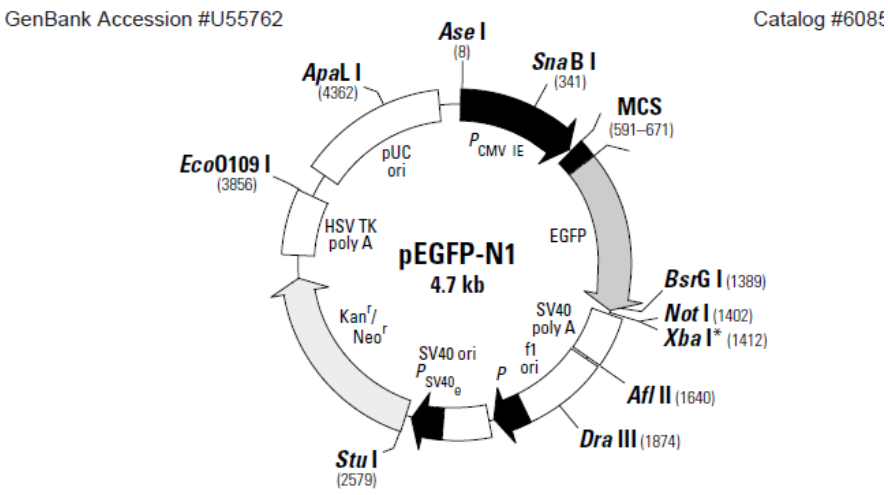
Appendix

Cloning and FAM198b GFP fusion

FAM198b is predicted to be a golgi membrane protein, to confirm this aFAM198b-GFP fusion was to be produced. However due to the short time frame for the project this work was not completed. Both an N terminal and C terminal GFP tag were to be generated as the structure of FAM198b was not known. Using this strategy it was hoped that at least one of the constructs would yield a functional protein.

pEGFP N1



Forward Primer

ECORI

5’ TAG TAG GAA TTC GCC ACC atg cgg cag aga aat gac ctg t 3’

34 bases

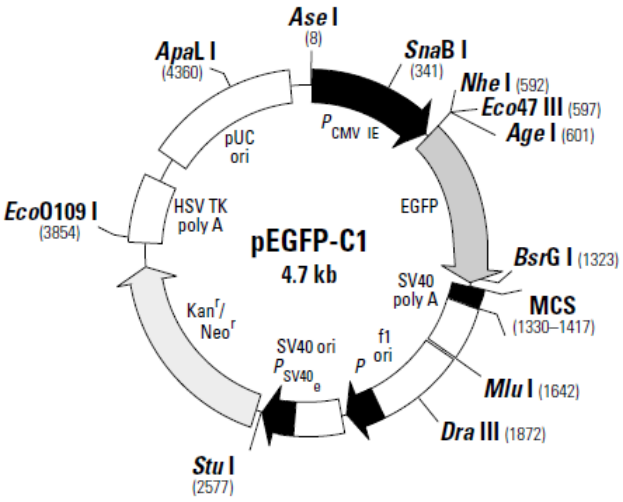
Reverse Primer

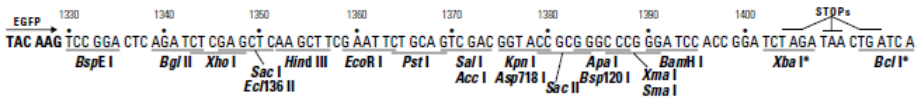
SALI

5’ CTA CTA GT CGA CGT ttc att cat agg taa gat tac t 3’

34 bases -

pEGFP C1





Forward Primer

ECORI

5’ TAG TAG G AAT TCT atg cgg cag aga aat gac ctg t 3’

Reverse Primer

SALI

5’ CTA CTA GTC GAC tca ttc att cat agg taa gat t 3’

There were some concerns that the C1 construct would not work because a bioinformatics program predicted FAM198b to have a cleavage site near the start of the FAM198b protein.

Project 2: Isolation and Culture of Mesenchymal Stem Cells for Craniofacial Tissue Regeneration

by

Klarke Michael Sample

A thesis submitted to the University of Birmingham for the degree of MASTER OF RESEARCH

Contents

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Introduction

The craniofacial tissues have been associated with a wide range of clinical manifestations. These can include acquired damage to the skull and facial tissues through a wide array of traumatic injuries, tumour invasion and congenital disease. Additionally malformation of the craniofacial tissues can be associated with many congenital abnormalities. Abnormalities and damage to the craniofacial tissues can be associated with many symptoms. The craniofacial tissues can be affected cosmetically and even functionally. For example congenital and acquired craniofacial deformities can be associated with an insufficiency of sight, sound perception, speech, breathing, mastication (chewing) and swallowing (Yoneno *et al*., 2005)

Corrective surgery has been attempted using materials from the iliac, often the hip bone, to create grafts. However this strategy requires the removal of a significant amount of tissue and sufficient donor material is not always possible. Additionally this degree of surgical intervention can result in an increased level of morbidity and mortality (Yoneno *et al*., 2005). To a degree these problems can be overcome through the practise of allotransplantation (the use of tissues from other individuals of the same species). However even this is associated with significant clinical limitations, largely due to the problems associated with immunogenicity and donor availability (Seidi *et al*., 2011).

Tissue engineering has the potential to overcome the limitations of the conventional therapeutic techniques. Not only could tissue engineering provide donor material with limited problems associated with immunogenicity. But also the grafts could be capable of integrating with host tissues more efficiently than a conventional graft (Yoneno *et al*., 2005;

Seidi *et al*., 2011). This paper will focus on the use of bone marrow stem cells (BMSCs) and the materials and techniques associated with tissue engineering. However the use of tissue engineering to overcome specific craniofacial abnormalities is beyond the scope of this investigation.

Tissue engineering is a branch of science which aims to utilise advances in the fields of molecular biology, cell biology, material science and bioengineering. The broad aim of tissue engineering is to repair or regenerate the function of tissues in areas that have been damaged. This can be achieved by either the *in vitro* generation of functional tissues by the time of implantation into a patient. Alternatively materials can be implanted into a patient, which allow for the generation of functional tissues *in vivo*. Tissue engineers have typically attempted to generate grafts containing a single tissue, for example cartilage, bone or skin. However interface tissue engineering is emerging as a field which aims to create functional tissues containing different tissues. This approach could be particularly effective for the reconstruction of areas requiring both hard and soft tissues (Seidi *et al*., 2011).

Tissue engineering can utilise small biopsies obtained from the patient as opposed to conventional surgical techniques. Cells can be isolated from the biopsies and cultured to obtain large numbers of cells. This approach could overcome the problems associated with the availability of donor material and immunocompatibility (George *et al*., 2006; Seidi *et al*., 2011). The isolation of stem cells which are capable of differentiating into many lineages could be very useful for the creation of these tissues. Bone marrow derived stem cells could be isolated from the patient, cultured on three-dimensional (3-D) bio-degradable scaffolds. The scaffolding could be created through the use of synthetic or organic materials. Tissue engineers aim to direct the formation of tissues and structures resembling those found in a

living person. This would be facilitated by utilising chemical and biochemical molecules or the properties of the scaffold itself to stimulate the growth of stem cells and direct their fate (Yoneno *et al*., 2005; George *et al*., 2006; Seidi *et al*., 2011).

With regard to stem cell therapy the use of the techniques associated with tissue engineering could be particularly effective. Stem cells have been shown to yield poor survival when injected directly into tissues. Largely due to the lack extracellular support and presence of necrotic tissue associated with areas of scarring and injury (Burns *et al*., 2009; Yamashita *et al*., 2009). The definition of a true stem cell is often debated in the literature. But stem cells are generally considered to be undifferentiated cells which are capable of maintaining a population of stem cells and spontaneously differentiating into other cell types (figure 1). Stem cells should also be capable of repopulating tissues regardless of whether significant damage has inflicted to the tissue or not (Stojanoski *et al*., 2009; Verfaille, 2009).

Many types of stem cells can be utilised by tissue engineers to produce tissues for craniofacial regeneration, but BMSCs (a variety of mesenchymal stem cell) could be especially useful. Mesenchymal stem cells are multipotent stem cells (stem cells which are capable of differentiating into a limited number of cell types), which are typically isolated from bone marrow and therefore should not raise ethical issues. However mesenchymal stem cells but can also be obtained from other sources such as placenta and adipose tissue. Mesenchymal stem cells are derived from the mesoderm (figure 2) and are particularly useful for craniofacial regeneration because they have been shown to spontaneously differentiate into cells contained within bone, cartilage and adipose tissues. All of which can be naturally involved in make up of a diverse range of craniofacial structures. Furthermore

bone marrow stem cells have been shown to differentiate into tissues outside of their own lineage, such as neurons, which are typically derived from the ectoderm. Mesenchymal stem cells are also favoured by tissue engineers because of their weak immunogenicity and immunomodulatory properties. Their immunomodulatory properties in particular could be very useful as inflammation could cause damage to the grafts (Yoneno *et al*., 2005; George *et al*., 2006; Schenke-Layland *et al*., 2007; Hicks and Jolkkonen, 2009).

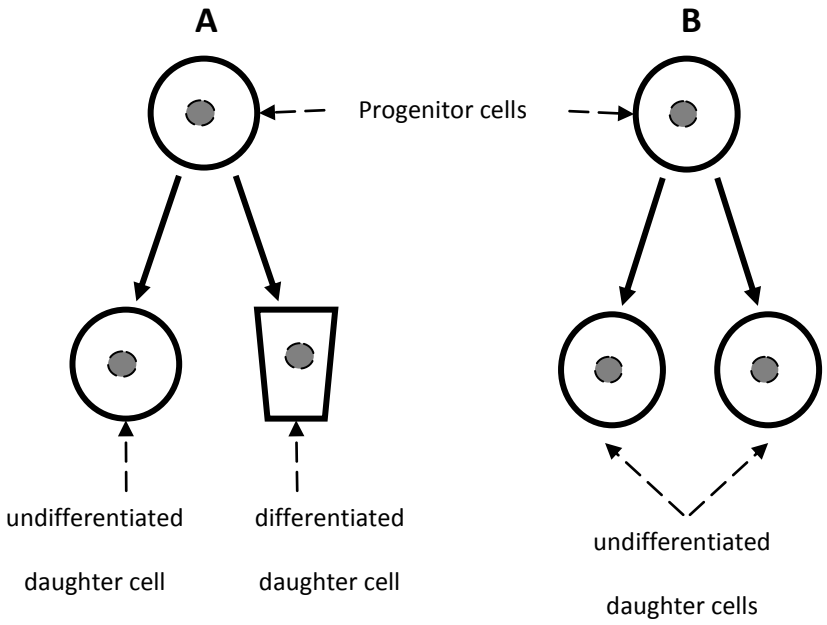

Figure 1: The division strategies employed by stem cells

A: Asymmetric Division: Two non-identical daughter cells are produced in this process. One cell differentiates into another cell type whilst the other would remain identical to the progenitor cell.

B. Symmetric Division: - Two daughter cells which both have identical when compared to the progenitor.

(Original diagram compiled using information from Stojanoski *et al*., 2009 and Verfaille, 2009)

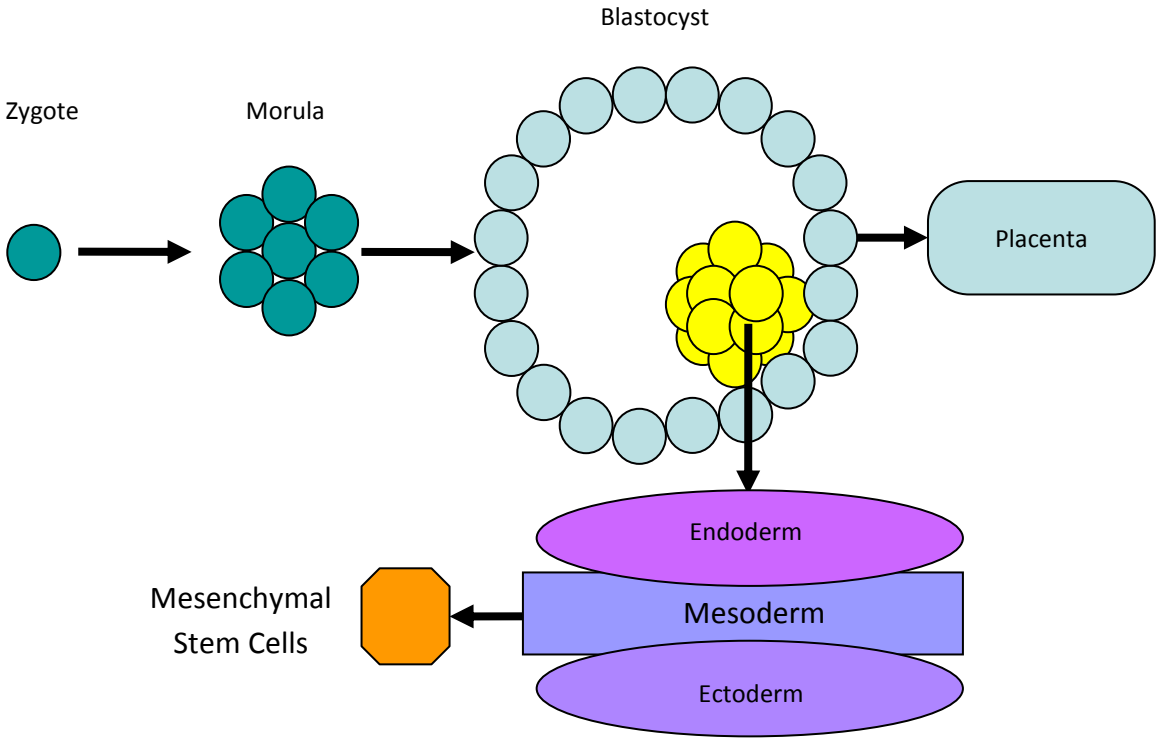

Figure 2: The formation of the germ layers and mesenchymal stem cells

A blastocyst is a hollow sphere of cells (blastoderm), which contains a layer of cells known as the inner cell mass. The blastoderm has the capacity to differentiate into all the cell types contained within the placenta. Whereas the inner cell mass can differentiate into over 200 cell types and forms the foetus. The inner cell mass will initially form three germ layers, the endoderm, mesoderm and ectoderm. (Original diagram compiled using information from Hwang *et al.*, 2009).

BMSCs can be differentiated into osteoblasts, chondrocytes and adipocytes within various 3-D cell scaffolds. The conventional method of gaining a specific tissue type has been to expose the BMSCs to molecules that stimulate differentiation within the collagen scaffold. These methods include exposing BMSCs to transforming growth factor-β1 to obtain chondrocytes, dexamethasone to obtain osteoblasts, insulin and indomethacin to obtain adipocytes, and 5-azacytidine to obtain myoblasts (Yoneno *et al.*, 2005).

Collagen scaffolds have great potential for these purposes as they form a honeycomb structure to which cells can attach, proliferate and differentiate within (George *et al*., 2006). Collagen has many advantages when compared to other scaffolding materials. Collagen is the most abundant protein in mammals and is a major component bone, cartilage and the extracellular matrix. Collagen has the relevant mechanical properties needed for a 3-D scaffold, with regard to plasticity and physiological stiffness. Furthermore collagen participates in the cell growth and differentiation *in vivo* (Yoneno *et al*., 2005).

There is increasing evidence in the scientific literature that cells are capable of responding to environmental signals of a physical nature. Substrate elasticity has been shown to influence a wide array of cellular behaviour. Substrate elasticity can affect cell morphology, survival potential, growth, migration and differentiation. Soft substrates induce cells to have lower cytoskeletal tension causing cells to appear round and small. It has also been shown that BMSCs cultured on thick or soft collagen gels can become quiescent. Whereas stiff substrates induce cells to form a rigid wide-spread morphology and express contractile stress fibres (Leong *et al*., 2010). A notable example of the ability of mesenchymal stem cells to react to an extracellular matrix constructed from collagen type 1 was presented by Engler *et al*. (2006). Mesenchymal stem cells demonstrated the potential to differentiated into different lineages such as neurons, myoblasts and osteoblasts when exposed to different substrate stiffness.

Schenke-Layland and colleagues (2007) described another particularly interesting case of extracellular matrix modulation and its effects on mouse embryonic stem cells (mESCs). It is widely believed that mESCs, derived from the inner cell mass have irreversibly committed to an epiblast lineage and that genetic manipulation would be required to trigger

differentiation into the placental lineages derived from blastoderm. However upon exposure to collagen IV, which is a component of the basement membrane, mESCs are capable of differentiating into cells associated with the placental lineage. This is not only interesting because it demonstrates the ability of stem cells to react to the extracellular matrix, but also because it challenges the definition of a pluripotent stem cell.

There are three main methods of containing cells within a hydrogel scaffold, including collagen scaffolds. The first method is matrix adhesion, where the cells bind within hydrogel foams. The hydrogel foams rely on the creation of a macroporous network, which can be created using the gas bubbles from sodium bicarbonate. When using matrix adhesion, the cells are usually allowed to attach onto the surface of the collagen gel and migrate into the gel (Jen *et al*., 1996).

The second method is known as matrix entrapment (or macroencapsulation), where cells are physically constrained within the hydrogel network. Collagen is a particularly suitable material for matrix entrapment because collagen naturally produces a crosslinked porous network, which allows for the transport of nutrients and removal of waste. Despite the restriction of migration this method has a couple of advantages, namely that *in vivo* the entrapped cells are protected from mechanical and immunological damage (Jen *et al*., 1996).

The third method is known as microencapsulation, which shares many properties of matrix entrapment. However microencapsulation relies upon the coating of single cells or small clusters of cells within a thin layer of semi-permeable scaffolding. Microencapsulation is generally used as a method of transplanting cells into a patient who requires the supply of hormones or other bioactive secreted products. (Jen *et al*., 1996). However matrix adhesion

and matrix entrapment are more appropriate techniques for the regeneration of craniofacial tissues.

Currently the ability of stem cells to react to the extra-cellular matrix has only been explored in two-dimensional (2-D) cell culture systems. This paper describes explores the preliminary stages of setting up a project to investigate the reaction of BMSCs to extracellular matrix modulation in a 3-D collagen culture system. This would involve defining the extracellular matrix conditions, cell densities and time required for any reactions to take place. Hypothetically BMSCs could react differently when exposed to 3-D gels of different thicknesses and stiffness (differential collagen concentrations). If in the longer term collagen density and gel thickness were to be found to impact upon potential for BMSC to differentiate into different lineages interface tissue grafts could potentially be produced. These grafts could be employed for craniofacial regeneration by utilising scaffolds constructed with collagen gels of different properties. It could even be possible to utilise both matrix entrapment and matrix adhesion, if these methods cause BMSCs to demonstrate different phenotypes.

Materials and Methods

Extraction of Collagen from rat tails

Materials	Source
Tails from 250-280g Wistar Hann rats	Aston University Animal House, Birmingham
Sterile Distilled Water	n/a
1% Glacial acetic acid	Sigma-Aldrich, UK

Methods

The tails were removed from 250-280g Wistar Hann rats. It is important to note that the tails were removed from rats that were sacrificed for other purposes, as it was not thought to be morally responsible to sacrifice rates only for the tails. The tails were skinned, tendons removed and placed in sterile water. To gain a near-pure collagen solution it is important to ensure that the tendons were free from other tissues. After washing three times in sterile water the tendons were removed from the water and weighed. For every 1g of tendon, 100ml of sterile water and glacial acetic acid was added to the water to give a final concentration of 1%. The solution was stored in a cold room at 4°C for 48 hours on a magnetic stirrer. Following the incubation the mixture was centrifuged at 10000 rpm for 90 minutes at 4°C, pellet discarded and the supernatant was stored at 4°C.

The Purity of Isolated Collagen

Materials	Source
Collagen solution from rat tails	n/a
Deionized Water	n/a
Tris-Acetate Poly Acrylamide Gel	(Invitrogen,)
NuPage LDS Sample Buffer (4X)	(Invitrogen,)
Tris-Glycine Native Sample Buffer (2X)	(Invitrogen,)
XCell SureLock™ Mini-Cell Electrophoresis System	(Invitrogen,)
Himark unstained molecular weight marker	(Invitrogen,)
Glacial Acetic Acid	Sigma-Aldrich, UK
Methanol	Sigma-Aldrich, UK
Coomassie blue stain R-250	Thermo Scientific

Methods

Poly Acrylamide Gel Electrophoresis (PAGE)

The PAGE was conducted as described in the manufacturer's instructions. It is important to note that a native PAGE was conducted, as opposed to a denaturing PAGE. This allows for the collagen monomers and dimers to be visualised. The wells were loaded with 20µL of the Himark unstained molecular weight marker, 10µL test mix, which included 1 µL of Collagen Stock Solution, 4 µL sterile water and 5µL of the native sample buffer. An additional test mix was made containing the collagen stock solution, which had been centrifuged for an additional 90 minutes at 14000rpm. This was conducted to determine whether improvements could be made for the solution purity.

Coomassie Blue Staining

Once the PAGE had been conducted the gel was removed from the gel cartridge and prefixed overnight in an appropriate volume of 50% methanol, 10% glacial acetic acid and 40% deionized

water. Following this incubation 0.25% Coomassie blue R-250 was added for 4 hours to stain the gel. The gel was then destained for 12 hours in 1L 5% methanol, 7.5% glacial acetic acid and 87.5% deionized water. At which point the gel could be imaged or stored in 7% glacial acetic acid.

Quantification of Collagen Concentration

Materials	Source
Collagen solution from rat tails	n/a
L-4-hydroxyproline	Sigma-Aldrich, UK
Chloramine-T	Sigma-Aldrich, UK
P-dimethylaminobenzaldehyde	Sigma-Aldrich, UK
Sodium acetate citric acid	Sigma-Aldrich, UK
Sodium Hydroxide	Sigma-Aldrich, UK
Glacial Acetic Acid	Sigma-Aldrich, UK
n-Propanol	Sigma-Aldrich, UK
Perchloric Acid	Sigma-Aldrich, UK
Sodium Chloride	Sigma-Aldrich, UK

Methods

Hydroxyproline assay

The hydroxyproline assay was conducted as described by Reddy and Enwemeka (1996). The collagen stock solution was compared to a standard curve of hydroxyproline. The standard curve was made up of 0.5, 1, 1.5, 2, 2.5, 3µg hydroxyproline in a total volume of 50µL.

Collagen Precipitation

The collagen was precipitated out from the solution to provide a simple method of validation for the hydroxyproline assay results. Collagen can be precipitated out from a solution by adding 200 µL of30% NaCl in 0.5N acetic acid for every 1mL collagen solution in a pre-weighed tube. This solution is

left over night before being centrifuged at 10000 rpm for 90 minutes at 4°C to pellet the collagen. Once the supernatant has been poured off the pellet can be left in the open tube for an hour to allow the remaining solution to evaporate and weighed.

Culture of Bone Marrow Stem Cells

Materials	Source
Femurs from 250-280g Wistar Hann rats	Aston University Animal House, Birmingham
Dulbecco's Modified Eagle's Medium(DMEM)	Sigma, UK
Fetal calf serum	PAA, The Cell Culture Co., UK
L-glutamine	Sigma, UK
Penicillin and Streptomycin	Sigma, UK
Trypsin-EDTA	Sigma, UK
FastRead Haemocytometer	Immune Systems, UK
Tryphan Blue	Sigma, UK
Leica DM IL inverted microscope	Leica Microsystems, USA

Methods

The isolation and growth of bone marrow cells was conducted using 1xDMEM supplemented with 20% FCS, 1% Pen/strep, 10% FCS and 4mM L-glutamine. The femurs were removed from the male Wistar Hann rats and soft tissue cleaned off before storing in sterile media. The condyle ends were removed and bone marrow flushed out in 5ml of media using a 23 gauge needle and 10 ml syringe and collected in a 15ml falcon tube. The bone marrow cells were then and transferred to a 75 ml tissue culture flask and made up to 10ml of sterile media and cultured at 37°C with 5% CO_2 in a humidified incubator. The media was replaced every two days until the cells reached confluence. At which point the cells were detached from the culture dish by washing with 1x phosphate-buffered saline (PBS) and incubated in 1xtrypsin-EDTA solution for 5 minutes in the conditions stated above. Once detached supplemented DMEM was added to the cells before being centrifuged at 250 RCF for

5 minutes, the pellet was resuspended in fresh supplemented DMEM and the cells were plated for further cultivation. The bone marrow stem cells were used by passage 2.

Preparation of Collagen Gel

Materials	Source
Collagen solution from rat tails	n/a
Dulbecco's Modified Eagle's Medium(DMEM)	Sigma, UK
Sodium bicarbonate	Sigma-Aldrich, UK
Sodium Hydroxide	Sigma-Aldrich, UK
Flat bottomed 24 well plates	Appleton Woods
Passage 2 Bone Marrow Stem Cells	n/a
FastRead Haemocytometer	Immune Systems, UK
Tryphan Blue	Sigma, UK
Leica DM IL inverted microscope	Leica Microsystems, USA

Methods

The collagen gels were made up in volumes suitable for a 24 well plate to test the reaction of bone marrow stem cells to different collagen concentrations and thicknesses. The specific concentrations of each reagent are detailed in table 1. The collagen solution was measured out into the 24 well plates, to which 1N Sodium hydroxide was added. 5xDMEM buffered with 1x Sodium bicarbonate containing $5x10^5$ cells (if required) was then added. The gel mix can be transferred, after being mixed well, to a humidified incubator at 37°C with 5% CO_2 for one hour. Examine the gel mix to ensure that the gel has formed formation; once the gels have solidified the gels can be immersed in complete DMEM. The media was changed every two days.

Condition	Materials	Quantity				Blank	Control
Thick Gel (1.3% Gel)	Well	1	2	3	4	5	6
	Collagen	400µL				400µL	0
	5xDMEM	560µL				560µL	0
	Cell/ml	5x10^5				0	5x10^5
	NaOH	40µL				40µL	0
	Total	1mL				1mL	0
Thin Gel (1.3% Gel)	Well	7	8	9	10	11	12
	Collagen	100µL				100µL	0
	5xDMEM	140µL				140µL	0
	Cell/ml	5x10^5				0	5x10^5
	NaOH	10µL				10µL	0
	Total	250µL				250µL	0
Condition	Materials	Quantity				Blank	Control
Low Collagen Concentration (1% Gel)	Well	13	14	15	16	17	18
	Collagen	150µL				150µL	0
	1% Acetic Acid	50µL				150µL	0
	5xDMEM	180µL				180µL	0
	Cell/ml	5x10^5				0	5x10^5
	NaOH	20µL				20µL	0
	Total	500µL				500µL	0
High Collagen Concentration (2% Gel)	Well	19	20	21	22	23	24
	Collagen	300µL				300µL	0
	1% Acetic Acid	0				0	0
	5xDMEM	180µL				180µL	0
	Cell/ml	5x10^5				0	5x10^5
	NaOH	20µL				20µL	0
	Total	500µL				500µL	0

Table 1: Constituents of the collagen gels prepared for the live dead assay

Cell viability within Collagen Gels

Materials	Source
Collagen Gels	n/a
LIVE/DEAD® Cell Viability Assay	Invitrogen, UK
Nikon TE2000-E Fluorescent Microscope	Nikon, UK
Nikon D70 Camera	Nikon, UK

Methods

Live Dead Assay

The DMEM was carefully aspirated from the surface of the collagen gels before immersing the gels in 0.2µg/ml Calcein acetoxymethylester (Calcein-AM) and 2.5µg/ml Propidium Iodide (PI) according to the manufacturer's instructions. Calcein-AM stain lives cells green and PI stains dead cells red, this can be imaged using a Nikon TE2000-E Fluorescent Microscope.

Results

The Purity of Isolated Collagen

Coomassie blue is a non-specific protein stain, which should stain all proteins present at a concentration of at least 0.5µg/cm2. Figure 3 demonstrates that the methodology to isolate a relatively pure collagen solution was successful and capable of achieving results similar to those published by Horiguchi *et al*. (2008).

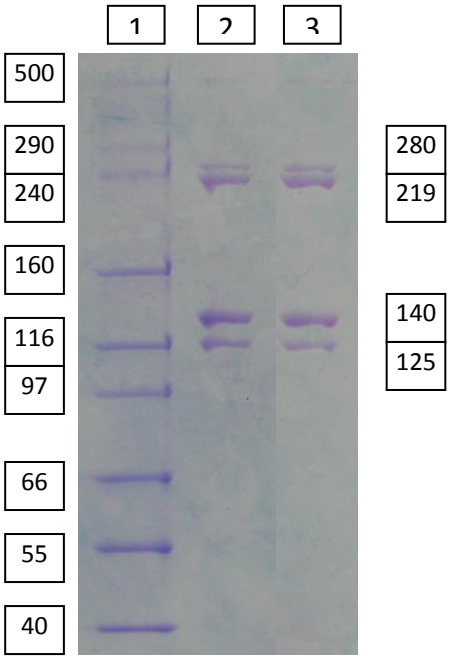

Figure 3: PAGE and Coomassie Blue staining to determine the purity of isolated collagen

Well Layout: 1 - Himark unstained molecular weight marker

2 - Collagen Stock Solution

3 - Collagen Stock Solution after additional centrifugation

Four bands can be identified clearly for each well containing a test sample, representing collagen type-1 alpha-1 (Col1a1) monomers (140 kDa), Col1a1 homodimers (280 kDa), collagen type-1 alpha-2 (Col1a2) monomers (125 kDa) and Col1a1/Col1a2 heterodimers (219 kDa). Additional centrifugation does not seem to enhance the purity of the collagen solution.

Quantification of Collagen Concentration

Hypoxyproline is a post translational product of proline hydroxylation catalysed by an enzyme prolyhydroxylase. This imino acid is thought to be exclusive to collagen. The concentration of Hydroxyproline within a sample can be used to estimate collagen concentration through the construction of a standard curve (figure 4).

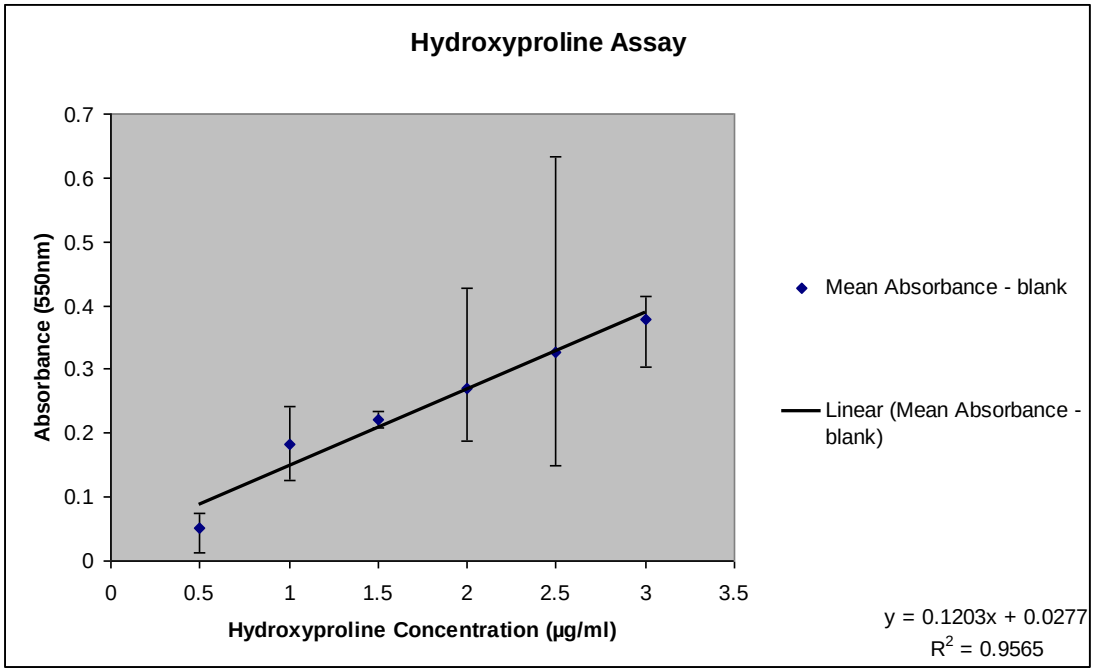

Figure 4: The standard curve produced from the hydroxyproline assay

The equation obtained from the standard curve can be applied to the absorbance obtained for the collagen stock solution to calculate the amount of hydroxyproline in the collagen stock solution:

Y = 0.1203X + 0.0277

(Y÷0.1203) - 0.0277 = X

(0.122÷0.1203) - 0.0277 = 0.9864µg/ml of hydroxyproline

Neuman and Logan (1950) presented that the collagen content can be determined by multiplying the hydroxyproline content of a sample by 7.46 because the hydroxyproline content of collagen in most mammals is 13.5% (100 ÷ 13.5% = 7.4):

0.9864 x 7.46 = 7.358544 µg/ml collagen

The collagen solution was predicted to be 7.358544µg/ml, which was lower than expected. To validate this result a collagen precipitation reaction was conducted which estimated the mean collagen concentration of the solution to be 33.339 mg/ml Collagen. Weighing the collagen precipitate is a crude method for determining the concentration of solutions. But this measurement is closer to the expected concentration than the hydroxyproline results, as collagen gels would not set at such a low concentration. For this reason the collagen solution was considered to be 33.339 mg/ml and the percentage of collagen per gel was calculated. The gels for testing the effect of collagen concentration were calculated to be 1% and 2% collagen gels, whilst the gels for testing the effect of gel thickness were calculated to be 1.3%.

Cell Survival

It was important to confirm the viability of the entrapped BMSCs over time to determine whether the collagen gels were capable of maintaining viable BMSCs or whether one condition was better than another (figure 5). The vast majority of the BMSCs entrapped within the collagen remained viable after 14 days in all the experimental conditions.

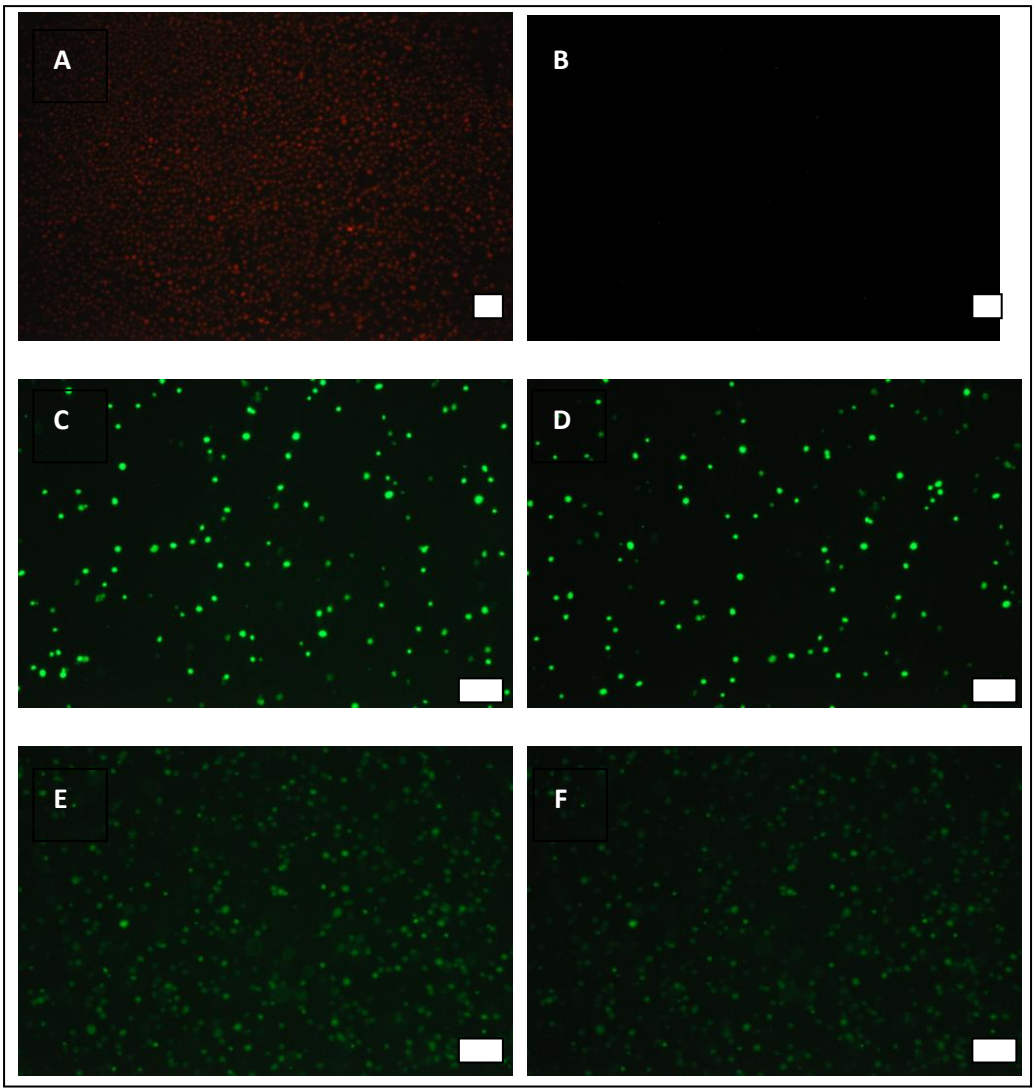

Figure 5: Live dead assay results for the cells after 14 days of being entrapped within a collagen gel

Representative images have been included in this figure for each of the different gel conditions. The live cells have been stained green with Calcein-AM and the dead cells have been stained red with PI. Each figure contains a 10µM scale bar.

A) A control group containing dead cells to verify the activity of PI

B) A gel containing no cells to control for background fluorescence

C) 1% Collagen gel

D) 2% Collagen gel

E) Thin (1.3%) collagen gel

F) Thick (1.3%) collagen gel

Discussion

The assays to quantify the concentration of collagen solutions demonstrate the need for further optimisation of the hydroxyproline assay. It is possible that the efficiency could be improved through the use of new reagents. For example if the calorimetric reagent, chloramine-T, has degraded the reaction efficiency could have been lowered due to the breakdown of chloramine-T to form hypochlorite.

Despite the inefficiency of the hydroxyproline assay, the results presented in this paper represent the successful setup and optimisation preliminary stages of an investigation. The protocols described in this could form the basis of further work to investigate whether changes to the extracellular matrix can stimulate the differentiation of BMSCs along specific lineages. The author believes that it would be interesting to explore whether BMSCs in entrapped dense collagen gels would preferentially differentiate in cells involved in bone formation. Furthermore, to explore whether BMSCs entrapped in low density collagen gels would preferentially differentiate in cells involved in adipose tissue formation.

As the properties of the extracellular matrix would be the variable factor if this project were to be continued, the differences between the different types of collagen gels must be characterised. This could be conducted using electron-microscopy to estimate the pore sizes within the collagen gels. It is expected that gels with a higher collagen concentration would have lower pore sizes than gels with lower collagen concentrations. Additionally rheology could be conducted to determine the mechanical strength of the gels. This testing could be used to determine whether the higher concentration of collagen results in greater crosslinking between the collagen molecules. The exact thickness of the collagen gels would also be determined.

Once the physical properties of the collagen gels had been quantified testing could be conducted to determine whether extracellular matrix modulation affects the BMSCs. In the first instance reverse transcription quantitative real time PCR (qPCR) could be conducted to assess specific changes in the transcriptome of the BMSCs relative to a house keeping gene such as GAPDH. It would be interesting to determine whether the entrapped cells have properties associated with a variety of markers including, but not limited to:

Proliferation - Proliferating Nuclear Cell Antigen (PNCA)

Quiescence - P53

Tissue Remodelling - Matrix metalloproteinase-1

BMSC characteristics - Nucleostemin

- C90/Thy1

Osteoblast Lineage - Collagen type 1 α1

- Bone Specific Protein

Adipocyte Lineage - Adipocyte fatty acid-binding protein 2

Chondrocyte Lineage - SOX9

Further studies at the RNA level could include the use of whole genome microarray technology to gain a full picture of the transcriptional changes occurring when BMSCs are exposed to extracellular matrix modulation. However this would be expensive and should only be used if significant changes have occurred within the cells, with respect to differentiation down a specific lineage.

Any results from the qPCR or microarray data could be confirmed using cell staining techniques such as the 5-bromo-2'-deoxyuridine (BrdU) proliferation assay (Roche, UK) or Von Kossa staining to detect mineralised cells such as those associated with bone and cartilage. Furthermore osteoblastic differentiation can be confirmed using energy-dispersive X-ray microanalysis to detect the calcium microcrystals synthesized by osteoblasts (George *et al*., 2006; Leong, 2010)

Should this project be successful these techniques could be applied to explore the effects of different types of extracellular matrix on BMSCs. For example there are many types of collagen, which are associated with a wide variety of tissues. It would be interesting to determine whether BMSCs would preferentially differentiation into chondrocytes when exposed to collagen types II, IX, X or XI. All of which are associated with the structural composition of cartilage. Furthermore the construction of different layers of types of collagen could prove to be useful in creating grafts containing different functional tissues.

Conclusion

Tissue engineering could potentially progress and present a viable alternative to transplantation. The implanted tissues could be constructed using synthetic or organic materials which can be grown after implantation into the required functionality. Alternatively these tissues could be engineered *in vitro* to form functional organs or tissues prior to implantation.

The type 1 collagen gels developed during this project are capable of supporting the viability of BMSCs. The protocols described in this thesis could form the basis for further study to explore the possibility producing scaffolds constructed with collagen gels of different properties and seeded with undifferentiated BMSCs to produce grafts comprising different layers of tissue. The grafts could be grown functional tissues *in vitro* or directly implanted whereby the graft would grow into the required functionality. These collagen gels are particularly suitable for the purpose of craniofacial regeneration not only because of their possible modulatory properties, but also their physical properties. Collagen gels are biocompatibility and have low immunogenicity properties. Furthermore collagen can be biodegraded by the body naturally; this means that graft can support the full incorporation of the graft into the host body. The host blood vessels would also be able to invade the graft to aid graft survivability. The honeycomb nature of the collagen gel provides a platform for the exchange of nutrients. Collagen gels can be created to imitate the structure of many tissue types and is capable of maintaining its structure without deforming or collapsing

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Appendix

Semi-Quantitative PCR

Semi quantitative PCR was conducted according to the following methods in an attempt to gain some preliminary results on the effects of 3-D extracellular matrix modulation. This portion of work was not completed due to persistent contamination in the negative control and possible non-specific binding or amplification of primer dimers during the Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) normalisation process.

Methods

RNA Isolation

Materials	Source
PureLink™ RNA Mini Kit	Ambion, UK
2-mercaptoethanol	Sigma-Aldrich, UK
70% ethanol (in RNase-free water)	n/a
18 gauge syringe needles	n/a

Method:

The BMSCs were lysed by passing the cells 5-10 times through an 18 gauge syringe needle. RNA was purified from the lysate using the RNeasy mini kit (including the optional DNase step) according to the manufacturer's instructions.

Generation of cDNA

Materials	**Source**
Omniscript RT Kit	Qiagen, UK

Method:

The Omniscript RT Kit was used to generate total complementary cDNA from the isolated RNA according to the manufacturer's instructions.

Normalising cDNA using GAPDH

Materials	Source
REDTaq	Ambion, UK
Forward and Reverse primers	Invitrogen, UK
cDNA	n/a
RNase free water	n/a
PCR Thermal Cycler 500 WMastercycler® Family	Eppendorf, UK
Agarose for electrophoresis	Sigma-Aldrich, UK
GelRed™	Biotium, UK
PCR Ranger 100 bp DNA Ladder	Norgen, UK
Electrophoresis tank	n/a

Primers	Sequence
GAPDH Forward Primer	5' - CGA TCC CGC TAA CAT CAA AT
GAPDH Reverse Primer	5' - GGA TGC AGG GAT GAT GTT CT

A REDTaq master mix made up by adding the following components:

Volume (µl)	Reagent
125	REDTaq
2	Forward and reverse primer mix (10µM)
<2	cDNA
25 - all of the above	RNase free water

For the first PCR reaction each tube contained 1.5µL of cDNA and 23.5µL of the REDTaq master mix. 6µL was removed after 30 and 40 cycles of the following PCR program:

94°C - 2 Minutes

94°C - 30 seconds
60°C - 20 Seconds
68°C -30 Seconds
} PCR Cycles

72°C -2 Minutes

A 1.5% agarose gel gel was made in 1x TAE buffer and 5µL of GelRed was added to the cooled gel before pouring. An 18 well comb was required. 6µL of PCR products and the PCR Ranger were loaded into the gel and ran for approximately half hour at 120 volts.

Semi quantification PCR bands

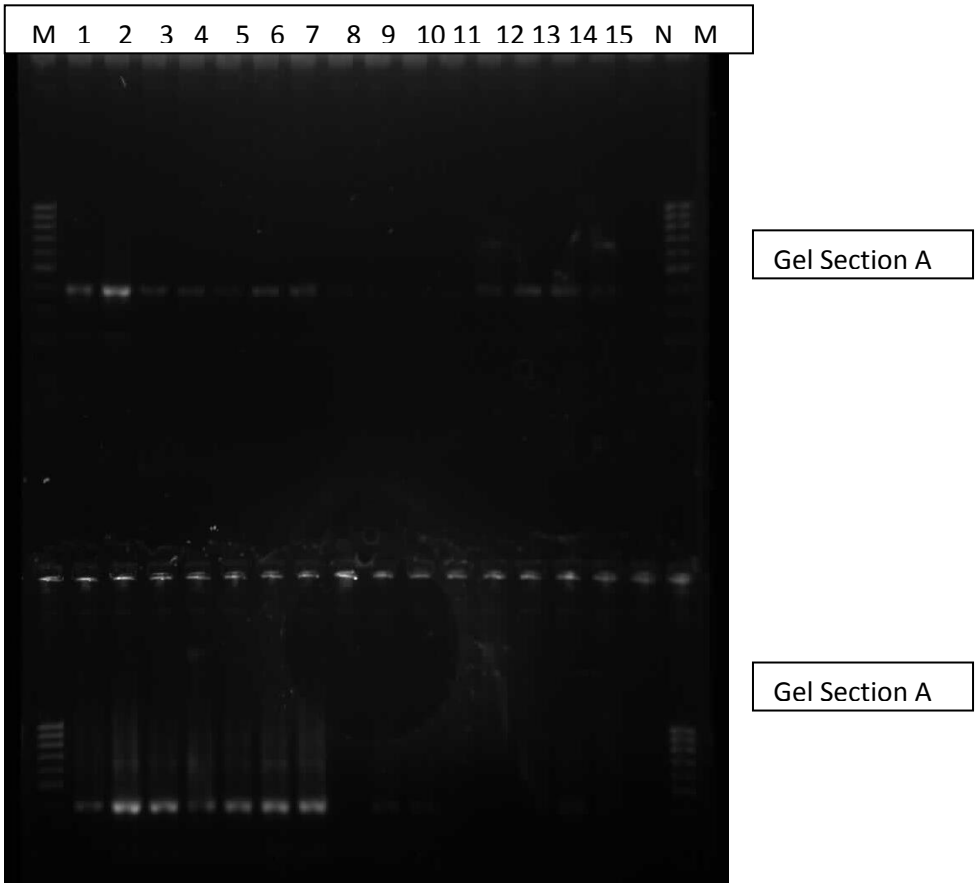
Materials	Source
Genesnap software	Syngene, UK
Gene Tool software	Syngene, UK

Images of the gels were taken using genesnap software and Gene Tool software was used to determine the intensity of the PCR product bands. These figures were used determine the amount of cDNA to use in the next PCR reaction.

Results

Well layouts:

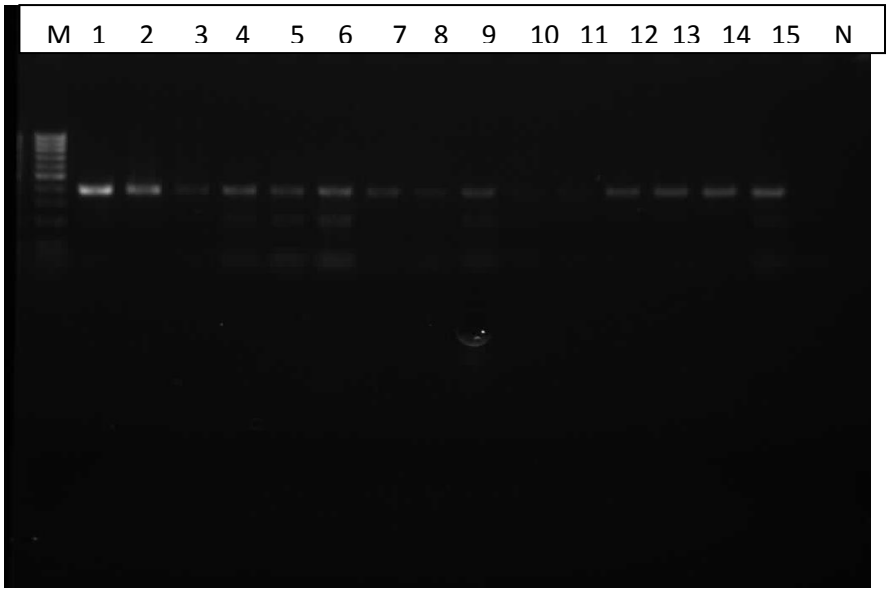
N - Negative Control
MW - PCR Ranger
1 - Freshly isolated BMSCs
2 - P1 BMSCs
3 - BMSCs after 7 days exposure to dexamethasone
4 - BMSCs after 2 days within Thick Gels
5 - BMSCs after 2 days within Thin Gels
6 - BMSCs after 7 days within Thick Gels
7 -BMSCs after 7 days within Thin Gels
8 - BMSCs after 14 days within Thick Gels
9 -BMSCs after 14 days within Thin Gels
10 - BMSCs after 2 days within 2% Gels
11 - BMSCs after 2 days within 1% Gels
12 - BMSCs after 7 days within 2% Gels
13 - BMSCs after 7 days within 1% Gels
14 - BMSCs after 14 days within 2% Gels
15 - BMSCs after 14 days within 1% Gels



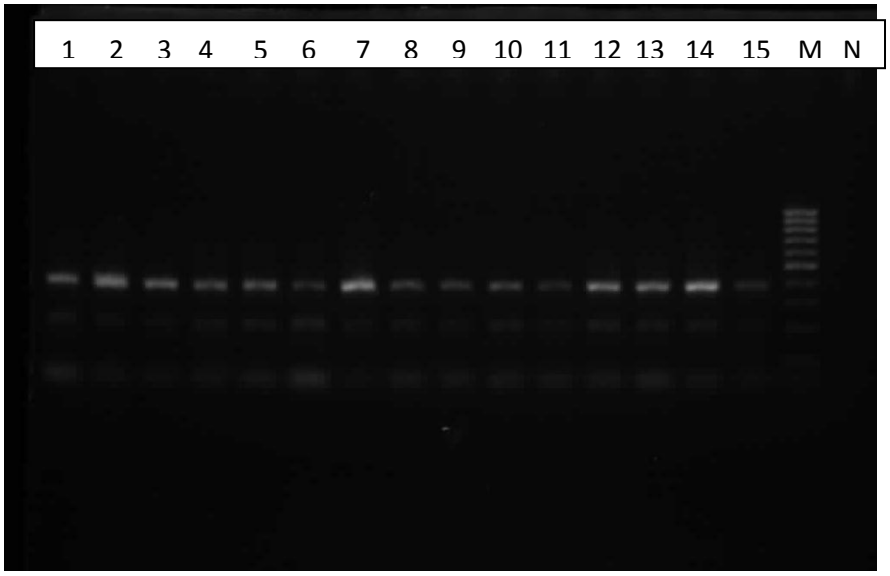
GAPDH normalisation 1 - PCR after 30 cycles (section a) and 40 cycles (section B)

The weakest bands could be visualised more easily at higher cycles, but so could undesired products.

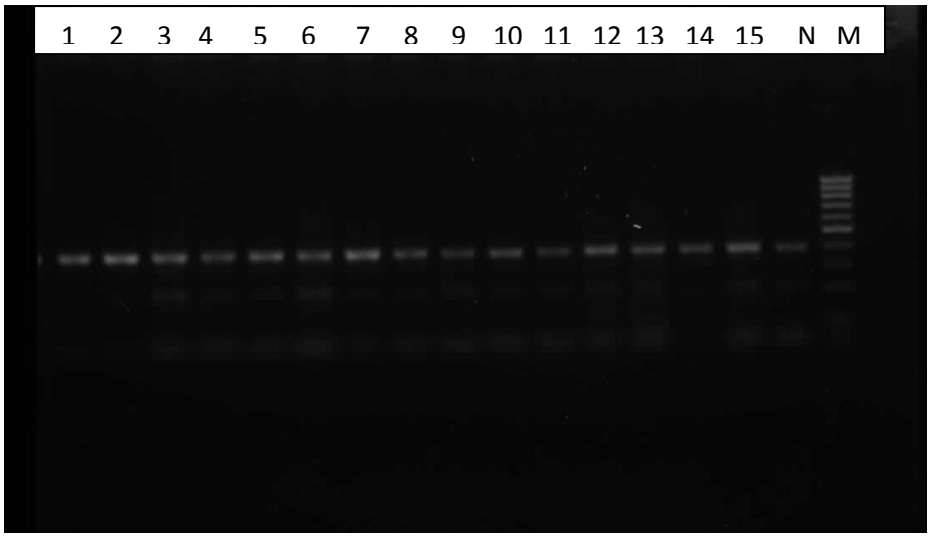
Therefore all further PCR programs utilised 35 cycles.



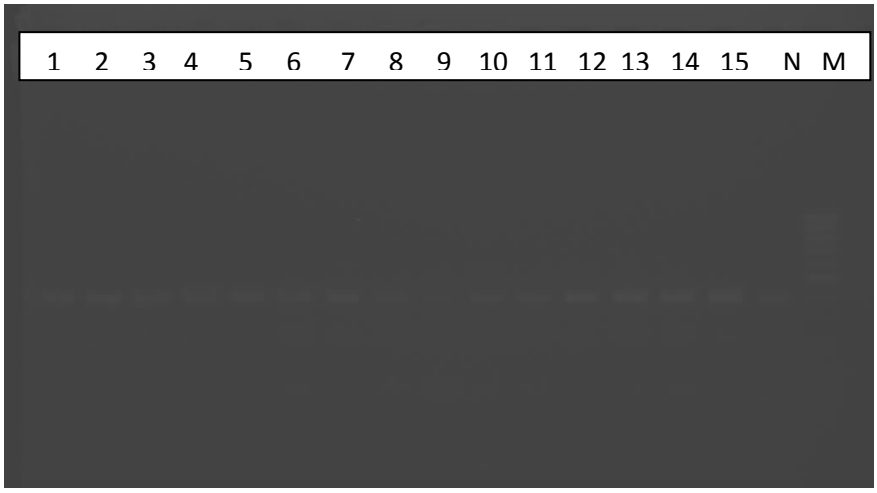
GAPDH normalisation 2



GAPDH normalisation 3



GAPDH normalisation 4



GAPDH normalisation 5