THE MOLECULAR PATHOGENESIS OF HAEMANGIOMA

By

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ABSTRACT

Haemangioma is a primary tumour of the microvasculature characterised by active angiogenesis and endothelial cell (EC) proliferation followed by slow regression or involution whereby the newly formed blood vessels are gradually replaced by fibro-fatty tissue. These developmental changes have been arbitrarily divided into the proliferative, involuting and involuted phases. The cellular and molecular events that initiate and regulate the proliferation and spontaneous involution of haemangioma remain poorly understood.

This study examined the expression of a number of genes known to be associated with angiogenesis. These include members of the signal transducers and activators of transcription (STAT) protein family of transcription factors, STAT-3 and STAT-1, and the endothelial receptor tyrosine kinases, VEGFR-1 and VEGFR-2. While STAT-3, STAT-1 and VEGFR-1 expression was detected in all phases of haemangioma, VEGFR-2 expression was found to be abundant only during the proliferative phase and decreased with ongoing involution.

In this study the cellular structures that form capillary-like outgrowths in an *in vitro* haemangioma explant model were characterised as haemangioma-derived mesenchymal stem cells (HaemDMSCs) while the cells obtained directly from dissociated proliferative haemangioma tissue were defined as haemangioma-derived endothelial progenitor cells (HaemDEPCs). This investigation showed that although the vascular endothelial growth factor (VEGF), a key growth factor for ECs, was able to maintain HaemDEPCs morphology and immunophenotype for a limited period, these cells eventually differentiated into HaemDMSCs, which subsequently differentiated into adipocytes. Furthermore, while VEGF induced significant capillary-like sprouting from tissue explants, both capillary-like sprouting and HaemDMSCs proliferation was inhibited by the addition of AG490, a Janus kinase (JAK) inhibitor which has also been shown to inhibit the STAT protein pathway. These findings indicate that the

development and differentiation of a progenitor cell and a stem cell population underlies the aetiopathogenesis of haemangioma and that VEGF and STAT signalling is involved in the programmed life-cycle of haemangioma.

The *in vitro* explant model for haemangioma offers an opportunity to study and identify novel treatment options for haemangioma. Interferon-alpha (IFN α) has been used to treat steroid-resistant haemangioma but is associated with serious side-effects. The tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) has been shown to specifically induce apoptosis of cancer cells while sparing normal cells. As IFN α has previously been shown to sensitise cells to TRAIL-induced apoptosis, this study examined the efficacy of low dose IFN α in combination with TRAIL in the *in vitro* explant model and also in the purified HaemDMSCs. Results showed that combining IFN α with TRAIL led to synergistic inhibition of capillary-like outgrowth. These results indicate that IFN α in combination with TRAIL serves as a potential treatment option for haemangioma. In contrast, HaemDMSCs were protected from TRAIL-induced killing. These cells were found to express high levels of the decoy receptors, osteoprotegerin (OPG) and decoy receptor 2 (DcR2). Increased OPG expression was also detected in the extracellular matrix and in the conditioned medium of HaemDMSCs. From these findings, we postulate that the increased level of extracellular OPG by HaemDMSCs is a stress response induced by their *in vitro* expansion and that secreted OPG functions as a protective shield preventing TRAIL action.

The empirical and unsatisfactory nature of the current therapies for haemangioma underscores the importance of a scientific approach to this common tumour. A better understanding of the molecular mechanisms that govern haemangioma is of both clinical and biological interest as it may provide vital information with therapeutic potential for haemangioma and also for other angiogenesis-dependent conditions.

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ABBREVIATIONS

ANG	Angiopoietin
APES	3-amino-propyltriethoxysilane
ApoJ	Apolipoprotein J
BCA	Bicinchoninic acid
BM	Basement membrane
bp	Base pairs
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
CA II	Carbonic anhydrase II
CL	Cell lysate
Col	Collagen
Ct	Cycle threshold
CVS	Chorionic villus sampling
CycA	Cylophilin A
DAB	Diaminobenzidine
DAPI	4',6-diamino-2-phenylindole
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EBM	Endothelial basal medium
EC	Endothelial cell
ECGS	Endothelial cell growth supplement
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EM	Electron microscopic
EMT	Endothelial to mesenchymal transdifferentiation
EPC	Endothelial progenitor cell
ERK	Extracellular signal regulated kinase
FADD	Fas-associated death domain
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FLT-4	Fms-related tyrosine kinase
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase

GLUT-1	Glucose transporter-1
H ₂ O ₂	Hydrogen peroxide
H&E	Haematoxylin and eosin
HaemDCs	Haemangioma explant-derived cells
HaemDEC	Haemangioma-derived endothelial cell
HaemDEPC	Haemangioma-derived endothelial progenitor cell
HaemDMCs	Haemangioma-derived mesenchymal cells
HaemDMSC	Haemangioma-derived mesenchymal stem cell
HGF	Hepatocyte growth factor
HPRT	Hypoxanthine phosphoribosyltransferase
HUVEC	Human vascular endothelial cell
IAP	Inhibitor of apoptosis protein
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IGF	Insulin growth factor
IHC	Immunohistochemistry
ISG	Interferon stimulated gene
JAK	Janus kinase
IL-6	Interleukin-6
Lam	Laminin
LDS	Lithium dodecyl sulphate
LeY	Lewis Y antigen
M	Medium
MC	Mast cell
min	Minutes
MCP-1	Monocyte chemoattractant protein-1
MeC	Mesenchymal cell
MMP	Matrix metalloprotease
MPC	Magnetic particle concentrator
MSC	Mesenchymal stem cell
mt Cyt <i>b</i>	Mitochondrial cytochrome b
NaBH ₄	Sodium borohydride
NaF	Sodium fluoride
Na ₂ B ₄ O ₇	Sodium tetra borate (Boric acid)
nm	Nanometer
NaO ₃ V	Sodium orthovanadate

OPG	Osteoprotegerin
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
Penstrep	Penicillin-Streptomycin
PFA	Paraformaldehyde
PI	Propidium iodide
PMSF	Phenylmethanesulphonyl fluoride
PPAR γ	Peroxisome proliferator-activated receptor- γ
PPIase	Peptidylprolyl cis-trans-isomerase
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RANK	Receptor activator of nuclear factor- κ B
RANKL	Receptor activator of nuclear factor- κ B ligand
RICH	Rapidly involuting congenital hemangioma
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
sec	Seconds
s.e.m	Standard error of the mean
siRNA	Short interfering RNA
SMA	Smooth muscle actin
SMC	Smooth muscle cell
STAT	Signal transducer and activator of transcription
STAT-3 + MC	STAT-3 positive mast cell
STAT-3 - MC	STAT-3 negative mast cell
TBS	Tris buffered saline
TBST	Tris buffered saline - Tween
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
T _m	Melting temperature
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
TL	Tissue lysate
VEGF	Vascular growth factor
VEGFR	Vascular growth factor receptor

VLA	Very late antigen
vWF	Von Willebrand factor
XIAP	X-chromosome-linked inhibitor of apoptosis protein

CHAPTER 1: GENERAL INTRODUCTION

1.1 HAEMANGIOMA

In 1982, Mulliken and Glowacki proposed to classify vascular anomalies into haemangioma or vascular malformations based on their biology (Mulliken & Glowacki, 1982). The International Society for the study of Vascular Anomalies adopted this classification for vascular anomalies in 1996 (Enjolras & Mulliken, 1997).

The term *haemangioma* refers to a rapidly growing vascular tumour of infancy (Mulliken & Glowacki, 1982) commonly known as *strawberry naevus* (Vikkula *et al.*, 1998). It is a benign tumour of the microvasculature in which there is initial excessive angiogenesis (new blood vessel growth) followed by slow spontaneous regression of the newly formed vessels within five to 12 years (Mulliken & Glowacki, 1982; Mulliken, 1988). Haemangioma does not occur during adolescence or adulthood (Mulliken, 1988).

1.1.1 THE INCIDENCE OF HAEMANGIOMA

Haemangioma is the most common tumour of infancy affecting up to 12% of Caucasians but is uncommon in other races (Mulliken, 1988; Hildano & Nakajima, 1972). An incidence of 0.8%, 1.4% and 3% has been reported in Japanese (Hildano & Nakajima, 1972), Black (Pratt, 1953), and Asian Indian (Tan, 2001), respectively. The incidence increases to 23% in premature infants with a birth weight less of than 1000 g (Amir *et al.*, 1996). Girls are three times more affected than boys (Hildano & Nakajima, 1972).

1.1.2. CLINICAL FEATURES

Haemangioma is distinct from vascular malformations. 70-90% of haemangioma appear by the fourth week of life (Mulliken, 1988; Simpson, 1959) with 30-40% being noticed at birth, usually as a pink macule, blanched area or telangiectasia surrounded by

a pale halo (Hildano & Nakajima, 1972; Payne *et al.*, 1966). The majority of lesions present as single localised cutaneous tumours in the head and neck region (60%) with the remainder being found in the trunk (25%) or the extremities (15%) (Finn *et al.*, 1983).

Haemangioma may grow to an extensive tumour in a local area or proliferate simultaneously at multiple sites in the body (Mulliken, 1988). They have been found in lymph nodes, thymus, spleen, liver, gastrointestinal tract, gallbladder, pancreas, adrenal glands, urinary bladder and lung (Edmondson, 1956; Cooper & Bolande, 1965; Burman *et al.*, 1967; Holden & Alexander, 1970; Cohen & Myers, 1986). Cases of haemangioma have also been reported in the brain, meninges and spinal cord (Cooper & Bolande, 1965; Burman & Mnasell, 1967; Cohen & Myers, 1986).

1.1.3 THE DIAGNOSIS

The diagnosis of haemangioma is well described (Mulliken, 1988; Pratt, 1953; Tan, 2001). This tumour usually first noticed as a pink macule (Figure 1.1-A), typically exhibits rapid growth during infancy, out of proportion to the child's growth. This rapid proliferation for the first 8-12 months is the hallmark of haemangioma. During this proliferative phase, the tumour assumes a bright crimson colour (hence the term *strawberry naevus*), is tense on palpation and is often tender to touch (Figure 1.1-B and -C) (Tan, 2001).

Following the proliferative phase, the tumour enters the involuting phase in which tumour growth is proportional to that of the child. During this phase, the lesion shrinks and softens. The crimson colour begins to fade becoming dull purple; the surface exhibits a mottled, greyish mantle with tiny white flecks (Figure 1.1-D). These cutaneous signs of involution start centrally and spread to the periphery. This takes at least 1-5 years. Once the haemangioma enters the involuted phase, which may take up to 10-12 years, its full life-cycle is spent (Takahashi *et al.*, 1994). The involved skin by haemangioma exhibits mild atrophy, has a wrinkled quality with few telangiectic

vessels and is slightly paler than the surrounding skin. There is often fibro-fatty tissue residuum with resultant contour deformity (Figure 1.1-E) (Tan, 2001). However, each lesion exhibits a spectrum of developmental changes, clinically and histologically (Mulliken, 1998; Tan, 2001).

50% of haemangioma show complete regression by the time the child is five years of age, and over 70% by the age of seven (Pratt, 1953; Simpson, 1959). Normal skin is restored in 50% of patients when involution is complete. Studies have also shown that the gender, race of the patient, size and site of the lesion, duration of the proliferative phase and clinical appearance of the lesion do not influence the rate of regression (Simpson, 1959; Finn *et al.*, 1983).

About 10-20% of lesions require treatment during the proliferative phase because they threaten life or function, or cause tissue distortion or destruction (Takahashi *et al.*, 1994; Mulliken *et al.*, 1995). Although all haemangioma eventually involute without treatment, they are disfiguring and may cause psychological distress, so intervention is required in a large number of patients (Tan, 2001).

1.1.4 DIFFERENTIAL DIAGNOSIS

‘Not all haemangioma look like strawberries’ (Mulliken, 1998). In the literature, almost all cutaneous vascular anomalies, at some point, have been called haemangioma. Terminological and clinical confusion between haemangioma and vascular malformations has been responsible for improper diagnosis, illogical treatment and misdirected research efforts (Mulliken & Glowacki, 1982; Mulliken *et al.*, 1995). An example of the similarity between a capillary-venous malformation that can look remarkably similar to a haemangioma that involves the skin and subcutaneous tissue is shown in Figure 1.2.

Figure 1.1: Natural progression of haemangioma on the right cheek and upper lip of a female patient. In Panels A-C, the haemangioma is in the proliferative phase. The lesion initially a pink macule noticed soon after birth then grew rapidly out of proportion of the growth of the child to assume a bright crimson colour. Photographs were taken 2 days after birth (A), at 1 month (B) and at 8 months (C). Panel D shows the lesion in the involuting phase at 18 months. During this phase, the lesion began to shrink and soften. The crimson colour changed to a dull purple and the surface exhibited a mottled, greyish mantle with tiny flecks. These cutaneous signs of involution began centrally and slowly spread to the periphery. Panel E shows the lesion at 6 years when it had fully involuted. The involved skin that remained had a wrinkled quality with mild atrophy and hypopigmentation and few telangiectic vessels. The fibro-fatty tissue residuum gave rise to distortion and contour deformity (E). Panel F shows the child 9 months after surgical excision of the residuum (F) (Courtesy of Dr Swee.T.Tan).

Figure 1.2: Not all strawberries are haemangioma. (A) A capillary-venous malformation behind the knee of a 1-year old child mimicking the appearance of a haemangioma. Unlike haemangioma, this lesion was present at birth and grew proportionately with the child. (B) A haemangioma in the right parotid region involving overlying skin and subcutaneous tissue in a 7-month old child. Spontaneous regression is expected in this lesion as in all haemangioma (Courtesy of Dr Swee.T.Tan).

1.1.5 CONGENITAL HAEMANGIOMA

There have been rare instances in which a fully-grown haemangioma is present at birth. These are known as congenital haemangioma (Boon *et al.*, 1996). Vikkula *et al* (1998) suggested that the trigger for these lesions occurred *in utero*. A subset of these lesions undergo rapid involution, usually within the first 14 months of life, leaving either atrophic or excess skin (Tan, 2001) (Figure 1.3) and are called rapidly involuting congenital hemangioma (RICH). There is also another type of congenital haemangioma called non-involuting congenital haemangioma (NICH). These lesions are present at birth but do not undergo post-natal regression (Mulliken & Enjolras, 2004). Some of the lesions have required steroid therapy or surgery because of their threat to life or function (Boon *et al.*, 1996).

Figure 1.3: A congenital haemangioma. Panel A shows the lesion on the left thigh of a 3-week old infant. Panel B shows the lesion at 14 months. By this time, spontaneous complete involution had occurred leaving behind slight hypopigmented skin. (Courtesy of Dr Swee.T.Tan).

1.1.6 COMPLICATIONS OF HAEMANGIOMA

The rapidly proliferating haemangioma may cause ulceration, obstruction of vital organs, tissue distortion or destruction, cardiac failure and skeletal changes (Tan, 2001).

Ulceration occurs in 5% of cases (Margileth & Museles, 1965) and is usually observed at the height of the proliferative phase (Bowers *et al.*, 1960). Ulcerated haemangioma are painful and is sometimes accompanied by secondary infection resulting in extensive necrosis and destruction of facial soft tissues (Tan, 2001). The incidence of ulceration is much higher in segmental hemangioma (Waner *et al.*, 2003)

A growing haemangioma can cause obstruction and tissue distortion as they encroach on a critical anatomical structure. For example, obstruction of the visual axis may cause deprivation amblyopia and the failure to develop binocular vision (Tan, 2001). Obstruction of the external auditory meatus can occur with haemangioma involving the parotid region (Figure 1.4-A and Figure 1.5-A). Hearing loss results if the lesion continues to grow beyond one year, when auditory conduction is necessary for normal speech development (Tan, 2001). Cardiac failure and skeletal changes such as overgrowth of the facial skeleton have also been described (Tan, 2001).

1.1.7 HEAD AND NECK HAEMANGIOMA

Haemangioma of the head and neck has been identified to favour certain sites on the face (Waner *et al.*, 2003). The importance of anatomic location is now well recognised as a major factor in determining whether complications such as visual axis occlusion, airway involvement, ulceration or permanent disfigurement may occur (Metry *et al.*, 2001; Garzon *et al.*, 2000; Zide *et al.*, 1997; Haik *et al.*, 1994). Waner *et al* (2003) categorised haemangioma into segmental and focal lesions.

Focal lesions are localised and tumour-like and most commonly involve the mid cheek, upper lip and upper eyelid (Waner *et al.*, 2003). An example of a focal lesion is depicted in Figure 1.6.

Segmental lesions are more widespread and plaque-like. The segmental distribution of these lesions is described as frontonasal, maxillary or mandibular and corresponds to the embryological mesenchymal prominences of the head and face. Ulceration and airway obstruction are more common in segmental lesions (Waner *et al.*, 2003). The female to male ratio of patients with segmental lesions is 5.7:1, almost twice that of patients with focal lesions (Waner *et al.*, 2003). Figures 1.1, 1.2-B, 1.4 and 1.5 show segmental haemangioma lesions.

Figure 1.4: A bilateral cervico-facial haemangioma. Panel **A** shows the lesion on a 1-year old girl with the 'beard' distribution, impinging on the upper airway. In Panel **B**, accelerated regression had occurred after a 9-month course of IFN α -2a therapy. Photograph was taken at age 1 year and 10 months (**B**) (Courtesy of Dr Swee.T.Tan).

Figure 1.5: A proliferating haemangioma on the left parotid region. Panel A shows a proliferating haemangioma in a 2-month old female infant with ulceration and obstruction of the auditory meatus. In Panel B, the lesion showed accelerated regression following systemic steroid therapy. Photograph was taken at 4 years and 3 months. At this stage, involution was complete leaving behind thin, atrophic and hypopigmented skin with some degree of fatty residuum (*Courtesy of Dr Swee.T.Tan*).

Figure 1.6: Natural progression of haemangioma near the right eye. The lesion initially a pink macule noticed soon after birth (A) became more prominent at 2 weeks of age (B) then grew rapidly to assume a bright crimson colour (C). Photograph was taken at 2 months (C). At 4 years, the lesion had fully involuted (D). The remaining fibro-fatty tissue residuum was then surgically removed (*Courtesy of Dr Swee.T.Tan*).

Segmental haemangioma have been associated with a clinically recognised set of structural malformations and anomalies designated as PHACE or PHACES syndrome (Frieden *et al.*, 1996; Reese *et al.*, 1993). This association is characterised by posterior fossa anomalies, *haemangioma* that are typically plaque-like and segmental in the cervicofacial area, arterial anomalies, cardiac and aortic arch defects, eye anomalies and sternal clefts or supraumbilical raphes. Brain abnormalities such as Dandy-Walker syndrome and cerebrovascular arterial anomalies have also been described (Adams & Lucky, 2006). A child with PHACES syndrome with segmental haemangioma associated with a sternal cleft is shown in Figure 1.7.

Figure 1.7: A patient with PHACES syndrome with segmental haemangioma associated with a sternal cleft. (Courtesy of Dr Swee.T.Tan).

1.1.8 TREATMENT

The majority of haemangioma are harmless cutaneous lesions that require no intervention and should be left to involute spontaneously (Margileth & Museles, 1965). However, treatment is necessary during the proliferative phase in 10-20% of cases because the tumour threatens life or function, causes tissue distortion and destruction (Takahashi *et al.*, 1994; Mulliken *et al.*, 1995). Various therapeutic modalities have been described, but pharmacological therapy with high dose systemic or intralesional corticosteroids is the first line of treatment (Mulliken *et al.*, 1995). Corticosteroids arrest growth in 30-90% of cases, however, side effects such as delayed skeletal growth, depressed immune function and personality changes limit their usefulness, particularly when prolonged treatment is required (Boon *et al.*, 1999).

In life-threatening cases that do not responded to steroids, IFN treatment has been used (Figure 1.5) (Ezekowitz *et al.*, 1992; Chang *et al.*, 1997). Both IFN α -2a and IFN α -2b are given subcutaneously with an average duration of 2 to 6 months (Drolet *et al.*, 1999; Garmendía *et al.*, 2001). The reported efficacy varies according to its definition in various series and ranges between 30-70% (Garmendía *et al.*, 2001). However, its use has been limited due to reports of severe neurotoxicity in the form of spastic diplegia in up to 20% of patients treated with IFN α -2a and in about 5% with IFN α -2b (Barlow *et al.*, 1998; Worle *et al.*, 1999; Dubois *et al.*, 1999). Chemotherapy has also been used to treat life-threatening, steroid-resistant haemangioma. Cyclophosphamide was used successfully in three infants with life-threatening, steroid-resistant haemangioma (Hurvitz *et al.*, 1986). A few reports on the use of vincristine also exist (Fawcett *et al.*, 2004). Other strategies that have been utilised, with varying degrees of success, include embolisation, surgery, laser therapy and sclerotherapy (Enjolras *et al.*, 1990; Jackson *et al.*, 1993; Tan, 2001). Surgery is considered an effective treatment for localised problematic proliferating haemangioma (Tan, 2001).

1.1.9 GENETICS IN HAEMANGIOMA

Although a family history has been noted in 10% of affected individuals (Margileth & Museles, 1965; Cheung *et al.*, 1997), it is generally regarded that haemangioma does not have a hereditary basis (Tan, 2001). However, being a Caucasian female with a positive family history apparently lowers the threshold for haemangiogenesis wherein extragenetic factors can trigger the appearance of haemangioma (Vikkula *et al.*, 1998).

1.1.10 HISTOPATHOLOGY

The pathogenesis of haemangioma is not well understood. The life cycle of haemangioma has been arbitrarily divided into three phases: (i) the *proliferative* phase, (ii) the *involuting* phase and (iii) the *involved* phase (Enjolras & Mulliken, 1997; Takahashi *et al.*, 1994, Tan, 2001). These phases describe the gross morphological changes associated with tumour onset, growth, and subsidence.

In the early proliferative phase, the lesions are highly cellular and consist of syncytial masses of plump ECs with tiny lumens (Tan, 2001). Occasional mitotic figures can be seen but the nuclei do not exhibit pleomorphism (Figure 1.8-A). Later in the proliferative phase, vascular channels are less compact and capillary-sized lumens can be seen lined with plump, mitotically active ECs and pericytes (Figure 1.8-B) (Vikkula *et al.*, 1998; Glukhova *et al.*, 1991). Multilamination of the basement membrane (BM) and accumulation of other cellular elements including mast cells (MCs), macrophages, fibroblasts, plasma cells, and interstitial cells are also seen (Vikkula *et al.*, 1998; Gonzalez-Crussi *et al.*, 1991; Glowacki *et al.*, 1982; Pasyk *et al.*, 1984; Dethlefsen *et al.*, 1986; Takahashi *et al.*, 1994; Tan *et al.*, 2004). As the haemangioma progresses to the involuting phase, the vascular lumens dilate, ECs flatten and the cellular elements are gradually replaced by fibro-fatty tissue, giving the haemangioma a lobular architecture (Figure 1.8-C). Progressive fibrosis is seen in the

late involuting phase (Figure 1.8-D and -E). In the involuted phase, marked fibrosis and a few thick-walled channels lined with flat endothelium are seen (Figure 1.8-F). A fully involuted haemangioma contains few capillary-like feeding vessels, and possess draining veins with flattened endothelia that lie in a stroma of fatty tissue containing collagen and reticulin fibres.

Figure 1.8: Histopathological features of haemangioma at different phases of development. A haematoxylin and eosin (H&E) stained haemangioma specimen from an 18-day old infant, showing proliferating endothelial masses with tiny lumens (arrows) (**A**). Occasional mitotic figures (insert: arrowhead) can be seen but nuclei do not exhibit pleomorphism (**A insert**). Later in the proliferative phase, vascular channels are less compact and capillary-sized lumens (arrows) can be seen lined with plump ECs in a specimen from a 5-month old child (**B**). In the early involuting phase, the vascular channels (arrows) are larger but there are still areas of endothelial proliferation (right upper corner) in this specimen from an 8-month old child (**C**). With further involution, the cellular elements are gradually replaced by fibro-fatty tissue (arrowheads). Progressive fibrosis is seen in this specimen taken from a 2 years and 9 months old child (**D**). Increasing fibrosis (arrowheads) with reduced cellularity occurs in the late involuting phase at 3 years and 6 months (**E**). Fibrosis replaced the cellular elements and few thick walled channels remain, lined with flat endothelium (arrows) in a specimen from an 8 years and 6 months old child (**F**). Original magnifications: 214X (insert: 640X) (*Courtesy of Dr Swee.T.Tan*).

Studies have also shown that although all samples predominantly exhibit the histological features characteristic of either the proliferative, involuting or involuted phases, distinct areas displaying proliferation or varying degrees of involution are evident in each lesion. This indicates a continuous spectrum of structural changes during the natural progression of haemangioma (Tan, 2001) (Figure 1.9).

Figure 1.9: A haemangioma on the elbow of a 13-month old girl. The image in panel **A**, shows the ‘Proliferative’ (P), ‘involuting’ (Ig), and ‘involved’ (Id) regions representing the different stages within a single lesion. Panel **B** shows an H&E stained tissue section of the biopsy taken from **A** clearly showing defined regions of proliferation (lower right) and involution (upper left). Original magnification: 214X (*Courtesy of Dr Swee.T.Tan*).

1.2. CURRENT THEORIES ON THE ORIGINS OF HAEMANGIOMA

1.2.1. PLACENTAL ORIGIN

North *et al* (2001) recently identified the expression of a distinct constellation of tissue-specific markers, co-expressed in both haemangioma and placental microvessels. They found that a set of placenta-associated vascular antigens including Fc γ RII and Lewis Y antigen (LeY) and placenta marker proteins including merosin and the glucose transporter-1 (GLUT-1) were expressed in 66 haemangioma lesions isolated from patients aged between 22 days and seven years.

It is now known that with the exception of GLUT-1 and merosin being present in brain microvessels, none of the other placenta-associated markers are expressed in the microvessels of normal skin. GLUT-1 is widely expressed early in development, but foetal endothelial GLUT-1 expression rapidly disappears except in microvessels of developing neuroepithelial tissues (Froehner *et al.*, 1998; Harik *et al.*, 1993; Mantych *et al.*, 1993_a; Mantych *et al.*, 1993_b). In the mature brain, a component of the blood brain barrier, necessitates high GLUT-1 expression for adequate blood:brain glucose transport (Pardridge *et al.*, 1990). In the placenta, GLUT-1 expression is required throughout gestation for adequate maternal-foetal glucose transfer (Jansson *et al.*, 1993), independent of developmental stage (Hahn *et al.*, 1995). Thus, North *et al* (2001) proposed that high GLUT-1 expression by haemangioma may permit tumour growth by providing fuel for mitosis.

Intense expression of merosin in a broad, continuous, band-like pattern encircling lesional capillaries in GLUT-1 positive haemangioma during all stages of proliferation and involution has been reported (Martin-Padura *et al.*, 1995; North *et al.*, 2001). Merosin expression is normally restricted to the nervous system, eyes (Villanova *et al.*, 1996) and placenta (Voit *et al.*, 1994). The merosin α 2 chain, one of ten distinct

laminin chains, is also present in certain nonvascular BM, including those of placental trophoblast, schwann cells, striated muscle and skin (Engvall *et al.*, 1990; Leivo *et al.*, 1988; Sewry *et al.*, 1996). The heterogeneity of laminin subtypes in the BM and the highly restricted expression of merosin suggests tissue-specific and developmental stage-specific functional diversity. Merosin is essential for stability and survival of fused myoblasts *in vitro*, by inhibiting apoptosis (Vachon *et al.*, 1996). Therefore, a role for merosin in the growth of haemangioma by inhibiting apoptosis has been suggested (North *et al.*, 2001).

The persistent expression of LeY and Fc γ RII in the endothelium of haemangioma and placental vessels is of unclear functional significance. LeY is a glycosylated oligosaccharide, expressed on the cell surface in a tissue-specific and stage-specific manner. It is important in cell-cell recognition and adhesion during development. Its expression has been associated with malignant transformation and progression (Lloyd *et al.*, 1987; Zhu *et al.*, 1995; Wakabayashi *et al.*, 1995) and also apoptosis (Hiraishi *et al.*, 1993; Minamide *et al.*, 1995). Fc γ RII is a low affinity Fc receptor normally expressed in macrophages, Langerhan cells, platelets and other leukocytes (Sedmak *et al.*, 1991; Pulford *et al.*, 1986). Its restricted expression to placental endothelium and its ability to bind only immunoglobulins suggest that it may be uniquely advantageous in placenta perhaps assisting clearance of immune complexes and/or maternal-foetal immunoglobulin transport (Sedmak *et al.*, 1991; Lang *et al.*, 1993).

North *et al* (2001) suggested that this unique phenotype shared between haemangioma and the placental foetal microvessels may be due to haemangioma ECs that originated from either invading angioblasts and aberrantly differentiated towards the placental microvascular phenotype. Alternatively, the ECs could have come from embolised placental cells or precursors that were dislodged into foetal circulation during gestation or at birth. Given the previously identified increased incidence of

haemangioma in infants exposed to chorionic villus sampling (CVS) (Burton *et al.*, 1995), North *et al* (2001) suggested that embolic placental ECs, already committed to the placental phenotype (GLUT-1/merosin/LeY/FcRII) could have reached foetal tissues from the chorionic villi through the right to left shunts of normal foetal circulation. In addition, Marchuk (2001) suggested that the local placental injury that usually occurs in CVS could result in increased intravascular shedding of placental cells from the chorionic villi into the foetal circulation. Furthermore, if the embolus contained a single EC or only a small number of ECs, this would be compatible with the clonality results of Boye *et al* (2001) (see section 1.2.2.1.2, page 19).

In contrast, the findings of Bree *et al* (2001) did not support the hypothesis that the placental trophoblast was the cell of origin for haemangioma as they lacked a villous architecture and did not express known placental trophoblastic markers. These investigators found no immunoreactivity for the trophoblastic markers, including human placental lactogen, placental alkaline phosphatase, and cytokeratins 7, 8, and 17, in 12 haemangioma tissue specimens from patients aged five days to two years.

1.2.2. EXPANSION OF ENDOTHELIAL CELLS IN HAEMANGIOMA: AN INTRINSIC OR EXTRINSIC DEFECT?

The process of foetal vascular development and angiogenesis is complex with several receptor tyrosine kinases (RTKs) and their ligands, namely angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and VEGF as known modulators (Cohen, 2002). Alteration in this system, at either the molecular or chromosomal level, has been suggested to account for the unregulated growth of blood vessels seen in haemangioma. In this paradigm, the rapid growth of these tumours in the postnatal period may be explained by the loss of angiogenic inhibitors derived from the placenta or mother (North *et al.*, 2002). This idea has lead to another hypothesis for the highly proliferating ECs seen in haemangioma as being derived from angioblasts, aberrantly switched to a

placental phenotype by somatic mutations and/or other abnormal inductive influences (an intrinsic defect). Furthermore, Bielenberg *et al* (1999) found that the epidermis overlying proliferating haemangioma showed altered expression of angiogenic factors. They suggested that the underlying defect may be extrinsic to the vascular ECs, which were responding to an aberrant exogenous growth signal (an extrinsic defect).

1.2.2.1. AN INTRINSIC DEFECT?

This theory suggests that the dramatic changes in endothelial behaviour in haemangioma may be caused by somatic mutations in a single EC which results in the disruption of normal controlled EC growth (Berg *et al.*, 2001; Boye *et al.*, 2001; Walter *et al.*, 2002).

1.2.2.1.1. Familial Studies

Although current evidence points towards the sporadic nature of haemangioma, a small number of families have been identified, wherein haemangioma and other vascular lesions segregated as a highly penetrant, autosomal dominant trait. Familial studies have suggested that the genetic alteration may be a somatic mutation or a common predisposing allele with incomplete penetrance (Vikkula, 2000; Tan, 2001). Families with suggestive autosomal dominant inheritance with incomplete penetrance and variable phenotypic expression have been identified (Blei *et al.*, 1998). These families represent, (i) subtypes of rare mutations causing autosomal dominantly inherited haemangioma; (ii) kindreds in which a common low penetrant mutation shows autosomal dominant pattern of inheritance; or (iii) families with random aggregation of a common genetic trait. In gene linkage studies involving three kindreds with familial haemangioma, Blei *et al* (1998) found linkage to the 5q chromosome. Walter *et al* (1999) further mapped this region to be 5q31-33. Candidate genes involved in blood vessel growth within this region include fibroblast growth factor-4 (FGF-4), platelet-derived growth factor- β (PDGF- β) and fms-related tyrosine kinase-4 (FLT-4). In

addition, a subsequent study by Berg *et al* (2001) found evidence for loss of heterozygosity on chromosome 5q ($p < 0.05$) in three out of six haemangiomas. They suggested that the deletion of a gene or genes in this chromosomal region may be caused by an intrinsic endothelial alteration rather than an external stimulus and that this somatic mutation may be the cause of sporadic haemangioma.

1.2.2.1.2. Haemangioma-derived Endothelial Cell Studies

Boye *et al* (2001) purified ECs from proliferating haemangioma (HaemECs) obtained from eight female patients and found that these HaemECs were clonal. These authors identified a non-random X chromosome inactivation pattern for the human androgen receptor gene locus and proposed a clonal origin for these cells. In contrast, the non-endothelial stromal cells isolated from the same lesions, were not clonal. These results demonstrated that clonality was restricted to the ECs within the lesion. They hypothesised that EC clonality may be due to somatic mutations in the genes of the ECs but not in other cell types constituting haemangioma. These investigators also suggested that although stromal cells and the overlying epidermis of haemangioma exhibited properties, including aberrant expression of angiogenic factors (Bielenberg *et al.*, 1999; Berard *et al.*, 1997), the ECs would not be expected to develop as clones of a single founder cell if they were merely responding to an externally derived angiogenic signal.

Boye *et al* (2001) found that HaemECs exhibited increased proliferation and migration in response to VEGF as compared to normal ECs. Furthermore, VEGF-induced migration in HaemDECs was stimulated instead of being inhibited by the angiogenesis inhibitor, endostatin. They suggested that alterations in the signalling pathways downstream of the VEGF receptor in HaemDECs, may be the result of somatic mutations that converted the inhibitory effect of endostatin to one of stimulation.

Walter *et al* (2002) also identified significant skewing toward one allele in haemangioma lesions. These authors performed clonality assays on DNA isolated from

haemangioma tissue subjected to gross dissection without the selective step of EC growth in culture and thereby avoided changes in cell composition that could occur with cell cultures.

Therefore, these studies provide strong support for the hypothesis that the clonal expansion and the abnormal proliferation of haemangioma is the result of one or more somatic mutations in key factors involved in angiogenesis. Although formal proof of this hypothesis awaits validation, Walter *et al* (2002) identified missense mutations in two VEGF receptors (VEGFRs), VEGFR-2 and VEGFR-3, which were only found in the haemangioma tissue and not in the patient matched normal skin. Therefore, these authors suggested that EC proliferation was the result of deregulated VEGF signalling.

There have also been reports on experimentally induced haemangioma lesions in mice. Williams *et al* (1989) showed that the injection of mouse ECs expressing the polyoma middle-T oncogene into adult mice resulted in the development of tumours that resembled haemangioma. These lesions were composed of host-derived ECs, apparently recruited by the injected oncogenic cells (Williams *et al.*, 1989). In contrast, Boye *et al* (2001) showed no evidence for additional EC recruitment by the original tumour ECs.

Li *et al* (2003) recently identified CD146 to be a possible marker for haemangioma ECs due to its reduced expression in these cells compared to the surrounding pericyte-like cells, and also to normal ECs. Studies have also shown that the ECs in haemangioma showed altered expression of several other angiogenesis-related molecules, such as E-selectin, TIE-2, VEGF, FGF-2 and insulin growth factor (IGF) (Kraling *et al.*, 1996; Yu *et al.*, 2001; Ritter *et al.*, 2001). These findings together with the down-regulation of CD146 expression suggest that haemangioma may be a consequence of a genetic alteration in haemangioma ECs (Li *et al.*, 2003).

1.2.2.2. AN EXTRINSIC DEFECT?

The extrinsic hypothesis invokes a defect in the local environment, which produces a signal or stimulus that causes haemangioma. For example, Berard *et al* (1997) found that stromal cells isolated from proliferating haemangioma and cultured *in vitro* released VEGF. Transcripts for the VEGFRs, VEGFR-1 and VEGFR-2 are abundantly expressed in proliferating haemangioma (Yu *et al.*, 2001), suggesting the high expression of these receptors in these lesions. Therefore, increased VEGF produced by stromal cells could accelerate the growth of haemangioma.

Bielenberg *et al* (1999) found a dramatic alteration in expression of IFNs in the epidermis overlying haemangioma. Compared with the epidermis of normal skin, there was a dramatic down-regulation of IFN β in the epidermis overlying proliferating phase haemangioma. However, gradual restoration of IFN β was observed as the lesion progressed to the involuting and involuted phases, with near-normal levels detected by five to 11 years of age. This dramatic down-regulation of IFN β expression coinciding with the proliferating phase of haemangioma provides another example of a possible extrinsic alteration in haemangioma.

1.2.3. THE ANGIOGENESIS CONCEPT

The concept that tumours are ‘angiogenic dependent’ was first proposed by Folkman and suggests new insights into the life cycle of haemangioma (Folkman, 1997; Folkman & Cotran, 1976). Angiogenic molecules are known to act on ECs and pericytes to initiate the formation of capillary networks (Risau *et al.*, 1988; Folkman & Klagsburn, 1987; Zetter, 1988; Breier *et al.*, 1992; Pepper *et al.*, 1992; Montesano *et al.*, 1986; Mignatti *et al.*, 1989). This process is tightly regulated by inhibitors of EC growth, which help to maintain the microvasculature in a quiescent state under physiological conditions (Mignatti *et al.*, 1989; Risau, 1995; Risau, 1997; Montesano *et al.*, 1986)

The angiogenesis concept proposes that capillaries are normally prevented from growing by physiological regulation of angiogenic factors. These factors are found in neoplastic tissue but are also present in normal tissue where neovascularisation does not occur (Folkman, 1996; Risau *et al.*, 1988; Montesano *et al.*, 1986). Release of stored angiogenic factors occurs in short bursts of capillary proliferation during wound healing, and in female, ovulation and menstruation (Augustin *et al.*, 1995; Klauber *et al.*, 1997). The rapidly growing haemangioma is reminiscent of capillary proliferation seen during wound healing and the neovascularisation associated with tumour growth. Amongst the plethora of angiogenic regulators, VEGF, VEGFRs, Ang-1, Ang-2 and their RTKs, TIE-1 and TIE-2, play crucial roles in maintaining the balance between vascular growth and regression (Ferrara *et al.*, 1992; Fong *et al.*, 1995; Shalaby *et al.*, 1995; Suri *et al.*, 1996; Carmeliet *et al.*, 1996_a; Hanahan, 1997; Maisonpierre *et al.*, 1997). Other factors such as FGF-2, transforming growth factor- β (TGF- β), PDGF and interleukin-6 (IL-6) are highly relevant as is the extracellular matrix (ECM) (Folkman & Klagsbrun, 1987; Tomanek and Schatteman, 2000; Haas *et al.*, 1999). Some of these factors are now known to be involved in both the proliferation and involution of haemangioma (Takahashi *et al.*, 1994; Jang *et al.*, 1998; Chang *et al.*, 1999; Bielenberg *et al.*, 1999) and are discussed in section 1.4, page 30.

1.3. CELLULAR COMPONENTS OF HAEMANGIOMA

1.3.1 ENDOTHELIAL CELLS

The hallmark of a growing haemangioma is the proliferating ECs forming syncytial masses with or without luminal formation (Tan *et al.*, 2000_a). Occasional mitotic figures are seen but the nuclei do not exhibit pleomorphism (Mulliken, 1988). As the lesion progresses, the later stages of the proliferating tumour consists of less compact vascular channels and capillary-sized lumens lined with highly cellular plump, proliferating ECs organised into lobules within a limiting membrane of reticulin fibres

(Mulliken, 1988). Confocal microscopy showed the three-dimensional images of these densely disorganised tortuous networks of vessels in proliferating haemangioma compared with normal tissues (Takahashi *et al.*, 1994).

The ECs within the growing lesion are proliferating as identified by tritiated thymidine autoradiography (Mulliken & Glowacki, 1982) and by immunohistochemical staining for proliferating cell nuclear antigen (PCNA) (Tan *et al.*, 2000_a; Tan, 2001). Studies showed that the majority of ECs in the proliferative and early involuting phase expressed PCNA but this number decreased with the on-going involution of haemangioma. PCNA staining in pericytes and MCs during the proliferative have also been identified (Hasan *et al.*, 2000_a).

Electron microscopic (EM) studies showed that the ECs in haemangioma exhibited characteristics of intracellular activity such as a convoluted nuclear membrane, swollen mitochondria, membranes of rough endoplasmic reticulum, and clusters of free ribosomes (Dethlefsen *et al.*, 1986; Hopfel-Kreiner, 1980). The luminal surface of these ECs exhibited thin projections whereas the basal side had thicker, club like projections (Dethlefsen *et al.*, 1986; Mulliken, 1988). In contrast, the lumen in involuting haemangioma contained EC remnants, often lined by only one or two ECs with signs of endothelial discontinuity and vessel degradation. EM of involuted haemangioma showed that the vessels had reverted to a more normal architecture and contained single layers of ECs surrounded by pericytes. From these findings Mulliken (1988) suggested that with development, haemangioma becomes more organised, as the lobular components were separated by fibrous septae that contained large calibre feeding and draining vessels.

ECs express specific markers that are used in their identification *in vivo* and in culture. Some of these include von Willebrand Factor (vWF), CD31 (Belloni & Tressler, 1990; DeLisser *et al.*, 1994), CD34 (Krause *et al.*, 1996), VEGFR-1 and

VEGFR-2 (Rettig *et al.*, 1992; Eichmann *et al.*, 1993; Quinn *et al.*, 1993; Peters *et al.*, 1993; Mustonen & Alitalo, 1995).

Studies have shown that although the ECs in haemangioma expressed CD31, vWF and urokinase in all phases, the levels of expression varied between phases. Takahashi *et al* (1994) showed that CD31 expression was significantly higher in the involuting phase compared to the involuted phase and vWF expression was higher in the proliferative and involuting phases but not in the involuted phase. Furthermore, Tan *et al* (2001_a) found that not every vessel in the involuted phase expressed these proteins. These findings are consistent with the current knowledge of these markers, i.e., they are not always expressed by ECs (Parums *et al.*, 1990; Kuzu *et al.*, 1992). Irregular vWF expression has also been identified in capillaries and tumour vessels (Garlanda & Dejana, 1997).

The glycoprotein, E-selectin is an EC-specific leukocyte adhesion molecule, involved in angiogenesis and is important in capillary tube formation (Nguyen *et al.*, 1993). High expression of E-selectin and VE-cadherin has been identified in the proliferative phase of haemangioma (Kraling *et al.*, 1996).

Expression of other endothelial markers such as CD34, intercellular adhesion molecule 1 (ICAM-1) and the very late antigen (VLA) integrin have also been identified in haemangioma. Martin-Padura *et al* (1995) showed that these markers were expressed in haemangioma to similar levels as in normal ECs. Therefore, they suggested that the abnormal EC proliferation in the proliferating lesion was more related to the release of local factors and fibrinolytic agents, rather than an altered endothelial phenotype.

GLUT-1 has recently been described as a marker for haemangioma. North *et al* (2000) showed that the ECs of haemangioma from all phases expressed GLUT-1 while GLUT-1 immunoreactivity was absent in other vascular lesions.

Li *et al* (2003) described the transmembrane glycoprotein, CD146 as another marker for haemangioma. This molecule is also considered a marker for ECs (Bardin *et al.*, 1996). It is involved in cell adhesion (Bardin *et al.*, 2001) and in the regulation, paracellular permeability and in signal transduction of ECs (Bardin *et al.*, 2001; Anfosso *et al.*, 1998; Anfosso *et al.*, 2001). Li *et al* (2003) found significantly low CD146 expression by the ECs of haemangioma biopsies from all phases of development. In contrast, the pericyte-like cells surrounding the endothelium in haemangioma showed strong immunoreactivity. Therefore, they suggested that CD146 expression may be useful in distinguishing HaemDECs from normal ECs (Li *et al.*, 2003). Their studies also showed that CD146 expression differed from previously described markers such as merosin, GLUT-1, FcγRII and LeY (North *et al.*, 2001; North *et al.*, 2000), in that, the reduced CD146 expression in haemangioma tissue was maintained even after the HaemDECs were isolated and cultured *in vitro* (Li *et al.*, 2003). On the other hand, while GLUT-1 and FcγRII were strongly expressed in fresh or formal in fixed haemangioma tissue, their levels of expression became indistinguishable from that of normal ECs in culture (Li *et al.*, 2003).

1.3.2. MAST CELLS

MCs are highly heterogeneous in terms of morphology, function and metabolic products (Katz *et al.*, 1991; Qu *et al.*, 1995). These cells are implicated in a variety of biological responses. They phagocytose, process antigens, produce cytokines and release a variety of preformed (e.g., histamine, proteoglycans and proteases) and newly formed (e.g., leukotrienes and prostaglandins) physiological mediators (Frangogiannis *et al.*, 1998). They carry an array of adhesion molecules, immune response receptors and other surface molecules that allow them to react to multiple specific and non-specific stimuli (Metcalf *et al.*, 1997). MCs are involved in immediate hypersensitivity reactions, host responses to parasites and neoplasms, immunologically non-specific inflammatory and fibrotic conditions, angiogenesis, tissue remodelling and wound

healing (Gordon *et al.*, 1990; Meininger & Zetter, 1992; Schwartz, 1994; Metcalfe *et al.*, 1997).

MCs are abundant within haemangioma tissues (Mulliken, 1988; Gonzalez-Cruss *et al.*, 1991; Glowacki *et al.*, 1982; Pasyk *et al.*, 1984). While Gonzalez-Cruss *et al.* (1991) and Glowacki *et al.* (1982) found MCs to be abundant in the proliferative phase, Takahashi *et al.* (1994) and Tan *et al.* (2000_a) showed that they were most prominent in the involuting phase, less in the involuted phase and least in the proliferating phase. Tan *et al.* (2000_a) demonstrated that the proportion of proliferating MCs was highest in the proliferative and lowest in the involuted phase.

All MCs contain the specific glycosylated serine protease, tryptase (MC_T) while a subgroup of MCs also express neutral protease like chymase (MC_{TC}) (Schwartz, 1994; Metcalfe *et al.*, 1997). Tan *et al.* (2000_a) showed that although haemangioma biopsies contained both MC types, the proportion of MC_{TC} increased from the proliferative through the involuting to the involuted phases (Tan *et al.*, 2000_a). This correlation of MC_{TC} with ongoing involution, paralleled that of progressive deposition of the ECM, indicated by increasing fibrosis and fatty deposition (Ruger *et al.*, 1994; Tan *et al.*, 2000_a). In addition, Hasan *et al.* (2000_b) found that although MC numbers increased four-fold following steroid treatment, the predominance of the histamine phenotype was unaltered.

1.3.2.1. ANGIOGENIC ROLES FOR MAST CELLS IN HAEMANGIOMA

The promotion of neovascularisation by MCs products, such as FGF-2 has been known for some time (Norrby *et al.*, 1990; Norrby *et al.*, 1994). MC accumulation has been correlated with normal and pathological angiogenesis (Meininger & Zetter, 1992). It has been suggested that MC products can act directly on ECs to stimulate their migration and/or proliferation or may act by degrading connective tissue matrix to provide space for neovascular sprouts (Meininger & Zetter, 1992). Qu *et al.* (1995) identified positive staining for FGF-2 within MCs of the proliferative phase but not in

the later phases of haemangioma and suggested that MCs may contribute to the growing haemangioma by releasing this polypeptide (Qu *et al.*, 1995)

An anti-angiogenic role for MCs in haemangioma has also been suggested. Tan *et al* (2004) postulated that although MCs were implicated with the development of haemangioma, MC secretion of angiogenic inhibitors such as IFN α , IFN β , IFN γ , and TGF- β may be involved in the regression of these lesions. However, they proposed that the involvement of MCs in the life cycle of haemangioma was complex and involved stimulators of angiogenesis in the proliferative phase but inhibitors in later phases.

1.3.2.2. ROLE OF MAST CELLS IN APOPTOSIS

Apolipoprotein J (ApoJ) is implicated in a variety of biological processes including membrane remodelling and apoptosis (Wong *et al.*, 1994). Hasan *et al* (2000_a) showed ApoJ staining within the MCs of haemangioma by tryptase and alcian blue-safranin (Csaba) double staining. They showed that expression of ApoJ in MCs was localised to the cytoplasmic granules which were also detected in the adjacent EC and capillary lumens. During the proliferative phase, only a small proportion of MCs were positive for ApoJ, while the proportion of ApoJ positive MCs increased with the involution of haemangioma. Due to the localisation of the ApoJ protein in MCs granules, these authors proposed that this protein may be synthesised and/or released by MCs. They also found ApoJ positive granules in the ECs and capillary lumens. Therefore, these authors suggested that MC secretion of ApoJ, an apoptotic modulator, may be involved in haemangioma involution.

1.3.3 OTHER CELLULAR COMPONENTS

1.3.3.1 MACROPHAGES AND MONOCYTES

All adult haematopoietic cells originate in the bone marrow and are derived from pluripotent haematopoietic stem cells (Buckland, 2002). Two major lineages then diverge in the bone marrow into the lymphoid and myeloid lineages. The common

lymphoid progenitor differentiates into B and T cells and the common myeloid progenitor differentiates to form the cells of the granulocyte/macrophage and the megakaryocyte/erythroid lineages including monocytes and macrophages (Akashi *et al.*, 2000). Monocytes then exit the peripheral circulation, enter tissues and differentiate into several cell types with varying functions. Macrophages, the major tissue-resident phagocytic cells of the immune system, are derived from monocytes (Rutherford *et al.*, 1993; Sunderkotter *et al.*, 1994).

Tissue macrophages are important mediators of physiological and pathological angiogenesis (Folkman & Klagsbrun, 1987; Isik *et al.*, 1996; Dipietro & Polverini, 1993_a; Dipietro & Polverini, 1993_b; Leibovich *et al.*, 1987; Leibovich & Wiseman, 1988; Thakral *et al.*, 1979; Hunt *et al.*, 1984; Polverini & Leibovich, 1984; Koch *et al.*, 1986; Leung *et al.*, 1989). They are important in the induction of new blood vessel growth during wound repair, inflammation and tumour growth (Sunderkotter *et al.*, 1994; Leibovich *et al.*, 1987). They produce soluble mediators such as FGF-2, TGF- α and - β , PDGF and VEGF (Dipietro & Polverini, 1993_b). Once activated, macrophages can also modulate the ECM composition and induce EC migration and proliferation (Sunderkotter *et al.*, 1994; Porras-Reyes *et al.*, 1991; Sunderkotter *et al.*, 1994).

Tan (2001) suggested an angiogenic role for macrophages in haemangioma. This was supported by results that showed increased macrophage infiltration during the proliferative phase but reduced numbers in the involuting and involuted phases.

Monocyte infiltration in haemangioma is suggested to be due to release of cell-derived chemotactic factors (Isik *et al.*, 1996). One candidate glycoprotein is the monocyte chemoattractant protein-1 (MCP-1) that is specific for monocytes and macrophages *in vitro* (Sunderkotter *et al.*, 1994; Yoshimura *et al.*, 1989; Rollins *et al.*, 1990). ECs, SMCs, fibroblasts and macrophages can produce MCP-1 (Isik *et al.*, 1996; Koch *et al.*, 1992). Isik *et al.* (1996) showed increased expression of MCP-1 mRNA by

ECs, SMCs and macrophages in the proliferative phase of haemangioma. They also showed that nine out of ten involuting lesions did not express mRNA for this molecule.

Ritter *et al* (2006) identified that haematopoietic cells of the myeloid lineage constituted a significant proportion of the cells in hemangiomas of the proliferative phase and suggested that these cells may be involved in promoting haemangioma possibly through expression of cytokines in response to hypoxia/ischemia.

These authors also observed that haemangioma ECs co-expressed a number of myeloid markers, demonstrating yet another unique feature of these cells. From these findings, Jia *et al* (2007) hypothesised that monocytes may be the precursors of haemangioma ECs.

1.3.3.2. PERICYTES

Interactions between ECs and pericytes are known to be important in the maintenance of vascular integrity (Furchgott & Zawadski, 1980). Pericytes control EC proliferation (Nehls & Drenckhahn, 1991; Orlidge & D'Amore, 1987) and serve as guiding structures, aiding the outgrowth of ECs (Nehls *et al.*, 1992). Pericytes also regulate capillary blood flow via a contractile mechanism (Paweletz & Knierim, 1989; Tilton *et al.*, 1979_a; Tilton *et al.*, 1979_b). Although pathological neovascularisation is associated with pericyte loss (Kuwabara & Cogan, 1963), failure of pericyte development during the angiogenic process results in the regression of the newly formed blood vessels (Lindahl *et al.*, 1997; Hirschi *et al.*, 1999).

Plump mitotically active pericytes line the microvessels of proliferating haemangioma (Takahashi *et al.*, 1994; Mulliken, 1988; Tan, 2001). Takahashi *et al* (1994) localised smooth muscle actin (SMA) to the cytoplasm of pericytes in all phases of haemangioma. They found that SMA expression was significantly higher in the involuting phase than in the proliferative and involuted phases. From their studies, Takahashi *et al* (1994) proposed an inhibitory role of pericytes in haemangioma.

1.3.3.3. PROTEASES

Takahashi *et al* (1994) showed increased expression of type IV collagenase during the proliferative phase of haemangioma. The expression of urokinase was also significantly higher during the proliferative and involuting phases than the involuted phase. High molecular weight (>150 kDa) metallomatrix proteases (MMPs) have been detected in the urine of patients with proliferating haemangioma (Marler *et al.*, 2000). These investigators suggested that proteolysis categorised by MMPs and type IV collagenase was required to provide space for the proliferating cells in haemangioma (Takahashi *et al.*, 1994; Marler *et al.*, 2000) and that the differentiating cells signalled an influx of MCs along with the autocrine induction of tissue inhibitor of metalloproteinase-1 (TIMP-1) expression. Furthermore, Takahashi *et al* (1994) showed that TIMP-1, while not detected during the proliferative phase, was readily detected in the pericyte cytoplasm of microvessels during the involuting phase.

TIMP-1 is well recognised for its role as an inhibitor of endothelial invasion into the yolk sac membrane and as a negative regulator of angiogenesis, by inhibiting angiogenic proteases, such as type IV collagenase (Takahashi *et al.*, 1996; Mignatti *et al.*, 1989; Takigawa *et al.*, 1990). Therefore, Takahashi *et al* (1994) suggested that TIMP-1 expression along with the presence of MC secreted angiogenic inhibitors, such as IFNs and TGFs, (Friesel *et al.*, 1987; Gordon *et al.*, 1990; Antonelli-Orlidge *et al.*, 1989) can result in the down-regulation of haemangiogenesis.

1.4. CYTOKINES AND GROWTH FACTORS IN HAEMANGIOMA

1.4.1. VASCULAR ENDOTHELIAL GROWTH FACTOR

VEGF is an important angiogenic cytokine that induces EC proliferation and differentiation (Risau W., 1995; Brown LF *et al.*, 1996). Sources of VEGF include

tumour cells, ECs and MCs. These cells rapidly release VEGF, apparently from a preformed pool (Boesiger *et al.*, 1998). Elevated serum levels of VEGF have been detected in patients with proliferating haemangioma compared with those with involuted lesions (Zhang *et al.*, 2005). Although *in situ* hybridisation and DNA microarray analysis showed low levels of VEGF mRNA in haemangioma (Ritter *et al.*, 2001; Yu *et al.*, 2001), immunohistochemical studies confirmed the presence of VEGF protein within the cytoplasm of ECs and pericytes during the proliferative phase (Takahashi *et al.*, 1994; Tan, 2000_a). Furthermore, Hasan *et al* (2000_b) found that VEGF transcript levels were not altered in steroid-induced involuting haemangioma *in vivo* and suggested that steroids act by modulating other angiogenic factors.

1.4.2. VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTORS

VEGF interacts with cells mainly through two RTKs, VEGFR-1 and VEGFR-2. VEGFR-1 signalling is essential for vascular remodelling during the early stages of embryogenesis (Fong *et al.*, 1995; Carmeliet *et al.*, 1996_b). VEGFR-2 is expressed primarily on ECs and plays a pivotal role in angiogenesis, by inducing EC differentiation and vasculogenesis in both normal and tumour tissue (Millauer *et al.*, 1996; Quin *et al.*, 1993). VEGFR-2 signalling also regulates proliferation and survival of ECs (Zachary & Gliki, 2001). While Brown *et al* (1996) showed high levels of VEGFR-1 and VEGFR-2 mRNA in proliferating haemangioma but not in involuting haemangioma, Yu *et al* (2001) showed that HaemDECs expressed increased mRNA levels for these receptors when in culture.

1.4.3. TIE RECEPTORS AND THEIR ANGIOPOIETIN LIGANDS

High expression of TIE-1, TIE-2 and Ang-2 but low expression of Ang-1 has been identified in HaemDECs (Yu *et al.*, 2001). They showed that TIE-2 mRNA and protein was up-regulated in response to Ang-1 in HaemDECs which also correlated

with increased survival of these cells. The authors confirmed increased TIE-2 expression in proliferating haemangioma by *in situ* hybridisation.

Yu *et al* (2001) also showed that Ang-2 mRNA was down-regulated in response to serum in HaemDECs, but not in normal ECs. They suggested that the regulation of the Ang/TIE system was altered in haemangioma. In addition, a mutation in the TIE-2 gene has been detected in haemangioma (Wang *et al.*, 2004).

1.4.4 FIBROBLAST GROWTH FACTOR-2

The production and metabolism of FGF-2 is considered to be involved in the pathogenesis of haemangioma (Takahashi *et al.*, 1994; Bielenberg *et al.*, 1999; Tan, 2001). Studies have shown increased expression of FGF-2 mRNA and protein in the proliferative phase of haemangioma (Takahashi *et al.*, 1994; Chang *et al.*, 1997). In addition, Tan (2001) identified positive staining for FGF-2 in MCs of both the proliferating and involuting phases. It has been suggested that FGF-2 may be synthesised by MCs and/or by other cells and stored in MCs (Tan, 2001). In addition, Hasan *et al* (2000) showed that the high urinary levels of FGF-2 noted in individuals with proliferating haemangioma (Dosquet *et al.*, 1998) was unaltered following successful steroid treatment as were the levels of FGF-2 mRNA transcripts within the lesion.

1.4.5 INSULIN GROWTH FACTOR-2

Another recently identified potential regulator of haemangioma growth is IGF-2. DNA microarray studies showed high expression of IGF-2 mRNA in proliferative lesions but low levels in involuting lesions. IGF-2 may be a reasonable putative regulator of haemangioma proliferation. As it is a known mitogen, IGF-2 can suppress apoptosis and regulate angiogenesis (Volpert *et al.*, 1996). Ritter *et al* (2002) showed that IGF-2 protein expression was localised to the tumour vessels and that the addition of recombinant IGF-2 protein stimulated vascular sprouting from explanted

haemangioma tissue. They also found that that IGF-2 expression was more correlated with the proliferative phase of haemangioma than VEGF. These authors suggested that high IGF-2 levels could increase expression of VEGF, thereby resulting in an increasingly favourable local environment for EC proliferation.

These results support a placental origin of haemangioma since IGF-2 is highly expressed and is important during embryonic development (Gruppen *et al.*, 1997; Demeestere *et al.*, 2004). Furthermore, as IGFs induce IFN γ expression (Tu *et al.*, 1999), Ritter *et al* (2001) suggested that sustained levels of IGFs could result in a shift in the balance of regulators in favour of involution.

1.4.6 OTHER FACTORS

1.4.6.1 PLATELET DERIVED GROWTH FACTOR, TRANSFORMING GROWTH FACTOR- β AND INTERLEUKIN-6

Hasan *et al* (2000_b) identified decreased transcription of PDGF, TGF- β 1, TGF- β 2 and IL-6 mRNA following successful steroid therapy for haemangioma. Therefore, these authors suggested that the angiostatic effects of steroids were in part due to the down-regulation of these factors.

1.5. EXTRACELLULAR MARKERS

The attachment of cells to the ECM is crucial for tissue integrity. Cells attach either directly to components of the collagen-rich interstitial matrix or to the BM, a more distinct sheath of the ECM that surrounds many kinds of tissue (Gumbiner BM, 1996). Mulliken (1988) found that multilaminated BMs which is a hallmark of a haemangioma in the proliferative phase, persisted through to the involuted phase.

Jang *et al* (1998) showed that in addition to growth factors, the ECM composition of a growing haemangioma was different to those undergoing spontaneous involution (see section 1.5.4, page 35).

1.5.1 INTERSTITIAL MARKERS

Tan *et al* (2000_a) showed that the interstitial collagen types I, III and V (Col-I, -III, -V) were present in the multilaminated BM, in the perivascular and in the stromal regions of haemangioma in all phases. They also found that collagen deposition increased in the stromal area with progression of haemangioma. These authors suggested that this pattern of collagen deposition was concomitant with the decreasing cellular content but increasing fibrotic nature of the later phases (Tan *et al.*, 2000_a).

1.5.2 BASAL LAMINA MARKERS

1.5.2.1 COLLAGEN TYPE IV

Tan *et al* (2000_a) showed that collagen type IV protein was present throughout the multilaminated BM in all three phases of haemangioma. In contrast, Ritter *et al* (2002) identified increased expression of collagen type IV mRNA and protein during the proliferative phase which decreased in the involuting phase. These authors suggested that the reduced levels of collagen type IV in the involuting and involuted phases are an effect, rather than a cause of involution (Ritter *et al.*, 2002).

1.5.2.2 LAMININS

11 different heterotrimeric assembly forms of laminin are known, each consisting of a unique combination of α , β and γ chains (Aumailley & Smith, 1998; Tan, 2001; Tan *et al.*, 2000_a).

Tan *et al* (2000_a) showed the presence of α_1 , β_2 and γ_1 chains but the absence of the α_2 , β_1 and γ_2 in the BM of microvessels in all three phases. From these findings together with the presence of the basement membrane-specific, heparan-sulphate proteoglycan perlecan (Noonan & Hassell, 1993) and the three-chain partially helical molecule collagen type IV (Sado *et al.*, 1998) in all phases of haemangioma, the authors concluded that the BM of haemangioma contained the same major constituents as normal vascular BMs.

1.5.3 COLLAGEN TYPE VIII

Collagen type VIII (Col-VIII) has been detected in the ECM of haemangioma in all phases as well as within the MCs (Tan *et al.*, 2000_a). Its intracellular localisation to MCs only in the early proliferative phase led to the hypothesis that Col-VIII may be synthesised by MCs during this phase but secreted into the pericellular and stromal region during involution (Tan *et al.*, 2000_a). Furthermore, Tan *et al* (2004) showed that the addition of Col-VIII antibodies to haemangioma cultures resulted in the disruption of newly developed capillary-like vessels. These results provide evidence for a functional role of Col-VIII in haemangioma.

1.5.4 VITRONECTIN

Plasma-borne molecules include fibronectin and vitronectin. Jang *et al* (1998) found that only vitronectin showed a marked difference in the deposition pattern in haemangioma. They found that haemangioma in the proliferative phase had extensive deposition of vitronectin in the subendothelial space, while those in the involuting phase did not. Furthermore, the vessels located in the adjacent normal dermis lacked vitronectin deposition. Therefore, these authors suggested that vitronectin deposition may serve as a marker for an actively proliferating lesion.

1.6. CELL ADHESION MOLECULES

1.6.1. INTEGRINS

High expression of the cell adhesion molecules, the $\alpha v\beta 3$ and $\alpha 5\beta$ integrins, have been reported in proliferating haemangioma with decreased abundance in the involuting and involuted phases (Ritter *et al.*, 2002; Nguyen *et al.*, 2004). Nguyen *et al* (2004) found that involuting lesions strongly expressed another integrin, $\beta 4$. They showed that while untreated proliferating lesions lacked $\beta 4$ expression, proliferative lesions treated with IFN α re-expressed $\beta 4$. This re-expression clinically corresponded to

the involution of haemangioma. Therefore, Nguyen *et al* (2004) suggested that both integrin molecules were involved in transducing mitogenic signals from ECM to the cell interior, which would either stimulate or inhibit proliferation of haemangioma.

1.7. THE INVOLUTION OF HAEMANGIOMA

The most curious biological characteristic of haemangioma is its spontaneous involution. Apoptosis is considered an important regulatory event in the involution of haemangioma (Razon *et al.*, 1998; Iwata *et al.*, 1996; Mancini & Smoller, 1996). In haemangioma, apoptosis begins prior to one year of age, increases five-fold during the involuting phase, and reaches a maximum at two years (Razon *et al.*, 1998). Iwata *et al* (1996) showed co-expression of the apoptosis-related antigen, LeY (also considered a tumour associated antigen) with the endothelial markers vWF, CD31 and CD34 and confirmed that at least some of the apoptotic cells in haemangioma are ECs.

1.7.1. APOPTOTIC PROTEINS IDENTIFIED IN HAEMANGIOMA

1.7.1.1. THE BCL-2 FAMILY PROTEINS

Bcl-2 and Bax protein expression have been implicated in the pathogenesis of haemangioma. Wang *et al* (2003) showed that Bax expression was higher in haemangioma compared to other vascular malformations and normal skin ($p < 0.01$) and that Bcl-2 was negative in all phases. However, other investigators have reported Bcl-2 expression in the proliferative phase and Bax in the involuting phase (Nakamura, 2000; Cheng *et al.*, 1999; Zhang *et al*, 2003; Dyduch *et al*, 2004).

Mancini & Smoller (1996) showed decreased Bcl-2 expression with age of the tumour. They found that the decrease in proliferation and Bcl-2 expression was detected much later in lesions, which exhibited a higher degree of vascularisation. Therefore, they suggested that such lesions would undergo longer periods of growth before entering the involuting phase.

1.7.1.2. APOLIPOPROTEIN J

ApoJ is a secreted glycoprotein for which many functions have been proposed (Jenne *et al.*, 1992) including apoptosis (Wong *et al.*, 1993). It is consistently detected in apoptotic cell death paradigms such as the *de novo* regression of the prostate and other hormone-dependent tissues and tumours (Tenniswood *et al.*, 1992; Wong *et al.*, 1993; French *et al.*, 1993; Wong *et al.*, 1994) and in the involuting mammary gland (Lee *et al.*, 1996).

Hasan *et al* (2000_a) found increased expression of ApoJ mRNA in involuting and involuted haemangioma while expression was undetected in proliferative lesions. Immunohistochemical analysis demonstrated increased positive granular staining for ApoJ localised to the MCs and the flattened endothelium of the involuting phase. However, the surrounding fibrofatty tissue was devoid of ApoJ. However, Tan, (2001) found that increased ApoJ expression was not associated with steroid-induced involution of haemangioma.

1.7.1.3. MITOCHONDRIAL CYTOCHROME B

Mitochondrial genes are associated with cell senescence and are proposed to play an important role in apoptosis (Zamzami *et al.*, 1997). Hasan *et al* (2001) were the first to report increased expression of mitochondrial cytochrome b (mt Cyt *b*) in spontaneous and steroid-induced involution of haemangioma. They also found that expression was low or completely absent in proliferative and involuted samples.

1.8. VASCULAR MORPHOGENESIS AND MAINTENANCE

1.8.1. THE MAMMALIAN VASCULAR SYSTEM

The mammalian vascular system is composed of interconnected channels that distribute oxygenated blood via arteries and capillaries (Sadler, 1985). These vessels

consist of three basic layers, viz., intima, media and adventitia (Figure 1.10) (Schoen & Cotran, 1999). The intima is the innermost layer consisting of a single layer of ECs and a thin underlying layer of connective tissue called the internal elastic lamina. The media is mainly composed of SMCs. Arteries contain abundant elastic matrix to accommodate arterial pressure but veins have only a thin media with thinner SMC layers. Capillaries lack media but are lined with scattered pericytes. The adventitia is the outermost layer composed of mainly connective tissue through which the vessels contact the surrounding matrix (Tan, 2001).

Figure 1.10: The basic structure of the blood vessel walls. Intima, media and adventitia are the three basic layers of the vessel wall of arteries, capillaries, veins and lymphatic vessels. Intima consists of a single layer of ECs and a thin underlying layer of connective tissue composed of elastic and collagen fibres. The media in the arteries and veins contain multiple layers of SMCs and elastic matrix. Capillaries lack media and are invested by scattered pericytes. The adventitia is mostly composed of connective tissue by which the vessels make contact with the ECM (*Image reproduced from Sheir et al., 2004*).

1.8.2. DEVELOPMENT OF THE MAMMALIAN VASCULAR SYSTEM

Early in embryonic development, vascular channels are formed by the differentiation of mesodermal cells into haemangioblasts (Folkman & D'Amore, 1996; Risau, 1995; Sadler, 1985). This *de novo* differentiation leads to the formation of blood islands, which are small aggregations or clumps of haematopoietic cells. While the cells

at the borders, differentiate into angioblasts and EC precursors that would eventually line the lumen, the cells in the center differentiate into blood cells (Risau, 1997). These islands fuse together to form the primitive vascular channels, i.e., the primary capillary plexus. This process is called *vasculogenesis* (Risau, 1988).

The plexus continues to undergo remodelling which involves the generation of more small and large vessels and also regression of some vessels. Some pruning steps take place such as lumen formation, peri-endothelial support cell recruitment and sprouting (Nakamura, 1988). These processes are collectively known as *angiogenesis* (Risau, 1988). These angiogenic processes ultimately lead to the formation of differentiated blood vessels in the early embryo (Risau, 1988). These processes are also involved with the angiogenic response in the adult (Tan, 2001).

The mesenchymal cells (MeCs) differentiate into SMCs (Nakamura, 1988; Kirby & Waldo, 1995). These cells migrate toward the evolving endothelial tubes and differentiate further into mural SMCs in veins and arteries, and into pericytes in the capillaries (Tan, 2001).

Figure 1.11.: Vasculogenesis and angiogenesis. Schematic overview of vasculogenesis and angiogenesis, showing coalescence of endothelial-cell precursors (angioblasts and haemangioblasts) and differentiation into ECs (A), which form the primitive vasculogenic networks (vasculogenesis) (B). Remodelling of these networks occurs through angiogenesis (C), which involves sprouting resulting in the formation of microvessels. (Image redrawn from Hendrix et al., 2003).

1.8.2.1 VASCULOGENESIS AND ANGIOGENESIS

Vasculogenesis is the process by which new blood vessels are formed by the differentiation and migration of endothelial progenitor cells to form interconnecting capillaries. Endothelial and haematopoietic cells share a common progenitor, the haemangioblast (Figures 1.12 and 1.13). These cells form aggregates in the developing yolk sac (also called the blood islands) and ablation of the transcription factor SCL present in these cells leads to failure in haematopoiesis and EC development (Robb *et al.*, 1995; Robb *et al.*, 1996; Sinclair *et al.*, 1999). Angiogenesis is the generation of new blood vessels from pre-existing blood vessels. The signals that regulate the migration and organisation of cells into tubes during vasculogenesis and angiogenesis are complex and few of the factors involved in these processes have been identified (Figures 1.12 and 1.13).

Figure 1.12: Roles of the vascular endothelial growth factor (VEGF)/VEGFRs) and the angiopoietin/TIE-2 systems in embryonic vascular development. (A) VEGF, VEGFR-1 and VEGFR-2, are critically involved in the formation of the primitive vascular system from differentiating mesodermal progenitors (vasculogenesis). (B) During the subsequent processes of angiogenesis and remodelling, VEGF and the VEGF receptors cooperate with the Ang-1/TIE-2 system. (C) The latter is also involved in the maturation of the developing vasculature, by mediating interactions between ECs and perivascular support cells. (Image redrawn from Breier, 2000).

Gene targeting experiments have provided insights into the functions of VEGF and VEGFRs during embryonic vascular development. VEGF is critically involved in early stage vascular development. Formation of blood vessels was found to be severely impaired in mice that lack VEGF, resulting in embryonic death around day 10 (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Other early stage processes of vascular development such as vasculogenesis, large vessel formation, capillary sprouting and the remodelling of the yolk sac vasculature are affected in VEGF knockout embryos. The severe haploid insufficient phenotype indicates that VEGF acts in a strictly dose-dependent manner to maintain angioblast differentiation and survival during early stage development. However, the vasculature becomes increasingly independent from VEGF after birth (Gerber *et al.*, 1999). Inactivation of VEGFR-2 also causes embryonic lethality around mid-gestation but the phenotypes of such mice are distinct from those lacking VEGF, thus indicating that both these factors exert different functions (Breier, 2000). Mice lacking functional VEGFR-2 show complete failure of vasculogenesis, EC differentiation and haematopoiesis (Shalaby *et al.*, 1995), consistent with its expression in the putative common precursor of endothelial and haematopoietic cells, the haemangioblast (Kataoka *et al.*, 1997). VEGFR-1 deficient mouse embryos have abnormally enlarged blood vessels (Fong *et al.*, 1995). Fong *et al.* (1999) found that VEGFR-1 and VEGFR-2 appears to exert opposite activities, in that VEGF-2 signalling was required for the emergence of mature ECs, while VEGFR-1 negatively regulated the commitment of angioblasts during vasculogenesis. VEGFR-3 was however identified as non important for vasculogenesis but plays a role in the remodelling of the vasculature and in the formation of lymphatic vessels (Veikkola *et al.*, 1999).

The angiopoietins, Ang-1 and Ang-2, also play a substantial role in angiogenesis (Hanahan, 1997). Ang-1, a chemotactic factor for ECs, supports and promotes vessel development (Figures 1.12, 1.13 and 1.14). Ang-1 is secreted from cells in the vicinity of developing blood vessels, and is considered as the agonistic TIE-2 ligand because

Ang-1 and TIE-2 knockout mice show a very similar phenotype. The targeted inactivation of Ang-1 or TIE-2 resulted in defective vascular development and subsequent embryonic death (Sato *et al.*, 1995; Suri *et al.*, 1996). In contrast to mice deficient for VEGF or the VEGFRs, vasculogenesis proceeds normally in Ang-1 and TIE-2 deficient mouse embryos, indicating that this signalling system functions downstream of the VEGF/VEGFR system. The most prominent defects in Ang-1 or TIE-2 deficient mouse embryos are the absence of heart trabeculae, the failure of remodelling of the yolk sac vasculature and the absence of capillary sprouts in the neural tube. The heart trabeculation phenotype indicates that Ang-1 and TIE-2 are involved in the recruitment of perivascular cells.

Figure 1.13: Growth factors and their receptors implicated in different stages of vasculogenesis and angiogenesis. Key: Ang-1 and -2 refer to angiopoietin-1 and -2 and TIE-2 to the angiopoietin receptor; FGF-2 is fibroblast growth factor-2 and FGFR is its receptor; VEGF is vascular endothelial growth factor and VEGFR-1 and VEGFR-2 its receptors. (Image redrawn from Charnock-Jones *et al.*, 2004)

The Ang-1/TIE-2 system mediates vessel maturation from simple endothelial tubes into more elaborate vascular structures composed of several cell types, and the maintenance of those vessels via the cell-cell and cell-matrix associations that produce

them. Consequently, Ang-1 may also help preserve EC quiescence. Moreover, Ang-1 also stimulates the formation of EC sprouts *in vitro*, and it acts synergistically with VEGF in this assay (Koblizek *et al.*, 1998). Therefore, it is likely that Ang-1 and VEGF co-operate to stimulate capillary sprouting in the developing organism. Based on the failure of blood vessels to interact properly with the underlying matrix or periendothelial supporting cells in Ang-1 deficient mouse embryos, it has been proposed that Ang-1 activity stabilizes blood vessels (Davis *et al.*, 1999) (Figure 1.14).

Ang-2 on the other hand, although analogous to Ang-1, it completely inhibits Ang-1 induced kinase activation of TIE-2 receptor on ECs (Witzenbichler *et al.*, 1998). Inhibition of TIE-2 kinase activity does not block EC proliferation and angiogenesis, but rather facilitates it (Hanahan, 1997). Focal expression of Ang-2 evidently blocks the Ang-1/TIE-2 signal, resulting in a loosening of this tight vascular structure and thereby exposing the ECs to activating signals from angiogenesis inducers, including VEGF. If VEGF (or another angiogenesis inducer) is present, the ECs become activated to migrate and proliferate, producing new capillary sprouts and in turn tubes. Furthermore, Ang-2 is uniformly expressed at high levels during vascular regression. This uniform presence of Ang-1 allows a shift in the local balance of Ang-1/Ang-2 back in favour of Ang-1, to effect maturation and stabilization of the newly formed vessels. Thus, there appears to be a collaboration between VEGF, Ang-2, and Ang-1 to elicit angiogenesis with vascular regression being associated with very high-level expression of Ang-2 in the absence of the activating (survival) signal from VEGF. This results in preventing the Ang-1 signal leading to catastrophic detachment from matrix and support cells, most likely with consequent apoptosis (Hanahan, 1997) (Figure 1.14)

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Figure 1.14. A model for the regulation of the vascular endothelium by VEGF, Ang-1 and Ang-2. All three ligands bind to their respective receptors and elicit distinct downstream cellular responses. VEGF binding to VEGFR-2 sends a classical proliferation signal. When first activated in embryogenesis, this interaction induces the birth and proliferation of ECs. In contrast, VEGF binding to VEGFR-1 elicits endothelial cell-cell interactions and capillary tube formation. Ang-1 binding to TIE-2 receptor recruits and maintains the association of peri-endothelial support cells (pericytes, smooth muscle cells, myocardiocytes), thus solidifying and stabilising a newly formed blood vessel. Ang-2 binds and blocks kinase activation in ECs. The Ang-2 negative signal causes vessel structures to loosen, reducing EC contact with matrix and disassociating peri-endothelial support cells. This loosening appears to render the ECs more accessible and responsive toward the angiogenic inducer VEGF (and other inducers). Ang-2 is expressed at uniformly high levels in vascular regression (e.g., in nonproductive ovarian follicles); the lack of VEGF co-expression suggests that loosening of cell-matrix interactions in the absence of a growth or survival signal elicits EC death, likely by apoptosis. Other factors may also play into these distinctive states (*Image redrawn from Hanahan, 1997*)

1.8.3. THE ANGIOGENIC CASCADE

The angiogenic process of vascular sprouting involves a cascade of events that include: (1) vasodilation and an increase in vascular permeability; (2) activation of proteases leading to degradation of the BM; (3) increase in EC proliferation; (4) migration of the ECs (probably towards an angiogenic stimulus); (5) assembly of ECs to form a tube with a lumen; (6) Recruitment of pericytes and MeCs to the outside of the capillary to form a stable vessel and finally (7) vessel maturation which involves inhibitory signals and signals required for the reconstitution and formation of the BM (Figure 1.15) (Tomanek *et al.*, 2000; Hirschi *et al.*, 1999).

Figure 1.15: Schematic representation of the angiogenic process. EC activation subsequent to exposure of an angiogenic stimulus is followed by BM degradation and the extension of the cytoplasmic processes in the direction of the stimulus (**A**). This is followed by EC migration into the surrounding matrix and formation of capillary sprouts. EC proliferation occurs just behind the migrating front and lumen formation begins in the proximal region of the sprout (**B**). Sprout maturation occurs with recruitment of MeCs to form peri-endothelial cells, reconstitution and formation of new BM which coincides with the cessation of cell migration and proliferation (**C**). (Courtesy of Dr Swee. T. Tan.)

1.8.4. ANGIOGENIC FACTORS IN BLOOD VESSEL DEVELOPMENT

Central to *vasculogenesis* and *angiogenesis* are the ECs. These cells migrate, proliferate and assemble into tubes with tight cell-cell connections to contain blood. MeCs are recruited then differentiate into the peri-endothelial support cells (pericytes for capillaries, SMCs for larger vessels and myocardiocytes in the heart), which provide maintenance and modulatory function to the vessels (Hanahan, 1997; Tomanek & Schattelman, 2000). The establishment and remodelling of blood vessels is controlled by paracrine and autocrine signals that mainly involve the binding of protein ligands to their respective RTKs expressed by vascular ECs (Folkman & Shima, 2000; Hanahan, 1997; Hanahan and Folkman, 1996) (Figure 1.16).

The generation of angioblasts from the mesoderm and the formations of ECs from these mesodermal cells during vasculogenesis or from pre-existing capillaries during angiogenesis (Folkman *et al.*, 1997; Tomanek & Schattelman, 2000) requires the action of the FGF family and in particular FGF-2 (Folkman & D'Amore, 1996; Flamme & Risau, 1992) (Figure 1.16).

VEGF is also an important regulator of both vasculogenesis and angiogenesis. Although both VEGFR-1 and VEGFR-2 are expressed on angioblasts and the ECs that arise from them, only VEGF binding to VEGFR-2 leads to EC proliferation (Shalaby *et al.*, 1995) (Figure 1.16). In contrast VEGF binding to VEGFR-1 is required for endothelial cell-cell interactions and capillary tube formation (Hanahan, 1997). Although disruption of the VEGFR-1 signal permits differentiation of ECs, it results in the formation of thin-walled vessels with a larger than normal diameter. Therefore, VEGFR-1 is said to be important with the later stages of vasculogenesis (Fong *et al.*, 1995).

Figure 1.16: The role of cytokines and cell-cell interactions in blood vessel formation (vasculogenesis and angiogenesis) is a culmination of a number of processes involving a variety of molecules and extensive interactions between a number of cell types (see text for details) (Reproduced with permission from Tan, 2001)

Ang-1 produced by MeCs is the chemotactic factor for ECs and also supports and promotes vessel development. Ang-1 binding to TIE-2 activates a signal from the ECs, which recruits undifferentiated MeCs to the forming vessel (Folkman *et al.*, 1997; Hanahan, 1997). Its signal is also required to maintain EC association with the differentiated peri-endothelial support cells, thereby stabilising the newly formed blood vessel (Hanahan, 1997) (Figure 1.16). Ang-2 on the other hand, is known as a natural antagonist of Ang-1. Ang-2 over expression blocks the stabilising signal of Ang-1 because Ang-1 can no longer induce TIE-2 receptor activation on ECs (Witzenbichler *et al.*, 1998). However, inhibition of TIE-2 kinase activity does not block EC proliferation and angiogenesis, instead facilitates it (Hanahan, 1997). This happens because the Ang-2 negative signal causes vessel structures to become loosened by the reduction of EC contact with the matrix and dissociation of peri-endothelial support cells. This loosening appears to render the ECs more accessible and responsive to VEGF (and other inducers) (Hanahan, 1997). It has also been suggested that high Ang-2 expression, loosening of cell-matrix interactions together with the absence of growth factors such as VEGF results in EC death most likely by apoptosis (Hanahan, 1997). Other modulators, some of which have been described in section 1.4 (page 30) also play into these distinctive states of angiogenesis (Plank & Sleeman, 2003).

Once the primitive vessel tubes are formed, they are remodelled to form the mature vessel bed. This remodelling involves the formation of the medial layer consisting of SMCs in large vessels and pericytes in the microvasculature, deposition of the BM and induction of tissue specific specialisation (Tan, 2001). Upon contact of EC with MeCs, the EC-derived TGF- β is activated from its latent state (Hirschi *et al.*, 1999; Folkman & D'Amore, 1996). The active TGF- β leads to the up-regulation of myosin and SMA which causes the MeCs to assume the morphology of pericytes or SMCs (Folkman & D'Amore, 1996). Failure of pericyte formation has been shown to cause regression of the naked nascent endothelial tubes (Folkman & D'Amore, 1996). EC-

MeC contact also suppresses cell proliferation and stabilises the mature vasculature (Tan, 2001) (Figure 1.16).

In addition to inducing differentiation of MeCs into pericytes or SMCs, the activated form of TGF- β also inhibits EC proliferation and stimulates matrix deposition (Hirschi *et al.*, 1999). Thus, a lack of local TGF- β production could permit increased proliferation of ECs. Furthermore, as TGF- β alters integrin expression, the poor vascular integrity in the TIE-2/Ang-null mice could be explained by the lack of EC-SMC contacts and lack of TGF- β (Folkman & D'Amore, 1996).

1.9 ROLE OF STAT PROTEIN IN TUMOURIGNESES

1.9.1 STAT PROTEINS

STAT proteins comprise a family of transcription factors that consists of seven different members: STAT-1, -2, -3, -4, -5A, -5B, and -6 (Darnell, 1997). Abundantly produced in many cell types, they are activated by over 40 ligands (Calo *et al.*, 2003). These include a series of extracellular signalling proteins such as cytokine, growth factors, and hormones that bind to specific cell-surface receptors (Horvath, 2000). Aberrant activation of STAT signalling can lead to the deregulation of crucial signalling pathways. In this way, STATs take part in tumourigenic cell transformation (Bowman *et al.*, 2000).

Cytokines that activate STATs include, type I cytokines (interleukins, neutrophilic factors, and hormones) and type II cytokines (IFNs). Growth factors also activate STAT proteins via their receptors. These include epidermal growth factor (EGF), hepatocyte growth factor (HGF), PDGF and VEGF (Korpelainen *et al.*, 1999; Bartoli *et al.*, 2000).

1.9.2 MECHANISMS OF STAT ACTIVATION

STAT proteins become activated by tyrosine phosphorylation of critical tyrosine residues. These residues are Tyr 701 for STAT-1, Tyr 690 for STAT-2, Tyr 705 for STAT-3, Tyr 693 for STAT-4, Tyr 694 for STAT-5 and Tyr 641 for STAT-6 (Becker *et al.*, 1998). The best-studied pathway for STAT activation is through the Janus kinases (JAKs). JAKs are constitutively associated with cytokine receptors. Ligand binding leads to dimerisation of the receptor and activation of the JAKs. These kinases once activated can trans-phosphorylate the critical tyrosine residues within the intracytoplasmic portion of the receptor chains to provide docking sites for the STAT proteins (Briscole *et al.*, 1996). STATs then make contact with the receptor through their SH2 domain. On binding, the JAKs subsequently tyrosine phosphorylate the 'docked' STAT at conserved tyrosine residues (Figure 1.17). Upon tyrosine phosphorylation, the STAT proteins dissociate from the receptor subunit and form dimers by virtue of reciprocal-SH2 phosphotyrosine interactions i.e., the SH2 domain of one STAT molecule binds with the SH2 domain of a second STAT molecule containing the phosphotyrosine (Chatterjee-Kishore *et al.*, 2000; Darnell, 1997).

STATs can form homodimers (most commonly) as well as heterodimers (Bromberg, 2001). Dimeric STATs are further phosphorylated, this time on serine, which makes it an active transcription factor. STAT dimers then translocate to the nucleus, bind to specific DNA-binding elements and activate gene transcription. Therefore, while tyrosine phosphorylation activates the STAT proteins, nuclear translocation and DNA binding is essential for it to effect gene regulation (Yahata *et al.*, 2003). Since STAT proteins lack a nuclear localisation signal, chaperone proteins have been suggested to assist nuclear translocation of STATs (Johnson *et al.*, 1998).

1.9.3 PHYSIOLOGICAL ROLE OF STATS

STATs play important roles in programming gene expression in biological events as diverse as embryonic development, organogenesis, regulation of cell growth and apoptosis (Horvath, 2000). Evidence for this comes from cell culture experiments, animal experiments and clinical observations on human cancer cells (Bromberg, 2001). While in normal cells ligand-dependent activation of STATs is a transient process, lasting for several minutes to several hours, many cancerous cell lines and tumors exhibit persistently tyrosine phosphorylated and thus constitutively activated STAT proteins. Furthermore, deregulated STAT protein signalling contributes to tumour development by bringing about the loss of immune surveillance (Bromberg, 2002).

Figure 1.17: Schematic representation of STAT activation via JAK activation. STAT-3 and STAT-1 activation is induced via signalling through IL-6 and IFN γ receptors. See text for details (*Reproduced from Kerr et al., 2003*).

STAT-3 activation is involved in early embryogenesis (Akira, 2000, Takeda *et al.*, 1997), in migration of skin epidermic cells and in skin remodelling during wound healing (Sano *et al.*, 1999). It is required for the inactivation of the macrophages and the inhibition of inflammatory cytokines in the T-helper cell response (Takeda *et al.*, 1999). STAT-3 activation is also required to maintain the pluripotent state of proliferating embryonic stem cells and for macrophage differentiation (Hirano *et al.*, 2000).

1.9.4 STAT-3 IN HUMAN ONCOGENESIS AND REGULATION OF APOPTOSIS

Persistent activation of STAT-3 plays an important role in preventing apoptosis in many large tumours and cell lines including lymphomas, leukemias, mycoses fungoides (Nielson *et al.*, 1999), multiple myeloma, brain (Bonni *et al.*, 1997; Ihara *et al.*, 1997), prostate, breast, lung, and head and neck cancers (Bromberg *et al.*, 2001). Studies have shown that the suppression of apoptosis by STAT-3 was due to its ability to induce expression of apoptosis suppressor proteins such as Bcl-2 and Bcl-xl (Catlett-Falcone *et al.*, 1999; Buettner *et al.*, 2002).

Various studies have shown that STAT-3 activation was required and sufficient to mediate cellular transformation (Bromberg *et al.*, 1998_a; Turkson *et al.*, 1998; Bromberg *et al.*, 1999; Ram *et al.*, 2000). A possible connection between STAT-3 and tumourigenesis is its association with c-Jun, the first discovered nuclear proto-oncogene (Bromberg, 2001).

Recently, Chan *et al* (2004) demonstrated that STAT-3-deficient mice were resistant to skin tumour development and suggested that STAT-3 was required for epithelial carcinogenesis by maintaining the survival of DNA-damaged stem cells and mediating the proliferation necessary for the clonal expansion of these cells during tumour promotion (Chan *et al.*, 2004). In addition, Zong *et al* (2000) found that endogenous IGF stimulation resulted in increased STAT-3 activation. Studies have also

shown that the stable dimerisation of STAT-3, caused by engineered mutations, was sufficient to transform NIH 3T3 cells (Bromberg *et al.*, 1999). All these findings have accentuated the importance of STAT-3 activation in inducing cell proliferation (Bromberg *et al.*, 1999).

1.9.5 STAT-1 IN GROWTH RESTRAINT, PROMOTER OF APOPTOSIS AND TUMOUR SUPPRESSION

STAT-1 is increasingly being established as a tumour suppressor since its activation is associated with growth arrest (Durbin *et al.*, 1996). Kaplan *et al* (1998) showed that STAT-1 deficient mice were more susceptible to tumour development and that the STAT-1^{-/-} genotype coupled with the removal of the tumour suppressor gene, p53, greatly increased spontaneous tumour formation (Kaplan *et al.*, 1998).

STAT-1 is now recognised as the principal target of IFNs (Schindler *et al.*, 1992; Darnell *et al.*, 1994; Stark *et al.*, 1998). Studies have shown that IFN α or IFN γ treatment does not inhibit the proliferation of STAT-1 deficient fibroblasts and that STAT-1 deficient osteosarcoma-derived cells do not undergo apoptosis in response to IFN γ or the tumour necrosis factor (TNF) (Su *et al.*, 1997; Sahni *et al.*, 1999; Migone, 1995). In addition, the defective growth of tumour cells in response to IFN γ has been attributed to STAT-1 activation (Chin *et al.*, 1997; Kumar *et al.*, 1997).

The involvement of STAT-1 in growth arrest and apoptosis in many cell types, seems to be due to its capacity to induce expression of pro-apoptotic mediators such as caspases and TRAIL (Choi *et al.*, 2003; Chin *et al*, 1997; Kumar *et al.*, 1997) while decreasing the expression of anti-apoptotic modulators such as Bcl-2 and Bcl-xl (Stephanou *et al.*, 2000).

1.10 AIM OF THIS PROJECT

The overall aim of this thesis is to study the cellular development of haemangioma and the signalling pathways that are involved in the proliferation and involution of haemangioma. The identification of novel treatment agents that may be associated with fewer side effects was also investigated. Studying the cellular processes involved in the biology of haemangioma will provide a better understanding of the formation of microvessels. This is of importance for both haemangioma growth, and also for angiogenesis associated with other proliferating tumours, as inhibiting the formation of new blood vessels is an important therapeutic target for reducing tumour growth.

1.10.1 OBJECTIVES

The specific objectives of this study were (1) to identify changes in expression of key transcription factors and RTKs involved in cellular proliferation and apoptosis during the natural progression of a haemangioma; (2) to study the expression patterns of TRAIL and its receptors in proliferating, involuting and involuted haemangioma and to elucidate the role of TRAIL receptor signalling in modulating haemangioma progression; (3) the utilisation of the newly developed *in vitro* model to evaluate the use of TRAIL as a potential treatment option for haemangioma; (4) the purification, culture and characterisation of haemangioma-derived cells *in vitro*; and (5) the identification of novel genes or cell types which may be important modulators of haemangioma development.

CHAPTER 2: METHODS

2.1 GENERAL METHODS

2.1.1 QUANTITATIVE REAL TIME PCR

2.1.1.1 AN INTRODUCTION TO QUANTITATIVE REAL TIME PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) assay was used in this study to quantify changes in mRNA expression. The basic principles, advantages, limitations and the special kinetics of qRT-PCR have been well adequately covered in numerous reviewed articles (Bustin, 2000; Giulietti *et al.*, 2001; Yin *et al.*, 2001; Rose'Meyer *et al.*, 2003). However, a brief introduction to qRT-PCR, the detection method used in this study and the limitations are described below.

qRT-PCR is now widely used to measure differences in mRNA expression due to its outstanding accuracy, broad dynamic range and sensitivity. It is also fast, highly reproducible and requires minimal amount of RNA (Freeman *et al.*, 1999; Heid *et al.*, 1996). qRT-PCR analysis allows direct quantification of specific PCR products during the exponential phase (i.e., at linearity) of the reaction, also taking into account the special amplification kinetics of each target.

For this study, the SYBR green dye was used as the reporter dye. This dye fluoresces on binding to double stranded DNA. Therefore, there is an increase in fluorescence of dye with the accumulation of PCR product which is reported after every cycle. Quantification is achieved by determining the cycle at which the fluorescence increases above an arbitrary threshold. This threshold can be manually changed for each experiment if necessary. The PCR cycle at which the fluorescence exceeds the chosen threshold, due to the linear amplification of the specific product is reported as the Ct value (Yin *et al.*, 2001). For most genes, an end point or plateau is reached in less than 40 cycles (depending on transcript abundance), whereby no further amplification can

take place due to competitive PCR effects. ΔCt values refer to the relative mRNA transcript levels for a particular candidate gene, whereby the Ct_x value for the reference or housekeeping gene is subtracted from the candidate gene Ct_y in relation to the total mRNA concentration and is given by the equation $\Delta Ct = Ct_y - Ct_x$. This means, a single Ct difference ($\Delta Ct = 1$) represents a two-fold difference in the amount of specific target (Livak & Schmittgen, 2001). Therefore, Ct values decrease linearly with increasing input target quantity and this can be used as a quantitative measurement of the input target (Giulietti *et al.*, 2001).

The limitation of SYBR Green I real time detection is that it does not differentiate between specific target and non-specific product. To overcome this limitation, DNA melt curves were analysed at the end of all qRT-PCR amplifications performed in this study. These are graphs obtained by plotting fluorescence as a function of temperature as the thermal cycler heats through the dissociation temperature of the product. The shape and position of this curve are functions of the GC/AT ratio, length and sequence and can be used to differentiate amplified products separated by less than 2°C in melting temperature. Analysis of melting curves can therefore distinguish desired from undesired products and also extend the dynamic range of template quantification (Ririe *et al.*, 1997) (see Appendix 1, Figure 1.2, page 270)

Relative quantification was the method chosen to quantify changes in mRNA levels in this study. Because this method quantifies the expression of the target gene with respect to a stably expressed reference gene (housekeeper gene), selection of an appropriate house keeping gene becomes important. House keeping genes are necessary for cell survival. The mRNA synthesis of these genes is considered to be stable between tissues and under different experimental treatments. However, numerous studies have shown that this is not always the case (Zhang & Snyder *et al.*, 1992; Bhatia *et al.*, 1994; Bereta and Bereta 1995; Chang *et al.*, 1998; Thellin *et al.*, 1999; Zhong & Simons, 1999; Schmittgen & Zakrajsek, 2000; Selvey *et al.*, 2001). In addition, the copy number

of the house keeping gene and the target gene should be similar so that measurements can be made on the same linear scale (Roche LC technical note 15/2002).

Quantification using a true reference gene allows for the normalisation of differences in the amount of amplifiable RNA or cDNA in individual samples generated by (i) different amounts of starting material, (ii) the quality of the starting material, (iii) enzymatic efficiency; and (iv) differences in RNA preparation and cDNA synthesis. This is because the reference gene is exposed to the same preparation steps as the gene of interest. When a single house keeping gene is used as a reference, differences in gene expression ratios may reflect regulation of the house keeping gene rather than the gene under investigation. Therefore, relating gene expression to several housekeepers in parallel has been suggested (Schmid *et al.*, 2003).

Some commonly used housekeeping genes include cyclophilin A (CycA) (Bjarnason *et al.*, 1998), glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (Petersen *et al.*, 1990), hypoxanthine phosphoribosyltransferase (HPRT) (Foss *et al.*, 1998) and 18SrRNA (Finnegan *et al.*, 1993). These genes were validated for use in this study (see Appendix 1, section 1.1.2.2, page 272).

2.1.1.2 RNA ISOLATION AND QUANTITATION

RNA from snap-frozen haemangioma tissue biopsies (1-2 mg of frozen tissue) and cell cultures (approximately 1×10^5 viable cells) was isolated by a modified protocol using a combination of TRIzol reagent (Invitrogen, Auckland, New Zealand) and the High pure RNA tissue Kit (Roche diagnostics, New Zealand, Ltd).

2.1.1.2.1 RNA extraction from snap frozen tissue biopsies.

Frozen haemangioma tissue was ground to a powder in liquid nitrogen and added to 750 μ l of Trizol. Samples were left in TRIzol overnight at 4°C. 350 μ l of chloroform was then added, mixed for 15 sec and left at room temperature for 10 min. The tubes were centrifuged at 4°C for 15 min at 13,000 X g and the aqueous phases (approx 500 μ l) containing the RNA were transferred into clean eppendorf tubes to which an equal volume of 70% ethanol was added. The entire mixture was then applied to the upper reservoir of an assembled High Pure filter and collection tube, and spun in a benchtop centrifuge at room temperature for 15 sec at 8,000 X g. The flowthrough was discarded and the filter and collection tube re-assembled. Any contaminating DNA was removed by adding 90 μ l of DNase I incubation buffer (1 M NaCl, 20 mM Tris-HCL, 10 mM $MnCl_2$ pH 7.0) with 10 μ l of DNase I onto the glass filter fleeces of the upper reservoir of each filter tube, and incubating for 15 min at 15-25°C. Next, 500 μ l of wash buffer I (5 M guanidine hydrochloride, 20 mM Tris-HCL, ethanol, pH 6.6) was added to the upper reservoir and spun for 15 sec at 8,000 X g. The flowthrough was discarded and 500 μ l of wash buffer II (20mM NaCl, 2mM Tris-HCL, ethanol, pH 7.5) was added, and spun for 15 sec at 8,000 X g. After discarding the flow-through, a final wash step using 300 μ l of wash buffer II was carried out and the filter tubes were inserted into sterile 1.5 ml eppendorf tubes. 50 μ l of elution buffer (nuclease-free, sterile, double distilled water) was applied to the upper reservoir of the filter tubes and incubated at

room temperature for 10 min. RNA was eluted by centrifugation for 1 min at 13,000 X g. RNA was stored at -80°C.

2.1.1.2.2. RNA extraction from cell cultures

To extract RNA from cell cultures, the culture media was removed from the dish and 1ml of TRIzol was pipetted directly into the culture dishes containing approximately 1×10^5 cells. The culture dish was swirled vigorously and RNA was purified from the TRIzol reagent as described in section 2.1.1.2.1 (page 58).

2.1.1.3 RNA QUANTIFICATION

The quantity and purity of each RNA extraction was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE). The absorbance was measured at 260 nm from 2 μ l of samples. RNA concentration was determined using the extinction coefficient for RNA of 40 μ g/ml and calculated using the following formula.

$$\text{Concentration (ng/}\mu\text{l)} = A_{260} \times \text{dilution} \times 40$$

The ratio of the absorbance at 260/280 nm was used to determine the purity of the RNA. Pure RNA has a ratio between 1.9-2.1. Early in the study the quality of the RNA samples were also determined by electrophoresis on agarose gels and by visualisation of the integrity of the 18S and 28SrRNA bands.

2.1.2 cDNA SYNTHESIS

cDNA was synthesised using a Superscript III cDNA synthesis kit (Invitrogen). cDNA was synthesised using either random hexamers or Oligo(dT)₂₀ as primers. Each reaction contained approximately 400 ng of RNA, 500 μ M dGTP, dCTP, dATP, dTTP and 2.5 μ M Oligo(dT)₂₀ or 250 ng of random hexamer primers. The RNA and primer mix was first denatured for 5 min at 65°C followed by incubation on ice for 1 min. A

reaction mixture containing 1X First-Strand buffer (50 mM Tris-HCL, pH 8.3, 75 mM KCL, 3 mM MgCl₂), 5 mM DTT, 200 U SuperScript III and 40 U RNaseOUT, was then added to each tube to a final volume of 20 µl and incubated for 1 hour (hr) at 50°C. cDNA was stored at -20°C until required.

Random hexamers primed cDNA templates were used in the housekeeping gene validation experiments where 18SrRNA was used as the internal reference gene and Oligo(dT)₂₀ was used for all subsequent amplifications.

2.1.3 PRIMER DESIGN AND RECONSTITUTION

PCR primers for this study were designed using the Oligo 6 software (Version 6.02, Molecular Biology Insights Inc., MN). Gene sequence information was obtained using nucleotide databases (www.ncbi.nlm.gov) and primer sequences were assessed for specificity using non-redundant basic local alignment search tools (www.ncbi.nlm.nih.gov/BLAST). Where possible, primers were designed to be intron spanning to differentiate between amplification from contaminating DNA and true cDNA amplified PCR product. A number of parameters were taken into account when the primer pairs were designed:

- The amplicon size ranged between 80-250 *bp*
- All primers were designed to have a GC content of 50-60%.
- All primer pairs had high melting temperatures (*T_m* of approximately 68°C) with the difference in *T_m* being not more than 2°C apart.
- Primers with stable 3' ends, stable hairpins with 5' overhangs, duplexes with 3' overlaps and mismatches at the 3' end were avoided as best as possible.

All the primers were synthesised by Invitrogen. Primers were reconstituted in 10 mM Tris, pH 8.0, 0.1 mM EDTA to a final concentration of 250 µM. A working concentration stock was then prepared for each primer pair containing 10 µM of

forward and reverse primer in a single tube, diluted in distilled water (dH₂O). Primer sequences and product sizes are given in Table 1.

<i>Gene</i>	<i>Primers: sense (+)</i> <i>Anti-sense (-)</i>	<i>Product size</i>
STAT-3	(+) 5'-GCAGAAAGATACGACTGAGG- 3'; (-) 5'-GCAGATCACCCACATTCAC- 3';	180 <i>bp</i>
STAT-1	(+) 5'-GGC AAA GAG TGA TCA GAA ACA A- 3' (-) 5'-GTT CAG TGA CAT TCA GCA ACT CT- 3'	118 <i>bp</i>
VEGFR-2	(+) 5' -ATTCCATGTCTCGGGTCCATT- 3' (-) 5' -CTTGCTGTCCCAGGAAATTCT- 3'	107 <i>bp</i>
CycA	(+) 5' -GGGGAGAAAGGATTTGGCTA- 3' (-) 5' -ACATGCTTGCCATCCACCC- 3'	259 <i>bp</i>
18SrRNA	(+) 5' -CTT AGA GGG ACA AGT GGC G- 3' (-) 5' -ACG CTG AGC CAG TCA GTG TA- 3'	110 <i>bp</i>
GAPDH	(+) 5' -TAAAGGGCATCCTGGGCTACACT- 3'; (-) 5' -TTACTCCTTGGAGGCCATGTAGG- 3'	200 <i>bp</i>
HPRT	(+) 5' -CCC CAC GAA GTG TTG GAT ATA A- 3' (-) 5' -GTC AAG GGC ATA TCC TAC AAC A- 3'	82 <i>bp</i>

Table 1: Primer sequences and cDNA amplicon size (in *bp*) used in this study.

<i>Gene</i>	<i>Primers: sense (+)</i> <i>Anti-sense (-)</i>	<i>Product size</i>	<i>Reference</i>
CD29	(+)5'-TCCAACCTGATCCTGTGTC-3' (-)5'-TCGTTGTTCCCATTCCTG-3'	88 bp	Gambero <i>et al.</i> , 2006
CD133	(+)5'-GCATTGGCATCTTCTATGGTT-3' (-)5'-CGCCTTGTCTCTGGTAGTGT-3'	170 bp	Liu <i>et al.</i> , 2006
CD44	(+)5'-AGAAGGTGTGGGCAGAAGAA-3' (-)5'-AAATGCACCATTTCTGAGA-3'	116 bp	Liu <i>et al.</i> , 2006
CD90	(+)5'-CGCTCTCCTGCTAACAGTCTT-3' (-)5'-CAGGCTGAACTCGTACTGGA-3'	143 bp	Liu <i>et al.</i> , 2006
PPAR γ	(+)5'-CACAAGAACAGATCCAGTGGTTGCAG-3' (-)5'-AATAATAAGGTGGAGATGCAGGCTCC-3'	101 bp	Redonnet <i>et al.</i> , 2002
Osteonectin	(+)5'-AGA AGC TGC GGG TGA AGA A-3' (-)5'-TGC CAG TGT ACA GGG AAG ATG-3'	127 bp	Yi-xiong <i>et al.</i> , 2006
VEGFR-1	(+)5'-TAC AAA GAA ATA GGG CTT CTG ACC-3' (-)5'-ATG GCC TCT AAG TAA TTT GAC TGG-3'	144 bp	Hong <i>et al.</i> , 2006

Table 1: Primer sequences and cDNA amplicon size (in *bp*) used in this study (contd).

<i>Gene</i>	<i>Primers: sense (+)</i>	<i>Product size</i>
	<i>Anti-sense (-)</i>	
TRAIL	(+) 5' -GAG AAC CTC TGA GGA AAC CATT- 3' (-) 5' -TTT CAT GGA TGA CCA GTT CAC C- 3'	256 bp
DR4	(+) 5' -TGT ACG CCC TGG AGT GAC AT- 3' (-) 5' -CAC CAA CAG CAA CGG AAC AA- 3'	115 bp
DR5	(+) 5' -CAA CGC TTC CAA CAA TGA- 3' (-) 5' - ACG TGC CTT CTT TAC ACT GA- 3'	119 bp
DcR1	(+) 5' -CCT GCA CCA TGA CCA GAG A- 3' (-) 5' -TGG CAC CAA ATT CTT CAA CA- 3'	168 bp
DcR2	(+) 5' - GCG GAG GAG ACA GTG ACC A- 3' (-) 5' - CCA GGA ACT CGT GAAGGA CA- 3'	227 bp
OPG	(+) 5' -GTC TTT GGT CTC CTG CTA- 3' (-) 5' -ACA CTC TCT GCG TTT ACT TT- 3'	151 bp

Table 1: Primer sequences and cDNA amplicon size (in *bp*) used in this study (contd).

<i>Gene</i>	<i>Primers: sense (+)</i> <i>Anti-sense (-)</i>	<i>Product size</i>
CD31	(+) 5' -CAG TGT CCC CAG AAG CAA AT- 3' (-) 5' -CTC CGA TGA TAA CCA CTG CAA- 3'	88 <i>bp</i>
GLUT-1	(+) 5' -CAG CCT GTG TAT GCC ACC ATT- 3' (-) 5' -TAG CGC GAT GGT CAT GAG TAT- 3'	155 <i>bp</i>
CD45	(+) 5' -TGT CTT TGA ACT GAG ACA TTC CA- 3' (-) 5' -TGC TCC ACA CTC CAG TTT GTA T- 3'	83 <i>bp</i>
Collagen-I	(+) 5' -CACTGGGTTCGGAGGAGAGT- 3' (-) 5' -CGGCACAAGGGATTGACAC- 3'	84 <i>bp</i>
Collagen-II	(+) 5' -ATGGAGACTGGCGAGACTTG- 3' (-) 5' -TGGACAGCAGGCGTAGGA- 3'	199 <i>bp</i>
Collagen-III	(+) 5' -GCGGATAGAGATGTCTGGAA- 3' (-) 5' -GAGGCTGTGGGCAAAC- 3'	157 <i>bp</i>
Collagen-VIII	(+) 5' -CTCCTCAGATGCCACCAC- 3' (-) 5' -TTCTTTGCCTTTCTTGGGTA- 3'	209 <i>bp</i>
Laminin-V	(+) 5' -AGGGCAACCAGATGAGCA- 3' (-) 5' -AGGAAGACAGCCGAGGAGAT- 3'	211 <i>bp</i>
IGF-2	(+) 5' -CCGTGCTTCCGGACAACCTC- 3' (-) 5' -TGGACTGCTTCCAGGTGTCA- 3'	72 <i>bp</i>
VEGF	(+) 5' -TGA GGA GTC CAA CAT CAC CAT- 3' (-) 5' -TGT GCT GTA GGA AGC TCA TCT- 3'	88 <i>bp</i>

Table 1: Primer sequences and cDNA amplicon size (in *bp*) used in this study (contd).

2.1.4 THE QUANTITATIVE REAL-TIME PCR ASSAY

qRT-PCR analysis was performed using the Platinum SYBR Green qPCR supermix UDG (Invitrogen) appropriately diluted to a final reaction volume of 20 μ l. Each reaction contained SYBR Green I, 1.5 U/ml Taq DNA polymerase, 20 mM Tris-HCL (pH 8.4), 50 mM KCL, 3mM MgCl₂, 200 μ M dGTP, dCTP, dATP and dTTP, 1 U/ml UDG, 0.1 μ M gene-specific primers with 1 μ l of template (reverse transcribed cDNA or purified PCR product) and 10 nM fluorescein (Invitrogen). All reactions were performed in duplicate. Controls included a no template (water) control and amplification from an equivalent amount of RNA that had not been reverse transcribed. Amplification was achieved with an initial denaturation at 95°C for 30 sec, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 20 sec and extensions at 72°C for 20 sec. Data was collected from both the annealing and extension steps. A melt-curve analysis was performed at the end of every amplification via a temperature gradient from 65 to 95°C in 0.5°C increment steps measuring fluorescence at each temperature for a period of 30 sec. The melt-curve analysis was performed to confirm the absence of spurious amplification products and primer dimers. Agarose gel electrophoresis was also performed on selected amplifications from each run to confirm that products of the expected size were amplified. Amplification products and the Ct values were detected using an iQ qPCR system and associated software (Bio-Rad Laboratories Pty Ltd, Auckland, New Zealand). The cycle numbers were calculated from areas of exponential amplification of the traces. The amount of mRNA expression was determined relative to a chosen internal house keeping gene included with each run. In some cases, the relative fold change in expression was also calculated using the formula.

$$\text{Fold change in expression} = 2^{-\Delta Ct}$$

2.1.5 HAEMANGIOMA SPECIMENS

All haemangioma specimens were obtained according to a protocol approved by the Wellington Ethics Committee. Part of each specimen was snap-frozen in liquid nitrogen for subsequent mRNA and protein analysis while the remainder was fixed in paraformaldehyde prior to wax embedding for immunohistochemistry. The haemangioma biopsies were categorised clinically into proliferating, involuting and involuted phases and the assignments confirmed by histological analysis as previously described (Tan_a *et al.*, 2000).

2.1.6 CELL CULTURE

2.1.6.1 CULTURE FACILITY SET-UP

All plasticware (except pipette tips and eppendorfs) and scalpels were obtained sterile from the manufacturers. The pipette tips, eppendorfs, and glass bottles, were sterilized by autoclaving at 120°C for 20 min before use. Spatulas and microforceps were wiped with 70% ethanol to prevent contamination. All handling of the tissues and transfer of all liquids were conducted under aseptic conditions in a sterile Email Air Handling Class II Biological safety cabinet (AES Environmental Pty LTD, Auburn Australia). Latex gloves were used at all times to prevent contamination.

Several different cell lines were used in the present project. Cell cultures were grown in a Heracell incubator (Kendro Laboratory Products GmbH, Hanau, Germany) at 37°C in humidified air with 5% CO₂; herein referred to as the cell culture incubator. All cell line stocks were stored at -80°C using 1 ml cryotubes (Greiner Bio-one, Frickenhausen, Germany) at 1x10⁶ cells/ml in 10% DMSO (dimethyl sulfoxide) and 90% heat-inactivated foetal calf serum (FCS) (Gibco-BRL, Invitrogen).

2.1.6.2 CELL LINES USED IN THIS STUDY

2.1.6.2.1 *HeLa cells*

HeLa cells are an immortalized adherent cervical cancer cell line isolated from Henrietta Lacks, who died from her cancer in 1951 (Master, 2002). HeLa cells were grown in Roswell Park Memorial Institute 1640 with Glutamine (RPMI 1640 + Glutamine, Gibco-BRL) medium supplemented with 10% FCS and 2% Penstrep (100 µg/ml streptomycin sulfate and 100 µg/ml penicillin G, Gibco-BRL), herein referred to as cell culture medium. Cells were maintained in either 25cm² or 75cm² sterile cell culture flasks (Greiner Bio-One) and were passaged by splitting the cells 1:10 twice weekly.

2.1.6.2.2 *3T3 fibroblasts*

The mouse fibroblast cell line, 3T3 fibroblasts, were obtained from Mr An Tan, Malaghan Institute of Research, Wellington, New Zealand. This is an immortalized cell line originally established from primary mouse embryonic fibroblast cells by two scientists called George Todaro and Howard Green in 1962 (Todaro & Green, 1963). 3T3 cells were grown in cell culture medium and maintained in a similar way as described for HeLa cells. 3T3 cells were passaged by splitting the cells 1:5 twice weekly.

2.1.6.2.3 *Human vascular endothelial cells*

Human vascular endothelial cells (HUVECs) were a kind gift from Dr Sarah Gunningham, Christchurch School of Medicine and Health Sciences, Otago University. HUVECs were grown in RPMI 1640 + glutamine, 10% FCS, 2% Penstrep and 30 µg/ml endothelial cell growth supplement (ECGS, Sigma-Aldrich, New Zealand). Cells were passaged 1:3 when the cells reached 80% confluency.

2.1.6.2.4 *Haemangi endothelioma cells*

The mouse derived haemangi endothelioma cell line (EOMA) was purchased from the American Type Culture Collection (ATCC). Cells were obtained frozen and thawed as described in section 2.1.6.3. EOMA cells were grown in cell culture medium and passaged by splitting the cells 1:5 twice weekly.

2.1.6.3. STANDARD CELL CULTURE TECHNIQUES

When required, cells frozen at -80 °C were thawed in a water bath at 37°C. Cells were then transferred to 15 ml centrifuge tubes (Greiner Bio-one) containing 10 ml of pre-warmed cell culture medium and centrifuged at 300 X g (1000 rpm) for 5 min at room temperature. Following removal of the supernatant, the cell pellet was resuspended in fresh cell culture medium and transferred to culture flasks for expansion.

To passage cells, the medium was removed and the cells were rinsed with sterile phosphate buffered saline (PBS) (Appendix 4, section 4.2.1). To dissociate the cells, an appropriate volume of pre-warmed trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen) (Final concentration of 0.05%-0.02%, respectively) was added, enough to cover the surface of the dish and the flasks were returned to the incubator for 2 - 5 min, or until the cells had detached. Media containing 5% FCS was then added at three times the volume of trypsin and cells were collected and transferred to 15 ml polypropylene centrifuge tubes. Cells were centrifuged at 300 X g (1000 rpm) for 5 min at room temperature. The supernatant was discarded, the cell pellet resuspended in fresh cell culture medium, and an appropriate volume of the cell suspension was transferred into new culture dishes.

When required the percentage of viable cells were counted after trypsinisation by the trypan blue dye exclusion using a haemocytometer. The pellet obtained after centrifugation following trypsinisation was resuspended in 500-1000 µl of fresh cell culture medium. A 10 µl sample of the suspended cells was diluted in 10 µl of 0.02%

trypan blue (Invitrogen) in PBS and 10 µl of this mixture was loaded into the groove of the haemocytometer. Only the cells which excluded the trypan blue dye were counted and the percentage of viable cells determined.

2.1.7 IMMUNOHISTOCHEMISTRY

2.1.7.1 PREPARATION OF SLIDES

For immunohistochemistry (IHC), slides were first cleaned with 70% ethanol, dried and submerged in a freshly prepared 2% solution of 3-amino-propyltriethoxysilane (APES, Sigma) prepared in acetone for 1 min. Slides were then transferred to a large tank of water to wash off any remaining APES and dried overnight in an oven at 37°C. The poly-cationic nature of APES allows interaction with the anionic sites of tissue sections resulting in strong adhesion, thus preventing loss of sections during the staining procedures (Howard & Bethell, 2001).

2.1.7.2 PARAFFIN EMBEDDED TISSUE SECTIONS

Although paraformaldehyde (PFA) fixation provides good tissue preservation and high resolution for light microscopy, it crosslinks proteins so strongly that antigenic sites become obscured thereby inhibiting or distorting immunoreaction. Unmasking of hidden antigenic epitopes was carried out using non-enzymatic methods as described in the following sections. 5 µm sections of formalin fixed paraffin embedded tissues were cut using a Reichert-Jung Biocut microtome (Leica Instruments, Nussloch, Germany), stretched in a 48°C water bath, transferred onto APES-coated glass slides and dried at room temperature overnight. Prior to immunostaining, paraffin embedded haemangioma biopsies were de-paraffinised in xylene (2 changes, 5 min each), re-hydrated through a graded ethanol (100%, 95%, 85% and 70%) series followed by a final rinse in dH₂O for 5 min.

2.1.7.3 CELL CULTURE PREPERATIONS

Cultured cells were plated onto 13 mm diameter coverslips (BDH, Palmerson North, New Zealand) which were cleaned and stored in a sealed container in 70% ethanol until needed. Prior to plating, the coverslips were flame-sterilised, and four coverslips were placed in each 35 mm diameter cell culture dish (Greiner Bio-one). The dishes containing the coverslips were then placed under UV light for 15 minutes.

After trypsinisation, approximately 1000-5000 cells were plated onto each coverslips in a total volume of 20 µl and allowed to attach for 15 minutes. The appropriate culture medium was then added to the dish and the cells were left overnight in the incubator.

2.1.8 STAINING PROCEDURES

2.1.8.1 HISTOCHEMISTRY

For histochemical analysis, tissue sections were stained with Mayer's haematoxylin and eosin (H&E) (Luna, 1992). Slides were deparaffined in xylene (2 changes, 5 min each), rehydrated through a graded ethanol series and rinsed in dH₂O. The slides were submerged in haematoxylin for 2 min and washed in running tap water for 5 min before counterstaining with eosin for 1 min. They were then dehydrated in 95% alcohol and absolute alcohol (2 changes for 5 min), cleared in xylene (2 changes for 3 min each) and mounted in D.P.X (BDH Chemicals Ltd, Poole, United Kingdom).

2.1.8.2 FLOURESCENT IMMUNOHISTOCHEMISTRY

Sensitive detection systems were employed to facilitate the immunolocalisation of some proteins. Sources and dilutions for the primary and secondary antibodies used in this study are given in Tables 2 and 3, respectively. Antibodies used in this study were raised in either, a mouse, a rabbit or a goat but are know to specifically recognise the human antigen.

Marker	Species	Antibody dilution	Technique employed	Source of primary antibody
STAT-1	Mouse	1:50 1:100 1:1000	IHC-P IHC-C WB	Cell Signalling, MA
STAT-3	Rabbit Mouse Mouse	1:30 1:100 1:1000	IHC-P,IHC-C IHC-C WB	Cell Signalling
STAT-5	Mouse	1:100	IHC-C	Invitrogen
VEGFR-2	Rabbit	1:30 1:1000	IHC-P,IHC-C WB	Cell Signalling
CycA	Rabbit	1:100 1:1000	IHC-P WB	BIOMOL Research Laboratories Inc, PV
GAPDH	Mouse	1:1000	WB	Abcam Limited, Cambridge, UK
DR4	Mouse	1:100 1:1000	IHC-P,IHC-C WB	Imgenex, SD
DR5	Mouse	1:200	IHC-P,IHC-C	Imgenex
DcR1	Rabbit	1:100	IHC-P,IHC-C	Imgenex
DcR2	Rabbit	1:200 1:1000	IHC-P,IHC-C WB	Imgenex
OPG	Mouse	5 µg/ml	IHC-P,IHC-C WB	R&D systems, MN
vWF	Rabbit	1:200	IHC-P,IHC-C	Dako Corp, Glostrup, Denmark
GLUT-1	Rabbit	1:100	IHC-P,IHC-C	Dako Corp
VEGFR-1	Goat	5 µg/ml 0.2 µg/ml	IHC-C WB	R&D systems

Table 2: Primary antibodies used in this study, their dilutions and sources.

Marker	Primary antibody	Antibody dilution	Technique employed	Source of primary antibody
p-STAT-3 (Tyr 705)	Rabbit Mouse	1:50 1:1000	IHC-P, IHC-C WB	Cell Signalling
p-STAT-1 (Tyr 701)	Goat	5 µg/ml	IHC-C	Santa Cruz Biotechnology, CA
p-STAT-5 (Tyr 695)	Rabbit	1:50	IHC-C	Cell Signalling
Vimentin	Mouse mAb	1:100	IHC-C	Dako Corp
α-SMA	Mouse mAb	1:100	IHC-C	Dako Corp
VEGF	Mouse	5 µg/ml	WB	R&D systems
PCNA	Mouse	1:50	IHC-P	Chemicon International, CA
CD34	Mouse	1:50	IHC-C	Dako Corp

Table 2: Primary antibodies used in this study, their dilutions and sources (*contd*). Key: IHC-C-Immunohistochemistry on cultured cells, IHC- Immunohisotchemistry on paraffin embedded tissue sections, FACS-Flow cytometry cell sorting, WB-Western blotting.

Secondary antibody	Antibody dilution	Source of antibody
Biotinylated sheep anti-goat IgG	1:50-IHC-C	Sigma
Sheep anti-goat Streptavidin	1:250-IHC-C	Invitrogen
AlexaFluor 555 conjugated IgG	1:2500-FACS	
Sheep anti-rabbit Cy3 conjugated IgG	1:250-IHC-P, IHC-C	Sigma
Sheep anti-mouse AlexaFluor 488 conjugated IgG	1:250-IHC-P, IHC-C	Invitrogen
Sheep anti-goat AlexaFluor 488 conjugated IgG	1:1000-WB	Invitrogen
Biotinylated goat anti-rabbit IgG	1:200-IHC-P	Sigma
Sheep anti-rabbit digoxigenin (DIG) conjugated IgG	1 µg/ml-IHC-P, IHC-C	Chemicon
Sheep anti-mouse DIG conjugated IgG	1:25- IHC-P, IHC-C	Roche Diagnostics
Sheep anti-mouse Fluorescein (FITC) conjugated IgG	1:100- IHC-P, IHC-C 1:1000- WB	Sigma
Sheep anti-DIG Rhodamine conjugated IgG	0.5-µg/ml-IHC-P, IHC-C	Roche Diagnostics
Biotinylated horse anti-mouse IgG	1:200-IHC-P	Vector Laboratories

Table 3: Secondary antibodies used in this study, their dilutions and sources. Key: IHC-C-Immunocytochemistry, IHC- Immunohistochemistry, FACS-Flow cytometry cell sorting, WB-Western blotting.

2.1.8.2.1 Paraffin embedded tissue sections

Following deparaffinisation and rehydration, slides were rinsed in dH₂O and then in tris buffered saline (TBS, pH 7.5, Appendix 4) containing 0.1% Tween-20 (BDH Chemicals) (TBST, Appendix 4) for 5 min each. To unmask cross-linked antigenic epitopes, the tissue sections were immersed in coplin jars containing boiling 10 mM sodium citrate (pH 6.0) for 10 min, except for VEGFR-2 staining for which a 1 mM EDTA (pH 8.0) solution was used. The coplin jars were placed in a pressure cooker containing boiling water and the lid of the cooker was loosely placed on top to contain the heat. The coplin jars were removed from the cooker and left to cool to room temperature for 40 min on the bench. The slides were then rinsed in TBST (2 changes, 5 min each). Autofluorescence from the tissue sections was quenched by washing the slides in three changes of freshly prepared 0.5% (w/v) sodium borohydride (NaBH₄) solution in TBS for 15 min each. Slides were then washed in TBST for 5 min and blocked with either 5% bovine serum albumin (BSA) or 5% goat serum prepared in TBST for 60 min at room temperature. This was followed by incubation with primary antibodies diluted in TBST containing either 1% BSA or 1% goat serum at 4°C overnight. Controls included the omission of the primary antibodies. The next day, sections were washed in TBST (3 changes, 5 min each) before incubation with the secondary antibodies diluted in TBST containing 1% BSA or 1% goat serum for 2 hours at room temperature. Slides were washed in TBST (3 changes, 5 min each) and the cell nuclei were counterstained with 4',6-diamino-2-phenylindole (DAPI; Boehringer Mannheim Biochemicals, IN) and mounted in Antifade (Vectorshield, Burlingame, CA) or ProLong® Gold Antifade (Invitrogen) (see section 2.1.8.3, page 75).

2.1.8.2.2 Cultured cells

Coverslips with the attached cells were removed from the culture medium and washed in TBS before being fixed. Cells were fixed in an ice cold solution of methanol

and acetone (1:1, v/v) for 10 min at -20°C except where indicated. Fixed cells were washed in TBST (2 changes, 5 min each). Boiling 10 mM sodium citrate, pH 6.0 or 1 mM EDTA, pH 8.0 (for VEGFR-2 staining) solution was poured over the coverslips and allowed to cool on the bench top for at least 30 min. Coverslips were washed in TBST (3 changes, 5 min each) and non-specific binding sites were blocked for 60 min at room temperature in TBST containing 5% BSA. Coverslips were then incubated in the respective primary antibodies diluted in TBST containing 1% BSA overnight at 4°C. The next day, the coverslips were rinsed in TBST and incubated in the respective secondary antibodies diluted in TBST containing 1% BSA for at least 1 hr at room temperature in a humidified chamber. Coverslips were washed in TBST (3 changes, 5 min each) and the cell nuclei were counterstained with DAPI or propidium iodide (PI) (Sigma) (see section 2.1.8.3) and mounted in Antifade onto clean microscope slides. In some cases, a post fixation wash in 4% PFA for 2 min was included after antibody incubation and prior to mounting in Antifade.

2.1.8.3 COUNTER STAINING OF THE NUCLEUS

DAPI was used to label the nucleus in fluorescently labelled samples. DAPI stock (100 µg/ml prepared in PBS, and stored in the dark at -20°C) was diluted immediately before use in TBST to a final concentration of 20 µg/ml. Tissue sections and cell culture samples were counterstained in DAPI for 10 min after fluorescent labelling, washed and mounted in Antifade.

For some experiments, the nuclei were stained with PI (10 µg/ml). PI stock was prepared in PBS to a final concentration of 2 mg/ml and stored in the dark at 4°C until required.

2.1.8.4 COLORIMETRIC VISUALISATION WITH DAB

Diaminobenzidine (DAB) colorimetric visualisation of immunostaining was

used to detect STAT-1 expression in haemangioma. STAT3, STAT-1 and CycA immunoreactive MCs were detected using DAB staining in conjunction with Csaba (alcian blue-safranin solution) staining. Five specimens each of proliferating, involuting and involuted haemangiomas were processed as for fluorescent immunohistochemistry, except the treatment with NaBH₄ was replaced with a 30 min incubation in 3% hydrogen peroxide (H₂O₂) prepared in methanol and slides were blocked with TBST containing 5% horse serum for 60 min at room temperature. Slides were incubated with either mouse anti-STAT-1, mouse anti-STAT-3 or rabbit anti-CycA primary antibodies followed by detection with a sensitive 3-step biotin-streptavidin/immunoperoxidase technique. Bound primary antibodies were detected by incubating the sections with either biotinylated anti-mouse or anti-rabbit antibodies for 1 hr at room temperature and then washed in TBST (3 changes, 5 min each). The Vecta stain *Elite* ABC reagent containing peroxidase-conjugated streptavidin (Vector Labs, CA) was then added to the section and incubated for 30 min at room temperature. All incubations were carried out in a humidified chamber. The sections were washed in TBST (3 changes, 5 min each) and immunoreactivity was detected by incubation with 0.05% 3'3'-diaminobenzidine tetrahydrochloride (Sigma), 0.01% H₂O₂ in 50 mM Tris HCL, pH 7.5 from 5-20 min. Stained sections were immersed in Csaba stain (see Appendix 4, section 4.2.10, page 299) for 15 min to identify MCs (Tan *et al.*, 2000). The sections were then rinsed thoroughly in tap water, dehydrated in tertiary butyl alcohol, cleared in xylol (BDH Chemicals) and mounted in D.P.X.

2.1.8.5 NEGATIVE CONTROLS

All immunostaining experiments included control sections which were processed in the same way as the test sections but excluded incubation with the primary antibody. For the DAB stained sections, the control slides were incubated with the DAB substrate for the same length of time as the test slides.

2.1.8.6 IMAGE ANALYSIS

Immunostained slides were viewed using an Olympus AX70 photomicroscope (Olympus Optical Co., Hamburg, Germany) equipped with appropriate narrow band fluorescence filters. Images were captured using an Olympus DP70 CCD camera. Some slides were visualised using a Leica TCS 4D confocal laser-scanning microscope fitted with a krypton/argon laser (Leica Lasertechnik, Heidelberg, Germany). For presentation purposes, images were background corrected and overlaid to produce dual colour images using Adobe Photoshop versions CS2 software.

2.1.9 WESTERN BLOTTING

2.1.9.1 SAMPLE PREPARATION FOR WESTERN BLOTS

2.1.9.1.1 *Tissue lysate*

Snap frozen tissue biopsies were weighed and crushed to a powder in liquid nitrogen and transferred to a sterile 1.5 ml eppendorf containing freshly prepared lysis buffer (50 mM Tris-Hcl pH 6.8 containing 5 mM EDTA, 2.5 mM NaF, 2 mM NaO₃V, 2 mM PMSF and 2% SDS) at 100 mg/ml. Activation of Na₃VO₄ is required before use (see Appendix 4, section 4.2.2, page 297). Tissue was further homogenised with a plastic pestle (Sigma) and boiled in a dry heat block for 10 min at 100°C. Lysates were then spun in a microcentrifuge at 14,000 rpm for 15 min at 4°C. Supernatants were transferred to fresh eppendorf tubes and stored at -20 °C until required for protein analysis.

2.1.9.1.2 *Cell lysate*

To prepare cell lysates for Western blotting, the cell culture media was first removed. The cells were rinsed with PBS and covered with 0.2 ml of freshly prepared lysis buffer. The cells were then mechanically detached from the cell culture dish using

a rubber cell scraper and spun in a microcentrifuge at 14,000 rpm for 15 min at 4°C. From this point the cell lysates were processed in the same way as the tissue lysates.

HeLa cell lysates were also prepared for Western blots. The cells were grown to 95% confluence in cell culture media in 100 cm² tissue culture dishes. The media was then replaced with fresh cell culture media containing IFN α -2b (Schering-Plough Ltd, New South Wales, Australia) at 10,000 U/ml and returned to the incubator for 10 min. Cells were then lysed and lysates were prepared for Western blotting as described above.

2.1.9.2 PROTEIN DETERMINATION

Protein concentrations in tissue and cell culture lysates were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Inc., WA). Serial dilutions of the BSA standard were prepared in lysis buffer ranging from 2 mg/ml to 15 μ g/ml and 25 μ l of each diluted BSA standard was added in duplicate into a 96-well plate. 25 μ l of each lysate was also analysed in duplicate along with 25 μ l of lysis buffer alone without protein. The BCA working reagent was made up as per manufacturer's instructions, as 50 parts solution A to 1 part solution B and 200 μ l was pipetted into each well. The solutions in the wells were mixed and the plate incubated for 30 min at 37°C. Colour development was measured in a VersaMAX multiwell plate spectrophotometer (Molecular Devices, CA). The absorbance was measured at a wavelength of 562 nm, blanked against lysis buffer alone containing no protein. Results were analysed with the Softmax Pro software programme (Molecular Devices) and the protein concentrations determined using a linear fit for the protein standards by plotting absorbance against concentration in μ g/ml.

2.1.9.3 GEL ELECTROPHORESIS

All reagents for protein electrophoresis were purchased from Invitrogen except where indicated. Samples were prepared for electrophoresis according to

manufacturer's instructions. Briefly, 5 µl NuPage® LDS sample buffer (4X), 2 µl NuPage® reducing agent (10X) with the appropriate volume of lysate was made up to 20 µl with deionised water. Samples were heated for 10 min at 70°C in a heat block, centrifuged and loaded into each lane of precast 4-12% gradient MES NuPAGE™ Bis-Tris gels alongside 7 µl of Sea Blue® Plus-2 pre-stained standard for size determination. The gel was run in 1XNuPage MES running buffer in a XCell SureLock™ Mini-Cell electrophoresis unit for 1 hr at 120 volts.

2.1.9.4 WESTERN BLOT TRANSFER

Following electrophoresis, the gel was soaked in Western blot transfer buffer (Appendix 4, section 4.2.6, page 298) for 10 min. An immobilon low fluorescence PVDF (polyvinylidene difluoride) membrane (0.2 µm pore size, Millipore Corporation, MA) was pre-soaked in methanol for 2 min, then 1 min in dH₂O and 5 min in transfer buffer before being placed onto the gel for transfer. The materials for the transfer were stacked in transfer buffer in a Western transfer apparatus (Appendix 4, section 4.2.23, page 301), avoiding air bubbles between the layers. The tank was filled with freshly prepared, ice cold transfer buffer, and the black side of the assembled cassette was placed nearest to the black electrode. Transfer was carried at 4°C in a mini-TRANSBLOT Cell transfer tank (BioRad Laboratories) for 6 hours at 100 volts onto the PVDF membrane.

2.1.9.5 IMMUNOSTAINING OF WESTERN TRANSFERRED PROTEINS

After protein transfer, the gels were stained with Coomassie Blue and destained (see Appendix 4, section 4.2.5, page 298). Transfer efficiency was determined by eye on examining the level of protein that remained in the gel post transfer. 95-99% transfer efficiency was assumed when all of the highest molecular weight markers of the protein ladder were successfully transferred onto the membrane. Following protein transfer, the membranes were rinsed in dH₂O and blocked, protein side up, in TBST

containing 5% non-fat milk for 1 hr at room temperature. All membrane incubations were performed on a rocker platform at room temperature unless otherwise specified. The blocking agent was removed and the membranes were washed in TBST (3 changes, 5 min each) followed by incubation with the respective primary antibodies. All primary antibodies were diluted 1:1000 in TBST containing 5% non-fat milk and incubated overnight at 4°C. The next day, the PVDF membranes were rinsed in TBST (3 changes, 5 min each) before incubation with secondary antibodies. Primary antibody binding was detected using either anti-mouse or anti-goat AlexaFluor 488 conjugated antibodies or with anti-rabbit Cy3 conjugated secondary antibodies. All secondary antibodies were diluted 1:1000 in TBST and incubated for 1 hr at room temperature. Following secondary antibody incubation, the membranes were rinsed in TBST (3 changes, 5 min each). Membranes were then scanned using a Fujifilm FLA-5100 imaging system (Fuji Photo Film Co Ltd., Tokyo, Japan).

2.1.9.6 ANALYSIS OF WESTERN TRANSFERRED PROTEINS

Image analysis and densitometry was performed using Image J software (National Institute of Health, MD). To obtain the band densities, the total area under the histogram for each band measurement was first calculated to obtain a band value. The same calculation was made on an equivalent area on the blot containing no band and was used as the background value. The background value was then subtracted from the band value to obtain a final band density measurement. This value was used for statistical analysis.

2.1.10 GENERAL MOLECULAR BIOLOGY TECHNIQUES

See Appendix 4, section 4.1 (page 294).

2.2 METHODS FOR CHAPTERS 3 AND 6

2.2.1 PRELIMINARY EXPERIMENTS

Methods pertaining to all preliminary experiments can be found in Appendix 1, section 1.1.1 (page 267).

2.2.2 GENE EXPRESSION CHANGES IN HAEMANGIOMA

2.2.2.1 QUANTITATIVE REAL TIME PCR

RNA was extracted from each of eight randomly selected proliferating, involuting and involuted haemangioma specimens. Oligo(dT)₂₀ primed cDNA was synthesised and qRT-PCR was performed for STAT-3, STAT-1, VEGFR-2, CycA and GAPDH according to sections 2.1.2 and 2.1.4 (pages 59 and 65). mRNA expression levels were determined relative to the internal reference gene GAPDH using the formula:

$$\Delta Ct = Ct_{(TEST\ GENE)} - Ct_{(GAPDH)}$$

The relative fold change in mRNA expression was calculated using the formula:

$$\text{Fold change in expression} = 2^{-\Delta Ct}$$

(Wang *et al.*, 2006; Miracle *et al.*, 2006; Ortiga-Carvalho *et al.*, 2005)

The data is expressed as the relative fold change in mRNA levels $\pm$ s.e.m.

2.2.2.2 WESTERN BLOTTING

Western blotting was performed as described in section 2.1.9 (page 77). Total protein was extracted and quantified from five proliferating, four involuting and five involuted haemangiomas biopsies and quantified using the BCA protein assay kit (section 2.1.9.2, page 78) and 50 μ g of total protein was separated on a 4-12% MES

NuPage gradient gel. Lysate (50 µg) from HeLa cells treated with IFN α -2b was included as a positive control. Following electrophoresis and transfer, membranes were blocked and incubated with primary antibodies against STAT-3, p-STAT-3 (Tyr 705), STAT-1, VEGFR-1, CycA and GAPDH at 4°C overnight followed by incubation with AlexaFluor 488 conjugated anti-mouse or anti-goat secondary antibodies. CycA immunoreactivity was detected by incubating the membranes with Cy3 conjugated anti-rabbit antibodies. Membranes were then washed and scanned on a fluorescent scanner.

2.2.2.3 FLOURESCENT IMMUNOHISTOCHEMISTRY

Sources and dilutions of the primary and secondary antibodies are given in Chapter 2, Tables 2 and 3, respectively and the general protocol used for immunostaining in section 2.1.8.2.1 (page 74). To facilitate the immunolocalisation of some proteins however, a 3-step detection system was employed as described in the text below.

Paraffin embedded haemangioma tissue sections (five from each phase) were immunostained with primary antibodies against VEGFR-2, STAT-3, p-STAT-3 (Tyr 705), CycA and GLUT-1. Staining for PCNA was undertaken in conjunction with that for STAT-3 and p-STAT-3 (Tyr 705) by incubating both primary antibodies together. Immunoreactivity for VEGFR-2, STAT-3 and p-STAT-3 (Tyr 705) was detected using an anti-rabbit DIG conjugate followed by an anti-DIG-Rhodamine conjugate for visualisation. Bound PCNA antibodies were detected using an anti-mouse FITC conjugate. Bound CycA and GLUT-1 antibodies were detected using an anti-rabbit Cy3 conjugate.

2.2.2.4 COLORIMETRIC VISUALISATION WITH DAB

DAB staining was used to detect STAT-1 expression in haemangioma. Following immunolabelling, the STAT-1 stained tissue sections were counterstained with the Csaba stain as described in section 2.1.8.4 (page 75). To identify MC

expression of STAT-3 and CycA, DAB staining in conjunction with Csaba staining was performed as described in section 2.1.8.4 (page 75).

2.2.2.5 CELL COUNTING

2.2.2.5.1 *STAT-3 and PCNA expression in haemangioma*

A minimum of 600 cells were counted from a typical ‘proliferative’ area (densely packed blood vessels with small lumens lined by plump ECs), an ‘involuting’ area (larger vessel lumens with flattened endothelia) and an ‘involuting’ area of each immunolabelled tissue section. Cells that stained positive for STAT-3, PCNA and STAT-3+PCNA were tabulated and the data plotted as the number of positively stained cells/unit area.

2.2.2.5.2 *STAT-3 and CycA expression by MCs in haemangioma*

At least 200 MCs were counted from proliferating and involuting specimens (four samples per phase). The data for STAT-3 and CycA staining within MCs is plotted as total number of MCs that were positive and negative for STAT-3 and CycA/unit area. The average number of positively stained MCs in each phase was evaluated statistically.

2.2.2.5.3 *p-STAT-3 (Tyr 705) and PCNA expression in haemangioma*

Five proliferative and three involuting specimens were counted as described in section 2.2.2.5.1. All cells that stained for p-STAT-3 (Tyr 705), PCNA and those that stained for both p-STAT-3 (Tyr 705) and PCNA were tabulated and the data from individual samples were plotted as histograms. ‘Proliferative’ areas from proliferating specimens and ‘involuting’ areas from involuting specimens were analysed from each section.

2.2.2.6 STATISTICS

All statistical analysis for qRT-PCR data was performed using the R package (R Development Core Team., 2005). The linear mixed effects method of analysis was used to allow for repeated measures on subjects (Pinheiro & Bates, 2000) and for each transcript, a sequential bonferroni adjustment was made for multiple testing. Data is represented as relative fold changes in mRNA expression $\pm$ standard error of the mean (s.e.m). SPSS software (version 12.0) was used to calculate the Spearman rank correlation coefficients between STAT-3 and VEGFR-2, STAT-3 and STAT-1 and between STAT-1 and VEGFR-2 mRNA expression. The MC data sets were analysed with the Binary Logistic Regression test using SPSS. The non-parametric Kruskal-Wallis one-way ANOVA by ranks test was applied to the band densities obtained from the immunoblotting data sets. The multiple comparisons test was then applied to determine where significant changes occurred. For all the statistical tests, significant changes were accepted at $p < 0.05$.

2.3 METHODS FOR CHAPTER 4

2.3.1 TISSUE CULTURE MEDIUM

MCDB 131 powder medium (Sigma) containing trace elements and L-glutamine was reconstituted with 1.18 g/L sodium bicarbonate (Sigma) to a final volume of 1 litre with dH₂O. To this, 2% Penstrep and 2.5 µg/ml Amphotericin B (Sigma) were added. The mixture was agitated vigorously to ensure complete solubilisation of the additives and the solution was filter sterilized through a 0.2 µm filter (Greiner Bio-One). The medium was either used immediately or stored at 4°C for up to eight weeks.

2.3.2 HAEMANGIOMA TISSUE SPECIMENS AND SAMPLE PREPARATION

Six proliferating and two involuting haemangioma biopsies were obtained according to protocols approved by the Wellington Ethics Committee. The specimens were kept moist and cleansed of all blood clots with PBS. Pieces of fresh operative haemangioma tissue were cut at room temperature into 1-mm explants and submerged in disinfecting media which was made up of MCDB 131 medium containing 1.18 g/L sodium bicarbonate, 10% Penstrep (500 µg/ml streptomycin sulfate, 500 µg/ml penicillin G), 25 µg/ml Amphotericin B and 2 mM L-glutamine (Gibco-BRL). Disinfection was carried out on a rocking platform for at least 2 hours at room temperature.

2.3.3 GEL PREPARATION AND *IN VITRO* TISSUE CULTURE

25 ml of the tissue culture medium prepared as described in section 2.3.2 was transferred to a 50 ml centrifuge tube (Greiner Bio-One) containing 75 mg of sheep fibrinogen I (Sigma) to a final concentration of 3 mg/ml. The tube was mixed gently for 2 min and 6 ml was transferred aseptically into 15 ml conical tubes, to which 0.5 U/ml

of human thrombin (Serva Electrophoresis GmbH, Heidelberg, Germany) was added. The tube was then mixed by inverting and 400 µl of the mixture was added into each well of a 24-well tissue culture plate (Greiner Bio-One). The plates were left in the hood for 20 min at room temperature to allow gel formation after which a piece of the haemangioma tissue specimen was placed on the centre of each gel. The tissue pieces were allowed to attach to gel surfaces for 20 min in the laminar hood. A second aliquot of fibrinogen-thrombin-medium mixture was then added so that the tissue fragment was sandwiched between two layers of fibrin gel (total volume 0.8 ml). Following gel formation, 1.5 ml of supplemented MCDB 131 medium was added to each well. The plates were incubated at 37°C in 3% CO₂/97% air in a humidified environment using a Heracell incubator (Kendo Laboratory Products).

This model was used to test the effect of AG490 on capillary-like outgrowth. From a stock solution of 1 mM AG490 (Sigma) dissolved in 100% DMSO (Sigma, Appendix 4 section 4.2.12, page 299), AG490 was diluted into both gel sandwiches and also into the liquid medium overlaying the cultures at the concentrations indicated. In addition, control cultures were treated with the same volume of DMSO as that used for the highest concentration of AG490. At least six cultures were used for each treatment type.

2.3.4 MODIFICATION OF THE *IN VITRO* TISSUE CULTURE MODEL

This model was used for the purification of the outgrowing cells from haemangioma explants. Following disinfection, the haemangioma explants were cultured as described above in section 2.3.3, except for a few modifications. These are, (i) 10% FCS (Gibco-BRL) was added to the tissue culture medium before use, (ii) 350 µl of the fibrinogen-MCDB 131-thrombin mixture was placed in each well of 24-well culture plate and the tissue explants were placed into the gel before the gel formed. Therefore, only one layer of gel was used for each explant. After gel formation, 500 µl

of the supplemented MCDB 131 containing 10% FCS was added to cover the gel in each well and the cultures were allowed to grow at 37°C under a 3% CO₂/97% air humidified environment. The culture medium was replaced every 3-4 days until the explant was used for dissociation.

This model was used to test the effect of VEGF and AG490 on haemangioma capillary-like outgrowth. Following three days of culture, only those explants which exhibited capillary-like outgrowth were selected. Culture media was removed and replaced with 500 µl of serum free MCDB 131 media containing 2% Penstrep. Following 24 hours of serum starvation, culture medium was removed once more and replaced with fresh serum free media containing either 50 ng/ml of VEGF (Sigma) and/or 50 µM of AG490. Cultures were maintained for a period of six days with the drug. Images of each explant were photographed before and after drug treatment for analysis.

2.3.5 QUANTITATION OF OUTGROWTH FROM TISSUE PIECES

Capillary-like outgrowth was recorded during the culture period by capturing images with an Olympus IX51 inverted microscope (upgraded to IX71) fitted with phase contrast objectives and a color view 1 camera (Tokyo, Japan). For most tissue pieces, the region of outgrowth from the original tissue piece exceeded the field view at X4 magnification. Therefore, a set of overlapping images were taken for each explant and Adobe Photoshop CS2 was used to generate a single image to include the entire region of outgrowth for analysis. Neovascular area was calculated by applying a grid (150 pixels/150 pixels) to each image and counting the boxes containing three or more vessels. The capillary-like outgrowth is expressed as a ratio of the area occupied by the neovessels to the area of haemangioma tissue from which they emanated (Hasan *et al.*, 2003; Hasan *et al.*, 2000).

2.3.6 ISOLATION AND CULTURE OF HAEMANGIOMA TISSUE-DERIVED CELLS

Within 10-15 days of culture, significant growth of cells from the tissue explants was observed. To purify these outgrowths from the haemangioma tissue explants, the culture medium was first removed. Sterilized (autoclaved and cleaned with 70% ethanol) forceps were used to remove the original tissue piece from the gel. Removing the tissue piece did not disturb the outgrowths which remained in the gel. The gel containing the outgrowths were then scraped off the well and digested in serum free RPMI media containing 1 mg/ml elastase (Worthington Biochemicals Corp, NJ) at 37°C for 2-5 min or until the gel was completely dissolved. The RPMI medium-elastase mixture was filter sterilized through a 0.2 μ M filter and warmed to 37°C before being used. On an average, the gels from three wells of a 24-well plate were digested in 2 ml of dissociation media. Once dissolved, enzyme activity was quenched by the addition of 3-4 ml of cell culture media (10% FCS, 2% penstrep in RPMI 1640 media) and the tubes were centrifuged at room temperature for 5 min at 300 X g (1000 rpm). The supernatant was then discarded and the cell pellet resuspended in 500 μ l of fresh cell culture medium. The percentage of viable cells was determined by trypan blue dye exclusion and seeded at a density of 1×10^4 viable cells/cm² in tissue culture flasks. Cells were incubated at 37°C in 97% humidity with 95% air and 5% CO₂ along with the other cell lines. The day after dissociation, the cell culture media was replaced with fresh media to remove non-attached, dead cells and cell debris. Following this, cultures were fed every 3-4 days and passaged 1:3 weekly or as required.

In addition, using previously described methods with a few modifications, the isolation of ECs from human haemangioma biopsies was also attempted (see section 2.3.16, page 99). This was to be used as a patient matched comparative cell line.

2.3.7 CHARACTERISATION OF HAEMANGIOMA CAPILLARY-LIKE OUTGROWTHS

2.3.7.1 MORPHOLOGICAL ANALYSIS

2.3.7.1.1 *Change in cell morphology*

To detect changes in morphology with passage number, the haemangioma explant-derived cells (HaemDCs) were photographed at each passage using an Olympus IX51 inverted microscope (upgraded to IX71) fitted with phase contrast objectives and a color view 1 camera (Tokyo, Japan).

2.3.7.1.2 *In vitro angiogenesis*

To test capillary-like formation *in vitro* approximately 3000 HaemDCs were resuspended in 300 μ l of cell culture media containing (3 mg/ml fibrinogen and 5 U/ml thrombin) and plated into 24-well culture dishes. The gel matrix was then overlaid with 500 μ l of cell culture media and maintained in 95% air, 5% CO₂ at 37 °C. HUVECs were also plated in parallel for comparison in cell culture media supplemented with 30 μ g/ml ECGS.

2.3.7.1.3 *Proliferation of HaemDCs*

HaemDCs were plated at a density of 1×10^4 cells per well into 24-well plates in cell culture medium. Cell numbers were counted using a haemocytometer at days 0, 2, 4, 6 and 9 and cell viability was assessed by the ability of the cells to exclude the trypan blue dye.

2.3.8 QUANTITATIVE REAL-TIME PCR

2.3.8.1 HaemDCs mRNA CHARACTERISATION

RNA was isolated from approximately 1×10^5 HaemDCs and qRT-PCR was performed as described in the General Methods (sections 2.1.1.2.2, 2.1.2 and 2.1.4,

pages 59 and 65). For mRNA characterisation experiments, qRT-PCR was performed for CD29, CD44, CD90, CD133, CD31, VEGFR-1, VEGFR-2, CD45, GLUT-1, Collagen-I, Collagen-II, Collagen-III, Collagen-VIII, Laminin-V, IGF-2 and VEGF. Primer sequences are listed in Table 1 (page 62). Changes in gene expression are reported relative to a GAPDH as ΔCt . Based on cell morphology and mRNA expression profiles, the HaemDCs were characterised as haemangioma-derived mesenchymal cells (HaemDMCs)

2.3.8.2 HaemDMCs DIFFERENTIATION EXPERIMENTS

Following adipocytic and osteocytic differentiation, mRNA was extracted from differentiated and undifferentiated cells and qRT-PCR was performed for PPAR γ and osteonectin using GAPDH as the internal reference gene. Changes in gene expression between treatment groups are reported as $\Delta\Delta Ct$ values $\pm$ s.e.m.

2.3.9 IMMUNOFLUORESCENT CHARACTERISATION OF HaemDMCs

Immunofluorescent staining of HaemDMCs, HUVECs and 3T3 fibroblasts was performed as described in section 2.1.8.2.2 (page 74). Fixed cells were incubated with primary antibodies overnight at 4°C against either α -SMA, vimentin, vWF, GLUT-1, CD34, OPG, VEGFR-1 or VEGFR-2. Immunoreactivity was detected using either an anti-mouse AlexaFluor 488 conjugate or an anti-rabbit Cy3 conjugate. To detect bound VEGFR-2 antibodies, coverslips were incubated with an anti-rabbit DIG conjugate followed by an anti-DIG-Rhodamine conjugate. VEGFR-1 immunoreactivity was detected using an anti-goat biotin conjugate followed by incubation with an AlexaFluor 555 streptavidin conjugate. All antibodies were diluted in TBST containing 1% BSA in a humidified chamber. For the EC specific markers, CD34, vWF, VEGFR-1 and VEGFR-2, HUVECs and 3T3 fibroblasts were stained in parallel as positive and negative controls, respectively. For all other markers, negative controls were included in

each staining procedure and consisted of HaemDMCs cultured on coverslips and processed in the same way as the test with the omission of the primary antibody.

Immunofluorescent detection of OPG was performed on confluent HaemDMCs cultured for at least eight days as described above. Dual staining was performed on HUVECs for vWF and OPG by incubating both primary antibodies together in TBST containing 1% BSA overnight at 4°C. OPG and vWF immunoreactivity was detected using anti-mouse AlexaFluor 488 and anti-rabbit Cy3 conjugated antibodies, respectively.

2.3.10 DIFFERENTIATION OF HaemDMCs

HaemDMCs at passage 3 were plated at 2×10^4 cells/cm² onto 13-mm diameter coverslips in 35-mm culture dishes and cultured in cell culture medium. At 90% - 95% confluence, cells were differentiated into adipocytes or osteocytes in specific induction media. Differentiation media was replaced every 2-3 days.

2.3.10.1 OSTEOGENIC DIFFERENTIATION

Osteogenic differentiation was induced by culturing HaemDMCs for a minimum of 14 days in osteogenic medium containing 10% FCS, 2% Penstrep, 100 nM Dexamethasone (Mayne Pharma Ltd, Victoria, Australia), 0.2 mM Ascorbic acid (Sigma) and 10 mM β -glycerophosphate (Sigma) in RPMI 1640 + glutamine media. Alizarin Red (BDH Laboratories chemicals group, Poole, UK) was used to detect calcium deposition indicative of osteogenesis on both uninduced and induced cells (Tropel *et al.*, 2004; Lee *et al.*, 2004; Sutherland *et al.*, 2005).

2.3.10.2 ADIPOGENIC DIFFERENTIATION

Adipogenic differentiation was induced by culturing HaemDMCs in adipogenic medium containing 10% FCS, 2% Penstrep, 100 nM Dexamethasone, 0.2 mM Ascorbic acid, 10 mM β -glycerophosphate, 5 μ g/ml insulin (Sigma) and 60 μ M indomethacin

(Sigma) in RPMI 1640 + glutamine media. Cells were cultured in adipogenic medium for a minimum of 21 days and stained with Oil-Red O (Sigma) to detect lipid laden adipocytes (Tropel *et al.*, 2004; Lee *et al.*, 2004; Sutherland *et al.*, 2005).

2.3.10.3 ENDOTHELIAL CELL DIFFERENTIATION

To induce EC differentiation, confluent HaemDMCs were cultured in RPMI medium containing 2% Penstrep and either 2% FCS or 10% FCS along with 50 ng/ml of VEGF (Oswald *et al.*, 2004). After 14 days of induction, cells were immunostained for EC specific markers, vWF, CD34 and VEGFR-2 as described in section 2.3.9 (page 90).

2.3.11 CELL CULTURE HISTOCHEMISTRY

2.3.11.1 ALIZARIN RED STAINING

Following osteocytic differentiation, cells were fixed with 4% PFA for 5 min and washed thoroughly in tap water. Cells were stained with Alizarin Red solution (pH 4.2, see Appendix 4, section 4.2.14, page 299) for 5 min. After removal of excess dye, the coverslips were dipped in acetone (20 dips) followed by a mixture of acetone-xylene (1:1) solution and finally in xylene alone. Coverslips were then mounted in D.P.X mounting medium.

2.3.11.2 OIL RED STAINING

Following adipocytic induction, cells were fixed in 4% PFA for 5 min and then thoroughly rinsed in tap water. Following a 5 min wash in 60% isopropanol, the cells were stained in a working solution of Oil Red O (see Appendix 4, section 4.2.15, page 300) for 15 min. Excess stain was then washed off by rinsing in 60% isopropanol. Cell nuclei were then lightly stained using Harris's haematoxylin and rinsed in dH₂O before being mounted in glycerine jelly (see Appendix 4, section 4.2.16, page 300).

As HaemDMCs differentiated into adipocytes and osteocytes (see Chapter 4), the cells are here in referred to as haemangioma-derived mesenchymal stem cells (HaemDMSCs).

2.3.12 EFFECTS OF VEGF AND AG490 ON HaemDMSCs

2.3.12.1 VEGF CELL PROLIFERATION ASSAY

The effect of VEGF on proliferation of HaemDMSCs and HUVECs was determined using the colorimetric EZ4U cell proliferation assay, according to manufacturer's instructions (Biomedica, Vienna, Austria). Briefly, 5000 cells in 50 μ l of RPMI media containing 0.1% FCS (for HaemDMSCs) or 2% FCS (for HUVECs) were seeded into 96-well plates and allowed to attach overnight. The next day, the cells were treated with 50 μ l of fresh media containing various concentrations of VEGF for the times indicated. Fresh media (50 μ l) containing VEGF was added to the HaemDMSCs cultures every 48 hours to maintain the VEGF concentration. At the end of the culture period, substrate was dissolved in 2.5 ml of activator and pre-warmed to 37°C. 20 μ l of this solution was added to each well and the cells were incubated at 37°C for 2 hours. Colour development was measured in a VersaMAX multiwell plate spectrophotometer. The absorbance was measured at wavelengths of 492 and 620 nm and blanked against wells containing medium but no cells. Results were analysed with the Softmax Pro software programme. Data is plotted as the mean absorbance value obtained from three wells per treatment group $\pm$ s.e.m.

2.3.12.2 EFFECT OF AG490 ON HaemDMSCS PROLIFERATION BY BrdU LABELLING

5×10^5 HaemDMSCs were plated onto 35 mm culture dishes in RPMI media containing 5% FCS and 2% Penstrep and allowed to adhere for 6 hours. Media was then replaced with fresh media containing 100 μ m or 50 μ m of AG490 diluted into the

media from a stock solution (see Appendix 4, section 4.2.12, page 299) and cells were returned to the incubator for 17 hours (an overnight incubation). Cells were given two pulses of BrdU (5-bromo-2-deoxyuridine, 20 nmol, Sigma) to achieve a final concentration of 10 μ M. BrdU was added at 3 hours intervals for the last 7 hours of a 24 hr incubation period with AG490. Cells were then trypsinized, pelleted and resuspended in 1 ml of TBS. Following a second centrifugation step, cells were resuspended in 300 μ l of TBS. Cells were then fixed in 70% ethanol by slowly adding 700 μ l of ice cold ethanol (100%) to the tube containing the cells while the tube was being vortexed. This was to avoid clumping of the cells on adding the fixative. Cells were fixed overnight at 4°C. Fixed cells were washed in cold TBS and digested in 0.5% Triton-X in 2 M HCL for 30 min at room temperature. Washed cells were treated with 0.1 M Na₂B₄O₇ (pH 8.5) to neutralise the samples and incubated with a biotin conjugated anti-BrdU antibody (1:100, Invitrogen) overnight at 4°C. The next day, cells were washed in TBS and incubated an AlexaFluor 555 streptavidin conjugate for 2 hours at room temperature. All antibodies were diluted in TBST containing 1% BSA. At least 1×10^4 cells were analysed for fluorescence per experimental condition on a FACScan (Becton Dickinson & Co, MA). To determine antibody specificity, BrdU unpulsed HaemDMSCs (1×10^4 cells) were also incubated with the anti-BrdU antibodies and treated in the same way as the experimental samples. The fraction of cells in S phase was determined from the number of cells that had incorporated BrdU using the CellQuest software (Becton Dickinson & Co).

2.3.12.3 EFFECT OF AG490 ON *IN VITRO* CAPILLARY-LIKE FORMATION AND QUANTITATION

To test the effect of AG490 on capillary-like formation by HaemDMSCs *in vitro*, 3×10^5 cells/ml were resuspended in RPMI culture medium containing 5% FCS, 2% Penstrep and 3 mg/ml fibrinogen along with either DMSO alone (control cultures) or AG490 at various concentrations. Immediately after adding 0.5 U/ml of thrombin to

the cell-media mixture, 300 μ l containing approximately 1×10^5 cells were plated into each well of a 24-well culture dish. Culture plates were maintained in the laminar flow hood until gel formation occurred after which the gel was overlaid with 500 μ l of RPMI media containing either vehicle alone (DMSO) or AG490 at the required doses. Cultures were maintained at 37°C in 5% CO₂/97% air for 12 days. During the culture period, half the media was replaced with fresh media containing the drugs at 72 hr intervals.

Capillary-like formation was quantified using the Image J software (National Institute of Health, MD). A counting frame (1000 μ m/1000 μ m) was applied to each image and the measure tool was used to calculate total capillary-like length. Cell nuclei present in each counting frame were counted using the cell counter tool.

2.3.13 WESTERN BLOTTING

2.3.13.1 EFFECT OF VEGF, AG490 AND IFN α -2b ON p-STAT-3 PROTEIN EXPRESSION

1×10^6 HaemDMSCs were plated onto 100 cm² culture dishes in cell culture medium and left to attach overnight. Culture media was then replaced with fresh media containing either 50 ng/ml of VEGF, 50 μ M AG490 or 10,000 U /ml of IFN α -2b and the dishes were returned to the incubator for the times indicated. For the combination treatment of AG490 and VEGF, the cells were pre-treated with AG490 for one hr prior to adding VEGF for an additional 45 min. Following drug treatments, cells were lysed and subjected to Western blotting as described in section 2.1.9 (page 77). 50 μ g of total protein from HaemDMSCs lysates and HeLa cell lysate (IFN α -2b treated for 10 min) was separated by electrophoresis and transferred onto PVDF membranes. The membranes were then blocked and incubated with mouse anti-p-STAT-3 (Tyr 705) and mouse anti-GAPDH primary antibodies. Anti-mouse AlexaFluor 488 conjugated secondary antibodies were used for detection. Following fluorescence scanning, the

membranes were stripped for 30 min (see Appendix 4, section 4.2.7 for the stripping buffer recipe, page 298) at room temperature and re-scanned to ensure signal from the p-STAT-3 protein bands were lost. Membranes were then probed with mouse anti-STAT-3 and goat anti-VEGFR-1 primary antibodies followed by incubation with anti-mouse and anti-goat AlexaFluor 488 conjugated secondary antibodies and scanned. Image analysis and densitometry was performed using the Image J software.

2.3.13.2 DETECTION OF VEGF IN THE CONDITIONED MEDIA OF HaemDMSCs

To detect if VEGF is secreted into the conditioned media by HaemDMSCs, 1×10^6 HaemDMSCs were plated onto 100 cm² culture dishes. On day 3, culture media was replaced with 10 ml of fresh serum free RPMI culture media containing 2% Penstrep. 24 hours later, the media was discarded and replaced with fresh serum free media. The cells were cultured for 96 hours after which the conditioned medium was collected in fresh tubes and the cells were overlaid with fresh cell culture medium containing 10% FCS and 2% Penstrep. HaemDMSCs were cultured for an additional 3 days and lysed as described in section 2.1.9.1.2 (page 77). Conditioned medium was collected and centrifuged to remove cell debris or particulate matter. The supernatant was then transferred to a pre-rinsed centricon-20 (Millipore Pty Ltd, Australia) and concentrated by centrifugation 4000 x g for 15 min to 200 µl (final volume). Concentrated medium was then precipitated using the ProteoExtract protein precipitation kit (Chemicon) as per manufacturer's instructions. Briefly, four volumes (800 µl) of precipitant was added to 200 µl of concentrated HaemDMSCs conditioned media and precipitated overnight at -20°C. The next day, precipitated protein was collected by centrifugation at 14,000 rpm for 10 min. The pellet was washed, resuspended in 50 µl of lysis buffer and quantitated as described in section 2.1.9.2 (page 78). Approximately 40 µg of total protein from HaemDMSCs conditioned media, 100 µg of haemangioma tissue, 100 µg of HaemDMSCs lysate was added to protein gel

loading buffer (10 X stock) (Appendix 4, section 4.2.20, page 301) in 1.5 ml microcentrifuge tubes to obtain a final concentration of at least 1X loading buffer along with 2.5 mM β -mercaptoethanol. Samples were boiled for 10 min at 100°C on a heated dry block and subjected to electrophoresis on 1.5 mm thick, 10% SDS polyacrylamide gels using a 4% stacking gel (Appendix 4, sections 4.2.18 and 4.2.19, pages 300 and 301) in a Miniprotean cell (BioRad Laboratories) along with 10 μ l of Magic Mark prestained molecular weight markers (Invitrogen). An additional gel containing HaemDMSCs and haemangioma tissue lysate, (100 μ g each) was run for the negative control. Gels were run for 120 min at 100 V in SDS running buffer (see Appendix 4, section 4.2.21, page 301).

Following gel electrophoresis and immunotransfer, the membranes were incubated with anti-mouse VEGF and anti-mouse GAPDH antibodies followed by anti-mouse AlexaFluor 488 conjugated secondary antibodies. The negative control membranes were incubated with secondary antibodies only. Antibody detection was analysed by scanning the membranes on a fluorescent scanner.

2.3.14 IMMUNOCYTOCHEMISTRY

2.3.14.1 EFFECT OF VEGF, IFN α -2b AND ADIPOCYTIC DIFFERENTIATION CONDITIONS ON STAT PROTEIN EXPRESSION

1x10⁵ HaemDMSCs were plated onto sterile 13 mm diameter coverslips in cell culture medium and allowed to attach overnight. Culture media was removed and replaced with fresh media containing either 50 ng/ml VEGF, 10,000 U/ml IFN α -2b or adipocytic differentiation media and the dishes were returned to the incubator for the times indicated. Culture media was then removed and the dishes were rinsed in PBS. Cells were fixed in 4% PFA for 5 min at 4°C. After washing in PBS (2 changes, 5 min), cells were permeabilised in 10% acetic acid prepared in 100 % methanol for 10 min at -20°C (Ma *et al.*, 2005). Cells were then washed in PBS and subjected to antigen

retrieval with 10 mM sodium citrate (pH 6) and blocking as described in section 2.1.8.2.2 (page 74). Coverslips were incubated with rabbit anti-p-STAT-3 (Tyr 705), rabbit anti-p-STAT-5 (Tyr 694), goat anti-p-STAT-1(Tyr 701), mouse anti-STAT-3 or mouse anti-STAT-5 primary antibodies. p-STAT-3 and p-STAT-5 expression was detected using an anti-rabbit-DIG conjugate followed by either an anti-DIG FITC conjugate or an anti-DIG rhodamine conjugate, while p-STAT-1 expression was detected using an anti-goat biotin conjugate and an AlexaFluor 488 streptavidin conjugate. STAT-3 and STAT-5 immunoreactivity was detected using an anti-mouse AlexaFluor 488 conjugate. Cell nuclei were stained using either PI (10 µg/ml) or DAPI (20 µg/ml) as described in section 2.1.8.3 (page 75) and the coverslips were mounted in Prolong Gold Antifade reagent (Invitrogen) on pre-cleaned glass slides.

Image analysis of p-STAT-5 (Tyr 694) nuclear translocation was performed using Image J software. At least 20 cells from each treatment group were identified and their nuclear boundaries traced. The intensity of p-STAT-5 (Tyr 694) immunoreactivity within these defined areas was measured and used as an index of STAT translocation (Ma *et al.*, 2005).

2.3.15 IMMUNOHISTOCHEMISTRY

Fluorescent immunohistochemical staining was performed on paraffin embedded haemangioma tissue sections (six from the proliferative phase and five from the involuting and involuted phases) as described in section 2.1.8.2.1 (page 74). Antigen retrieval was performed by immersing the slides in boiling 10 mM sodium citrate (pH 6.0) for 10 min. Sections were incubated with rabbit anti-vWF, mouse anti-PCNA and mouse anti-OPG primary antibodies. Bound primary antibodies were detected with anti-rabbit Cy3 and anti-mouse AlexFluor 488 conjugated secondary antibodies.

2.3.16 ISOLATION OF ENDOTHELIAL CELLS AND ENDOTHELIAL PROGENITOR CELLS FROM PROLIFERATING HAEMANGIOMAS

Approximately 1 g of proliferating haemangioma tissue was cut into 1 mm² and disinfected for at least 4 hours as described in section 2.3.2 (page 85). Tissue pieces were then pelleted by centrifugation for 5 min at 300 X g and resuspended in serum free RPMI medium. Following a second centrifugation step, the tissue was resuspended and digested in serum free RPMI media containing 0.5 mg/ml Collagenase P (Roche) and 2% Penstrep for 30 min in a water bath maintained at 37°C. The digested tissue was then passed through a 45 µm cell strainer (Greiner Bio-one) and the collagenase activity was quenched by adding FCS to 10% of the total volume. The undigested tissue was transferred to a fresh 50 ml falcon tube using the digestion medium, gently homogenised in a glass homogeniser and then allowed to digest for an additional 30 min. The cells were strained and the collagenase activity quenched as above. The single cell suspension was washed in FACS buffer (Appendix 4, section 4.2.13, page 299) and counted using a haemocytometer. The ECs were isolated with CD31 coated magnetic dynal beads (Invitrogen) using approximately five beads per cell. The required volume of coated beads was added to the cells and the final volume was made up to 300 µl with FACS buffer and transferred to a FACS tube. The cell bead mixture was then incubated at 4°C on a tube rotator for 1 hr. The FACS tube was placed into a magnetic particle concentrator (MPC) and the beads were allowed to collect to the magnet. The remaining FACS buffer containing the unbound fraction was removed. The tube was then removed from the MPC and the beads were resuspended in fresh cell culture media and plated onto 1% gelatine coated culture dishes (see Appendix 4, section 4.2.11, page 299).

The bead purification step was omitted in later experiments due to low yield. From then on the entire single cell suspension obtained after digestion was directly plated onto 1% gelatine coated dishes and cultured in RPMI media containing 20% FCS, 2% Penstrep and 15 µg/ml ECGS (Sigma; Appendix 4, section 4.2.17, page 300).

2.3.17 STATISTICS

To test for an effect of VEGF and AG490 on capillary-like outgrowth a 2-way ANOVA was used. To test for an effect of various concentrations of AG490 on capillary-like outgrowth and for the effect of VEGF on HUVEC and HaemDMSCs proliferation, a 1-way ANOVA was used. All ANOVA tests were followed by Bonferroni's post hoc test to determine where significant differences between experimental groups occurred. For these experiments, the statistical tests were performed on the data from replicate wells set up with biopsies from a single patient. Therefore, since all the samples tested came from a single patient and were performed on the same day, a normal distribution can be assumed. This allows the use of the parametric method of statistical testing.

Statistical analysis on p-STAT-5 nuclear translocation was performed using the non-parametric Mann-Whitney *U* test. For all other data sets, the Kruskal-Wallis by ranks test was performed followed by multiple comparisons to determine where significant effects occurred. For these data, although the cell line used was consistent, normal distribution cannot be assumed as the experiments were performed on different days. Therefore, non-parametric statistical tests by ranks were applied. For all statistical tests, differences with *p* values of < 0.05 were considered significant.

2.4 METHODS FOR CHAPTER 5

2.4.1 QUANTITATIVE REAL TIME PCR

RNA was extracted from frozen haemangioma tissue and from HaemDMSCs as described previously in the section 2.1.1.2 (page 58). Oligo (dT)₂₀ primed cDNA was synthesised and qRT-PCR was performed for TRAIL, DR4, DR5, DcR1, DcR2 and OPG according to sections 2.1.1.2.1, 2.1.2 and 2.1.4 (pages 58, 59 and 65). Primer sequences are listed in Table 1 (page 63). Changes in TRAIL and TRAIL-Rs mRNA expression between phases are expressed relative to that for GAPDH as Δ Ct values. Changes in gene expression between haemangioma tissue samples and HaemDMSCs are expressed as $\Delta\Delta$ Ct values $\pm$ s.e.m.

2.4.2 FLOURESCENT IMMUNOHISTOCHEMISTRY

Four proliferative paraffin embedded haemangioma biopsies were immunostained as described in section 2.1.8.2.1 (page 74). All slides except those which were stained for DR5 were subjected to heat induced antigen retrieval by boiling in 10 mM sodium citrate (pH 6.0) for 10 min. No antigen retrieval was required for DR5 detection. All slides were blocked in TBST containing 5% BSA for 60 min which was followed by incubation with primary antibodies overnight at 4°C against either mouse anti-DR4, mouse anti-DR5, rabbit anti-DcR1, rabbit anti-DcR2 or mouse anti-OPG. The next day, the slides were washed and incubated with secondary antibodies as follows, (i) for DR4 detection, slides were incubated with an anti-mouse DIG conjugate followed by an anti-DIG Rhodamine conjugate, (ii) for DR5 and OPG detection, slides were incubated with an anti-mouse AlexaFluor 488 conjugate and (iii) for DcR1 and DcR2 detection, slides were incubated with an anti-rabbit DIG conjugate followed by an anti-DIG FITC conjugate (see Tables 2 and 3 for antibody dilutions, pages 71 and 73).

Immunofluorescent staining of HaemDMSCs was performed as described in section 2.1.8.2.2 (page 74). Fixed cells were incubated with primary and secondary antibodies as described above except for DR4 detection. Following incubation with anti-DR4 antibodies, coverslips were washed and incubated with an anti-mouse AlexaFluor 488 conjugate.

All slides and coverslips were counterstained with either DAPI or PI (see section 2.1.8.3, page 75). Negative controls were included in each staining experiment in which the primary antibody was omitted.

2.4.3 EFFECT OF INTERFERON AND TRAIL ON HAEMANGIOMA CAPILLARY-LIKE OUTGROWTH

Proliferative haemangioma biopsies were cultured as described in section 2.3.3 (page 85). For drug treatments, TRAIL (250 ng/ml, BIOMOL) and IFN α -2b (4600 U/ml and 23000 U/ml; Schering-Plough Pty Ltd, Wellington, New Zealand) was dissolved in the fibrinogen-thrombin-medium mixture and in the supplemented medium. A minimum of six wells were used for each treatment type and 18 wells were used as the untreated control cultures. Microvessel outgrowth was analysed on day 9 in culture as a ratio of the area occupied by the neovessels to the area of haemangioma tissue (section 2.3.5, page 87) from which they emanated (Hasan *et al.*, 2003; Hasan *et al.*, 2000) and is expressed as % Control $\pm$ s.e.m.

2.4.4 EFFECT OF IFN α -2b AND TRAIL ON HaemDMSCs

The effect of IFN α -2b and TRAIL on HaemDMSCs was determined using the colorimetric EZ4U cell proliferation assay, according to the manufacturer's instructions (Biomedica). Briefly, 5000 cells were seeded in 50 μ l of RPMI media containing 0.1% FCS into each well of a 96-well plate and allowed to attach overnight. The next day, the cells were treated with 50 μ l of fresh media containing either TRAIL (250 ng/ml)

and/or IFN α -2b (4600 U/ml and 23000 U/ml). Fresh media (50 μ l) containing the drugs were added to each well after 48 hours. Following 96 hours of incubation with the drugs, the EZ4U substrate was dissolved in 2.5 ml of activator and prewarmed to 37°C. 20 μ l of this solution was added to each well and the cells were incubated at 37°C for 2 hours. Absorbance was read at 492 and 620 nm. The experiment was performed with HaemDMSC isolates from three separate patients and the results are expressed as % Control $\pm$ s.e.m.

2.4.5 WESTERN BLOTTING

2.4.5.1 DR4 AND DcR2 DETECTION

Protein was extracted from snap frozen haemangioma biopsies of the same patients that were used for the *in vitro* explant model, as described in section 2.1.9.1.1 (page 77). Protein from HaemDMSCs was extracted using the same protocol with the following exceptions. 100 cm² tissue culture dishes containing HaemDMSCs at 90% confluence were used from each patient. Culture media was removed and the cells were rinsed once with PBS. 50 μ l lysis buffer was added to each plate and the cells were mechanically scrapped off the dish using a rubber cell scraper. Cell lysate was transferred to a new eppendorf tube. From here on, cell lysates were treated in the same way as tissue lysates. 50 μ g of total protein from cell and tissue lysate was subjected to Western blotting as described in section 2.1.9 (page 77). Immunoblots were incubated with either mouse anti-DR4 or rabbit anti-DcR2 along with mouse anti-GAPDH primary antibodies followed by incubation with anti-mouse and/or anti-rabbit AlexaFluor 488 conjugated secondary antibodies. Antibody binding was detected as described in section 2.1.9.5 (page 79). The negative control membranes were treated in the same way as the test with the omission of primary antibody incubation.

2.4.5.2 OPG DETECTION

To detect OPG protein, HaemDMSC conditioned media and cell lysates were prepared as described in section 2.3.13.2 (page 96). Following gel electrophoresis and immunotransfer, the membranes were incubated with anti-mouse OPG and anti-mouse GAPDH antibodies followed by anti-mouse AlexaFluor 488 conjugated secondary antibodies. The negative control membranes were incubated with secondary antibodies only. Antibody detection was analysed by scanning the membranes on a fluorescent scanner.

2.4.6. STATISTICS

To test for significant changes in TRAIL and TRAIL receptor mRNA expression between phases, the R package (R Development Core Team., 2005) was used. The linear mixed effects method of analysis was used to allow for repeated measures on subjects (Pinheiro & Bates, 2000) and for each transcript, a sequential Bonferroni adjustment was made for multiple testing. To test for changes in mRNA expression between HaemDMSCs and haemangioma tissue biopsies, the non-parametric Friedman's test for paired samples was applied to the $\Delta\Delta C_t$ values obtained for each gene. To test for an effect of TRAIL and IFN α -2b on capillary-like outgrowth and on HaemDMSCs proliferation, a 2-way ANOVA was used. As multiple ANOVA tests were performed for each treatment, sequential bonferroni adjustments were applied to the resultant p values. Pairwise comparisons were performed between each treatment group using the Bonferroni's post hoc test. The Mann Whitney U test was applied to the band densities obtained from the immunoblotting data sets. Differences with p values of < 0.05 were considered significant.

CHAPTER 3: ROLE OF STAT PROTEINS AND VEGFR-2 IN HAEMANGIOMA

3.1 INTRODUCTION

Given the high frequency of deregulated STAT-3 and STAT-1 signalling in tumors (see section 1.9, page 49) and the elevated levels of VEGF found in proliferating haemangiomas (Takahashi, *et al.*, 1994; Tan *et al.*, 2000_a), a model for the involvement of these genes in haemangioma was proposed (Figure 3.1). This model suggests that increased expression of VEGFR-2, STAT-3 and the activated form of STAT-3, p-STAT-3 (Tyr 705) is associated with the proliferative phase whereas the involuting phase involves apoptosis induced by increased STAT-1 signalling.

Figure 3.1: Proposed model for the role of STAT-3, p-STAT-3 (Tyr 705), STAT-1 and VEGFR-2 in haemangioma.

3.2 AIMS

The aim of this study was to test the model proposed in section 3.1 (Figure 3.1). This study investigated whether increased VEGFR-2, STAT-3 and p-STAT-3 (Tyr 705) expression was present during the proliferative phase of haemangioma and if increased STAT-1 expression was present during involution. qRT-PCR was used to determine changes in mRNA expression of STAT-3, STAT-1 and VEGFR-2 in proliferating, involuting and involuted haemangioma specimens. To correlate changes of mRNA with protein abundance, Western blotting and IHC procedures were used to examine protein expression, distribution and state of phosphorylation.

3.3 RESULTS

3.3.1 PRELIMINARY EXPERIMENTS

Preliminary experiments were conducted to assess the amplification kinetics of the qRT-PCR amplification assays used in this study. All amplifications were linear over a 10,000-fold range and only primer sets with greater than 96% efficiency were used. Preliminary experiments determined that GAPDH was the most suitable internal reference gene to use in qRT-PCR of those tested (18SrRNA, HPRT and CycA), as its expression did not vary significantly between the phases of haemangioma when normalised to total RNA content (Appendix 1, section 1.1.2.2, page 272). The mean Ct value for GAPDH was 20 (s.e.m. = 1.0) using 25 ng of input RNA.

In addition, specificity of the various antibodies used in this study was validated by immunohistochemical and Western blotting studies on cell lines and tissue sections known to express those proteins (see Appendix 1, section 1.1.2.3, page 275).

3.3.2 GENE EXPRESSION CHANGES IN HAEMANGIOMA

3.3.2.1 CHANGE IN mRNA EXPRESSION

The relative change in mRNA expression for STAT-3, STAT-1 and VEGFR-2 was determined for five proliferating, eight involuting and seven involuted haemangioma samples. Proliferating haemangioma specimens expressed more STAT-3 mRNA than involuting and involuted specimens, with a significant difference detected between proliferating and involuted lesions ($p < 0.05$; Figure 3.2). Expression of VEGFR-2 mRNA was significantly greater in proliferating haemangioma compared with both involuting and involuted lesions ($p < 0.05$ and $p < 0.01$, respectively; Figure 3.3). No statistically significant difference in STAT-1 mRNA expression was detected between proliferating and involuting lesions, however, significantly lower levels were detected in involuted lesions compared to both, proliferative and involuting lesions ($p < 0.05$, Figure 3.4)

Figure 3.2: Changes in STAT-3 mRNA expression. The mRNA levels for STAT-3 were determined for five proliferating, eight involuting and seven involuted specimens relative to GAPDH. Proliferating haemangioma specimens expressed significantly higher levels of STAT-3 mRNA than involuted specimens (* $p < 0.05$).

Figure 3.3: Changes in VEGFR-2 mRNA expression. VEGFR-2 mRNA levels were determined in the same samples as in Figure 3.2. Proliferating haemangioma specimens expressed highest levels of VEGFR-2 mRNA which decreased in the involuting and involuted specimens (* $p < 0.05$, ** $p < 0.01$).

Figure 3.4: Changes in STAT-1 mRNA expression. mRNA levels for STAT-1 were determined in five proliferating, eight involuting and seven involuted specimens relative to GAPDH. Proliferating and involuting samples expressed more STAT-1 mRNA compared to involuted samples (* $p < 0.05$).

In addition, mRNA expression of VEGFR-2 and STAT-3 varied proportionately across all the three phases ($r = 0.820$; $p < 0.001$; Figure 3.5). Graphs showing the correlation between STAT-3 with STAT-1 mRNA ($r = 0.692$; $p < 0.001$) and VEGFR-2 with STAT-1 mRNA ($r = 0.75$; $p < 0.001$) are given in Appendix 1, Figure 1.9, page 280. The raw ΔCt values obtained for STAT-3, VEGFR-2 and STAT-1 mRNA from proliferating, involuting and involuted haemangioma biopsies are given in Table 4, page 111.

As the method used in this study reports gene expression changes relative to the internal reference gene (GAPDH) using the same amount of input mRNA, it circumvents the possibility of identifying false changes in gene expression.

Figure 3.5: Correlation between STAT-3 and VEGFR-2 mRNA expression in proliferating, involuting and involuted haemangiomas. The mRNA levels for STAT-3 and VEGFR-2 were determined from haemangioma biopsies from the different clinical phases using qRT-PCR. The threshold value (C_t) was determined for each gene and then expressed as a difference (ΔC_t) between that for the target gene and that for internal house-keeping gene, GAPDH. Low ΔC_t values reflect greater mRNA expression. Proliferating haemangiomas ($\triangle$) had greatest STAT-3 and VEGFR-2 expression compared with involuting (+) and involuted ($\diamond$) lesions. VEGFR-2 and STAT-3 mRNA expression were correlated (Spearman rho $r = 0.893$; $p < 0.001$).

	Phase	Δ STAT-3	Δ VEGFR-2	Δ STAT-1
Patient-1	Proliferating	1.1	3.3	5.2
Patient-2	Proliferating	2.1	3.8	4.9
Patient-3	Proliferating	1.5	3.6	4.7
Patient-4	Proliferating	-2.3	3.1	3.6
Patient-5	Proliferating	1.45	-1.1	-1
Patient-6	Involuting	0.8	4.2	-0.8
Patient-7	Involuting	1.85	6.3	2
Patient-8	Involuting	4.2	12.8	4.9
Patient-9	Involuting	0.65	3.7	1.8
Patient-10	Involuting	3	6.2	5.5
Patient-11	Involuting	2.95	8.9	4.5
Patient-12	Involuting	2.2	8.5	3.4
Patient-13	Involuting	-0.1	2.8	0.7
Patient-14	Involuted	4.4	14.4	7.2
Patient-15	Involuted	4.4	13.3	7.9
Patient-16	Involuted	5.1	10.7	6.4
Patient-17	Involuted	4.4	10.1	8.6
Patient-18	Involuted	4.6	11.25	7.1
Patient-19	Involuted	2.6	7.6	3.8
Patient-20	Involuted	4.2	10.7	6.1

Table 4: Δ Ct values for STAT-3, VEGFR-2 and STAT-1 obtained from proliferating, involuting and involuted haemangioma biopsies.

3.3.2.2 DIFFERENCES IN PROTEIN EXPRESSION BY WESTERN BLOTTING OF HAEMANGIOMA SPECIMENS

Antibody specificity was confirmed by using HeLa cells as a positive control. Immunoreactive bands detected on all immunoblots were at the expected molecular weights. In addition, negative controls in which the primary antibody was omitted, showed minimal staining. Therefore, all immunoreactivity observed in this study was presumed to be due to specific labelling of the primary antibodies. All band density analyses were made on the same gel.

3.3.2.2.1 *STAT-3 AND p-STAT-3 (TYR 705)*

To determine whether changes in STAT-3 mRNA expression correlated with changes in its protein abundance, the same samples used for mRNA analysis were analysed by Western blotting. Lysates prepared from four proliferating, three involuting and three involuted haemangioma biopsies were analysed for STAT-3 protein expression. As discussed in Chapter 1, p-STAT-3 (Tyr 705) is the activated form of STAT-3. Levels of p-STAT-3 (Tyr 705) were studied in five proliferating, three involuting and five involuted haemangioma biopsies. All samples showed STAT-3 and p-STAT-3 (Tyr 705) immunoreactive bands at 92 kDa as expected (Wu *et al.*, 2003). No statistically significant difference in STAT-3 protein abundance was found between proliferating, involuting and involuted specimens (Figure 3.6-A and -B).

Immunoblotting for p-STAT-3 (Tyr 705) showed that the amount of p-STAT-3 (Tyr 705) was greatest in proliferating haemangioma and decreased in involuting and involuted lesions, however, this difference in expression was not statistically significant ($p = 0.23$) as very little p-STAT-3 (Tyr 705) was detected (approximately 5% of that of IFN α -2b stimulated HeLa cells) (Figure 3.6-C and -D).

Figure 3.6: Western blotting for STAT-3 and p-STAT-3 (Tyr 705). 50 μ g of total protein from proliferating, involuting and involuted haemangioma lesions were subjected to immunoblotting as described in section 2.2.2.2. Membranes were incubated with either mouse anti-STAT-3 or mouse anti-p-STAT-3 (Tyr 705) along with mouse anti-GAPDH antibodies. With equal protein loaded from each sample (**A**), no statistically significant change in total STAT-3 protein expression was found between phases as determined by densitometry analysis (**B**). Although abundance of p-STAT-3 (Tyr 705) decreased with phase (**C**), this decrease failed to reach statistical significance. In addition, p-STAT-3 (Tyr 705) expression levels were significantly lower within haemangioma biopsies compared to IFN α -2b stimulated HeLa cells (**D**).

3.3.2.2.2 *STAT-1*

STAT-1 protein expression was studied in five proliferating, three involuting and four involuted haemangioma biopsies. Immunoreactive bands were detected at approximately 90 kDa as expected (Lemmink *et al.*, 2001). Although there appeared to be a decrease in relative STAT-1 protein expression with ongoing involution (Figure 3.7-A), this change did not achieve significance (data not shown).

3.3.2.2.3 *VEGFR-1*

Five proliferating, three involuting and four involuted haemangioma biopsies were examined for VEGFR-1 protein expression. Immunoreactivity was detected at 180 kDa as expected (Takahashi & Shibuya, 1997). No significant change in VEGFR-1 expression was detected between the lesions (Figure 3.7-B and -C).

Figure 3.7: Western blotting for STAT-1 and VEGFR-1 expression in proliferating, involuting and involuted haemangioma lesions. 50 μ g of total protein from proliferating, involuting and involuted haemangioma lesions were subjected to immunoblotting as described in section 2.2.2.2. The membranes were blocked and probed with mouse anti-STAT-1 or goat anti-VEGFR-1 antibodies along with mouse anti-GAPDH antibodies. (A) STAT-1 expression within the haemangioma lesions was found to be significantly lower compared to IFN α -2b stimulated HeLa cells (A). No statistically significant difference in STAT-1 (A) and VEGFR-1 (B) protein expression was found between proliferating, involuting and involuted specimens (C).

3.3.2.3 IMMUNOHISTOCHEMICAL ANALYSIS

3.3.2.3.1 *STAT-3 EXPRESSION*

Immunohistochemical staining of haemangioma specimens (five samples per phase) showed that STAT-3 was readily detectable in all three phases of haemangioma (Figure 3.8). Proliferating lesions showed highest STAT-3 expression in the plump ECs lining the densely packed blood vessels of the proliferative phase (Figure 3.8-A and -D). Some of these cells were found to be actively proliferating as they co-expressed PCNA (Figure 3.8-A and -D, yellow arrows). Panels-B and -E show STAT-3 staining in the flattened endothelium lining the large dilated vessels and in the pericellular regions of the involuting phase. There were also fewer PCNA-positive cells present during the involuting phase (Figure 3.8-B and -E, yellow arrows). Minimal STAT-3 expression was detected in involuted lesions which were devoid of any proliferating PCNA-positive cells. The same pattern of staining was observed when a different STAT-3 antibody was used.

The decrease in STAT-3 expression seen between phases in Figure 3.8 appears to be due to a decrease in cell number as apposed to a decrease in STAT-3 staining intensity per cell.

Figure 3.8: Immunohistochemical localisation of STAT-3 and PCNA in paraffin embedded haemangioma biopsies. Proliferating (A&D), involuting (B&E) and involuted (C&F) haemangioma sections were stained with rabbit anti-STAT-3 and mouse anti-PCNA antibodies as described in section 2.2.2.3. Bound STAT-3 antibodies were detected using an anti-rabbit DIG conjugate and an anti-DIG-Rhodamine conjugate. Bound PCNA antibodies were detected using an anti-mouse FITC conjugate. Cell nuclei were counterstained with DAPI (blue). Panels D, E & F are enlargements of the areas outlined in A, B & C, respectively. Proliferating lesions (A&D) contained the greatest number of PCNA stained nuclei (light blue staining, yellow arrows) and showed the greatest STAT-3 expression (red staining). Involuting (B&E) and involuted lesions (C&F) contained fewer PCNA positive cell nuclei, and showed less staining for STAT-3 protein. Red blood cells in the lumen of the blood vessels appear green, due to autofluorescence. Scale bars: 50 μ m.

3.3.2.3.2. VEGFR-2

Greatest VEGFR-2 immunoreactivity was detected in proliferating lesions, less in the involuting, and the least in the involuted specimens (Figure 3.9). Within the proliferative lesions, VEGFR-2 staining was restricted to the cells lining the blood vessels (Figure 3.9-A, white arrows). These cells have been characterised as plump ECs with “immature” morphology, that is, large nuclei and scant cytoplasm, lining small nascent vessels but also in the interstitial regions (Yu *et al.*, 2004). Serial sections stained with anti-vWF antibody showed a similar staining pattern for vWF (Figure 3.9-D) and VEGFR-2 (Figure 3.9-B). These results indicated that most of the VEGFR-2 positive cells also expressed vWF. However, not all vessels in the involuting haemangiomas expressed VEGFR-2 (Figure 3.9-B, yellow arrows) with almost negligible staining in the involuted lesions (Figure 3.9-C). Although the IHC technique is not considered to be quantitative, different staining intensities for VEGFR-2 were observed within a single field of view. Figure 3.9-B shows that within an involuting lesion, the small vessels continue to express high levels for VEGFR-2 (white arrows) while the larger, more established or ‘mature’ vessels show decreased immunoreactivity for VEGFR-2 (yellow arrows).

Figure 3.9: Immunohistochemical localisation of VEGFR-2 in haemangioma. Proliferating (**A**), involuting (**B&D**) and involuted (**C**) haemangioma biopsies were stained with rabbit anti-VEGFR-2 antibodies (**A-C**) or rabbit anti-vWF antibodies (**D**) as described in section 2.2.2.3. Bound VEGFR-2 antibodies were detected using an anti-rabbit DIG conjugate and an anti-DIG-Rhodamine conjugate (**A-C**). vWF immunoreactivity was detected using an anti-rabbit Cy3 conjugate (**D**). Red blood cells in the lumen of the blood vessels appear red, due to autofluorescence. Cell nuclei were counterstained with DAPI (blue). VEGFR-2 expression (red staining) was found to be the greatest in the proliferating lesions (**A**, white arrows) and less in involuting lesions (**B**, white arrows) with immunoreactivity localized to the cells surrounding the vessel lumens (white arrows). Note some large vessels in the involuting do not stain for VEGFR-2 (**B**; yellow arrows) whereas other smaller vessels in the vicinity still express VEGFR-2 (**B**; white arrows). There was negligible VEGFR-2 expression in involuted lesions (**C**). Immunoreactivity for vWF (red) was also localised to the cells surrounding the blood vessel lumens (**D**) indicating that VEGFR-2 expression may be predominantly expressed by the ECs in haemangioma. Scale bars: 50 μ m.

3.3.2.3.3 *STAT-1*

The anti-STAT-1 antibody used in this study did not yield a detectable signal on immunofluorescently labelled tissue sections. Therefore, STAT-1 immunoreactivity was visualised using a peroxidase conjugated secondary antibody in combination with DAB as the enzyme substrate.

STAT-1 immunoreactivity was present in the plump ECs lining the vessels as well as in the interstitium of the proliferative phase (Figure 3.10-A) and decreased in the involuting phase (Figure 3.10-B). The difference in STAT-1 expression between the proliferative and involuting phase may be merely a consequence of decreased cellular content in the involuting lesions as compared with proliferating lesions. Involved lesions (Figure 3.10-C) showed almost negligible STAT-1 immunoreactivity, which was similar in staining intensity to the negative control (Figure 3.10-D). Some MCs in the proliferative and involuting phases also expressed STAT-1 (Figure 3.10- A and –B, black arrows).

STAT-1 protein abundance was also analysed by Western blotting (see section 3.3.2.2.2, Figure 3.7-A, page 115). From a total of 12 samples, only one proliferative lesion exhibited a strong immunopositive band for STAT-1. Weak bands were detected from two other proliferating lesions and one involuting lesion. The IHC and Western blotting results therefore suggest that haemangioma express low levels of STAT-1.

3.3.2.3.4 *GLUT-1*

GLUT-1 has been identified as an immunohistochemical marker expressed in all three phases of haemangioma but not in other vascular malformations (North *et al.*, 2000). Some of the haemangioma biopsies used in this study were stained with rabbit anti-GLUT-1 antibodies. An example of GLUT-1 immunoreactivity on haemangioma samples is shown in Figure 3.11.

Figure 3.10: Immunohistochemical localisation of STAT-1 in haemangioma. Proliferating (A), involuting (B) and involuted (C) haemangioma tissue sections were stained with anti-STAT-1 antibodies and counterstained with Csaba to identify MCs (light blue) as described in section 2.2.2.4. Most STAT-1 immunoreactivity (brown staining) was detected in the ECs surrounding the blood vessels of the proliferative phase (A) and the involuting phase (B). Some STAT-1 expression was also detected in the MCs (light blue staining, black arrows). Weak STAT-1 expression was present in the involuted phase with only a few positively stained flattened endothelia (C). An involuting haemangioma section used as the negative control showed no non-specific staining of the STAT-1 antibody (D). Note the increase in MC numbers in the involuting phase (B&D). Immunoreactivity was detected with DAB and visualised using bright field microscopy. Scale bars: 50 μ m.

Figure 3.11: Intense GLUT-1 immunoreactivity in a proliferating haemangioma. Sections were stained with rabbit anti-GLUT-1 antibodies as described in section 2.2.2.3. Bound GLUT-1 antibodies were detected with an anti-rabbit Cy3 conjugate (red). (A) GLUT-1 immunoreactivity (red staining) was detected on the cells that surround the blood vessels. Panel B shows the overlaid image of the DAPI stained cell nuclei (blue) and the GLUT-1 positive vessels (red). Scale bars: 50 μ m.

3.3.2.3.5 MAST CELLS EXPRESS STAT-3

Csaba staining was used to identify MCs (Rüger *et al.*, 1994) and showed that there was a three-fold increase in MC numbers in involuting haemangiomas compared with proliferating haemangiomas, and that the MCs were predominantly of biogenic amine phenotype throughout the development of haemangioma (Figures 3.12). These findings are consistent with those previously reported (Tan *et al.*, 2000_a, Tan, 2001).

This study showed that MCs in both the proliferative and involuting phases expressed STAT-3 protein. Of 200 MCs counted, those that stained positive for STAT-3 (STAT-3 + MCs) and negative (STAT-3 – MCs) were identified in the proliferative and involuting phases. Although the total number of MCs was higher in the involuting phase than the proliferative phase, the proportion of STAT-3 expressing MCs was highest during proliferative phase and decreased in the involuting phase (Figure 3.12-C and Table 5). This change was found to be statistically significant with $p < 0.05$.

Developmental Phase	Number of STAT-3 + MCs	% STAT-3 + MCs
Proliferative	195.33 $\pm$ 6.6 ($n = 30$),	90.5
Involuting	164.5 $\pm$ 5.5 ($n = 10$),	76.1
Proliferative vs Involuting	$p < 0.05$	

Table 5: The total number and percentage of STAT-3 positive MCs in proliferating and involuting haemangioma. Values are mean $\pm$ s.e.m; n indicates the average number of fields counted at a magnification of X40.

C

Figure 3.12: Mast cells present in proliferating and involuting haemangiomas express STAT-3. STAT-3 immunoreactivity was detected by DAB staining (brown) and counterstained with Csaba to identify the MCs (light blue). Double labelling of a proliferating (A) and an involuting (B) haemangioma with STAT-3 antibodies and Csaba stain demonstrated the presence of STAT-3 protein within MCs (black arrows). (C) The black columns show STAT-3 positive MCs and the white columns show STAT-3 negative MCs. Fewer MCs were present in proliferating lesions per unit area compared to involuting lesions which contained a three-fold increase in the number of MCs per unit area. However, 90% of MCs in proliferating lesions expressed STAT-3 while only 76% of MCs expressed STAT-3 in the involuting phase. This change reached statistical significance with * $p < 0.05$. Scale bars: 50 μm.

3.3.2.4 STAT-3 AND p-STAT-3 (TYR 705) IMMUNOLOCALISATION

Confocal microscopy was used to examine the intracellular localisation of STAT-3 and p-STAT-3 (Tyr 705) within proliferating haemangioma biopsies. Tissue sections were double labelled with PCNA and STAT-3 or p-STAT-3 (Tyr 705). STAT-3 expression was detected in the cytoplasm of both proliferating and non-proliferating cells (Figure 3.13, red staining). While immunoreactivity for p-STAT-3 (Tyr 705) was localised to the nucleus across all haemangioma tissue sections examined, p-STAT-3 (Tyr 705) staining was detected in both proliferating (PCNA positive) (Figure 3.14) and non-proliferating cells (PCNA negative) (data not shown). Figure 3.14 shows a region on a proliferating haemangioma tissue section where p-STAT-3 (Tyr 705) expression was detected within the nuclear compartment of PCNA positive cells.

Figure 3.13: Confocal serial optical sections through a STAT-3 and PCNA dual labelled haemangioma section. (A-E) Bound STAT-3 antibodies were detected using an anti-rabbit DIG conjugate and anti-DIG-Rhodamine conjugate (red). Bound PCNA antibody was detected using an anti-mouse FITC conjugate (green). Note the localisation of STAT-3 to the cytoplasm clearly distinct from the PCNA positive nuclei. Panel B shows STAT-3 immunoreactivity on PCNA negative cells (white arrows) and Panel D shows STAT-3 immunoreactivity on PCNA positive cells (yellow arrows).

Figure 3.14: Confocal images through a p-STAT-3 (Tyr 705) and PCNA dual-labelled proliferating haemangioma section. (A) Bound p-STAT-3 (Tyr 705) antibodies were detected using an anti-rabbit DIG conjugate and an anti-DIG-Rhodamine conjugate (red). (B) Bound PCNA antibodies were detected using an anti-mouse FITC conjugate (green). Cell nuclei were counterstained with DAPI (blue). Panels A and B show p-STAT-3 (Tyr 705) (white arrow heads, red) and PCNA (white arrow heads, green) staining within the cell nuclei, respectively. Panel C is the overlay of A and B showing co-localisation of p-STAT-3 and PCNA to the nucleus (yellow). Although in this image, p-STAT-3 (Tyr 705) appears to co-localise with PCNA, this was not always case in other regions of the same section and on other haemangioma tissue sections. Scale bars: 20 μ m.

3.3.2.5 STAT-3 PROTEIN EXPRESSION CORELLATES WITH PROLIFERATION

To investigate if STAT-3 expression correlates with proliferation, immunohistochemical staining for STAT-3 was performed in conjunction with PCNA on haemangioma tissue sections (three from each phase). PCNA is a nuclear antigen expressed maximally in the S phase of the cell cycle (Kurki *et al.*, 1986). In order to use IHC to make a quantitative measurement of protein abundance, different areas on the same slide were analysed. This eliminates variables relating to tissue or slide processing and preparation. Although the haemangioma biopsies used in this study were clinically assigned to proliferative, involuting or involuted phases, there existed regions within a single tissue section that exhibited the characteristics of each phase. Therefore, this provided an opportunity to adopt IHC staining to quantitatively measure the correlation of STAT-3 expression with proliferation. As described in section 2.2.2.5.1. (page 83), a ‘proliferative’ area (densely packed blood vessels with small lumens lined by plump ECs), an ‘involuting’ area (larger vessel lumens with flattened endothelium) and an ‘involved’ area within the same tissue section was chosen using the DAPI channel. At least 600 cells were counted from each ‘area’ and the number of STAT-3-positive, PCNA-positive and those cells that expressed both STAT-3 and PCNA were tabulated and expressed as the number of positively stained cells per unit area (Figures 3.15 and 3.16). The results from additional lesions analysed are shown in Figures 1.10 and 1.11 in Appendix 1 (pages 281 and 282). The cells counted represent all positively stained cells across the lesions and not only the ECs. Results showed that STAT-3 and PCNA protein expression concurred with the mRNA study in that the proliferative areas of proliferating haemangiomas had more PCNA stained nuclei and the highest number of STAT-3 positive cells compared with the involuting and involuted areas on the same section. However, not all PCNA-positive cells expressed STAT-3 and vice versa.

A

Proliferating haemangioma

B

	Proliferative area	Involuting area	Involved area
STAT-3	68%	55.5%	25.7%
PCNA	15%	25%	11.3%
STAT3+PCNA	7%	10%	4.5%

Figure 3.15: STAT-3 expression in different areas of proliferating haemangioma. (A) Results shown here are representative of three different lesions analysed. Black columns show STAT-3-positive cells, white columns show PCNA-positive cells and grey columns show the number of cells that stain positive for STAT-3 and PCNA (STAT3+PCNA). ‘Proliferating’ areas within a proliferating lesion contained the most number of STAT-3-positive cells which decreased in the ‘involuting’ and ‘involved’ areas. No significant differences in the number of proliferating cells (PCNA-positive) between the ‘proliferating’ and ‘involuting’ areas suggests that the lesion on the whole is highly proliferative. ‘Involved’ areas contained few STAT-3 and PCNA-positive cells. **(B)** Percentage of cells that stained positive for STAT-3, PCNA and those that expressed both STAT-3 and PCNA (STAT3+PCNA).

A

Involuting haemangioma

B

	Proliferative area	Involuting area	Involved area
STAT-3	90%	47.3%	26.7%
PCNA	13%	4.5%	2.4%
STAT3+PCNA	12%	3.6%	1.9%

Figure 3.16: STAT-3 expression in different areas of involuting haemangioma. (A) Results shown here are representative of three different lesions analysed. Black columns show STAT-3-positive cells, white columns show PCNA-positive cells and grey columns show the number of cells that stain positive for STAT-3 and PCNA. 'Proliferating' areas within an involuting lesion contained the most number of STAT-3-positive cells which decreased in the 'involuting' and 'involved' areas. There were also fewer PCNA-positive cells in the 'involuting' area as compared to the 'proliferative' area. The 'involved' areas were devoid of any PCNA expression. 'Involved' areas contained very few STAT-3-positive cells. **(B)** Percentage of cells that stained positive for STAT-3, PCNA and those that expressed both STAT-3 and PCNA (STAT3+PCNA).

3.3.2.6 p-STAT-3 (TYR 705) EXPRESSION DOES NOT CORRELATE WITH PROLIFERATION

This study found that p-STAT-3 (Tyr 705) immunoreactivity was not evenly distributed across haemangioma tissue sections but was found in distinct but random regions. To investigate if p-STAT-3 (Tyr 705) expression correlates with proliferation, immunohistochemical staining for p-STAT-3 (Tyr 705) was performed in conjunction with PCNA on five proliferating and three involuting haemangioma tissue samples. Positively stained cells in three fields of view were counted as described in section 2.2.2.5.3 (page 83). The counted cells in Figure 3.17 include all p-STAT-3 (Tyr 705) and PCNA-positive cells and were not limited only to the ECs in the lesions. Proliferating lesions contained the highest number of PCNA-positive cells as compared to involuting lesions (Figure 3.17). However, there was no obvious correlation between PCNA expression and p-STAT-3 (Tyr 705) expression. Figure 3.17 shows that proliferating lesions contained more p-STAT-3 (Tyr 705) expressing cells than involuting lesions and that the decrease in p-STAT-3 (Tyr 705) did not correlate with PCNA expression. In addition, the staining pattern of p-STAT-3 (Tyr 705) did not correlate with either age or treatment regime of the patient. Therefore, these results indicate a role for p-STAT-3 (Tyr 705) in signalling pathways other than proliferation in haemangioma.

A

Expression of p-STAT-3 (Tyr 705)
and PCNA in haemangioma

B

Age	p-STAT-3 (Tyr 705)	PCNA	% p-STAT-3 (Tyr 705) + PCNA
4 months	143	164	33
5 months	74	139	4.4
5 months	191	129	4.2
6 months	45	100	1.4
11 months	234	102	3.7
1yr and 1 month	27	55	2.5
1yr and 3 months	18	44	3.3
4 yrs and 9 months	56	34	14

Figure 3.17: p-STAT-3 (Tyr 705) expression does not correlate with proliferation. p-STAT-3 (Tyr 705) and PCNA-positive cells were examined in proliferating and involuting haemangiomas as described in section 2.2.2.5.3. (A) Black columns show p-STAT-3 (Tyr 705)-positive cells and white columns show PCNA-positive cells. Proliferative lesions contained the highest number of PCNA-positive cells which decreased in the involuting phase. Although proliferating lesions contained more p-STAT-3 (Tyr 705)-positive cells, its expression did not correlate with PCNA expression in that p-STAT-3 (Tyr 705) was not expressed by the same cells that expressed PCNA. (B) Number of positively stained cells for p-STAT-3 (Tyr 705) and PCNA and the percentage of cells that expressed both p-STAT-3 (Tyr 705) and PCNA (p-STAT-3 (Tyr 705 + PCNA) within three fields of view (600 μm²).

3.4 DISCUSSION

The proliferative phase of haemangioma is characterised by excessive angiogenesis (Mulliken *et al.*, 1995). In section 3.1, a model was proposed in which increased VEGFR-2, STAT-3 and p-STAT-3 (Tyr 705) expression was hypothesised to be associated with haemangioma proliferation and increased STAT-1 expression with haemangioma involution. The following sections will discuss and relate the results of this study to the model described in section 3.1.

3.4.1 IS VEGFR-2, STAT-3, p-STAT-3 (Tyr 705) SIGNALLING INVOLVED IN HAEMANGIOMA PROLIFERATION?

Given that the growth of many tumours is dependent on VEGF/VEGR-2 signalling (Kim *et al.*, 1993; Chen *et al.*, 2004; Strawn *et al.*, 1996, Dreves *et al.*, 2000) and that VEGFR-2 expression is found to be three to five-fold higher in ECs of tumour vasculature compared with normal vasculature (Plate *et al.*, 1994), this study sought to determine if expression of VEGFR-2 differed between the different phases of haemangioma.

Consistent with previous reports, this study found that proliferating haemangioma lesions expressed highest levels of VEGFR-2 mRNA which decreased in involuting and involuted lesions. IHC staining showed that VEGFR-2 protein concurred with mRNA levels, i.e., the plump ECs lining small nascent vessels in the proliferative phase strongly expressed VEGFR-2. While all the vessels stained positive in proliferating lesions, the number and intensity of VEGFR-2 positive blood vessels decreased in the involuting lesions, with the involuted lesions being completely devoid of any VEGFR-2 immunoreactivity. These results confirm that increased VEGFR-2 expression may be crucial for haemangioma proliferation. This pattern of VEGFR-2 expression is also consistent with the increasing evidence of its importance in tumour vascularisation, growth, and metastasis (Millauer *et al.*, 1994; Bernatchez *et al.*, 1999).

VEGFR-2 activates STAT proteins (Bartoli *et al.*, 2000; Bartoli *et al.*, 2003). In particular, STAT-3 has been shown to be activated by EC receptors such as Ang-2 and VEGFR-2 (Korpelaine *et al.*, 1999; Bartoli *et al.*, 2000; Niculescu *et al.*, 1999; Bartoli *et al.*, 2003; Schaefer *et al.*, 2002). While numerous studies have found STAT-3 activation in a variety of human tumours including breast, prostate, head and neck and ovarian cancer (Garcia *et al.*, 2001; Grandis *et al.*, 2000; Dhir *et al.*, 2002; Song & Grandis, 2000), other investigators have reported that STAT-3 activation was required and sufficient to mediate cellular transformation (Bromberg *et al.*, 1998; Turkson *et al.*, 1998; Bromberg *et al.*, 1999; Ram *et al.*, 2000). In addition, Chen *et al* (2004) showed that a strong correlation existed between VEGF, VEGFR-2, STAT-3 and p-STAT-3 expression in ovarian epithelial carcinoma. A role for STAT-3 has also been identified in vasculogenesis as well as angiogenesis. Osugi *et al* (2002) showed that cardiac-specific activation of STAT-3 promoted vascular formation in the heart while Funamoto *et al* (2000) found that STAT-3 signalling could control vessel growth during cardiac remodelling. In addition, Valdembrì *et al* (2002) found that STAT-3 activation induced vessel sprouting from chicken aorta rings. Chen *et al* (2007) have also recently reviewed the role of STAT-3 in angiogenesis.

This study found that proliferating haemangioma expressed highest levels of STAT-3 mRNA with decreased abundance in involuting and involuted lesions. In addition, a strong correlation between VEGFR-2 and STAT-3 mRNA expression was identified. This suggests that VEGFR-2 signalling may be important in driving EC proliferation through STAT-3 activation, although direct evidence of this relationship was not investigated at this stage of the study. A more functional role for STAT-3 is discussed in section 4.3.4 (page 163). Increased transcription of STAT-3 and VEGFR-2 mRNA is also indicative of the rapid turnover occurring in the proliferative phase. Double staining IHC results showed that ‘proliferating’ areas of haemangioma tissue sections taken from lesions in the proliferative and involuting phase contained the

highest number of STAT-3 cells and also the highest proportion of proliferating nuclei (PCNA-positive) as compared to the involuting and involuted areas. IHC results also showed that some of these proliferating cells (PCNA-positive) also expressed STAT-3. These findings support the role of STAT-3 in haemangioma proliferation.

In addition, 90% of MCs present in the proliferative phase expressed STAT-3, and although the number of MCs increased approximately three-fold in involuting lesions, only 70% of the MCs in the involuting phase expressed STAT-3. The promotion of neovascularisation by MC products such as FGF-2 has been known for some time (Norrby, 1994; Norrby *et al.*, 1990). The positive staining for STAT-3 in MCs in both proliferative and involuting lesions suggests that STAT-3 may be synthesised and stored by MCs. A similar role for MCs in the synthesis and/or storage of other angiogenic proteins has been previously described (Tan, 2001; Tan *et al.*, 2000_a, Tan *et al.*, 2004).

Although IHC showed that the number of STAT-3 expressing cells was highest in the proliferative phase and that fewer STAT-3 expressing cells were present in involuting and involuted lesions, Western blotting showed that abundance of STAT-3 protein per cell remained unaltered between the proliferating, involuting and involuted lesions. This discrepancy may be due to the basic biology of these lesions. Through-out this study, involuting and involuted lesions contained more fibro-fatty deposits and lysates from these lesions contained less total protein per mg of tissue as compared to proliferating lesions. Therefore, larger volumes of protein lysate from the involuting and involuted lesions were loaded on the gels during Western blotting to maintain equal protein loading. These results mean that although the number of STAT-3 expressing cells is highest in the proliferative phase compared to the involuting and involuted phases, total STAT-3 protein abundance per cell does not change with the phases. Interestingly, Yang *et al* (2005) showed that unphosphorylated STAT-3 can also drive

gene expression of proteins such as MET and MRAS, both of which are present at high levels in many cancers.

Phosphorylation of STAT-3 at tyrosine-705 is generally considered to be a prerequisite for STAT-3 activation in tumours (Shi *et al.*, 2002). This tyrosine residue is constitutively phosphorylated during viral-induced angiogenesis and transformation in Kaposi's sarcoma (Burger *et al.*, 2005). Of particular importance to this study is the fact that this residue is phosphorylated by VEGF signalling. Bartoli *et al* (2000) showed that STAT-3 (Tyr 705) phosphorylation occurred within 60-90 min of VEGF stimulation in bovine ECs. In addition, Johnson *et al* (1998) showed that STAT protein nuclear translocation was required for their transcriptional ability.

Consistent with the conventional model of p-STAT-3 (Tyr 705) activation in oncogenesis, this study identified that proliferative haemangioma contained more p-STAT-3 (Tyr 705) protein than involuting and involuted lesions. Interestingly, although p-STAT-3 (Tyr 705) immunoreactivity was nuclear in all phases, its expression was not restricted to proliferating (PCNA positive) cells. Although unexpected, these results can be explained by the involvement of p-STAT-3 (Tyr 705) in cellular processes other than proliferation, such as differentiation and apoptosis (Deng *et al.*, 2000; Zhao *et al.*, 2002). Although expression of p-STAT-3 (Tyr 705) expression was initially thought to be associated with the proliferation of haemangioma, the results from this study indicate a role for p-STAT-3 (Tyr 705) in the involution of haemangioma. This hypothesis is discussed further in other sections of this thesis (see section 4.3.6, page 179).

VEGFR-1 protein expression was also investigated in haemangioma biopsies in proliferating, involuting and involuted haemangioma biopsies. This receptor was studied because it was found to be expressed by HaemDMSCs (section 4.3.3.4, page 153). VEGFR-1 expression in haemangioma is discussed in Chapter 4.

3.4.2 IS STAT-1 INVOLVED IN HAEMANGIOMA INVOLUTION?

STAT-1 is considered a tumour suppressor as it inhibits growth and acts as a pro-apoptotic factor (Calo *et al.*, 2003). STAT-1 activation is required for the anti-proliferative effects of IFN α as well as the apoptotic effect of TNF α (Bromberg *et al.*, 1996; Kumar *et al.*, 1997). The loss of STAT-1 signalling enhances oncogenesis (Kaplan *et al.*, 1998).

Due to the involvement of STAT-1 in growth arrest and apoptosis in many cell types (Chin *et al.*, 1996; Chin *et al.*, 1997; Kumar *et al.*, 1997), a role for STAT-1 signalling in the involution of haemangioma was proposed (Figure 3.1). STAT-1 mRNA and protein expression patterns were investigated in this study. qRT-PCR results showed that proliferating and involuting haemangiomas expressed more STAT-1 mRNA than involuted lesions. This decrease in mRNA levels correlated with a decrease in STAT-1 protein expression. IHC showed that proliferating lesions contained more STAT-1 protein primarily localised to the cells lining the blood vessels and also in some of the interstitial cells. Although IHC detected STAT-1 protein in proliferating and involuting haemangioma, Western blotting showed that the overall expression of STAT-1 was low. These results suggest that even though STAT-1 is classically considered to be a tumour suppressor (Kaplan *et al.*, 1998) and a regulator of apoptosis (Chin *et al.*, 1997; Kumar *et al.*, 1997), it may not be an important factor in regulating the biology of haemangioma.

3.4.3 LIMITATIONS OF THIS STUDY

The major limitation of this study is that the samples available consisted of only formalin-fixed paraffin embedded haemangioma tissue sections and fresh snap frozen tissue biopsies. These biopsies were taken from different individuals at different developmental ages. In addition, although each lesion was clinically categorised as being proliferative, involuting or involuted, it is known that any individual

haemangioma lesion can exhibit characteristics typical of all three phases (Tan, 2001). Although each of the biopsies predominantly represented one phase of haemangioma (i.e., either proliferating, involuting or involuted) it is possible that the tissue sample contained regions from the other phases as well. This becomes important during qRT-PCR and Western blotting studies when gene expression analyses are done on snap frozen tissue samples. With IHC, however, it is possible to visually identify and discriminate between the different phases.

A second limitation of this study is that the results obtained in this chapter by qRT-PCR, IHC and Western blotting techniques identify correlations in gene expression. Ideally, experiments need to be performed on an appropriate cell line derived from haemangioma in order to provide functional evidence for the importance of VEGF and STAT signalling in these lesions.

CHAPTER 4: ROLE OF STEM CELLS IN HAEMANGIOMA

4.1 INTRODUCTION

The precise causes for haemangioma remain unknown, however, considerable progress has been made towards understanding the cellular and molecular events that govern its life cycle. The early proliferating tumour is characterised by excessive angiogenesis (Takahashi *et al.*, 1994; Kraling *et al.*, 1996; Bielenberg *et al.*, 1999). Several angiogenic factors such as VEGF, FGF-2, PDGF, TGFs and IL-6 (Folkman & Klagsbrun, 1987; Folkman, 1997) are now known to be important regulators of haemangioma development (Hasan *et al.*, 2000_b; Takahashi *et al.*, 1994; Chang *et al.*, 1999). In addition to increased expression of receptors crucial in angiogenesis, genetic alterations and somatic mutations in VEGFRs have been reported (Walter *et al.*, 2002; Blei *et al.*, 1998; Walter *et al.*, 1999; Nguyen *et al.*, 2004; Yu *et al.*, 2001; Ritter *et al.*, 2002). Recently, the presence of endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs) and clonal expansion of ECs with aberrant properties have been identified in proliferating haemangioma (Yu *et al.*, 2004_a; Yu *et al.*, 2006; Yu *et al.*, 2001, Li *et al.*, 2003, Khan *et al.*, 2006; Boye *et al.*, 2001; Walter *et al.*, 2002).

Primary cultures derived from haemangioma are useful models for *in vitro* studies because it allows the addition of factors to the medium or components of the medium to be changed and their effects evaluated. However, as a single haemangioma lesion consists of a highly heterogeneous population of cells, the purification and establishment of a primary cell line is not straightforward and may often contain contamination with other cell types. Other disadvantages of using a cell culture model include the formation of abnormal cell architecture and changes in gene expression (Kivell, 2003).

A scientific approach to the study of the pathogenesis of haemangioma has been hampered by the absence of a suitable model for haemangioma. Previous experimental models include the induction of 'haemangioma' in mice by transgenic ECs (Dubois-Stringfellow *et al.*, 1994), ECs that have been transformed by the polyoma middle T oncogene (Sage, 1992; Williams *et al.*, 1989) or ECs obtained from murine (Meininger *et al.*, 1995) and human haemangioma (Boye *et al.*, 2001). ECs have also been successfully cultured from human haemangioma on plasma clots (Mulliken *et al.*, 1982). The limiting factor of these models is the absence of other cellular elements and ECM characteristics of human haemangioma as well as the necessity of plasma (Tan, 2001). Therefore, these models are not ideal for investigating the effect of modulating molecules.

In 2000, Tan *et al* (2000_b) developed a novel human *in vitro* model for haemangioma, which overcomes some of the deficiencies associated with the above systems. In this model, freshly obtained haemangioma biopsies are embedded between two fibrin gel layers and cultured in serum free conditions. Within days, a complex network of capillary-like outgrowth emanates from the tissue fragment. In addition, the serum free condition of this model allows for agents such as growth factors, drugs or antibodies to be added to the cultures so that their effect on capillary-like outgrowth can be studied. However, one limitation of this model is that cell-specific signal transduction pathways, in response to drugs or other factors added to the medium, cannot be easily investigated.

4.2 AIMS

The capillary-like outgrowth emanating from the haemangioma biopsy in the explant model has previously been characterised as ECs (Tan *et al.*, 2000_b; Ritter *et al.*, 2000). As these cells exhibit an aggressive proliferative and migratory characteristic, it is hypothesised that these cells are involved in driving the proliferation and involution of haemangioma. The aim of this study was to purify, characterise and establish primary cultures of these cells in order to further elucidate the mechanisms regulating the programmed biological behaviour of haemangioma. A functional role for the importance of VEGF and STAT signalling in haemangioma was also investigated using the *in vitro* explant model and the purified cultures.

4.3 RESULTS

4.3.1 MORPHOLOGICAL OBSERVATIONS OF THE *IN VITRO* HAEMANGIOMA CULTURE

Proliferative haemangioma tissue fragments were cultured using a previously described method (Tan *et al.*, 2000_b). 90-95% of the explants gave rise to a complex array of microvessels emanating from its perimeter within 3-4 days. Early on in this study, involuting and involuted haemangiomas were also cultured. However, outgrowths from these cultures had fewer sprouts, which were not seen until after 10-12 days in culture. This difference in growth rates between proliferative, involuting and involuted haemangiomas has been reported previously (Tan *et al.*, 2000_b). The capillary-like outgrowth is shown in Figure 4.1. Additional images can be found in Appendix 2, Figure 2.1, page 284.

Several other observations were made from these explanted cultures. The capillary-like structures were often noted to emanate from the tissue perimeter and follow a circular path surrounding the original tissue piece (Figure 4.2-A and Appendix 2, Figure 2.2, page 285). This ring like formation was sometimes seen even before any sprouting had occurred (Figure 4.2-B). Removal of the original tissue piece was followed by the disruption and the eventual disintegration of the newly formed outgrowths (Figure 4.2-C). 95% of the cultures gave rise to the cell type shown in Figure 4.1 and Figure 4.2-A. However, in 4-5% of the cultures, a different cell type was seen to grow out, sometimes along with another cell type (Figure 4.2-D and Appendix 2, Figure 2.3, page 285). This cell type was also seen to rapidly proliferate and form a sheet-like layer within the fibrin gel (Figure 4.2-E). Morphologically, these cells look similar to ECs, however, confirmation of this requires immunohistochemical analysis, which was not performed during this study. In addition, occasionally both cell types grew out from some explants (Figure 4.2-F).

Figure 4.1: Haemangioma explant model. Phase contrast microscopy of the explant cultures at high magnification showing the individual cell structures growing out from the haemangioma tissue explant. Scale bars: (A) 500 μm , (B) 200 μm .

Figure 4.2: Morphological characteristics of the haemangioma explant model. Proliferating haemangioma biopsies were cultured as described in section 2.3.3. **(A)** Capillary-like structures that emanated from the explanted tissue followed a circular pattern of growth surrounding the original tissue. Photograph was taken on day 14 in culture. **(B)** A ring like clearing was sometimes observed around the explant even before any capillary-like growth. Photograph was taken on day 2 in culture. **(C)** Capillary-like structures did not survive when the original haemangioma tissue piece was removed. Photograph was taken on day 2 after the tissue piece was removed. **(D)** 4-5% of cultured proliferating haemangioma explants gave rise to another cell type emanating from its surface. These cells exhibited the morphology similar to ECs. Photograph was taken on day 16 in culture. **(E)** When present, the second cell type continued to grow forming a sheet like layer in the gel matrix. **(F)** Some explants gave rise to two cell types with different morphologies emanating from the tissue surface. Photograph was taken on day 7 of culture. Scale bars: (A,B,C&E) -1000 μm , (D&F) – 200 μm , respectively.

4.3.2 MODIFICATION OF THE *IN VITRO* EXPLANT MODEL

In addition to culturing haemangioma explants as described by Tan *et al* (2000_b), a modification of this system was also used in this study. Preliminary experiments showed that this system (see section 2.3.4, page 86) yielded capillary-like sprouts within the same time frame as the sandwich model. The main difference between the two systems is that the modified system consisted of the haemangioma tissue piece embedded into a single gel layer which was then covered with liquid medium. This modified system offered several advantages.

In testing for the effects of various agents on capillary-like outgrowth, this model, (i) allowed easy access of the agent to the explanted haemangioma biopsy; (ii) was more economical as it required less culture media and agents to be added; and (iii) was less time consuming and tedious. This system was also more suited for the purification of the capillary-like outgrowths as lower volumes of the fibrin gel used was easily digested with elastase.

4.3.3 PURIFICATION AND CHARACTERISATION OF THE CAPILLARY-LIKE OUTGROWTHS

4.3.3.1 CELL CULTURE CONDITIONS

Haemangioma explant-derived primary cells (HaemDCs) were isolated from explanted cultures of six proliferating and two involuting haemangioma biopsies (see section 2.3.6, page 88 for details on the isolation procedure). The adherent cells were culture-expanded in RPMI 1640 + L-glutamine medium supplemented with 10% FCS and 2% antibiotics.

Although previous studies have shown that primary cell cultures often require special growth conditions such as the use of high serum concentrations (20%) and additional supplements including ECGS and FGF-2, these factors were not required for the survival and proliferation of HaemDCs. Preliminary experiments showed that

although there was no indication of apoptosis or necrosis when the cells were maintained in medium containing 1-2% FCS, the rate of proliferation was slow. Cells cultured in medium containing 0-0.1% FCS survived for approximately 6-8 days after which the cells showed signs of senescence and eventually death (data not shown).

Coating the cell culture dish with a protein substrate to aid cell attachment is also a common practice for primary cell culture. Preliminary experiments determined that although the haemangioma-derived cell cultures attached well to all the substrates tested in this study including 10 $\mu\text{g}/\text{cm}^2$ Fibronectin (Sigma), 1% Gelatine (commercially available from the super-market), Collagen (Sigma) and Poly-D-lysine (kindly provided by Dr Bronwyn Kiwell, Victoria University of Wellington), their use was found to be unnecessary and was therefore discontinued.

4.3.3.2 MORPHOLOGY AND GROWTH CHARACTERISTICS

Phase contrast microscopy of HaemDCs demonstrated a homogeneous population of fibroblast-like, spindle-shaped cells at passage 1 (Figure 4.3-B), which formed colonies when grown at low density. In later passages (> passage 5), the cells began to display a broadened, flat morphology (Figure 4.3-C), which is typical of MSCs (Tropel *et al.*, 2004). With the appearance of the flattened star-shape morphology, the cultures grew exponentially, requiring weekly passages. They could be expanded for more than 15 doublings without any more changes in morphology and cryopreservation did not affect their growth or morphology (data not shown).

The HaemDCs also exhibited angiogenesis-like characteristics *in vitro*. When plated back into a fibrinogen-media gel matrix, the cells formed capillary-like structures (Figure 4.3-D), which continued to develop with time and resulted in substantial formation of capillary-like structures by day 20 in culture (Figure 4.3-E). HUVECs, which are normal ECs, did not form these capillary-like structures even after 20 days in culture (Figure 4.3-F).

Figure 4.3: Morphological characterisation of HaemDCs. (A) Phase contrast microscopy of a proliferating haemangioma explant in culture with capillary-like outgrowths emanating from the original tissue piece. (B) Cells purified from the solubilised gel matrix and cultured *in vitro* exhibited fibroblast-like spindle shape morphology at passage 1. (C) At passage 5, cells appeared flat and broadened. (D) The purified cells formed capillary-like like structures when cultured in a 3-dimensional medium based gel matrix which continued to develop with time (image shown is after 15 days in culture) (E). (F) HUVECs did not form capillary-like structures when plated into the medium-based gel matrix. Scale bars: (A) 500 μm , (B-F) 250 μm .

To examine the population doubling time of these primary cultures, cell isolates from two proliferative and one involuting haemangioma were plated at the same density and grown under identical conditions. The cultures derived from the proliferative lesions were assayed at passage 5 while the cultures from the involuting lesions were at passage 2. Cell numbers were determined on days 0, 2, 4, 6 and 9 and doubling time was found to be approximately 4 days (Figure 4.4).

Figure 4.4: Population doubling time of HaemDCs. Cell isolates derived from two proliferative haemangioma lesions (pink and blue line) and one involuting lesion (yellow line) were plated into 24-well tissue culture plates at a density of 1×10^4 cells/well. Cell numbers were counted at days 0, 2, 4, 6 and 9 and plotted as number of cells $\times 10^4$. The results are presented as the mean $\pm$ s.e.m of triplicate determinations.

4.3.3.3 mRNA CHARACTERISATION

To determine whether the HaemDCs isolated from cultured explants were mesenchymal, as suggested by their morphology and growth characteristics, qRT-PCR for lineage-specific transcripts was undertaken. Analysis of mRNA transcripts was assessed by analysing the threshold cycle (Ct) obtained for each gene by qRT-PCR from HaemDCs isolated from four proliferating haemangiomas and one involuting haemangioma relative to the house-keeping gene, GAPDH. HUVECs and 3T3

fibroblasts were also analysed in parallel for comparison. Table 6 shows that the HaemDCs expressed transcripts for the mesenchymal markers, CD29, CD44 and CD90, but did not express transcripts for the EC markers, CD31 and VEGFR-2, or the haematopoietic antigen, CD45. Low level expression of transcripts for VEGFR-1 was detected in the HaemDCs tested. The HaemDCs also expressed mRNA for GLUT-1 which is the marker used to differentiate haemangiomas from other vascular malformations (North *et al.*, 2000). Although HUVECs expressed transcripts for CD29 and CD44, CD90 mRNA was not detected. In addition, while HUVECs expressed transcripts for the EC markers VEGFR-2 and CD31, 3T3 fibroblasts did not express any of the above markers with the exception of CD44. These results indicate that the HaemDCs isolated from haemangioma explants express a distinct profile of mRNA transcripts for a range of MSC markers that is different from both normal ECs and fibroblasts.

In addition, qRT-PCR showed that HaemDCs isolates from four different patients expressed transcripts for VEGF, Collagen-I, -II, -III and VIII but not Laminin-V. Low levels of IGF-2 mRNA were also detected. In contrast, 3T3 fibroblasts did not express mRNA for any of these genes. An example of the typical mRNA expression profile obtained for these markers in HaemDCs and their corresponding biopsies of origin is given in Appendix 2, Figure 2.4, page 286. The raw ΔC_t values (relative to GAPDH) for all cell lines analysed for these markers can be found in Appendix 2, Table 2.1, page 287.

Phase	HaemDCs	Passage	GLUT-1	Stem cell marker CD133	MSC markers			EC markers		Haematopoietic antigen	
					CD29	CD44	CD90	VEGFR-1	VEGFR-2	CD31	CD45
Prolif	Patient A	3	ND	-	2.9	4.4	5.2	10.1	-	-	-
Prolif	Patient A	9	ND	-	2.9	3.7	4.6	9.8	-	-	-
Prolif	Patient B	3	ND	-	1.9	3	2.8	ND	-	-	-
Prolif	Patient C	3	7	-	1	2.5	2.2	10.2	-	-	-
Prolif	Patient D	3	5.5	-	1.2	3.7	2.6	ND	-	-	-
Ig	Patient F	3	7.9	-	2.2	3.2	2.4	9.9	-	-	-
	HUVECS	12	9.7	-	2.3	3.2	-	9.8	9.6	2.2	-
	3T3 Fibroblasts		-	-	-	4.1	-	-	-	-	-

Table 6: mRNA characterisation of HaemDCs. mRNA expression for GLUT-1, the stem cell marker, CD133, the MSC markers, CD29, CD44 and CD90, the EC markers, CD31, VEGFR-1 and VEGFR-2 and the haematopoietic antigen, CD45 was analysed in HaemDCs isolated from four proliferating haemangioma lesions (Patient A-D), one involuting haemangioma lesion (Patient F), HUVECs and 3T3 fibroblasts. RNA was extracted from 1×10^5 cells, reverse transcribed into cDNA and analysed by qRT-PCR as described in section 2.3.8.1. Expression of transcripts was determined relative to GAPDH (Δ Ct). The table gives the Δ Ct values obtained for each gene. HaemDCs expressed mRNA for a number of markers characteristic of MSCs including CD29, CD44 CD90 but did not express mRNA for the EC markers, CD31, VEGFR-1 and VEGFR-2 or the hematopoietic marker, CD45. HaemDCs and HUVECs expressed mRNA for the the haemangioma antigen, GLUT-1. None of the cell lines expressed mRNA for the stem cell marker, CD133. Key: ‘-’ shows no amplification and therefore absence of mRNA transcript; ND indicates not determined.

4.3.3.4 IMMUNOHISTOCHEMICAL CHARACTERISATION

Immunofluorescent labelling of lineage-specific markers revealed that all cells stained for vimentin (Figure 4.5-A) and GLUT-1 (Figure 4.5-B) while only some cells were positive for α -SMA (Figure 4.5-C). In addition, the HaemDCs did not express any of the commonly used EC markers, vWF, CD34 or VEGFR-2 (Figure 4.6 and Figure 4.7). As positive and negative controls in the same experiment, immunostaining for vWF, CD34 and VEGFR-2 was carried out on HUVECs and 3T3 fibroblasts, respectively. Immunoreactivity for VEGFR-1 was detected on HaemDCs and HUVECs but not on 3T3 fibroblasts (Figure 4.7). VEGFR-1 expression by HaemDCs was confirmed by Western blotting (Figure 4.12).

Figure 4.5: Immunohistochemical localisation of vimentin, α -SMA and GLUT-1 on HaemDCs. HaemDCs were fixed and immunostained for vimentin, α -SMA and GLUT-1 as described in section 2.3.9. Vimentin and α -SMA expression was detected using anti-mouse AlexaFluor 488 conjugated antibodies (green staining) and GLUT-1 expression was detected with an anti-rabbit Cy3 conjugate (red staining). Cell nuclei were counterstained with DAPI (blue). All HaemDCs expressed the MSC marker, vimentin (A) and the haemangioma marker, GLUT-1 (C) while only some cells showed immunoreactivity for another MSC marker, α -SMA (B). Scale bars: (A-B) 100 μ m, (C) 50 μ m.

Figure 4.6: Immunohistochemical localisation of vWF and CD34 on HUVECs, HaemDCs and 3T3 fibroblasts. HUVECs (A&D), HaemDCs (B&E) and 3T3 fibroblasts (C&F) were fixed and labelled with rabbit anti-vWF (A-C) and mouse anti-CD34 (D-F) antibodies as described in section 2.3.9. Bound antibodies were detected using either an anti-rabbit Cy3 (red) or an anti-mouse AlexaFluor 488 conjugate (green). Cell nuclei were counterstained with DAPI (blue). HUVECs were used as a positive control and expressed both vWF (A) and CD34 (D) while no immunoreactivity was detected for vWF and CD34 on the HaemDCs (B&E, respectively) and the cell line used as a negative control, the 3T3 fibroblasts (C&F, respectively). Scale bars: 50 μ m.

Figure 4.7: Immunohistochemical localisation of VEGFR-2 and VEGFR-1 on HUVECs, HaemDCs and 3T3 fibroblasts. HUVECs (**A&D**), HaemDCs (**B&E**) and 3T3 fibroblasts (**C&F**) were fixed and labelled with rabbit anti-VEGFR-2 (**A-C**) and goat anti-VEGFR-1 antibodies (**D-F**). Bound antibodies were detected using either an anti-rabbit DIG conjugate followed by an anti-DIG-Rhodamine (red) conjugate or an anti-goat biotin conjugate followed by an AlexaFluor 555 streptavidin conjugate (red). Cell nuclei were counterstained with DAPI (blue). HUVECs used as a positive control, expressed both VEGFR-2 (**A**) and VEGFR-1 (**D**). HaemDCs and 3T3 fibroblasts did not express VEGFR-2 (**B&C**, respectively). Positive staining for VEGFR-1 was detected on HaemDCs (**E**) but was absent on the negative control cell line, 3T3 fibroblasts (**F**). Scale bars: (A-C) 50 μ m, (D-F) 20 μ m.

Taken together, the qRT-PCR and IHC data supports the assertion that the primary cultures obtained from the capillary-like outgrowths from haemangioma explants are of mesenchymal origin and are now referred to as haemangioma-derived mesenchymal cells (HaemDMCs)

Although the HaemDMCs were found to express VEGF and collagen mRNA, protein expression was absent. Western blotting (Figure 4.8) and immunofluorescence staining (data not shown) of HaemDMCs showed that the cells neither expressed nor secreted VEGF. 50 ng and 25 ng of purified recombinant VEGF₁₆₅ along with total protein from HaemDMCs conditioned media (40 µg), HaemDMCs (100 µg) and proliferative haemangioma tissue (100 µg) lysates were subjected to Western blotting under reducing conditions. The resulting immunoblot was probed with an anti-VEGF antibody that recognises three isoforms of VEGF-A, VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉. Immunoreactive bands corresponding to a molecular weight of 23 kDa were detected only with the purified VEGF protein (Figure 4.8).

In addition, picrosirius red staining of HaemDMCs showed no collagen fibres either in the cells themselves or in their ECM (data not shown). An involuting haemangioma section was used as a positive control and showed expression of collagen fibers (Figure 4.9). Staining of collagen fibres with picrosirius red allows the visualisation of individual collagen fibres on the basis of double refraction of light against a black background, when epipolarisation microscopy is used (Junqueira *et al.*, 1979).

Figure 4.8: Western blotting for VEGF-A. Total protein extracted from the conditioned media of HaemDMCs, from HaemDMCs and from proliferating haemangioma tissue was subjected to Western blotting as described in section 2.3.13.2. Lane: 1) 50 ng of purified VEGF₁₆₅; 2) 25 ng of purified VEGF₁₆₅; 3) 40 µg of total protein from the medium of HaemDMCs conditioned over a 96 hour period; 4) 100 µg of total protein from HaemDMCs and 5) 100 µg of total protein from a proliferative haemangioma tissue sample. Following electrophoresis and transfer onto a PVDF membrane, the membrane was blocked and probed with an antibody that recognises the VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉ isoforms of VEGF-A. Specific immunoreactivity was detected at 23 kDa from the purified VEGF protein but was absent on the other samples (black arrow).

Figure 4.9: Picrosirius red stained section of an involuting haemangioma tissue section. Under a polarised light source, the haemangioma tissue section stained with picrosirius red showed the presence of collagen fibers. In the image above, thick fibers appear red or yellow while thin fibers appear green. Scale bar: 500 µm.

4.3.3.5 MULTILINEAGE DIFFERENTIATION POTENTIAL OF HaemDMCs

To determine the multilineage capacity of HaemDMCs cultured cells were differentiated toward the adipogenic and osteogenic lineages using lineage-specific induction factors. To induce adipogenesis, cells were cultured in medium containing Dexamethasone, ascorbic acid, β -glycerophosphate, insulin and indomethacin. Differentiation into adipocytes was detected within 15 days of induction and continued to accumulate over time (Figure 4.10-D). A significant fraction of the cells contained multiple, intracellular lipid-filled droplets that accumulated Oil-Red O (Figure 4.10-E). No lipid droplets were observed in undifferentiated HaemDMCs (Figure 4.10-A). Adipocytic differentiation potential of HaemDMCs was maintained even after 10 passages, indicating their phenotypic stability and their ability to undergo self-renewal *in vitro*.

Differentiation into osteoblasts was induced *in vitro* by treating cells with ascorbic acid, β -glycerophosphate and Dexamethasone. To confirm osteogenic differentiation, calcification of the ECM was assessed using Alizarin Red stain. While no calcification was observed in undifferentiated cells (Figure 4.10-A) the HaemDMCs readily differentiated into osteocytes. Figures 4.10-B and -C show mineralization of the ECM produced by cultured HaemDMCs that was revealed by Alizarin Red staining. Consistent with previous findings, differentiation required the cells to be plated at high confluency (greater than 2×10^4 cells/cm²) (Tropel *et al.*, 2004).

HaemDMCs isolated from proliferating and involuting haemangioma biopsies showed similar osteogenic differentiation potential (Figure 4.10-B and -C, respectively). However, HaemDMCs isolated from proliferating haemangiomas readily differentiated into adipocytes by day 15 (Figure 4.10-E) while HaemDMCs derived from involuting lesions took approximately 60 days to produce any lipid vacuoles (Figure 4.10-F).

In contrast, HaemDMCs isolated in this study did not differentiate into ECs or express EC-like markers when cultured in the presence of VEGF (data not shown).

Confirmation of differentiation into adipocytes and osteoblasts was investigated by qRT-PCR analysis of transcripts for the peroxisome proliferator-activated receptor (PPAR- γ), a key regulatory gene of adipogenesis (Camp *et al.*, 2002), and osteonectin, a gene expressed by osteoblasts (Jundt *et al.*, 1987). Table 7 shows the expression levels of these genes in mesenchymal cell isolates from five proliferating and one involuting haemangioma. Gene expression was determined relative to GAPDH (Δ Ct) in undifferentiated versus differentiated cells ($\Delta\Delta$ Ct). Expression of PPAR- γ increased as expected (approximately 16-fold) while that for osteonectin did not alter significantly. Differentiation experiments were replicated with HaemDMCs isolates from five different proliferative biopsies and two involuting biopsies. These data are consistent with the HaemDMCs being haemangioma-derived mesenchymal stem cells (HaemDMSCs), which are here after referred to as such. An example of the typical change in mRNA expression for osteonectin and PPAR γ , before and after induction, from one experiment is shown in Figure 4.11.

Phase	HaemDMCs	Passage	$\Delta\Delta$ Ct PPAR- γ	$\Delta\Delta$ Ct Osteonectin
Prolif	Patient A	3	4.7	3.4
Prolif	Patient B	3	4.3	-1.5
Prolif	Patient C	3	5.5	1.7
Prolif	Patient D	3	4.9	-0.2
Prolif	Patient E	3	2.9	-1
Ig	Patient F	3	3.3	-0.4
Mean ($\pm$ s.e.m.)			4 ± 0.4	0.3 ± 0.8

Table 7: PPAR- γ and osteonectin mRNA expression. HaemDMCs isolated from five proliferating (Patient A-E) and one involuting haemangioma (Patient F) were differentiated into adipocytes and osteoblasts. qRT-PCR was performed to identify changes in PPAR- γ and osteonectin mRNA expression in undifferentiated cells versus differentiated cells ($\Delta\Delta$ Ct)

Figure 4.10: Differentiation potential of HaemDMCs. HaemDMCs purified from a proliferating (**B,D&E**) and an involuting haemangioma (**C&F**) were plated onto glass coverslips at 2×10^4 cells/cm² and cultured in osteocytic or adipocytic differentiation media as described in section 2.3.9. HaemDMSCs purified from proliferating (**B**) and involuting (**C**) lesions differentiated into osteocytes after 14 days of induction as depicted by Alizarin Red staining. HaemDMCs also differentiated into lipid laden adipocytes. Phase contrast image of adipocyte formation in culture by HaemDMCs (**D**). Cytoplasmic lipid vacuoles were identified by staining with Oil Red O (**E&F**). HaemDMCs from proliferative lesions (**E**) more readily differentiated into adipocytes than HaemDMCs from involuting lesions (**F**). Panel **E** shows increased adipocyte formation by HaemDMCs from a proliferative lesion after only 21 days of induction while Panel **F** shows adipocyte formation by HaemDMCs from an involuting lesion after 50 days in induction media. Undifferentiated HaemDMCs isolated from a proliferating haemangioma showed no staining with Alizarin Red and Oil Red O (**A**). Scale bars: 50 μ m.

Figure 4.11: Validation of adipocytic and osteocytic differentiation of HaemDMSCs. Differentiation into adipocytes and osteocytes was validated by qRT-PCR performed for PPAR γ and osteonectin, respectively. Changes in mRNA expression are expressed relative to that for GAPDH (the internal reference gene) as Δ Ct values. Black columns indicate PPAR γ expression and white columns indicate osteonectin expression. An increase in mRNA expression was observed for both genes in differentiated HaemDMSCs compared to undifferentiated cells. A lower Δ Ct indicates higher gene expression while a higher Δ Ct indicates low expression.

Figure 4.12: Western blot verification of VEGFR-1 and STAT-3 expression by HaemDMSCs. Western blotting was performed as per section 2.3.13.1. Each lane contains 50 μ g of total protein from HaemDMSCs. Following electrophoresis and transfer onto a PVDF membrane, the membrane was blocked with goat anti-VEGFR-1, mouse anti-STAT-3 and mouse anti-GAPDH antibodies followed by incubation with anti-goat and anti-mouse AlexaFluor 488 conjugated secondary antibodies. The resulting blot confirmed expression of VEGFR-1 and STAT-3 by HaemDMSCs as signal was observed at the appropriate sizes of 180 kDa and 92 kDa, respectively. GAPDH was included as the loading control.

4.3.4 ROLE OF VEGF AND STAT SIGNALLING *IN VITRO*

4.3.4.1 EFFECT OF VEGF AND AG490 ON CAPILLARY-LIKE OUTGROWTH

The importance of VEGF and STAT signalling on ‘microvessel’ outgrowth was investigated using the explant model developed by Tan *et al* (2000_b) and also using the modified culture system developed in this study (section 2.3.4, page 86). Explants treated with 50 ng/ml of VEGF exhibited a higher degree of capillary-like outgrowth after six days in culture compared to controls ($p < 0.01$). This dramatic sprouting was mitigated by the addition of 50 μ M of AG490, an inhibitor of STAT protein phosphorylation (Huang *et al.*, 2006; Andl *et al.*, 2004), in the culture medium (Figure 4.13-A). Among the sprouting explants, the VEGF treated group was observed to be more advanced in terms of number and length of sprouts. In addition, cultures treated with AG490 and VEGF showed significantly less capillary-like growth than untreated cultures ($p < 0.01$) (Figure 4.13-A). This suggests that the inhibitory effect of AG490 can overcome the stimulatory effect of VEGF on capillary-like outgrowths. Figure 4.13-A shows the average capillary-like outgrowth of explanted haemangioma tissue obtained from two separate patients. Duplicate wells containing an explanted tissue piece were used for each treatment.

Explants were also cultured with various concentrations of AG490 diluted into the gel matrix and in the overlying medium for a period of ten days. Addition of 10 μ M and 50 μ M AG490 at the beginning of the culture inhibited sprouting with significance achieved at $p < 0.05$ and $p < 0.001$, respectively (Figure 4.13-B). Results shown in Figure 4.13-B are representative of two separate experiments conducted with haemangioma biopsies obtained from two individual patients. Triplicate wells were used for each treatment with at least six wells used as control cultures.

A

B

Figure 4.13: Effect of AG490 and VEGF on capillary-like outgrowth from proliferating haemangioma explants. Proliferating haemangioma explants were cultured as outlined in sections 2.3.3 and 2.3.4. In all experiments, DMSO was added to the untreated control cultures at the same volume as that used for the highest concentration of AG490. (A) Significant increase in capillary-like outgrowth was observed with the addition of VEGF (50 ng/ml) to the cultures above that of the controls. This stimulatory effect of VEGF was mitigated by including AG490 (50 µM). In addition, including AG490 along with VEGF decreased capillary-like growth compared with untreated cultures. Capillary-like outgrowth was quantified 6 days post treatment. (B) Pieces of proliferating haemangioma biopsies were cultured in the presence of AG490 at various concentrations and capillary-like growth was assayed after 9 days in culture. Capillary-like outgrowth was significantly inhibited by 50 µM and 10 µM AG490. * represents significance at $p < 0.05$, ** at $p < 0.01$ and *** at $p < 0.001$.

4.3.4.2 EFFECT OF VEGF AND AG490 ON HaemDMSCs.

To investigate if VEGF signalling through STAT proteins is involved in the proliferation of HaemDMSCs, cells were incubated with VEGF at various concentrations known to stimulate proliferation of ECs and the extent of proliferation was measured using the EZ4U assay. Although VEGF induced proliferation of HUVECs (Figure 4.14-A), it did not stimulate the proliferation of HaemDMSCs even at low serum concentrations (Figure 4.14-B).

Figure 4.14: VEGF stimulates proliferation of HUVECs but not HaemDMSCs. HUVECs (**A**) and HaemDMSCs (**B**) were treated with the indicated concentrations of VEGF and the extent of cell proliferation was measured using the EZ4U assay as described in section 2.3.12.1. The increase in cell number was measured by the increase in the absorbance values of HUVEC cultures treated with various concentrations of VEGF for 72 hours (**A**). VEGF did not stimulate proliferation of HaemDMSCs proliferation as assayed from the absorbance readings (**B**). Data shown in (**B**) is representative of two separate experiments performed with HaemDMSCs isolates from two haemangioma patients set up in triplicate. HUVECs were cultured in RPMI medium containing 2% FCS for 72hrs and HaemDMSCs in 0.1% FCS for 6 days. ** $p < 0.01$.

In addition, treatment with VEGF neither induced p-STAT-3 phosphorylation (Figures 4.15) nor caused its nuclear translocation (Figure 4.16). Figure 4.15 shows that in comparison with IFN treated HeLa cells, unstimulated HaemDMSCs express very low levels of p-STAT-3 (Tyr 705) and that VEGF treatment does not significantly alter p-STAT-3 (Tyr 705) expression levels. Identical results were obtained when the experiment was performed on two separate occasions with HaemDMSCs isolates from two different haemangioma biopsies (from two patients).

In addition, no significant change in p-STAT-3 (Tyr 705) expression was noted when HaemDMSCs were treated with AG490 (50 μ M) for 24 hours (Figure 4.15). Immunoblotting for total STAT-3 and GAPDH protein confirmed equal protein loadings.

A

B

Figure 4.15: VEGF does not induce p-STAT-3 (Tyr 705) expression in HaemDMSCs. HaemDMSCs isolated from a proliferating haemangioma were treated with either VEGF (50 ng/ml), AG490 (50 μ M) alone or both for the indicated time periods and subjected to Western blotting as described in section 2.3.13.1. HeLa cells stimulated with IFN α -2b (10,000 U/ml) for 10 min were included as a positive control. Immunoblots were probed with mouse anti-p-STAT-3 (Tyr 705) and mouse anti-GAPDH antibodies. **(A)** Western blot analysis showed that p-STAT-3 (Tyr 705) expression was not induced after VEGF stimulation in whole cell lysates. Membranes were then stripped and re-probed with an anti-STAT-3 antibody. The levels of STAT-3 and GAPDH expression were determined as a control for equivalent protein loading. **(B)** Graphical representation of p-STAT-3 (Tyr 705) expression by densitometry. In comparison with IFN treated HeLa cells, unstimulated HaemDMSCs expressed very low levels of p-STAT-3. The slight decrease in p-STAT-3 (Tyr 705) expression identified with VEGF treatment was not considered significant due to the low abundance of p-STAT-3 (Tyr 705) expression in the unstimulated cells to begin with.

Localisation of p-STAT-3 (Tyr 705) in response to VEGF treatment was determined by confocal microscopy. No change in p-STAT-3 (Tyr 705) expression or localisation was detected with VEGF treatment (Figure 4.16-A). Expression pattern of p-STAT-1 (Tyr 701) and p-STAT-5 (Tyr 694) was also investigated in HaemDMSCs with and without VEGF treatment. Immunoreactivity for p-STAT-1 was observed to be cytoplasmic in both unstimulated and VEGF stimulated HaemDMSCs (Figure 4.16-A). Although immunoreactivity for p-STAT-5 was undetectable in resting HaemDMSCs, there was an increase in p-STAT-5 (Tyr 694) immunoreactivity within the nuclear compartment following a 30 min incubation with VEGF (Figure 4.16-A). p-STAT-5 (Tyr 694) immunoreactivity detected within the nuclei of HaemDMSCs after VEGF treatment was significantly greater than that detected in the unstimulated cells ($p < 0.001$) (Figure 4.16-B).

A

B

Figure 4.16: Immunolocalisation of p-STAT-3 (Tyr 705), p-STAT-1 (Tyr 701) and p-STAT-5 (Tyr 694) in response to VEGF. Panel A: Confocal microscopy was used to detect immunoreactivity for p-STAT proteins (green) in unstimulated HaemDMSCs (A-C) and HaemDMSCs stimulated with VEGF (50 ng/ml) for 30 min (D-F). Cells were fixed and stained as described in section 2.3.14.1. Cell nuclei were counterstained with PI (red). In untreated cells, immunoreactivity for p-STAT-3 (Tyr 705) (A) and p-STAT-1 (Tyr 701) (B) was mainly cytoplasmic while staining for p-STAT-5 (Tyr 694) (C) was almost negligible. After 30 min of VEGF stimulation an increase in p-STAT-5 (Tyr 694) immunoreactivity was detected which was found to be predominantly within the nuclear compartment (yellow) (F). Overlapping of the green (p-STATs) and the red (nucleus) colors (yellow) indicates the presence of p-STAT proteins within the nuclei of HaemDMSCs. No significant change in p-STAT-3 (D) or p-STAT-1 (E) expression or localisation was detected. Panel B: Graphical representation of the increase in p-STAT-5 (Tyr 694) expression detected within the nuclei of HaemDMSCs following VEGF treatment as determined by densitometry analysis, *** $p < 0.001$. Scale bars: 50 μ m

To test if STAT signalling is involved in HaemDMSCs proliferation, cells were treated with AG490 in the presence of the thymidine analogue, BrdU. Studies have shown that as BrdU is readily incorporated into the DNA of replicating cells and that quantifying BrdU incorporation can be used as a measure of cell proliferation (Porstmann *et al.*, 1985).

Interestingly, after 24 hours of treatment with AG490, the number of BrdU positive cells significantly decreased indicating a delay in the accumulation of cells in S phase (Figure 4.17-A). A slight increase in the number of cells in G1 phase of the cell cycle, with minimal effects on G2/M phase of the cell cycle was also observed (data not shown). Results shown in Figure 4.17-A are representative of at least five individual experiments.

In addition, the effect of AG490 on vessel formation was investigated using the *in vitro* assay described in section 2.3.12.3 (page 94). A dose dependent decrease in vessel formation was observed when this assay was performed with medium containing both 5% (Figure 4.17-B) and 1% FCS (data not shown). The cultures in 5% FCS were assayed after a period of 14 days while those in 1% FCS were analysed after 21 days. In addition to measuring vessel formation, cell nuclei were also counted for each treatment group using counting frames of 1000 cm² (see section 2.3.12.3, page 94). A dose dependent decrease in cell number was observed with AG490 treatment (Figure 4.17-C). Figure 4.17-B and -C show the results from two individual experiments.

Figure 4.17: The effect of AG490 on HaemDMSCs. (A) AG490 inhibited proliferation of HaemDMSCs by stalling cell cycle progression. HaemDMSCs were treated with 100 μ M or 50 μ M AG490 for 24 hrs in culture media containing 5% FCS. Cells were pulsed with BrdU for the last seven hours of treatment with AG490 and fixed in ethanol. Following incubation with an anti-BrdU antibody, antibody binding was analysed by flow cytometry. A minimum of 1×10^6 cells were analysed per experimental condition and the fraction of cells in S phase were determined by BrdU incorporation. Results obtained are representative of five individual experiments. (B) AG490 inhibited vessel formation of HaemDMSCs. 1×10^5 HaemDMSCs were plated into each well of a 24-well culture dish containing either vehicle alone (DMSO), 100 μ M AG490 or 50 μ M AG490 dissolved in the culture media. After 12 days in culture, vessel formation was assessed as described in section 2.3.12.3. A significant decrease in vessel formation was detected with AG490 at both 100 μ M and 50 μ M. (C) AG490 inhibited proliferation of HaemDMSCs. The total cell nuclei within the same area as that used for assessing vessel formation was counted. Results showed that the number of cell nuclei also decreased with an increase in AG490 concentration. All values are expressed as the mean $\pm$ s.e.m. Results shown in (B) and (C) are representative of two individual experiments set up in duplicate. Control cultures were set up in quadruplicates. * $p < 0.05$, ** $p < 0.01$.

A

B

C

4.3.5 OSTEOPROTEGERIN EXPRESSION IN HAEMANGIOMA.

Osteoprotegerin (OPG) is involved in osteoclast differentiation and activation (Simonet *et al.*, 1997; Tsuda *et al.*, 1997; Fuller *et al.*, 1998). See section 5.3.5 (page 227) for more details on why OPG expression was investigated in HaemDMSCs. qRT-PCR revealed that HaemDMSCs expressed high levels of OPG mRNA (Figure 5.4-A and Appendix 3, Figure 3.5). Immunohistochemical staining revealed that HaemDMSCs expressed large amounts of OPG protein in the ECM but not within the cells (Figure 4.18-D). Consistent with previous reports, OPG co-localised with vWF in HUVECs (Figure 4.18-C) (Zannettino *et al.*, 2005). However, HUVECs expressed less OPG protein (Figure 4.18-B) than HaemDMSCs (Figure 4.18-B). Since HaemDMSCs expressed high levels of OPG protein, it was hypothesised that OPG might serve as a marker for MSCs within haemangioma tissues *in vivo*.

Immunohistochemical staining of haemangioma tissue sections showed that proliferating lesions contained more OPG positive cells (Figure 4.19-B, green staining) than involuting lesions (Figure 4.19-E) while involuted lesions contained no OPG staining cells (Figure 4.19-H). Dual immunostaining for vWF and OPG revealed that most vessels in the proliferating lesions expressed vWF but in a punctate manner (Figure 4.19-A, white arrows) and that these cells strongly expressed OPG (Figure 4.19-B, white arrows). Late proliferative and early involuting phases contained regions that exhibited punctuate vWF (Figure 4.19-D, white arrows) and high OPG staining (Figure 4.19-E, white arrows). These lesions also contained vessels that strongly expressed vWF (Figure 4.19-D, yellow arrows) ,which did not express OPG (Figure 4.19-E, yellow arrows). In addition, the vessels in the late involuting and involuted phases that strongly expressed vWF (Figure 4.19-G, yellow arrows) did not express OPG (Figure 4.19-H, yellow arrows). Dual staining for vWF and PCNA indicated that regions with punctate, weak vWF staining and high OPG expression also contained more PCNA

positive cells than regions with strongly staining vWF and no OPG expression (Figure 4.20).

Figure 4.18: Immunohistochemical localisation of vWF and OPG on HUVECs and OPG on HaemDMSCs. IHC was performed on HUVECs (**A-C**) fixed in a mixture of methanol and acetone. Cultures were stained with rabbit anti-vWF and mouse anti-OPG antibodies as described in section 2.3.11. HaemDMSCs were fixed and stained with mouse anti-OPG antibodies (**D**). Bound vWF primary antibodies were detected using an anti-rabbit Cy3 conjugate (red) and bound OPG antibodies were detected with an anti-mouse AlexaFluor 488 conjugate (green). Panel **C** is the merged image of Panels **A** with **B**. Cell nuclei were counter-stained with DAPI (blue). HUVECs expressed vWF (**A**, red staining) and OPG (**B**, green staining) which was found to colocalize (**C**, yellow staining). Merging images with red and green staining appears yellow. HaemDMSCs expressed high levels of extracellular OPG (**D**, green staining). Scale bars: 100 μ m.

Figure 4.19: Confocal immunohistochemical localisation of vWF and OPG in haemangioma. Proliferating (A-C), involuting (D-F) and involuted (G-I) paraffin embedded haemangioma tissue sections were stained with rabbit anti-vWF (A,D&G) and mouse anti-OPG (B,E&H) antibodies. Bound vWF and OPG antibodies were detected using an anti-rabbit-Cy3 conjugate (red) and an anti-mouse AlexaFluor 488 conjugate (green), respectively. Panels C, F and I are the merged images of Panels A with B, D with E, and G with H, respectively. Most cells lining the blood vessels in proliferating lesions showed punctate immunoreactivity for vWF (A, white arrows). The same cells also showed strong staining for OPG (B, white arrows). Involuting lesions contained regions that showed punctate vWF staining (D, white arrows). These regions expressed high levels of OPG (E, white arrows). However, these lesions also contained vessels that showed intense immunoreactivity for vWF (D, yellow arrows) but were negative for OPG (E, yellow arrows). Vessels in involuted lesions that strongly stained for vWF (G, yellow arrows) were negative for OPG (H, yellow arrows). Scale bars: 50 μ m.

Figure 4.20: Immunohistochemical localization of vWF and PCNA on paraffin embedded haemangioma tissue biopsies. Proliferating (**A-C**), involuting (**D-F**) and involuted (**G-I**) haemangioma biopsies were stained with rabbit anti-vWF antibodies and mouse anti-PCNA antibodies. Bound vWF and PCNA antibodies were detected using an anti-rabbit-Cy3 conjugate (red) and an anti-mouse AlexaFluor 488 conjugate (green) respectively. (**A**) Proliferating lesions showed punctate immunoreactivity for vWF (white arrows) and contained the highest number of PCNA positive cells (green staining). (**B**) In involuting lesions, the vessels that exhibited punctate staining for vWF (white arrows) also contained some PCNA positive cells. But vessels that stained strongly for vWF (yellow arrows) did not contain any cells that express PCNA. (**C**) Vessels in involuted lesions that strongly expressed vWF were devoid of PCNA positive cells. Scale bars: 50 μ m.

4.3.6 EFFECT OF INTERFERON AND DIFFERENTIATION MEDIA ON STAT PROTEIN EXPRESSION IN HaemDMSCs

IFN α -2b treatment significantly decreased proliferation of HaemDMSCs (see section 5.3.4, page 224). In addition, treatment of HaemDMSCs with 10,000 U/ml of IFN α -2b for 15 min resulted in a dramatic increase in p-STAT-3 (Tyr 705) expression (Figure 4.21-A) accompanied by its nuclear translocation (Figure 4.21-B). In addition, increased expression and nuclear translocation above that of the untreated controls was seen for p-STAT-5 (Tyr 694) and p-STAT-1 (Tyr 701) (Figure 4.21-B) in IFN α -2b treated cultures. These results lead us to the hypothesis that STAT signalling might be involved in haemangioma differentiation.

To test this hypothesis, STAT-3, STAT-5, p-STAT-3 (Tyr 705) and p-STAT-5 (Tyr 694) expression and localisation was analysed in HaemDMSCs treated with adipocyte induction media for 30 min, 4 hours and 24 hours. No significant change in expression or localisation of p-STAT-3 (Tyr 705) and p-STAT-5 (Tyr 694) was observed in treated versus the untreated cells at all time points (Appendix 2, Figure 2.5, page 288).

In addition, the effects of IFN α -2b and AG490 were tested on the rate of adipogenesis. HaemDMSCs were cultured with differentiation media containing either, 100 U/ml IFN α -2b, 15000 U/ml of IFN α -2b, 5 μ M AG490 or 20 μ M of AG490. These differentiation conditions were maintained for a period of 16 days with media being changed every 2-3 days. No significant increase in adipocyte formation was observed with either dosage of IFN α -2b above that of the control cultures containing differentiation media alone. In addition, neither dose of AG490 was able to block lipid-laden vacuole formation (data not shown).

Figure 4.21: IFN α -2b induces an increase in p-STAT protein expression and nuclear translocation in HaemDMSCs. (A) HaemDMSCs were treated with IFN α -2b for the times indicated. Unstimulated and HaemDMSCs stimulated with 10,000 U/ml of IFN α -2b for 5, 10 and 15 min were lysed and subjected to Western blotting as described in section 2.3.13.1. While unstimulated HaemDMSCs expressed low levels of p-STAT-3 (Tyr 705), a time dependent increase in p-STAT-3 (Tyr 705) expression was detected with IFN α -2b stimulation. (B) Confocal immunohistochemical localisation of p-STAT proteins (green) in HaemDMSCs stimulated with 10,000 U/ml IFN α -2b for 15 min (D-F) in comparison with control unstimulated HaemDMSCs (A-C). Cell nuclei were counterstained with PI (red). Unstimulated HaemDMSCs expressed low levels of p-STAT3 (Tyr 705) (A), p-STAT-5 (Tyr 694) (B) and p-STAT-1 (Tyr 701) (C) (green) detected only in the cytoplasm. Following IFN α -2b treatment, immunoreactivity for p-STAT3 (Tyr 705) (D), p-STAT-5 (Tyr 694) (E) and p-STAT-1 (Tyr 701) (F) was detected within the nuclei of HaemDMSCs (yellow). Overlapping of the green (p-STATs) and the red (nucleus) colors (yellow) indicates the presence of p-STAT proteins within the nuclei of HaemDMSCs. Some immunoreactivity for p-STAT-5 and p-STAT-1 was also detected in the cytoplasm of the IFN α -2b stimulated HaemDMSCs (E&F, respectively). Scale bars: 50 μ m.

4.3.7 ENDOTHELIAL PROGENITOR CELLS IN HAEMANGIOMA

Using previously described methods with a few modifications, the isolation of ECs from human haemangioma biopsies was attempted. This was to be used as a patient matched comparative cell line. However, these attempts were futile as cell yield was low after the isolation step with CD31 coated beads. These cells did not proliferate well and died following the first passage (See Appendix 2, section 2.1, page 289)

However, good yield was obtained when the bead isolation was omitted. The morphology exhibited by the cells obtained from digested proliferating haemangioma tissues was distinct from HaemDMSCs in that these cells had round central bodies with some cytoplasmic projections (Figure 4.22). This morphology has been shown to be typical of EPCs (Ashara *et al.*, 1997; Kalka *et al.*, 2000). Immunohistochemical staining performed on these cells 48 hours after dissociation showed that the cells expressed typical EC markers, vWF (Figure 4.23), VEGFR-1 (Figure 4.25-A) and more importantly VEGFR-2 (Figure 4.25-B). These results are consistent with an EC phenotype. Therefore, these cells are here in referred to as haemangioma-derived EPCs (HaemDEPCs).

Figure 4.22: EPC-like cells obtained from a proliferating haemangioma. The cells obtained from collagenase treated proliferating haemangioma tissue were plated onto gelatin coated culture dishes. These cells exhibited EPC-like morphology i.e., they contained round central bodies with some cytoplasmic projections. Photograph was taken on day 2 in culture. Scale bar: 500 μ m.

Figure 4.23: Immunohistochemical localisation of vWF and OPG on HaemDEPCs. On day 2 in culture, HaemDEPCs were fixed and stained with rabbit anti-vWF and mouse anti-OPG primary antibodies as described in section 2.3.9. Bound anti-vWF antibodies were detected with an anti-rabbit Cy3 (red) conjugate and bound anti-OPG antibodies were detected with an anti-mouse AlexaFluor 488 (green) conjugate. Cell nuclei were counterstained with DAPI (blue). Positive immunoreactivity was detected for vWF (red) and OPG (green) on all HaemDEPCs present on the coverslip. Scale bar: 50 μ m.

Interestingly, on day 5 in culture, HaemDEPCs had changed to a MSC-like morphology, i.e., they appeared flat and broadened (Figure 4.24-A). At this stage, immunohistochemical analysis showed that they no longer expressed vWF (Figure 4.24-B) and VEGFR-2 (Figure 4.25-D). However, the cells continued to express VEGFR-1 (Figure 4.25-C) and high levels of extracellular OPG (Figure 4.24-B) which again is typical of HaemDMSCs as shown earlier (Figure 4.18-C). Furthermore, the MSC nature of the spontaneously differentiated cells was confirmed by the ability of the cells to form adipocytes (Figure 4.26). These results indicate that there is an EPC to MSC transdifferentiation process occurring *in vitro*.

A

B

Figure 4.24: Change in HaemDEPC morphology and immunophenotype on day 5 in culture *in vitro*. (A) Phase contrast microscopy showing MSC like morphology, i.e., cells appeared flat and broadened. These cells were fixed and stained with rabbit anti-vWF and mouse anti-OPG primary antibodies as described in section 2.3.9. Bound anti-vWF antibodies were detected with an anti-rabbit Cy3 (red) conjugate and bound anti-OPG antibodies were detected with an anti-mouse AlexaFluor 488 (green) conjugate. Cell nuclei were counterstained with DAPI (blue). At this point in culture, vWF expression (red) was lost while high levels of extracellular OPG staining was observed (B). Note the OPG staining pattern (green) is similar to that detected on HaemDMSCs (Figure 4.18).. Scale bars: (A) 100 μm , (B) 50 μm .

Figure 4.25: Immunohistochemical localisation of VEGFR-1 and VEGFR-2 on HaemDEPCs. HaemDMSCs were fixed and stained with goat anti-VEGFR-1 and rabbit anti-VEGFR-2 antibodies as described in section 2.3.9. Bound VEGFR-1 antibodies were detected with an anti-goat biotin conjugate followed by a Streptavidin 555 conjugate (red) and bound VEGFR-2 antibodies were detected with an anti-rabbit DIG conjugate followed by an anti-DIG FITC conjugate (green). Cell nuclei were counterstained with DAPI (blue). HaemDEPCs expressed VEGFR-1 and VEGFR-2 on day 2 in culture (**A** & **B**, respectively). The cells continued to express VEGFR-1 on day 5 (**C**) but lost VEGFR-2 expression by day 5 in culture (**D**). Scale bars: 20 μ m.

Figure 4.26: Adipocytic differentiation of the MSCs transdifferentiated from HaemDEPCs. The MSCs-like cells shown in Figure 4.24-A differentiated into adipocytes when cultured in adipocytic induction media as described in section 2.3.10.2. Lipid-laden vacoules (circular bodies) appeared within two weeks of induction. Scale bar: 200 μm .

In addition, when a second dish containing HaemDEPCs was cultured in media containing 50 ng/ml of VEGF from day 2 after dissociation, some cells continued to exhibit the EPC morphology (Figure 4.27-A). These cells expressed both vWF (Figure 4.27-B) and OPG (Figure 4.27-C). However, not all cells cultured in VEGF were able to maintain their EC phenotype as some cells present in the culture dish were morphologically and immunohistochemically similar to HaemDMSCs (data not shown). These results indicate that the spontaneous differentiation of HaemDEPCs to HaemDMSCs could be delayed with addition of VEGF thereby indicating the importance of this growth factor in HaemDEPCs survival and proliferation, but VEGF alone is not sufficient to retain the endothelial properties of these cells and that other growth factors such as FGF-2 and IGF-2 may be required. Furthermore, these results were replicated from a proliferating haemangioma biopsy obtained from a second patient.

Figure 4.27: HaemDEPCs maintain their endothelial phenotype when cultured in VEGF. (A) Phase contrast microscopy showing HaemDEPCs maintained in 50 ng/ml of VEGF for ten days. The cells appeared morphologically similar to those from day 2 after dissociation (Figure 4.22). Immunohistochemical staining for vWF (B) and OPG (C) on HaemDEPCs maintained in VEGF for ten days. The cells were fixed and stained with rabbit anti-vWF and mouse anti-OPG primary antibodies as described in section 2.3.9. Bound vWF antibodies were detected with an anti-rabbit Cy3 conjugate (red) and bound OPG antibodies were detected using an anti-mouse AlexaFluor 488 conjugate (green). Cell nuclei were counterstained with DAPI (blue). Cells expressed vWF (B, red) and some OPG (C, green). Note OPG expression is very different from that seen on HaemDMSCs (Figure 4.18-D).. Scale bars: (A) 500 μ m, (B&C) 50 μ m.

CHAPTER 4.4: DISCUSSION

4.4.1 CHARACTERISATION OF HAEMANGIOMA EXPLANT OUTGROWTHS

The lack of an ideal model system for haemangioma has led to empirical and unsatisfactory therapies (Mulliken *et al.*, 1995; Tan *et al.*, 2000_b). However, an *in vitro* model that mimics, as closely as possible, the features of haemangioma was developed (Tan *et al.*, 2000_b) based on the models using the rat aortic rings (Nicosia & Ottinetti, 1990) and human placenta (Brown *et al.*, 1996). In this model, freshly resected haemangioma tissue placed into fibrin gels, develop outgrowths or ‘microvessels’ within days of culture. Although these outgrowths have been reported to be ECs (Tan *et al.*, 2000_b), other reports have shown that these outgrowing cells express EC-specific markers only in the area immediately adjacent to the explanted tissue (Ritter *et al.*, 2002). This suggests that the cells may be dedifferentiating and down-regulating these proteins as they acquire a migratory phenotype.

During this study, a method was developed for the purification and culture of these haemangioma explant outgrowths *in vitro*. This study characterised these cells to be MSCs rather than ECs. MSCs derived from proliferating and involuting haemangiomas exhibited fibroblast-like morphology in early passages and a flat broadened morphology in later passages. qRT-PCR showed that the cells expressed mRNA transcripts for the MSC markers, CD29, CD44 and CD90 (Pittenger *et al.*, 1999) but not CD133 which identifies haemopoietic precursor cells and EPCs (Hilbe W *et al.*, 2004). The cells did not express mRNA transcripts for CD31, CD45 and VEGFR-2 and were not immunohistochemically positive for vWF, VEGFR-2 or CD31, indicating that they were not contaminated with cells of haematopoietic or endothelial origin. These results and the finding that the cells expressed mRNA and protein for the haemangioma marker, GLUT-1, confirmed that the cells were haemangioma-derived MSCs.

Furthermore, consistent with previous reports, the HaemDMSCs expressed α -SMA and vimentin, which have been used to characterise MSCs (Sutherland *et al.*, 2005; Yu *et al.*, 2006).

As no MSC-specific markers currently exist, rigorous identification of MSCs requires demonstration of their capability to differentiate along specific mesenchymal lineages. Consistent with previous reports of the presence of MSCs in haemangioma (Yu *et al.*, 2006), the HaemDMSCs isolated in this study exhibited capacity for self-renewal and the ability to differentiate into multiple mesenchymal cell lineages, adipocytes and osteoblasts, when cultured in specific induction media. The increase in osteonectin and PPAR γ mRNA expression identified post-induction validated the differentiation of the HaemDMSCs into adipocytes and osteocytes, respectively. PPAR γ is a key regulatory gene of adipogenesis (Camp *et al.*, 2002) while osteonectin is considered a differentiation marker for bone cells, as its expression has been identified in osteoblasts, young osteocytes and also in osteoprogenitor cells (Jundt *et al.*, 1987).

Similar to bone marrow-derived MSCs (Okuyama *et al.*, 2006), the HaemDMSCs expressed mRNA and protein for VEGFR-1. However, unlike bone-marrow derived MSCs (Oswald *et al.*, 2004), HaemDMSCs did not exhibit EC-like characteristics when cultured in high concentrations of VEGF. In addition, Yu *et al.* (2001) showed that haemangioma-derived ECs did not express mRNA for VEGF. However, the HaemDMSCs isolated in this study expressed high levels of VEGF and IGF-2 mRNA, as identified by qRT-PCR. Both these factors are potent stimulators of angiogenesis known to be up-regulated during the proliferative phase of haemangioma *in vivo* (Takahashi *et al.*, 1994; Ritter *et al.*, 2002). These data suggest that the MSCs present in haemangioma may be an important source of angiogenic regulators that govern haemangioma progression. This study also found that HaemDMSCs expressed mRNA for Collagen -I, -II,-III and -VIII but did not express or secrete collagen protein when cultured *in vitro*. As these collagen fibres are present in all three phases of

haemangioma (Tan *et al.*, 2000_a), it may be postulated that in the *in vivo* environment, HaemDMSCs may be involved in synthesising some of the ECM proteins in haemangioma.

4.4.2 A ROLE FOR VEGF AND STAT SIGNALLING IN HAEMANGIOMA *IN VITRO*

As discussed in Chapter 3 of this thesis, VEGF and STAT protein signalling may be important in haemangioma proliferation. To obtain functional evidence for this, VEGF and STAT signalling was studied in both the haemangioma tissue explants and HaemDMSCs. To elucidate the importance of STAT protein signalling, a JAK/STAT inhibitor, AG490, was added to haemangioma explants and its effect on capillary-like outgrowth was studied. The precise role of VEGF detected in proliferative haemangioma (Takahashi *et al.*, 1994; Tan *et al.*, 2000_a) is unknown. In this study, addition of VEGF to haemangioma explants stimulated significant sprouting of outgrowths from haemangioma tissues. This effect was mitigated by addition of AG490 along with VEGF in the cultures. In addition, incubating the explants with various concentrations of AG490 resulted in decreased levels of sprouting. These data suggest a model of haemangioma progression in which an important element is the relationship between VEGF and STAT proteins.

The role of VEGF and STAT protein signalling was also studied in the purified HaemDMSCs cultures. With respect to the VEGFRs, HaemDMSCs expressed only VEGFR-1 but not VEGFR-2, while normal ECs (HUVECs) expressed both receptors. This study found that while addition of VEGF significantly induced the proliferation of HUVECs, it had no effect on HaemDMSCs proliferation. These results were not surprising as previous studies have shown that the activation of VEGFR-2 was required for EC proliferation and migration, while the activation of VEGFR-1 resulted in EC differentiation (Neufeld *et al.*, 1999).

The observation that proliferation was substantially inhibited by AG490 confirms the importance of STAT protein signalling in HaemDMSCs proliferation. Furthermore, blocking STAT signalling reduced vessel formation of HaemDMSCs, when cultured in a 3-dimensional fibrin matrix *in vitro*. Similar to the decrease in vessel formation, a dose dependent decrease in cell number was observed with AG490 treatment. These results suggest that the decrease in vessel formation may simply be a secondary effect resulting from decreased cellular proliferation in the presence of AG490.

Various studies have shown that AG490 inhibited phosphorylation and activation of STAT-1, STAT-3 and STAT-5 (Zhang *et al.*, 2000; Andl *et al.*, 2004; Maziere *et al.*, 2001; Hattori *et al.*, 2001; Yamaura *et al.*, 2003; El-Adawi *et al.*, 2002; Banes *et al.*, 2003), however, it is now known that AG490 can affect different members of the STAT family depending on the cell type. Andl *et al* (2004) showed that AG490 abolished the migration of EPCs by decreasing STAT-1 phosphorylation, while other investigators showed that it inhibited the proliferation of ovarian, breast, pancreatic and renal cell cancer cells by decreasing STAT-3 phosphorylation and STAT-3 DNA binding activity (Burke *et al.*, 2001; Horiguchi *et al.*, 2002; Huang *et al.*, 2006). Mascareno *et al* (2001) showed that AG490 decreased Ang-2 mRNA expression in ischaemic hearts by inhibiting activation of STAT-5 and STAT-6 and Seki *et al* (2000) showed that AG490 blocked Ang-2 induced STAT-3 phosphorylation and SMC proliferation. The results obtained with AG490 in this study reveal the importance of STAT protein signalling in HaemDMSCs and in haemangioma. However, further investigation is required to identify the member(s) of the STAT family affected by AG490 in HaemDMSCs.

Studies in other cell culture systems showed increased phosphorylation and nuclear translocation of STAT-3, STAT-5 and STAT-1 following VEGF treatment (Lu *et al.*, 2006; Bartoli *et al.*, 2003; Yahata *et al.*, 2003; Korpelainen *et al.*, 1999). This

study showed that HaemDMSCs expressed both STAT-3 and STAT-5 protein. Although a small fraction of STAT-3 was phosphorylated at the steady state, VEGF treatment failed to alter the levels and nuclear translocation of both p-STAT-3 (Tyr 705) and p-STAT-1 (Tyr 701). In contrast, increased expression of p-STAT-5 (Tyr 694) was detected within the nuclei of HaemDMSCs following VEGF stimulation.

It is now known that the outcome of STAT signalling is determined by its integration with other activated signal transduction pathways. For example, Korpelainen *et al* (1999) showed that although all three VEGFRs (VEGFR-1, VEGFR-2 and VEGFR-3) activated both STAT-3 and STAT-5, only VEGFR-1 signalling induced the expression of a cell cycle inhibitor protein, p21 in a STAT-5 dependent manner. They found that although VEGFR-2 and VEGFR-3 signalling suppressed p21 expression, their activity was overcome by increased STAT-5 activation.

This study found that VEGF did not induce HaemDMSCs proliferation or have an effect on p-STAT-3 (Tyr 705) and p-STAT-1 (Tyr 701) signalling. However, as VEGF induced the nuclear expression p-STAT-5 (Tyr 694) and since HaemDMSCs expressed VEGFR-1, it is interesting to speculate that VEGF signalling through p-STAT-5 is required for cellular processes other than proliferation such as cell migration or cell adhesion. Furthermore, since STAT-5 signalling is involved in cell differentiation (Dai *et al.*, 2007; Hoshino & Fujii, 2007) and adipogenesis (Nambu-Wakao *et al.*, 2002), a role for STAT-5 as a mediator of haemangioma involution was tested in this study. These results are discussed in section 4.4.5 (page 212).

4.4.3 ROLE OF OSTEOPROTEGERIN IN HAEMANGIOMA

This is the first study to report expression of osteoprotegerin (OPG) in haemangioma. OPG, a member of the TNF receptor superfamily (Simonet *et al.*, 1997) is a secreted glycoprotein that exists as a 60 kDa monomer and a 120 kDa disulfide-linked dimer. OPG is critical in the control of bone remodelling by inhibiting

osteoclastogenesis. It does this by binding the receptor activator of nuclear factor- κ B ligand (RANKL) and thereby preventing the interaction of receptor activator of nuclear factor- κ B (RANK) and RANKL (Lacey *et al.*, 1998; Yasuda *et al.*, 1998). Transgenic mice over-expressing OPG have increased bone mass (Simonet *et al.*, 1997) while OPG-deficient mice have decreased bone density (Bucay *et al.*, 1998; Mizuno *et al.*, 1998; Yun *et al.*, 2001). Osteoclasts are responsible for the resorption of bone during normal bone metabolism and for the destruction of bone seen in a variety of pathologies, such as rheumatoid arthritis (Haynes *et al.*, 2003)

In addition to bone, OPG mRNA expression is found in a number of tissues, including lung, heart, kidney, liver, stomach, intestine, brain, spinal cord and the thyroid gland (Simonet WS *et al.*, 1997; Yasuda *et al.*, 1998). This wide tissue distribution of OPG suggests its involvement in processes in addition to bone turnover. This notion is supported by the findings of Yun *et al* (2001) who showed that OPG regulated B cell maturation and development. Collin-Osdoby *et al* (2001) showed that OPG was involved in the inflammatory functions of ECs. Furthermore, Price *et al* (2001) showed that administration of OPG prevented vascular calcification induced by treatment with warfarin and supra-physiological doses of vitamin D in rats. Therefore, these studies provide evidence for the protective vascular effects of OPG. In addition, Hofbauer & Schoppet (2004) have reviewed the links between OPG and vascular disease.

Consistent with previous reports that showed OPG mRNA and protein expression by cultured MSCs and marrow stromal cells (Le Blanc *et al.*, 2004; Neville-Webbe *et al.*, 2004), the HaemDMSCs isolated in this study expressed high levels of OPG mRNA and secreted abundant levels of OPG protein which appeared to be associated with the ECM. This extracellular pattern of OPG expression has also been noted in human osteoblast-like cells (Atkins *et al.*, 2002). Because the HaemDMSCs expressed high levels of OPG, it was hypothesised that OPG may serve as a potential marker for MSCs within the haemangioma tissue *in vivo*. In addition, as various studies

have shown that proliferating haemangioma contain ECs that express vWF, double labelling immunohistochemical analysis for vWF and OPG was performed in this study with an aim to differentiate between ECs and MSCs within haemangioma lesions of various developmental stages. High OPG expression was identified in the cells surrounding the blood vessels. These cells also exhibited weak (punctate) staining for vWF. These results suggest that these cells are either immature ECs (the punctate staining pattern may represent the immature state of the ECs) or perhaps MSCs which have the capacity to differentiate into ECs. Alternatively, it may be possible that the MSCs express low levels of vWF when present within the tissue but lose vWF expression when cultured *in vitro*. Findings to support this hypothesis include those of Nagaya *et al* (2004) who showed that MSCs expressed vWF and were involved in neovascularisation in the infarcted heart.

In addition, the regions with high OPG expression and punctate vWF staining also contained more PCNA-positive cells. This indicated that these regions were rapidly proliferating. Importantly, as the lesions progressed towards the involuting phase, OPG and PCNA expression decreased concomitant with increased vWF staining. High PCNA expression in proliferating lesions with decreased abundance in involuting lesions has already been shown in Chapter 3. These results indicate that OPG protein expression correlates with proliferation.

Because of the high levels of extracellular OPG staining identified on HaemDMSCs and the high OPG expression detected in haemangioma tissue sections, we hypothesise that in haemangioma, OPG acts as a survival factor and affords protection against apoptosis *in vivo* and *in vitro*. Previous studies have implicated OPG as a mediator of cell survival. Malyankar *et al* (2000) showed that purified OPG protected ECs from apoptosis induced by growth factor deprivation. Cross *et al* (2005) showed that OPG stimulated EC growth, as well as the formation of cord-like structures on a matrigel substrate to a similar extent as VEGF. In addition, the increased

extracellular OPG secretion by HaemDMSCs could also be a stress response associated with the *in vitro* culture of these cells.

The cells isolated from dissociated tissue also expressed OPG at similar levels to HUVECs rather than the high extracellular levels described for HaemDMSCs. Together these data suggest that the dissociated tissue contains abundant EPCs rather than the HaemDMSCs. This discrepancy can be accounted for by the observation that the putative EPCs spontaneously differentiated in culture to form MSCs, and that the resulting haemangioma-derived MSCs were able to differentiate into adipocytes and osteoblasts as for the HaemDMSCs. It has been previously suggested that adipocytes and ECs have a common progenitor (Planat-Benard *et al.*, 2004). This data supports this postulate as the EPCs identified in haemangioma were able to form adipocytes. We suggest that the EPCs are stem cells that give rise to MSCs which are able to form adipocytes and osteoblasts. While these putative endothelial stem cells are pluripotent and are capable of forming self-renewing MSCs, the self-renewing potential of the endothelial stem/progenitor cells was not demonstrated in this study. The observation that VEGF treatment of HaemDEPCs was able to inhibit the spontaneous differentiation to MSCs suggests a role for VEGF in EC maturation in haemangioma.

4.4.4 THE ROLE OF MSCs AND EPCs in HAEMANGIOMA

To place the results of this study into context, an introduction to MSCs and EPCs is described in sections 4.4.4.1 and 4.4.4.2. In addition, several models for haemangioma progression were hypothesised from the findings of this study together with the previous identification of EPCs and MSCs in haemangioma (Yu *et al.*, 2004_a; Yu *et al.*, 2006). These models are discussed in section 4.4.4.3.

4.4.4.1 MESENCHYMAL STEM CELLS

Stem cells are an attractive source of cells for tissue engineering because of their unique biological properties. Stem cells have been isolated from a variety of somatic

tissues (Korbling, *et al.*, 2003). MSCs represent a subset of these cells residing in the bone marrow. Other sources of MSCs included peripheral blood (Pittenger *et al.*, 1999; Zvaifler *et al.*, 2000), umbilical cord blood (Kim *et al.*, 2004) and also connective tissue sites including the dermis, muscle, adipose, trabecular bone, and synovial membranes (Tuan *et al.*, 2003). MSCs are also present in term cord blood (Zvaifler *et al.*, 2000), in foetal organs (such as liver, BM, and kidney), the blood of preterm foetuses (Erices *et al.*, 2000; Campagnoli *et al.*, 2001; Almeida-Porada *et al.*, 2002) and the placenta (Fukuchi *et al.*, 2004).

MSCs can be expanded in the laboratory and can be induced to differentiate along multiple lineages giving rise to cartilage, bone, fat, muscle, ECs and vascular tissue *in vitro* and *in vivo* (Jiang *et al.*, 2002; Abedin *et al.*, 2004; Phinney, 2002; Devine *et al.*, 2003), displaying the important properties of self-renewal and plasticity of stem cells.

One of the most interesting characteristics of MSCs is their ability to home to sites of tissue damage or inflammation (Devine *et al.*, 2001). Homing of MSCs have been demonstrated in the settings of bone fracture, cerebral ischemia and the infarcted heart (Mosca *et al.*, 2000; Devine *et al.*, 2001; Devine *et al.*, 2002; Price *et al.*, 2006). Saito *et al* (2002) demonstrated that when MSCs were administered intravenously, these cells engrafted within regions of myocardial infarction and a distinct subpopulation participated in angiogenesis. Pittenger & Martin (2004) showed that the “window” of MSC homing was limited i.e., if MSC administration was delayed until two weeks after infarction, no significant cardiac engraftment was observed and that most of the cells had returned to the bone marrow. In addition, Rombouts & Ploemacher (2003) showed that the *in vitro* expansion of MSCs diminished the efficacy of injury-induced homing.

The possible involvement of MSCs in neovascularisation has been recently proposed due to their capacity to contribute to tumour angiogenesis *in vivo* (Reyes *et al.*, 2002) and due to their plasticity to differentiate into endothelial-like cells (Reyes *et al.*,

2001). Tang *et al* (2003) showed that neovascularisation induced by MSCs involved secretion of paracrine factors by the MSCs themselves. This resulted in vessel sprouting and incorporation of MSCs into newly forming blood vessels. In addition, Silva *et al* (2004) found increased vascularity and improved cardiac function with the implantation of MSCs into chronically ischaemic myocardium. These authors showed that implanted MSCs homed to the site of injury and differentiated into both, ECs and SMCs, which formed the vessel walls.

MSCs have also been shown to differentiate into ECs when cultured in the presence of VEGF (Oswald *et al.*, 2004). Wu *et al* (2005) showed that when MSCs were co-cultured with mature ECs, about 25% of the MSCs began to express KDR but not vWF. Their morphological appearance was also different from that of the adjacent mature ECs. Previous studies have shown that ECs go through different stages of differentiation. They initially express VEGFR-2, one of the earliest differentiation markers for ECs, which plays an important role in ECs growth and vasculogenesis, followed in sequence by TIE-2, TIE-1, VE-cadherin, CD34 and vWF (Vittet *et al.*, 1996; Yamashita *et al.*, 2000). Wu *et al* (2005) suggested that MSCs possess the milieu-dependent differentiation potential along endothelial lineage when co-cultured with mature ECs. These authors also found that the VEGFR-2-positive MSCs cells did not co-express CD105 or CD166. These results indicate that the cells lose the phenotypic characteristics of MSCs as they differentiate toward ECs. In addition, Wu *et al* (2005) showed that the addition of anti-VEGF antibodies inhibited the differentiation of MSCs, indicating the important role of VEGF in EC differentiation.

4.4.4.2 ENDOTHELIAL PROGENITOR CELLS

EPCs were first discovered by Ashara *et al* (1997) as a population of cells that co-expressed EC specific markers such as vWF (Shi *et al.*, 1998) and VEGFR-2 (Peichev *et al.*, 2000) along with the stem cell marker, CD133 (Fargeas *et al.*, 2003).

Since then, EPCs have been found in the bone marrow, blood circulation, foetal liver and skeletal muscle (Peichev *et al.*, 2000; Majka *et al.*, 2003).

EPCs are involved in tissue neovascularisation, post-natal organ generation, arteriogenesis and also tumour angiogenesis (Rafii *et al.*, 2002; Rafii & Lyden, 2003; Fuchs *et al.*, 2001; Asahara *et al.*, 1997; Al-Khalidi *et al.*, 2003; Kocher *et al.*, 2001; Orlic *et al.*, 2001; Jackson *et al.*, 2001; Kinnaird *et al.*, 2004; Carmeliet & Luttun, 2001). Incorporation of EPCs and MSCs into sites of physiological and pathological neovascularisation *in vivo* has also been reported (Asahara *et al.*, 1997; Asahara *et al.*, 1999; Shi Q *et al.*, 1998). Studies have also shown that EPCs are highly proliferative. These cells form new blood vessels *de novo* as well as enhance neovascularisation when administered systemically (Rafii & Lyden, 2003; Kalka *et al.* 2000).

Tepper *et al.* (2005) showed that hypoxia initiated a cellular cascade with localised migration and proliferation of bone marrow-derived EPCs *in vitro* and *in vivo*, which resulted in angiogenesis. In animal models of myocardial infarction, the injection of *ex vivo* expanded EPCs significantly improved blood flow and cardiac function (Kawamoto *et al.*, 2001; Kocher *et al.*, 2001). Furthermore, Lyden *et al.* (2001) showed that impaired recruitment of bone marrow-EPCs blocked tumour angiogenesis and growth. Various studies also showed that terminally differentiated mature ECs did not improve neovascularisation (Kalka *et al.*, 2000; Kocher *et al.*, 2001; Hur *et al.*, 2004).

4.4.4.3 PROPOSED MODELS FOR HAEMANGIOMA

4.4.4.3.1 The Original Model

One of the main findings from this study is that MSCs rapidly grow out from explanted haemangioma biopsies. This suggests that MSCs are present at high numbers in haemangioma. The initial clinical description of a promontory mark of haemangioma as ‘an anemic nevi’ or ‘area of low blood flow’ suggests that tissue hypoxia, a powerful stimulus for neovascularisation, may be involved (Al Buainian *et al.*, 2003; Hamlat *et*

al., 2005). A recent study has also reported that proliferating haemangiomas contain large numbers of haematopoietic cells of the myeloid lineage which are known to migrate to zones of hypoxia (Ritter *et al.*, 2006). Additionally, Kleinman *et al* (2007) demonstrated that hypoxia-induced factors which are important for post natal vasculogenesis are upregulated in children with proliferating haemangiomas. These include hypoxia inducible factor-1 α (HIF-1 α), stromal cell-derived factor-1 α (SDF-1 α), matrix metalloproteinase-9 (MMP-9) and VEGF-A. Kleinman *et al* (2007) also suggested that even though overexpression of these factors can occur in normoxic tissues in certain types of cancers (Zhong *et al.*, 1999), the lack of increased HIF-1 α expression in involuting haemangioma biopsies indicates that physiological mechanisms are active in haemangioma and that these lesions are truly hypoxic. There is also data to suggest that hypoxia may be the initial stimulus for chorangioma, a tumour of the placenta that is histologically similar to haemangioma (Ogino *et al.*, 2003).

The original model proposes the recruitment of MSCs to the site of the haemangioma as a result of hypoxia. The recruited MSCs then express high levels of VEGF and other growth factors which act in an autocrine manner to induce MSC differentiation into ECs. This is supported by the findings of Annabi *et al* (2003) who showed that in a hypoxic environment MSCs secreted increased levels of VEGF that in turn induced MSC migration in an autocrine manner. They also showed increased expression of matrix metalloproteinase-1 (MMP-1) during hypoxia and suggested that this protein was involved in MSCs-mediated tube formation. However, the HaemDMSCs isolated in this study failed to differentiate and exhibit EC characteristics when cultured in the presence of 50 ng/ml VEGF along with low serum (2%) and normal serum (10%) concentrations. These culture conditions have been previously described for MSC differentiation into ECs (Oswald *et al.*, 2004; Wu *et al.*, 2005). These results suggest that HaemDMSCs either lose their ability to differentiate into ECs when in culture *in vitro* or that a second progenitor cell type is responsible for the origin

of ECs in haemangioma. Based on these findings, a second model involving EPCs was hypothesised for haemangioma progression.

4.4.4.3.2 An Alternative Model

The ECs in early proliferating haemangioma are described as ‘angioblastic’ and are similar to embryonic rather than neonatal vascular ECs based on their morphology and protein expression patterns (Malan, 1974; Pack & Miller, 1950; Dosanjh *et al.*, 2000). These findings suggest that haemangioma contain primitive ECs. Increased levels of circulating EPCs have been noted in haemangioma patients (Kleinman *et al.*, 2003). Furthermore, Yu *et al* (2004_a) isolated EPCs from proliferating haemangioma by purifying a population of cells that expressed the EC marker, VEGFR-2 and the stem cell marker, CD133. They speculated that these EPCs were responsible for the uncontrolled clonal expansion of ECs in haemangioma.

The cellular pathways of EPC mobilisation and trafficking are becoming clear. HIF-1 α , SDF-1 α and VEGF-A are now known to be capable of enhancing the mobilisation and recruitment of EPCs to hypoxic tissues during post natal vasculogenesis (Ceradini *et al.*, 2004; Rafii *et al.*, 2003; Kalka *et al.*, 2000; Park *et al.*, 2004; Forsythe *et al.*, 1996). Increased expression of these mediators has been recently identified in proliferating haemangiomas (Kleinman *et al.*, 2007).

In the alternative model for haemangioma (Figure 4.28), both MSCs and EPCs are recruited to the site of the haemangioma as a result of hypoxia during the proliferative phase. Both cell types express high levels of OPG that prevents them from undergoing apoptosis during this phase. The MSCs secrete growth factors such as VEGF which first induce EPC proliferation followed by EPC differentiation into mature ECs as the lesion progresses towards involution. The presence of EPCs together with the possibility that MSCs can then themselves differentiate into vascular smooth muscle cells now creates a perfect environment to form functional capillary networks *in vivo*. This phenomenon has been recently shown by Melero-Martin *et al* (2007). These

authors found that implantation of EPCs with SMCs subcutaneously into immunodeficient mice resulted in the formation of an extensive blood vessel network within one week. Wu *et al* (2004) showed that EPC-derived ECs had the ability to assemble into a microvascular-like network when seeded on three-dimensional glycolic scaffolds along with SMCs.

The alternative model is based on results from other studies that have reported the differentiation of EPCs into mature ECs (Gehling *et al.*, 2000; Peichev *et al.*, 2000). In addition, studies have also reported the secretion of angiogenic factors such as VEGF by MSCs (Hamano *et al.*, 2000; Villars *et al.*, 2000). The VEGF family of polypeptides includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PlGF (Korpelainen EI & Alitalo K, 1998; Veikkola *et al.*, 2000; Veikkola & Alitalo, 1999). The VEGF-A gene can be alternatively spliced resulting in gene products corresponding to amino acids 121, 165, 189 and 206 main splice forms in humans. VEGF-A is expressed during MSC-derived osteoblastogenesis (Furumatsu *et al.*, 2003; Mayer *et al.*, 2005). Mayer *et al* (2005) showed that mRNA for VEGF₁₆₅, VEGF₁₂₁ and VEGFR-1 increased during osteogenesis and also under hypoxic conditions. Furthermore, this secreted VEGF induced increased EC sprouting. Therefore, these authors suggested that human MSCs secreted VEGF-A as an angiogenic factor in a differentiation dependent manner. In addition, VEGF has been shown to mobilise EPCs from bone marrow (Asahara *et al.*, 1997) and also cause EPC maturation to ECs (Ehrbar *et al.*, 2005).

qRT-PCR results from this study showed that HaemDMSCs expressed VEGF and IGF-2 mRNA transcripts to similar levels as that seen in the original haemangioma tissue. Both these factors have been identified as modulators of haemangioma in the proliferative phase *in vivo* (Takahashi *et al.*, 1994; Ritter *et al.*, 2000). Since Yu *et al* (2001) found that the isolated ECs from proliferating haemangiomas expressed very low levels of VEGF transcript, we hypothesised that the HaemDMSCs may be the source for at least some of the angiogenic modulators of haemangioma. However, Western

blotting experiments showed that the secreted isoforms of VEGF-A, VEGF₁₂₁ and VEGF₁₆₅ were not present in the conditioned medium of the cultured HaemDMSCs. In addition, Western blotting results showed that neither VEGF₁₂₁, VEGF₁₆₅ nor VEGF₁₈₉ were detected within HaemDMSCs themselves or in proliferative haemangioma tissue.

Other studies that reported on the high expression of VEGF protein during the proliferative phase (Tan *et al.*, 2000_a; Takahashi *et al.*, 1994) did not provide details on the member(s) of the VEGF family detected in their studies. Although, all members show some degree of sequence homology, the anti-VEGF antibody used in this study was specific for the VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉ isoforms of VEGF-A. Therefore, these findings suggest that VEGF expression seen in haemangioma may be that of the other members of the VEGF family and not the VEGF isoforms of VEGF-A which were tested for, in this study.

Yu *et al* (2006) first speculated that MSCs were the source of adipocytes that appeared during involution of haemangioma. Consistent with this theory, this investigation found that MSCs rapidly grew out from explanted haemangioma tissue pieces and that these HaemDMSCs maintained their adipocytic and osteocytic differentiation properties *in vitro* (The Alternative Model; Figure 4.28). These findings further support the model in which MSCs are likely to be precursors of the adipocytes during involution of haemangioma *in vivo*.

Figure 4.28: An Alternative Model For Haemangioma. This model suggests that there are two main cell types, the MSCs and EPCs which are involved in the pathogenesis of haemangioma. Both cell types may be recruited to the site of the haemangioma perhaps, in response to hypoxia. Both cell types express increased levels of OPG and exhibit punctate immunoreactivity for vWF indicating their immature state. High OPG expression prevents apoptosis during the proliferative phase. This model suggests that the MSCs are the main source for a variety of angiogenic modulators such as VEGF and IGF-2. These growth factors secreted by MSCs can in turn act on the recruited EPCs to enhance their survival, proliferation and also induce EPC maturation to ECs. This model also proposes that a yet-to-be identified signal is involved in inducing MSCs differentiation into the adipocytes which is seen during involution and that when a proliferating haemangioma explant is cultured *in vitro*, it is the MSCs that rapidly grow out from the tissue piece. These HaemDMSCs maintain their ability to differentiate into adipocytes and osteocytes *in vitro*. The role of IFNs and STAT proteins in haemangioma is discussed further in section 4.4.5.

Haemangioma

4.4.4.3.3 The Final Model

Towards the end of this study, tissue from a proliferating haemangioma was treated with collagenase to obtain a single cell suspension and the cells were cultured *in vitro*. These cells had a distinct appearance in that they had round central bodies with some cytoplasmic projections. This morphology has been shown to be typical of EPCs (Asahara *et al.*, 1997; Quirici *et al.*, 2001). After two days in culture, cells obtained directly from dissociated proliferative tissue biopsies were also immunophenotypically similar to EPCs as they stained for vWF, VEGFR-1 and VEGFR-2 which is consistent with previous findings (Ziegler *et al.*, 1999). VEGFR-2 is one of the earliest markers expressed on EPCs. In addition, although these cells expressed OPG, the pattern of OPG expression was not extracellular as seen in MSCs, but was similar to the OPG-vWF staining pattern seen on normal ECs or HUVECs.

Following continued culture, an interesting change in cell morphology to that of the flat, broadened shape of MSCs was noted within five days. The cells now lost vWF expression and exhibited the MSC-like extracellular pattern of OPG expression. Furthermore, these cells differentiated into adipocytes under appropriate conditions which confirmed their plasticity and mesenchymal characteristics. However, when the same cells were cultured in VEGF for eight days, some cells retained their EPC-like morphology and continued to express both vWF and OPG, while some others showed MSC morphology, did not express vWF and expressed high levels of extracellular OPG.

From these results, we propose a final model for haemangioma in which the HaemDEPCs represent the primary and most important component in a proliferating haemangioma (Figure 4.29). In this model, the high levels of VEGF present within the lesions during the proliferative phase are involved in EPCs survival, proliferation and/or maturation of ECs. Results from this study also suggest that VEGF alone may not be enough to maintain the endothelial phenotype of the HaemDEPCs and that other growth factors such FGF-2 or IGF-2 may be required. High levels of both these growth factors

have been identified during the proliferative phase and have been found to decrease with involution (Tan *et al.*, 2000_a; Ritter *et al.*, 2001). Khan *et al* (2006) also isolated HaemDEPCs from proliferative haemangioma biopsies and maintained them in culture medium containing various factors including VEGF, FGF-2, IGF-2, hydrocortisone, heparin, ascorbic acid, and cAMP. These authors did not provide any information on the consequence of removing these factors.

In this study, all the HaemDEPCs cultured *in vitro* spontaneously differentiated into MSCs in the absence of VEGF. We established this phenomenon as a transdifferentiation process as opposed to a situation in which one cell type outgrows another within the same culture dish. This was based on the following reasons. In the absence of VEGF, a change in cell morphology was observed to be uniform across the entire dish with every single cell exhibiting a flat, broadened shape within five days in culture. If two cell types were present and one cell type was outgrowing the other, the presence of both cell types would have been seen sometime during the two-five day culture period but this was not the case. In contrast, both cell types (EPCs and MSCs) were observed in the second situation examined in this study i.e., culturing HaemDEPCs in the presence of VEGF. This suggests that VEGF along with other factors may be required for HaemDEPCs to maintain their endothelial phenotype.

In the model shown in Figure 4.29, we hypothesise that involution of haemangioma is triggered by a decrease in the levels of VEGF (and other growth factors). The consequence of this is that the EPCs no longer survive and proliferate as ECs but transdifferentiate into MSCs that further differentiates into the adipocytes seen in the involuting and involuted phases of haemangioma. Furthermore, when haemangioma biopsies are cultured *in vitro*, we believe that it is the EPCs that migrate out but as they migrate further away from the tissue they are no longer exposed to the growth factors present with the original lesion (i.e., VEGF and others). This results in their transdifferentiation from EPCs to MSCs. It was these MSCs that were isolated

from the *in vitro* haemangioma model and characterised in this study (see section 4.4.1, page 189). These migrating MSCs retain their ability to form both adipocytes and osteocytes *in vitro*. This is consistent with the findings of Ritter *et al* (2001) who identified that the sprouting outgrowths contained ECs only in the area immediately adjacent to the explant tissue whereas cells further away from the tissue no longer expressed these markers. The ECs in their study were characterized by their expression for CD31 and CD34 as well as for the positive staining for *G. simplicifolia* isolectin, a documented endothelial stain (Faraggiana *et al.*, 1989; Sahagun *et al.*, 1989; Hansen-Smith *et al.*, 2001).

This kind of endothelial to mesenchymal transdifferentiation (EMT) occurs in response to TGF- β signalling in the developing heart (Ramsdell & Markwald, 1997; Boyer *et al.*, 1999). Frid *et al* (2002) found that a small percentage of cells in EC preparations from bovine arteries can undergo EMT. Dvorin *et al* (2003) showed that in addition to ECs, EPCs were also capable of transdifferentiating into a mesenchymal phenotype, in response to TGF- β . Camenisch *et al* (2002) showed that once EMT took place, endothelial functions such as the ability to form tube-like structures and to adhere leukocytes in response to inflammatory signals, were lost.

Finally, Paruchi *et al* (2006) have shown that clonal ECs isolated from the pulmonary valves of a nine month-old infant exhibited EMT in response to TGF- β . However, they stated that the resulting MeCs failed to differentiate into adipocytes or osteocytes. In contrast, the HaemDMSCs isolated in this study, could undergo adipocytic differentiation under appropriate conditions. This suggests that these transdifferentiated cells are MSCs and exhibit MSC-like properties.

From these results we hypothesise that the cells obtained by the direct dissociation of a proliferating haemangioma biopsy are EPCs as they could differentiate from an endothelial phenotype expressing all the ECs markers into MSCs. Furthermore, the pattern of EC marker expression seen on the HaemDEPCs was

consistent with that detected *in vivo* by immunohistochemical analysis of haemangioma tissue sections.

The results from this investigation also provide evidence for the role of VEGF in maintaining the endothelial characteristics of HaemDEPCs. Results showed that with the withdrawal of VEGF, the HaemDEPCs differentiated into HaemDMSCs that further differentiated into the adipocytes seen in involuting and involuted haemangioma.

Chapter 3 (section 3.3.2.2.3, page 114) described the expression of VEGFR-1 in all phases of haemangioma whereas VEGFR-2 expression decreased with involution. VEGFR-1 expression detected on both EPCs and MSCs isolated in this study indicates that its constant expression may be required for the differentiation processes occurring throughout the different developmental phases of haemangioma progression, i.e. EPCs to mature ECs and EPCs to MSCs to adipocytes. In contrast, VEGFR-2 expression detected only on EPCs suggests that activation of this receptor, in response to the high levels of VEGF, could be responsible for EPCs survival and proliferation during the proliferative phase (The Final Model, Figure 4.29).

Finally, although CD133 expression by HaemDEPCs was not directly investigated in this study, the ability of these cells to differentiate from one cell type to another meets the criteria of a stem cell (Lovell & Mathur, 2004). A point to note here is that CD133 expression is now known to be quickly down-regulated in culture (Wu *et al.*, 2004). To our knowledge there have not been any previous reports of an EPC to MSC transdifferentiation process occurring in haemangioma. Therefore, these findings represent novel insights into the pathogenesis of haemangioma. In the final model, we hypothesise that the EPC to MSC differentiation process governs haemangioma biology (Figure 4.29).

Figure 4.29: The Final Model For Haemangioma. In this model, the EPCs represent the primary component of a proliferating haemangioma. These cells express OPG which acts as a survival factor. They also show punctuate or weak immunoreactivity when immunostained for vWF. This punctuate staining may represent their immature developmental state. During the proliferative phase, high levels of growth factors, such as VEGF present in the lesion act on the EPCs to enhance their survival and induce their proliferation. VEGF may also play a role in EPC maturation into ECs. These mature ECs exhibit intense immunoreactivity when immunostained for vWF, seen as the lesion progresses towards involution. The decreasing concentrations of VEGF and other growth factors in the involuting phase results in the differentiation of the EPCs to MSCs. These MSCs can then further differentiate into the increasing numbers of adipocytes seen in the involuting and involuted phases of haemangioma. In addition, this model suggests that when proliferative haemangioma explants are cultured *in vitro*, the EPCs grow out but as they migrate further away from the original tissue and are thereby exposed to low VEGF concentrations, the cells transdifferentiate into MSCs. These HaemDMSCs maintain their ability to differentiate into adipocytes and osteocytes *in vitro*. Finally, although the findings from this investigation did support the notion that IFN α -2b can accelerate adipogenesis, it would be interesting to further investigate the role of STAT proteins in haemangioma adipogenesis.

Haemangioma

4.4.5 ROLE OF INTERFERONS AND STAT PROTEINS IN HAEMANGIOMA ADIPOGENESIS

Results from this study also provide evidence for the involvement of STAT proteins and IFN signalling in HaemDMSCs (see section 4.3.6, page 179). The identification of increased expression of IFN stimulated genes together with the increased expression of IFN β in the dermis overlying involuting and involuted haemangioma (Ritter *et al.*, 2000; Bielenberg *et al.*, 1999) suggests that IFN signalling may be important in driving haemangioma involution. As IFN α -2b has been used as treatment for haemangioma, we hypothesise a model for haemangioma in which expression of IFNs in the overlying dermis of involuting and involuted haemangioma activates the STAT proteins which then causes and/or accelerates MSC adipogenesis (Figures 4.28 and 4.29). Several members of the STAT family play a role in the regulation of genes that contribute to the phenotype of the mature adipocyte (Stephens *et al.*, 1996; Stephens *et al.*, 1999; Floyd and Stephens, 2003).

In order to provide functional evidence for the role of IFNs and STAT proteins in adipogenesis, adipocytic differentiation of HaemDMSCs was induced in presence of IFN α -2b (15,000 U/ml) and AG490 (20 μ M). As both drugs inhibited HaemDMSCs proliferation (see section 4.3.4.2, page 165, for AG490 and Chapter 5, section 5.3.4, page 224, for IFN α -2b), the doses selected for the differentiation experiments were chosen with the rationale being that the drugs should have a biological effect on their target proteins without significantly affecting cell proliferation. Failure to detect accelerated adipogenesis with IFN α -2b may be due to several possibilities, (i) the physiological concentrations of IFNs in haemangioma is currently unknown therefore the arbitrarily chosen IFN α -2b concentration used in the differentiation experiments of this study, may not have been high enough to induce adipogenesis above that of the control, (ii) this study did not examine the effects of other IFNs such as IFN β and IFN γ which also activate STAT proteins (Darnell, 1997; Durbin *et al.*, 1996; Meraz *et al.*,

1996). Given that IFN β expression has been detected in the dermis overlying involuting haemangioma (Bielenberg *et al.*, 1999), it may be useful to test its effect on HaemDMSCs adipogenesis in future experiments. In addition, as stated in section 4.4.2. (page 191), AG490 affects different STAT proteins depending on the cell type. The identification of adipocytes in the presence of AG490 suggests that the particular STAT protein members that are involved in HaemDMSC adipogenesis are not inhibited by AG490.

CHAPTER 5: *TRAIL* - A POTENTIAL TREATMENT FOR HAEMANGIOMA?

5.1 INTRODUCTION

Although most haemangiomas require no intervention (Margileth *et al.*, 1965), treatment is necessary in 10 to 20% of cases during the proliferative phase because of their location, size, or behaviour (Takahashi *et al.*, 1994; Mulliken *et al.*, 1995). The mainstay treatment for haemangioma during the proliferative phase is pharmacologic therapy with glucocorticoids being the first line of treatment (Mulliken *et al.*, 1995). However, not all lesions respond to steroids. IFN α is considered the second-line of treatment and is indicated for lesions that fail to respond to steroid therapy (Mulliken *et al.*, 1995; White *et al.*, 1991; Spiller *et al.*, 1992; Blei *et al.*, 1993; Ohlms *et al.*, 1994), with accelerated regression seen in 90% of cases (Mulliken *et al.*, 1995). However, IFN treatment is accompanied by serious side-effects (Vial & Descotes, 1994; Barlow *et al.*, 1998; Dubois *et al.*, 1999). Most children given IFN α develop fevers for the first one-two weeks of therapy (Tan, 2001). Reversible toxicoses such as transient neutropenia, anaemia and a five-fold increase in liver transaminases is also seen (Tan, 2001). Significant neurotoxicity also occurs with spastic diplegia in 10% of cases (Barlow *et al.*, 1998). The neurological sequelae are arrested by cessation of treatment. In some cases they resolve completely but in others varying degrees of residual deficit persists (Tan, 2001).

The actual mechanism of action of IFNs in the regression of haemangioma is unknown. IFNs are a family of cytokines which are classified into two groups, type I (IFN α and IFN β) and type II (IFN γ). In addition to their anti-viral effects, IFNs exhibit pleiotropic biological activities, including anti-tumor, anti-proliferative and

immunomodulatory effects (Morris and Zvetkova 1997; Gresser *et al* 1997; Stark *et al.*, 1998) and transcriptionally regulate over 100 genes (Stark *et al.*, 1998).

IFNs act as apoptosis-inducing cytokines on various cancer cell lines (Chawla-Sarkar *et al.*, 2003), including multiple myeloma, (Chen *et al.*, 2001), melanoma (Chawla-Sarkar *et al.*, 2001), and ovarian carcinoma (Morrison *et al.*, 2002). Greater cell growth-inhibitory effects of IFN β compared with IFN α has been shown consistently (Borden *et al.*, 1982; Rosenblum *et al.*, 1990; Johns *et al.*, 1992). IFNs have also been shown to up-regulate the expression of different apoptosis-related proteins including death receptors and their respective ligands, caspases and several members of the Bcl-2 family in a variety of cells (Ossina *et al.*, 1997; Spanaus *et al.*, 1998). Transcriptional induction of TRAIL has been found to be one of the earliest events following IFN administration (Chen *et al.*, 2001; Sato *et al.*, 2001). IFN β pre-treatment has been found to sensitise human melanoma cells to TRAIL-induced apoptosis (Chawla-Sarkar *et al.*, 2002; Leaman *et al.*, 2002), which were otherwise resistant to TRAIL treatment alone.

TRAIL is a member of the TNF superfamily, that induces rapid apoptosis in a wide variety of transformed cell lines, but appears to have little or no detectable toxicity on normal cells *in vitro* and *in vivo* (Walczak *et al.*, 2000; Pitti *et al.*, 1996; Walczak *et al.*, 1999). TRAIL binds to four distinct membrane-bound receptors, DR4 (TRAIL-R1), DR5 (TRAIL-R2), DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and a soluble receptor, OPG. These receptors are expressed by tumour, transformed and normal cells (Griffith *et al.*, 1999; Walczak *et al.*, 1997; Green, 2000; Spierings *et al.*, 2004). TRAIL induces apoptosis through the activation of DR4 and DR5, which engage the ‘cell-extrinsic’ apoptosis pathway (LeBlanc & Ashkenazi, 2003; Kelly and Ashkenazi, 2004) (Figure 5.1). TRAIL binding, leads to the formation of the death inducing signalling complex (DISC) consisting of the apoptosis ‘initiating’ proteases, caspase-8 and caspase-10, and an adaptor molecule, the Fas-associated death domain (FADD). Once recruited, the activated caspase-8 and -10 in turn activate ‘effector’ caspases, including caspase-3, -6

and -7, which then execute the cell's apoptotic demise (Sprick *et al.*, 2000; Kischkel *et al.*, 2000). In some cancer cell lines, TRAIL-induced caspase-3 activation is further augmented through engagement of the cell-intrinsic apoptosis pathway (LeBlanc *et al.*, 2002; Deng *et al.*, 2002; Ravi and Bedi, 2002) (Figure 5.1). In this case, caspase-8 or caspase-10 cleaves and activates the pro-apoptotic Bcl-2 family member Bid, which then interacts with two other pro-apoptotic members, Bax and Bak, to induce the release of cytochrome *c* and Smac/Diablo from the mitochondria. Cytochrome *c* together with Apaf-1 activates caspase-9, which contributes to further activation of caspase-3, -6 and -7. Smac/Diablo binds to inhibitor of apoptosis proteins (IAPs), such as X-chromosome-linked IAP (XIAP). This prevents the IAPs from physically binding caspase-3, hence promoting further caspase-3 activation. In certain cancer cells, Bax is essential to connect the extrinsic and intrinsic pathways. Loss of function mutations have been shown to render cells resistant to TRAIL. However, the resistance associated with Bax inactivation can be circumvented by pre-exposing the cells to chemotherapy, which up-regulates levels of DR5 and Bak (Kelley & Ashkenazi, 2004)

Binding of TRAIL to DcR1, DcR2 and OPG does not transduce the death signal as they do not contain a functional cytoplasmic death domain and are therefore considered as competitive decoys receptors for TRAIL (Griffith *et al.*, 1998; Ashkenazi *et al.*, 1999; Degli-Esposti, 1999; Ashkenazi & Dixit, 1998). Interestingly, DcR1 and DcR2, as anti-apoptotic genes, are expressed in many normal tissues, but their expression are frequently lost in various types of human cancers (Ashkenazi & Dixit, 1998; Kelley Ashkenazi, 2004).

In addition to inducing apoptosis in cancer cell lines (Mitsiades *et al.*, 2001; Naka *et al.*, 2002), recombinant soluble TRAIL shows remarkable efficacy against tumour xenografts of colon carcinoma (LeBlanc *et al.*, 2002; Ashkenazi *et al.*, 1999), breast carcinoma (Walczak *et al.*, 1999), multiple myeloma cells (Mitsiades *et al.*, 2001) and glioma cells (Fulda *et al.*, 2002; Pollack *et al.*, 2001).

Furthermore, the potential for TRAIL to act synergistically with other anti-cancer therapies is of particular interest. TRAIL has been shown to act synergistically when combined with IFN α (Shigeno *et al.*, 2003), genotoxic agents including doxorubicin, cisplatin or etoposide (Arizono *et al.*, 2003; Bouralexis *et al.*, 2003; Miao *et al.*, 2003; Hotta *et al.*, 2003; Jones *et al.*, 2003; Xu *et al.*, 2003), DNA-damaging drugs (Morrison *et al.*, 2002; Ashkenazi *et al.*, 1999), radiotherapy (Gong & Almasan 2000; Chinnaiyan *et al.*, 2000; Ramp *et al.*, 2003), and cyclooxygenase-2 inhibitors (Totzke *et al.*, 2003).

IFN α was found to sensitise cells to TRAIL-induced apoptosis through an up-regulation of DR4 and DR5 (Oshima *et al.*, 2001; Shigeno *et al.*, 2003). In addition, Kumar-Sinha *et al* (2002) showed that a synergistic apoptotic response was achieved in breast cancer cells with the combined treatment of IFN α and TRAIL. In certain tumour cell lines, IFN induced TRAIL expression by the tumour cells themselves. This resulted in TRAIL-mediated apoptosis by an autocrine or paracrine mechanism (Chen *et al.*, 2001; Oshima *et al.*, 2001). Therefore, from these studies, Kumar-Sinha *et al* (2002) have suggested that IFNs may 'prime' cells for TRAIL induced apoptosis.

Figure 5.1: Activation of the cell-extrinsic and cell-intrinsic apoptosis pathways by TRAIL (*Image re-drawn from Ashkenazi & Kelley, 2004*). TRAIL binding to the death receptors (DR4 and DR5) activates the cell-extrinsic pathway and is sufficient to trigger apoptosis in some cell types, whereas in other cell types amplification of this pathway through the intrinsic pathway is needed to commit the cell to apoptosis. Crosstalk between these two pathways involves cleavage of Bid and subsequent activation of Bax and Bak. See text for details.

5.2 AIMS

As described in section 5.1, various reports of the anti-tumour effects of IFN α have been documented. In addition, IFN α has been shown to be effective as an angiogenic inhibitor in a variety of angiogenic diseases (Gutterman, 1994). Furthermore, the high expression of IFN β in the dermis overlying involuting and involuted haemangioma but not in the dermis overlying proliferating lesions, together with the efficacy of IFN α in the treatment of haemangioma, suggests that this cytokine may play an important role in haemangioma involution. Unfortunately, the significant side effects associated with high dose IFN therapy especially neurotoxicity has limited its use for haemangioma (Tan, 2001). In this study, it was hypothesised that if a lower dose of IFN α shows improved efficacy in combination with another agent, it may be of greater clinical value.

Therefore, in this study the efficacy of the combined treatment of IFN α -2b with TRAIL was investigated. mRNA levels of TRAIL and its receptors were investigated using qRT-PCR in proliferating, involuting and involuted haemangioma biopsies. IHC analyses of the TRAIL receptors (TRAIL-Rs) on paraffin embedded haemangioma tissue sections were performed. Using the *in vitro* model (described in Chapter 4), the effect of TRAIL and IFN α -2b treatment on haemangioma capillary-like growth was investigated.

5.3 RESULTS

5.3.1 mRNA EXPRESSION OF TRAIL AND ITS RECEPTORS

Relative changes in mRNA expression for TRAIL, DR4, DR5, DcR1, DcR2 and OPG was determined in 11 proliferating, 4 involuting and 6 involuted haemangioma samples relative to GAPDH as ΔCt values. The average ΔCt values obtained for each gene per phase are presented in Table 8.

TRAIL was abundantly expressed in all three phases of haemangioma at similar levels. The expression profile for TRAIL receptors was similar for the proliferating and involuted samples. DR4 mRNA was the most abundant and was expressed in all three phases at greater levels than DR5 (at least 30-fold more). Surprisingly, DR5 expression could not be detected in the involuting samples although it was detected in the proliferating and involuted specimens ($p < 0.05$) albeit at low levels. The decoy receptors DcR1, DcR2 and OPG were detected at low but comparable levels within all three phases.

Phase	TRAIL	DR4	DR5	DcR1	DcR2	OPG
Proliferating (n=11)	4.3 $\pm$ 0.5	8.1 $\pm$ 0.2	14.3 $\pm$ 0.7	9.5 $\pm$ 0.7	9.5 $\pm$ 0.3	10.7 $\pm$ 0.5
Involuting (n=4)	5.2 $\pm$ 0.3	9.9 $\pm$ 0.3	> 18	13.6 $\pm$ 0.3	12.1 $\pm$ 0.4	11.7 $\pm$ 0.7
Involuted (n=6)	3.4 $\pm$ 1.4	8.3 $\pm$ 3.4	13.6 $\pm$ 5.6	10.2 $\pm$ 4.2	10 $\pm$ 4.1	8.8 $\pm$ 3.6

Table 8: Expression of TRAIL and TRAIL receptors mRNA in haemangioma. Relative expression levels of mRNA for TRAIL and TRAIL receptors in 11 proliferating, 4 involuting and 6 involuted specimens determined by qRT-PCR using GAPDH as the internal house-keeping reference gene. Values are expressed as the average ΔCt value obtained for each gene $\pm$ s.e.m. Lesions from all phases expressed high levels of TRAIL mRNA and moderate levels of DR4, DcR1, DcR2 and OPG mRNA. Very low levels of DR5 mRNA were detected in all samples.

5.3.2 PROTEIN EXPRESSION OF TRAIL AND ITS RECEPTORS

Immunohistochemical staining revealed a positive reaction for all five TRAIL receptors in proliferating haemangioma tissue sections. Confocal laser scanning showed that proliferating haemangioma lesions expressed low levels of DR4 (Figure 5.2-A, white arrows) and DR5 protein (Figure 5.2-B, white arrows), moderate levels of DcR1 protein (Figure 5.2-C, white arrows), high levels DcR2 (Figure 5.2-D, white arrows) and OPG protein (Figure 5.6-A and -B). While staining for DR4, DR5, DcR1 and DcR2 was essentially cytoplasmic in all lesions analysed (Figure 5.2, white arrows), immunoreactivity for OPG was detected in the cytoplasm (Figure 5.6, yellow arrows) as well as within the nuclear compartment in some cells (Figure 5.6, white arrows). Negative controls included immunostaining without primary antibody incubation and showed no staining throughout the study. In addition, antibody specificity for DR4 and DcR2 was determined by Western blotting (Figure 5.7). As expected, immunoreactive bands were detected for DR4 and DcR1 at 55 kDa and 32 kDa, respectively (Grataroli *et al.*, 2002; Vindrieux *et al.*, 2002). Unfortunately, antibody specificity for the anti-DR5 and anti-DcR1 antibodies could not be verified, as the antibodies did not perform well on Western blots.

Figure 5.2: Confocal immunohistochemical localisation of TRAIL receptors in haemangioma. Paraffin embedded proliferating haemangioma tissue sections were stained with primary antibodies against DR4 (**A**), DR5 (**B**), DcR1 (**C**) and DcR2 (**D**) as described in section 2.4.2. Bound DR4 antibodies were detected using an anti-mouse DIG conjugate and an anti-DIG-Rhodamine conjugate (red). Bound DR5 antibodies were detected using an anti-mouse AlexaFluor 488 conjugate (green). Bound DcR1 and DcR2 antibodies were detected using an anti-rabbit-DIG and an anti-DIG-FITC conjugate (green). Cell nuclei were counterstained with either DAPI (blue) or PI (red). Positive immunoreactivity was detected for all four TRAIL receptors. Red staining indicates DR4 expression (Panel **A**), while green staining indicates DR5 (Panel **B**), DcR1 (Panel **C**) and DcR2 (Panel **D**) expression. White arrows show cytoplasmic staining detected for all receptors. Scale bars: 50 μm .

5.3.3 EFFECT OF INTERFERON AND TRAIL ON CAPILLARY-LIKE GROWTH

Freshly resected haemangioma tissue has been shown to develop outgrowths or 'microvessels' within days of placing in culture (Tan *et al.*, 2000_b). Using this system, the effect of exogenously applied TRAIL (250 ng/ml) and a low and high dose of IFN α -2b (4600 U/ml and 23000 U/ml, respectively), singly and in combination was tested on proliferating haemangioma tissue explants isolated from five different patients (Figure 5.3-A). Haemangioma explants when grown in the absence of TRAIL and IFN under serum-free culture conditions grew well and gave rise to numerous microvessel-like outgrowths by nine days in culture. Figure 5.3-A graphically represents the extent of microvessel outgrowth for all five patients relative to the control (C). TRAIL treatment (T) alone had negligible effect on microvessel outgrowth in all five patients. Except for the tissue explants that were treated with TRAIL (T) alone, all other treatments resulted in significant inhibition of microvessel outgrowth compared to untreated control (C) cultures ($p < 0.01$). In addition, while low dose of IFN α -2b (I_L) inhibited outgrowth ($p < 0.01$), IFN α -2b (I_L) when used in conjunction with TRAIL (T+ I_L) resulted in a synergistic inhibition of microvessel outgrowth ($p < 0.001$) such that the level of inhibition was similar to that seen with high dose IFN α -2b treatment (I_H) alone. While cultures treated with high dose IFN α -2b (I_H) showed reduced microvessel outgrowth ($p < 0.001$), the combined treatment with TRAIL and high dose IFN α -2b (T+ I_H) reduced microvessel outgrowth further but not to a statistically significant extent.

5.3.4 EFFECT OF INTERFERON AND TRAIL ON HaemDMSCs

The EZ4U assay was used to test the effect of TRAIL (250 ng/ml) and IFN α -2b (4600 U/ml and 23000 U/ml) on HaemDMSC proliferation. No effect was detected at the end of a 48 hour incubation period. HaemDMSCs were also treated with IFN α -2b and TRAIL for 96 hours in a similar manner as the tissue explants. Figure 5.3-B shows

that similar to the tissue explants, treatment with TRAIL alone did not inhibit proliferation of HaemDMSCs. While IFN α -2b treatment (low dose and high dose) significantly inhibited HaemDMSC proliferation compared to untreated cultures ($p < 0.01$), the combined treatment of TRAIL with low dose of IFN α -2b (T+I_L) did not show a synergistic effect on HaemDMSC proliferation (Figure 5.3-B) as that seen with the cultured tissue explants shown in Figure 5.3-A. All proliferation assays were performed on the purified cells from at least three different patients. Cultures were assayed in triplicate for each treatment type.

Figure 5.3: Effect of TRAIL and IFN α -2b on haemangioma cultures, *in vitro*. The effect of 250 ng/ml TRAIL (T), 4600 U/ml IFN α -2b (I_L) and 23000 U/ml IFN α -2b (I_H) was tested on haemangioma capillary-like outgrowth (A) and HaemDMSC proliferation (B). (A) Capillary-like outgrowth was calculated by the area occupied by the outgrowing vessels relative to the area of the tissue from which they emanated and is expressed as % Control. Data presented shows the average results obtained following nine days of treatment from five different proliferating haemangioma patients $\pm$ s.e.m. (B) Extent of HaemDMSC proliferation following 96 hours of treatment with TRAIL and IFN α -2b. Proliferation was assessed as a direct measure of absorbance and the results are presented as % Control. Data shown in Panel B represent the average results obtained from three individual experiments performed with HaemDMSC isolates from three proliferating haemangioma patients $\pm$ s.e.m. To detect statistically significant changes, a 2-way ANOVA was applied to all data sets. ** represents significance at $p < 0.01$.

5.3.5 mRNA AND PROTEIN EXPRESSION OF TRAIL AND ITS RECEPTORS IN HaemDMSCs

The difference in response to the combined treatment of TRAIL and IFN α -2b on the tissue explants compared with the HaemDMSCs was speculated to be due to differences in TRAIL receptor expression. Using qRT-PCR, the expression profiles of mRNA for TRAIL and TRAIL receptors in HaemDMSCs and the tissue pieces from which they were isolated were compared. The difference in mRNA expression between the tissue and the cultured cells normalised to expression of GAPDH as Δ Ct values (Figure 5.4-A) is graphically represented as the average $\Delta\Delta$ Ct values for the six biopsies analysed (Figure 5.4-B). Small differences in expression of mRNA for DR4, DR5 and DcR1 were found but there was a large induction of OPG expression ($p < 0.001$) and DcR2 ($p < 0.01$) decoy receptors in the HaemDMSCs. This increase in decoy receptor expression was also matched by a similar decrease in TRAIL expression ($p < 0.001$) by the cultured cells. The raw Δ Ct values are listed in Table 3.1, Appendix 3, page 292.

A

	TRAIL	DR4	DR5	DcR1	DcR2	OPG
HaemTissue	5.1	7.6	12.6	8.4	8.7	10.7
HaemDMSCs	15.9	11.3	14.7	10.9	5.7	4.3

B

Figure 5.4: Differences in mRNA levels for TRAIL and TRAIL receptors in haemangioma tissue and HaemDMSCs. Relative mRNA levels for TRAIL and TRAIL receptors were determined by qRT-PCR from six proliferating haemangioma tissues and the corresponding HaemDMSC isolates from each tissue. (A) The table gives the average Δ Ct values obtained for each gene from tissue pieces (HaemTissue) and cell isolates (HaemDMSCs). (B) Differences in TRAIL and TRAIL receptor expression between the HaemDMSC cell lines relative to tissues were determined as $\Delta\Delta$ Ct values. Data presented shows the average $\Delta\Delta$ Ct values obtained for each gene between tissue and cells. A low value indicates higher expression of the gene in the tissue while a high value shows higher mRNA expression of that gene by HaemDMSCs. Significant differences in gene expression between tissues and cells were detected using the Friedmann's non-parametric test for paired samples, where ** represents significance at $p < 0.01$ and *** at $p < 0.001$.

IHC analysis showed strong positive cytoplasmic immunoreactivity for DR4, DR5 and DcR2 on HaemDMSCs (Figure 5.5-A, -B and -D, respectively). Low levels of DcR1 were also detected (Figure 5.5-C). As mentioned in Chapter 4, HaemDMSCs showed strong extracellular staining for OPG (Figure 5.6-C) which was clearly different from cytoplasmic (Figure 5.6-A and -B, yellow arrows) and nuclear staining (Figure 5.6-A and -B, white arrows) seen within the tissue lesion *in vivo*. In addition, OPG expression by HaemDMSCs was found to increase with time (data not shown).

Figure 5.5: Confocal immunohistochemical localisation of TRAIL receptors on HaemDMSCs. Cells were fixed and stained with antibodies against DR4 (A), DR5 (B), DcR1 (C) and DcR2 (D) as described in section 2.4.2. Bound primary antibodies were detected using either an anti mouse AlexaFluor 488 conjugate (green) or a FITC conjugated anti-DIG secondary antibody (green). Cell nuclei were counterstained with PI (red). Green staining shows high expression for DR4, DR5 and DcR2 but low expression of DcR1 in HaemDMSCs. Scale bars: 50 μm.

Figure 5.6: Confocal immunohistochemical localisation of OPG in haemangioma. Paraffin embedded proliferating haemangioma tissue sections (A&B) and HaemDMSCs were stained with a mouse anti-OPG antibody as described in section 2.4.2. Immunoreactivity was detected using an anti-mouse AlexFluor 488 conjugate (green). Cell nuclei were counterstained with DAPI (blue). In proliferative haemangioma lesions (Panels A&B), OPG expression was detected in the cytoplasm (yellow arrows) as well as within the nuclear compartment (white arrows). HaemDMSCs showed strong extracellular staining for OPG (Panel C, green staining). Scale bars: 50 μm .

To determine whether changes in DR4 and DcR2 mRNA expression correlated with changes in its protein abundance, the same samples were used for immunoblotting as described in section 2.4.5.1 (page 103). The immunoblots shown in Figure 5.7 clearly shows that although equal amounts of total protein (100 µg/well) were loaded from each sample, HaemDMSCs expressed greater amounts of GAPDH protein compared to proliferating haemangioma tissues. No significant difference in total DR4 protein expression was detected between the cells and tissues (Figure 5.7-A). Consistent with mRNA studies, DcR2 protein expression was significantly higher in HaemDMSCs than in the tissues ($p < 0.05$; Figure 5.7-B and -C).

Figure 5.7: DR4 and DcR2 protein expression in haemangioma. DR4 (**A**) and DcR2 (**B**) protein expression was examined in proliferative haemangioma tissue biopsies and HaemDMSCs by Western blotting as described in section 2.4.5.1. Each gel contained 50 µg of total protein extracted from haemangioma tissues from four patients and five HaemDMSCs cell lines. Membranes were incubated with mouse anti-DR4 or rabbit anti-DcR2 antibodies followed by AlexaFluor 488 conjugated anti-mouse or anti-rabbit secondary antibodies. Immunoreactive bands were detected for DR4 and DcR2 at 55 kDa and 32 kDa, respectively, corresponding to the expected molecular weight for these proteins. Membranes were also probed with mouse anti-GAPDH antibodies. The immunoblots showed that HaemDMSCs expressed greater amounts of GAPDH protein than tissue biopsies. No change in total DR4 protein expression was found between cells and tissues. In contrast, HaemDMSCs expressed greater amounts of DcR2 protein than haemangioma biopsies. (**C**) Densitometry analysis of DcR2 band densities confirmed that the increase was significant with $p < 0.05$ (*). Negative controls were included in each experiment in which lysate from haemangioma tissue and HaemDMSCs were immunoblotted with secondary antibody only. No non-specific immunoreactive bands were detected on the negative control immunoblots therefore validating the specificity of the anti-DR4 and anti-DcR2 primary antibodies.

5.3.6 HaemDMSCs SECRETE OPG INTO THE CULTURE MEDIUM AND OPG ANTIBODY SPECIFICITY

To determine whether HaemDMSCs secrete and release OPG when cultured *in vitro*, Western blot analysis was performed using the MAB8051 anti-OPG antibody (R&D Systems) as described in section 2.4.5.2 (page 104). Under reducing conditions, the immunoblots showed that the culture media conditioned over a 96 hour period, contained detectable levels of the monomeric form of OPG (60 kDa) (Figure 5.8-A, red arrow). In addition, a band at approximately 220 kDa was also detected in the conditioned media of HaemDMSCs (Figure 5.8-A, black arrow). Both bands were detected when the experiment was repeated with conditioned media obtained over a 72 hour culture period (Appendix 3, Figure 3.1, page 293). In addition, no bands were detected when unconditioned media was analysed in the same way as conditioned media (Appendix 3, Figure 3.1, page 293). These results suggest that perhaps the 220 kDa band represents a novel hyper-glycosylated form of OPG. The dimeric form of OPG has been shown to be 120 kDa and was not detected in either experiment.

In addition, HaemDMSCs lysate and haemangioma tissue lysate were also run on the same gel and subjected to immunoblotting. Bands at both 60 kDa and 220 kDa were detected from HaemDMSCs lysate (Figure 5.8-A, red and black arrows, respectively). In order to determine the specificity of antibody binding, immunoblotting was performed with the omission of the primary antibody and incubation with secondary antibodies only. The resulting immunoblot showed no bands at either 60 kDa or 220 kDa using HaemDMSCs lysates (Figure 5.8-B). Furthermore, GAPDH immunoreactivity was not detected from the conditioned media (Figure 5.8-C, black arrow). These results indicate that the OPG protein detected in the media was not the result of HaemDMSCs lysing during the experiment

Waterman *et al* (2007) recently showed that the MAB8051 anti-OPG antibody recognises a 30 kDa band in addition to OPG in breast cancer tissues and prostate

cancer cell lines. Using MALDI-TOF, these authors identified that the 30 kDa band corresponded to carbonic anhydrase II (CA II). In this study, only the haemangioma tissue lysates showed an immunoreactive band at 30 kDa with this antibody (Figure 5.8, green arrow). Since neither the conditioned medium nor the HaemDMSCs lysates showed this 30 kDa band, it was confirmed that the immunohistochemical studies performed for OPG on HaemDMSCs was not detecting CA in the cultured cells but was specific for OPG or at the least, a protein that contains an OPG domain. However, caution is required in interpreting results of OPG immunohistochemical analysis in paraffin embedded haemangioma tissue sections because of the non-specific binding of the MAB8051 anti-OPG antibody on haemangioma tissue lysate.

Figure 5.8: OPG protein expression in HaemDMSCs conditioned media, in HaemDMSCs and in a proliferative haemangioma tissue biopsy. (A) 40 μ g of total protein from the medium of HaemDMSCs conditioned over a 96 hour period (M), 100 μ g of total protein from HaemDMSCs (CL) and proliferating haemangioma tissue lysate (TL) were analysed by SDS-PAGE and immunoblotted with the mouse anti-OPG MAB8051 primary antibody. (B) A negative control immunoblot containing lysate from HaemDMSCs (CL) and haemangioma tissue lysate (TL) was probed with secondary antibody only. The test sample showed immunoreactive bands at 60 kDa (red arrow) and at 220 kDa (black arrow) from the conditioned media and HaemDMSCs. These bands were absent in tissue lysate and on the negative control. In contrast, a band at 30 kDa was detected from tissue lysate (green arrow) which was absent in the conditioned media, the HaemDMSCs lysates and the negative control. (C) The test immunoblot was probed with anti-GAPDH antibodies. Absence of an immunoreactive band for GAPDH (38 kDa) from conditioned media (M) indicates that the band corresponding to OPG protein (60 kDa, red arrow) from the media did not come from lysed cells.

5.4 DISCUSSION

5.4.1 THE EFFECT OF TRAIL AND INTERFERON ON HAEMANGIOMA CAPILLARY-LIKE OUTGROWTH

The cellular and molecular biology of haemangioma and its spontaneous and therapy-induced regression is still largely unknown. Understanding the molecular pathways that are active in these tumours could lead to the development of more effective therapies, as molecules that are involved in proliferation or involution could be targeted.

Recombinant TRAIL protein offers great promise as a cancer therapy as it shows the unique property to destroy several types of tumour cells but spares most normal cells (Smyth *et al.*, 2003). TRAIL also induces the expression of a cohort of IFN stimulated genes (ISGs) including IFN β , caspase 7 and STAT-1 which are involved in apoptosis. This suggests the existence of molecular cross-talk between the TRAIL death receptor and IFN signalling pathways (Kumar-Sinha *et al.*, 2002). Increased expression of ISGs has been identified during haemangioma involution. In addition, apoptosis has been shown to increase five-fold in haemangioma undergoing regression (Razon *et al.*, 1998). It is therefore interesting to postulate that TRAIL receptor signalling may be a regulator of haemangioma progression, presumably through the induction of apoptosis of the ECs that form the microvasculature characteristic of haemangioma. Given the potential use of TRAIL in anti-cancer therapy, this study investigated the effects of TRAIL and IFN α -2b on haemangioma capillary-like outgrowth when added singly and in combination. The expression of TRAIL and TRAIL-Rs in proliferating haemangioma biopsies and on HaemDMSCs was also investigated.

This study showed that although proliferating haemangioma expressed protein for the functional receptors for the TRAIL ligand, DR4 and DR5, TRAIL alone did not affect rate of capillary-like outgrowth. However, combining TRAIL with a low dose

IFN α -2b resulted in a synergistic inhibitory effect on capillary-like outgrowth. In addition, for some explants, this inhibition was similar to the inhibition caused by high dose IFN α -2b alone. These results suggest the possibility of substituting high dose IFN α -2b therapy with the combined treatment of TRAIL with a low dose of IFN α -2b, thereby possibly reducing the harmful side effects seen clinically with IFN treatment.

This study also investigated the effect of TRAIL and IFN α -2b on HaemDMSCs proliferation. Although TRAIL and IFN α -2b added singly inhibited HemDMSCs proliferation to a certain extent, the synergistic effect of TRAIL with IFN was not detected. qRT-PCR and immunohistochemical analysis showed that although the cells expressed DR4, DR5 and DcR1, the expression of DcR2 and OPG was greatly up-regulated in the cultured cells.

The molecular mechanism by which DcR2 confers resistance to TRAIL-induced apoptosis remains unclear (Almasan & Ashkenazi, 2003; Bouralexis *et al.*, 2003; Davidovich *et al.*, 2004). Various studies have found that DcR2 overexpression protected cells from TRAIL-induced apoptosis (Degli-Esposti *et al.*, 1997; Marsters *et al.*, 1997). In addition, decreased DcR2 expression caused due to the hypermethylation of the DcR2 gene has been identified in a number of tumours and tumour cell lines including breast, lung, prostate, bladder, cervical cancer, ovarian, lymphoma, leukaemia and multiple myeloma and neuroblastomas (Shivapurkar *et al.*, 2004; Van Noesel *et al.*, 2002). Mérimo *et al* (2006) showed that DcR2 impaired TRAIL-DISC processing and initiator caspase activation while Meng *et al* (2000) showed that the over-expression of DcR2 delayed p53-induced apoptosis in human colon cancer cells. Liu *et al* (2005) reported that while DcR2 over-expression in human lung cancer cells was protective against chemotherapeutic agent-induced apoptosis, silencing of DcR2 expression enhanced apoptosis. These findings support the possibility that the increased expression of DcR2 by HaemDMSCs may be to some extent protecting them from apoptosis.

During this study, increased extracellular OPG deposition by HaemDMSCs was consistently detected by immunohistochemical studies. From these results, we hypothesise that HaemDMSCs secrete increased levels of OPG, which then acts as a protective shield around the cells preventing apoptosis. We also postulate that increased OPG production is a stress response that results from culturing these cells *in vitro* i.e. outside their normal environment. Previous studies have shown that the *in vitro* culture of primary cell lines can result in the formation of abnormal cell architecture and can cause significant changes in gene expression (Kivell, 2003). Kanzaki *et al* (2006) found that application of a cyclical tensile strain up-regulated OPG mRNA expression in human periodontal ligament cells (hPLDCs). Yamamoto *et al* (2006) also found that hPLDCs exposed to mechanical stress as hydrostatic pressure showed increased OPG mRNA expression compared to the untreated cells. In addition to increased OPG mRNA expression, Tang *et al* (2006) showed increased concentrations of OPG protein in the conditioned medium of MC3T3-E1 osteoblastic cells subjected to cyclic tensile strain. The presence of secreted OPG in the conditioned media of HaemDMSCs and the increased expression of OPG by HaemDMSCs are consistent with these findings.

In contrast, although immunohistochemical studies showed OPG expression within the haemangioma lesions (Figure 5.6), Western blotting analysis failed to detect an immunoreactive band at the correct size using haemangioma tissue lysates (see section 5.3.6, page 233). These data suggest that proliferating haemangioma lesions do not express high levels of OPG and this could explain why the combined treatment of IFN α -2b and TRAIL resulted in the synergistic inhibition of capillary-like growth from explanted biopsies.

To date, the physiological functions of the multiple TRAIL-Rs remain obscure. Although various studies have suggested that the ratio of death and decoy receptors are critical in the regulation of the apoptotic pathway (Ashkenazi, 2002; Bouralexis *et al.*, 2003), other investigators have shown that there is no correlation between TRAIL

receptor expression and susceptibility to TRAIL-induced apoptosis (Keane *et al.*, 1999; Wen *et al.*, 2000; Knight *et al.*, 2001). In addition, several other mechanisms of resistance to TRAIL have been proposed in different cell types including cFLIP, a caspase-8 and a caspase-10 related molecule that lacks catalytic activity and acts as a competitive inhibitor of death receptor-mediated apoptosis (Tschopp *et al.*, 1998; Leverkus *et al.*, 2000). XIAP can also bind to caspase-3 and caspase-9 and inhibit their activity (LeBlanc & Ashkenazi, 2003; Salvesen, 2002; MacFarlane *et al.*, 2002). Furthermore, sensitivity of some cell types to TRAIL is hinged on the balance between pro-apoptotic (e.g., BAX and BAK) and anti-apoptotic (e.g. Bcl-2 and Bcl-xl) members of the Bcl-2 family (Kelly & Ashkenazi, 2004).

There is mounting evidence to suggest that the interaction of OPG with TRAIL is biologically important, particularly in *in vitro* culture systems. OPG has been shown to protect serum-starved ECs and fibroblast-like synovial cells from TRAIL-triggered cell death (Pritzker *et al.*, 2004; Miyashita *et al.*, 2004). OPG also acts in a paracrine and autocrine manner by binding TRAIL to promote the survival of prostate cancer cells (Holen *et al.*, 2002), breast cancer cells (Neville-Webbe *et al.*, 2004), multiple myeloma cells (Shipman & Croucher, 2003) and ameloblastoma cells (Sandra *et al.*, 2006). Although further studies are required to elucidate the ability of OPG to counteract the biological activity of TRAIL *in vivo*, it is clear that the relative concentrations and the expression patterns of OPG in the local environment is a key determinant in TRAIL-mediated cell toxicity. Based on these findings, we hypothesise that the HaemDMSCs isolated in this study are resistant to TRAIL mediated apoptosis due to increased protein levels of OPG and DcR2. However, further experiments would be required to confirm this. This would include testing the effect of TRAIL on HaemDMSCs transfected with short interfering RNA (siRNA) for the OPG and DcR2 genes.

5.4.2 OPG ANTIBODY SPECIFICITY

OPG is secreted as a covalent, disulfide-linked homodimer, which has been shown to be the predominant extracellular form (Simonet *et al.*, 1997). While the homodimer has a molecular weight of 120 kDa, immunoblotting for OPG under reducing conditions shows an immunoreactive band at 60 kDa corresponding to the OPG monomer (Haynes *et al.*, 2003). In addition to the monomeric and dimeric forms of OPG, alternative splicing of the OPG gene has been shown to give rise to three mRNA isoforms: mature mRNA and two splice variants (Morinaga *et al.*, 1998). The splice variant sequences contain a premature stop codon and are predicted to encode truncated proteins of 30-40 kDa.

There have been conflicting reports on the specificity of the MAB 8051 antibody. This antibody was used in this study to detect OPG protein expression. Although Haynes *et al* (2003) showed that the antibody detected both, the monomeric and the dimeric forms of OPG, Waterman *et al* (2007) advised caution when using this antibody. Their studies showed that the antibody, in addition to OPG, also detected a 30 kDa band on immunoblots that corresponded to CA II. To determine the specificity of the MAB 8051 antibody, Western blotting was performed in this study with HaemDMSCs lysates, HaemDMSCs conditioned media and haemangioma tissue lysates.

Consistent with the findings of Waterman *et al* (2007), an immunoreactive band at 30 kDa was detected when haemangioma tissue lysates were subjected to immunoblotting. However, this band was not detected using either HaemDMSC lysates or the HaemDMSC conditioned medium run on the same gel and therefore subjected to the same conditions as the tissue lysates. In contrast, the HaemDMSCs lysates and HaemDMSCs conditioned media showed the presence of an immunoreactive band at 60 kDa which corresponds to the molecular weight of the native OPG monomer. Furthermore this band was absent on haemangioma tissue lysates. However,

haemangioma tissue lysates did not show any immunoreactive bands at the appropriate size. Therefore, we conclude that the immunohistochemical studies performed during this study using the MAB 8051 anti-OPG antibody may be non-specifically cross reacting to CA II within the haemangioma tissue sections.

CA II is a highly conserved enzyme which catalyses the hydration of carbon dioxide and the dehydration of bicarbonate. In addition to normal cells (Onji, 1999), increased CA II expression is detected in brain tumors (Parkkila *et al.*, 1995), leukemia (Leppilampi *et al.*, 2002), the tumour endothelium of melanoma, renal cell carcinoma, oesophageal and lung cancers (Yoshiura *et al.*, 2005). CA II is considered an important factor for tumour cell growth as rapidly proliferating cells have a requirement for a high influx of bicarbonate to support their metabolic processes (Bianchi *et al.*, 2005). Increased CA II expression has been reported in acidic and hypoxic conditions mimicking a tumour environment (Yoshiura *et al.*, 2005). Furthermore, CA II inhibitors can inhibit the growth of cancer cell lines such as prostate and breast cancer cells and cell lines (Supuran & Scozzafava, 2000; Mallory *et al.*, 2005). Consistent with the function of this enzyme, the high expression of CA II identified within the proliferative phase seems logical, as most cells in this phase, being highly proliferative would require increased levels of CA II. In contrast, minimal proliferation in the involuting and involuted phases corresponds to reduced CA II expression.

However, it is important to note that the epitope recognised by the MAB 8051 anti-OPG antibody within haemangioma tissue sections still differentiated between the cells that exhibited punctuate vWF immunoreactivity and those that showed strong vWF immunoreactivity (see section 4.3.5, page 174). Therefore, this antibody may be useful in determining the ‘maturity’ of the ECs within haemangioma.

Finally, an additional immunoreactive band was detected with both HaemDMSCs lysates and HaemDMSCs conditioned medium and had a molecular weight of 220 kDa. Two groups have investigated the domains of OPG and their

importance to function. These studies have shown that the first four domains of OPG (encompassing the TNF receptor-like region) were required for activity in an osteoclastogenesis assay while the last intra-chain disulfide bond was critical for OPG function (Simonet *et al.*, 1997; Yun *et al.*, 1998). Cundy *et al* (2002) reported a mutant form of OPG that resulted from the loss of an aspartate residue. They showed that this mutant form was secreted and was hyperglycosylated. It is known that glycosylation is important for correct folding, trafficking, stabilisation and protection from proteases, as well as for interaction with other molecules (Dwek, 1998). The primary sequence of OPG contains six potential glycosylation sites but the degree of glycosylation at each of these sites *in vivo* has not been investigated previously (Cundy *et al.*, 2002).

Since the band at 220 kDa was repeatedly detected on the immunoblots from two separate experiments performed during this study and was not detected when unconditioned media was analysed in the same way, we can conclude that this band may represent a novel glycosylated form of the OPG protein. However, deglycosylation experiments or carbohydrate detection methods would need to be performed to confirm this. We hypothesise that the protein at 220 kDa being detected by the antibody must at least contain an OPG domain.

CHAPTER 6: CYCLOPHILIN A IN HAEMANGIOMA

6.1 INTRODUCTION

CycA, a ubiquitously distributed protein, belongs to the immunophilin family (Galat, 1993) and is defined on the basis of its binding to the potent immunosuppressive drug cyclosporine A (Fruman *et al.*, 1992; Liu *et al.*, 1991). Intracellular CycA has peptidylprolyl cis-trans-isomerase (PPIase) activity, which is crucial for folding and isomerisation of many proteins. (Galat, 1993; Kofron *et al.*, 1991). CycA also acts as a potent chemoattractant for monocytes (Sherry *et al.*, 1992), neutrophils (Sherry *et al.*, 1992; Xu *et al.*, 1992), eosinophils (Xu *et al.*, 1992) and T cells (Allain *et al.*, 2002) *in vitro*. Xu *et al* (1992) showed that CycA also elicited an inflammatory response characterised by a rapid influx of neutrophils when injected *in vivo*.

Initially believed to exist solely as an intracellular protein, various studies have shown that CycA is secreted by cells in response to inflammatory stimuli (Sherry *et al.*, 1992; Xu *et al.*, 1992). Jin *et al* (2000) showed that CycA functioned as a secreted oxidative-stress-induced growth factor from rat SMCs and mediated extracellular signal regulated kinase (ERK1/2) activation and SMCs growth. They also found that recombinant CycA mimicked the effects of secreted CycA. Seko *et al* (2004) found that CycA was secreted by cardiac myocytes in response to hypoxia/reoxygenation and that CycA secretion resulted in the activation of the ERK1/2 pathway and increased Bcl-2 expression. These authors suggested a protective role for CycA against oxidative stress-induced apoptosis. In addition, Yang *et al* (2005) showed that CycA induced EC and SMC proliferation while Jin *et al* (2004) showed that it activated ECs in a pro-inflammatory manner.

6.2 AIMS

In this study, CycA was first investigated as a house keeping gene. However, preliminary qRT-PCR results identified that CycA mRNA levels differed between the three developmental phases (see Appendix 1, section 1.1.2.2, page 272). Therefore, CycA expression was investigated as a test gene. qRT-PCR was used to measure changes in levels of CycA mRNA while Western blotting and IHC techniques were used to measure CycA protein abundance in proliferating, involuting and involuted haemangioma samples.

6.3 RESULTS

6.3.1 CHANGE IN mRNA EXPRESSION

The relative change in mRNA expression for CycA was determined for proliferating, involuting and involuted haemangioma samples (eight samples per phase) by qRT-PCR as described in section 2.2.2.1 (page 81). Expression of CycA mRNA was highest in proliferating haemangiomas and decreased in involuting and involuted lesions with a significant difference detected between proliferating and involuting lesions ($p < 0.05$) (Figure 6.1).

Figure 6.1: Changes in CycA mRNA expression. mRNA levels for CycA were determined for eight proliferating, eight involuting and eight involuted haemangioma samples relative to GAPDH. Proliferating samples expressed more CycA mRNA than involuting and involuted lesions. Significance was achieved between the proliferative and involuting phases (* $p < 0.05$).

6.3.2. PROTEIN EXPRESSION

6.3.2.1 WESTERN BLOTTING

Some of the samples used for qRT-PCR were examined by Western blotting to identify if CycA protein abundance correlated with changes in mRNA expression. Figure 6.2 shows immunoreactive bands for CycA protein detected at 18 kDa, as expected (Hamilton & Steiner, 1998), from four proliferating, three involuting, three involuted haemangioma biopsies and HeLa cells. Immunoblots were also analysed for GAPDH to confirm equal amounts of total protein. Although CycA mRNA levels varied with phase, no statistically significant difference in CycA protein expression was found between the specimens (Figure 6.2).

Figure 6.2: Western blotting for CycA in haemangioma. 50 µg of total protein from proliferating, involuting and involuted haemangioma lesions were subjected to immunoblotting as described in section 2.2.2.2. Membranes were incubated with rabbit anti-CycA and mouse anti-GAPDH antibodies. With equal protein loaded from each sample (as determined by GAPDH immunoreactivity), no change in total CycA protein expression was found between phases.

6.3.2.2 IMMUNOHISTOCHEMICAL ANALYSIS

6.3.2.2.1 *CycA* EXPRESSION

Immunohistochemical staining of haemangioma specimens (five samples per phase) showed that CycA was detectable in all phases of haemangioma (Figure 6.3, red staining). CycA protein was detected in the plump ECs of the proliferative phase (Figure 6.3-D, white arrows) and in the flattened ECs of the involuting (Figure 6.3-E, white arrows) and involuted phases (Figure 6.3-F, white arrows). In addition, the cells in the fibrofatty residuum of the involuting and involuted phases also expressed CycA (Figure 6.3-E and -F, respectively, yellow arrows). The decrease in CycA expression seen between phases in Figure 6.3 appears to be due to a decrease in cell number as opposed to a decrease in CycA staining intensity per cell.

Figure 6.3: Immunohistochemical localisation of CycA in haemangioma. Proliferating (A&D), involuting (B&E) and involuted (C&F) paraffin embedded haemangioma sections were stained with a rabbit anti-CycA antibody. Bound antibodies were detected using an anti-rabbit Cy3 conjugate (red). Cell nuclei were counterstained with DAPI (blue). Panels D, E & F are the enlargements of the areas outlined in A, B & C, respectively. CycA expression (red staining) was detected in the plump ECs of the proliferative phase (D, white arrows) and also in the flattened ECs of the involuting and involuted phases (E&F, respectively, white arrows). Involuting (E) and involuted (F) lesions also contained CycA positive cells in the interstitium (yellow arrows). Scale bars: 50 μ m.

6.3.2.2.2 MAST CELLS EXPRESS CycA

To detect if MCs express CycA, DAB colorimetric visualisation of CycA immunoreactivity in conjunction with Csaba staining was performed as described in section 2.2.2.4 (page 82). Of the 200 MCs counted, those that stained positive for CycA (CycA + MCs) were identified in the proliferative and involuting phases. Consistent with earlier results (see section 3.3.2.3.5, page 122), a three-fold increase in the number of MCs was identified in involuting samples as compared to proliferating samples. However, the proportion of MCs expressing CycA did not vary with phase (Table 9, Figure 6.4)

Developmental Phase	Number of CycA + MCs	% CycA + MCs
Proliferative	169 $\pm$ 2.3 ($n = 25$),	81
Involuting	177 $\pm$ 6.5 ($n = 7$)	85
Proliferative vs Involuting $p < 0.1$		

Table 9: CycA expression by MCs in haemangioma. CycA positive and negative MCs were counted from five samples of the proliferative and involuting phases as described in section 2.2.2.5.2. Of a total of 200 MCs counted from each lesion, the values in the table gives the number of CycA positive MCs (CycA+Mcs) and the percentage of CycA expressing MCs (% CycA+Mcs) . Values are the mean (of five lesions) $\pm$ s.e.m; n indicates the total number of fields of view counted at a magnification of X40.

Figure 6.4: MCs in proliferating and involuting haemangiomas express CycA. CycA immunoreactivity was detected by DAB staining (brown) and counterstained with Csaba to identify the MCs (light blue). Double labelling of a proliferating (**A**) and an involuting (**B**) haemangioma with CycA antibodies and Csaba stain demonstrated the presence of CycA protein within MCs (black arrows). In Panel **B**, the yellow arrows show few MCs that do not express CycA. (**C**) The black columns show CycA positive MCs and the white columns show CycA negative MCs. As identified earlier in this study, involuting lesions contained approximately three-fold more MCs per unit area as compared to proliferating lesions. However, approximately 80% of MCs present in proliferating and involuting lesions expressed CycA. Scale bars: 50 μ m.

A

B

C

6.4 DISCUSSION

This is the first study to examine CycA expression in haemangioma. This study found that proliferating lesions expressed highest levels of CycA mRNA with decreased abundance in involuting and involuted haemangiomas. However, Western blotting studies showed that total protein levels of CycA did not differ between the phases. IHC confirmed this finding. Immunohistochemical staining showed that CycA immunoreactivity was detected in lesions from all three phases and that as a consequence of a decrease in cell number between phases, more CycA staining was present in proliferating lesions as compared to involuting and involuted lesions.

Although the findings from this study are purely descriptive, due to the presence of CycA in all phases of haemangioma together with its known function in protein remodelling and refolding as well as in EC proliferation and apoptosis, a model for the role of CycA in haemangioma was hypothesised and is depicted in Figure 6.5.

In this proposed model, a hypoxic event induces high expression and secretion of CycA from various cell types within the lesion. Secreted CycA then acts in a paracrine manner to induce further EC proliferation during the proliferative phase. With the initiation of involution, CycA functions as PPIase and participates in the tissue remodelling events that characterises the regression of haemangioma. The presence of CycA in MCs is of unclear significance. Perhaps the influx of MCs during the involuting phase still expressing CycA is required to maintain CycA protein levels within the lesion. A second role for CycA in the involuting phase of haemangioma may be in the induction of EC apoptosis. This is supported by the findings of Jin *et al* (2004) who showed that CycA can induce EC apoptosis similar to the proapoptotic effect of TNF α . However, further investigation is required to determine the precise role(s) of CycA in the pathogenesis of haemangioma.

Figure 6.5: A role for CycA in haemangioma progression. See text for details.

CHAPTER 7: KEY FINDINGS AND FINAL CONCLUSIONS

7.1 THE SIGNIFICANCE OF THIS RESEARCH

Over the past two decades, various studies have identified a number of cellular and extracellular markers associated with haemangioma. However, the molecular mechanisms that govern haemangioma proliferation and its subsequent involution are still largely unknown. This research aimed to enhance the current understanding of the molecular basis of haemangioma, as well as the complexity of cell signalling in the biology of this tumour. In this study, quantitative and qualitative differences in expression were investigated for a set of genes that were chosen because of their association with tumour susceptibility and angiogenesis. It was hoped that results of this research would identify novel markers that would lead to the development of more effective treatments. This study was also conducted in order to develop a working model for the biology of haemangioma. The following is a summary of some of the novel findings of this study.

7.1.1 KEY FINDINGS

7.1.1.1 VEGF, VEGFRs AND STAT PROTEINS IN HAEMANGIOMA

Using an *in vitro* model, that mimics as closely as possible the features of haemangioma, this study confirmed VEGF to be an important factor governing haemangioma proliferation. The addition of exogenous VEGF resulted in significant outgrowth of capillary-like structures from haemangioma explants. These results are consistent with the known role of VEGF as an angiogenic factor (Ferrara, 2000) as well as its high expression during the proliferative phase of haemangioma (Takahashi *et al.*, 1994; Tan, 2001). In addition, proliferating lesions expressed highest levels of VEGFR-2 as compared to involuting and involuted lesions. These findings confirm that

VEGF/VEGFR-2 signalling is involved in the proliferation of haemangioma. In contrast, the presence of VEGFR-1 in all phases of haemangioma suggests that VEGFR-1 signalling may be required during the entire life cycle of haemangioma. As VEGFR-1 is involved in cellular differentiation (Neufeld *et al.*, 1999), it is postulated that VEGFR-1 activation may be involved in transforming the highly cellular content of the proliferative phase into the fibro-fatty residuum of the involuting and involuted phases.

This study identified that STAT protein signalling is required for capillary-like outgrowth from explanted haemangioma tissues and for the proliferation of haemangioma tissue-derived cells. Of the seven members in the STAT family of transcription factors, STAT-3 expression was examined in haemangioma samples due to its association with tumour proliferation (Bromberg *et al.*, 1998). Although there were high levels of STAT-3 in proliferating lesions, total abundance of STAT-3 per cell did not vary with the different phases. This suggests that STAT-3 abundance decreases with ongoing involution of haemangioma and that this decrease is concomitant with a reduction in cell number in involuting and involuted lesions. In addition, VEGF did not activate STAT-3 in cells purified from haemangioma lesions *in vitro* and activated STAT-3 did not correlate with proliferation *in vivo*. STAT-1 was also investigated due to its association with apoptosis. However, all lesions examined in this study showed low expression for STAT-1. These findings suggest that although STAT-3 and STAT-1 signalling may be occurring in haemangioma, these transcription factors are not critical regulators of haemangioma. However, preliminary experiments conducted in this study showed that STAT-5 activation is associated with VEGF signalling in haemangioma. Since STAT-5 is now known to be critical in cell transformation, its activators and down-stream targets in haemangioma biology warrants further investigation.

7.1.1.2 THE USE OF TRAIL AS TREATMENT FOR HAEMANGIOMA

Pharmacological therapy is the mainstay treatment for haemangioma during the proliferative phase with steroids being the first line of treatment and IFN α , the second choice, both of which were discovered serendipitously. These treatments are slow and are associated with serious side-effects. Surgery and laser therapy are indicated if pharmacological therapy fails. However, all these treatment modalities are empirical measures and leave definite sequelae (Tan, 2001). Therefore, one of the aims of this investigation was to use the *in vitro* haemangioma model (Tan *et al.*, 2000_b) to help elucidate the mechanisms of action of existing therapies, such as IFN α , and also identify novel agents for the treatment of haemangioma. Findings from this research identified TRAIL in combination with a low dose of IFN α as a potential treatment for proliferating haemangioma. Results showed that the combined effect of TRAIL with a low dose of IFN α acted synergistically and inhibited capillary-like outgrowth from haemangioma explants to a similar extent as the singly added high dose of IFN α . Since TRAIL is known to be non-toxic to normal cells, this novel finding of its effect against haemangioma capillary-like outgrowth deserves further investigation.

7.1.1.3 THE IDENTIFICATION OF STEM CELLS AND THEIR ROLE IN HAEMANGIOMA PROGRESSION

The proliferative phase of haemangioma is characterised by rapid proliferation of ECs with the accumulation of other cellular elements involved in angiogenesis including MCs, pericytes and macrophages (Takahashi *et al.*, 1994). However, the most curious biological characteristic of haemangioma is the spontaneous regression. Although various cell types and cell-specific markers have been identified in haemangioma, the mechanisms of origin of the heterogeneous cell population, their post-natal presentation and their distinctive pattern of limited growth and involution are poorly understood.

In this study, EPCs and MSCs were purified from haemangioma tissues. We postulate that abnormal stem cell development and differentiation underlies the aetiopathogenesis of haemangioma. Although EPCs and MSCs have been previously reported in haemangioma (Yu *et al.*, 2004_a; Yu *et al.*, 2006), this study showed that the cellular structures that rapidly emanated from explanted proliferative haemangioma biopsies were MSCs as they readily differentiated into osteocytes and adipocytes *in vitro*. However, the cells obtained from the direct dissociation of proliferating lesions were characterised as immature EPCs. These EPCs then further differentiated into MSCs with continued culture *in vitro*. This *de novo* transdifferentiation process *in vitro* may be due to the culture of the EPCs in the absence of growth factors which were present in the original lesion. This study also found that the MSCs that differentiated from EPCs maintained their plasticity into adipocytes *in vitro*. Since adipocyte deposition is associated with the spontaneous involution of haemangioma, we hypothesise that this process of transdifferentiation from EPCs to MSCs and MSCs to adipocytes regulates the biology of haemangioma.

7.1.1.4 HOW DO THE FINDINGS FROM THIS STUDY FIT IN WITH THE CURRENT KNOWLEDGE OF HAEMANGIOGENESIS?

The high incidence of haemangioma in the head and neck region together with the solitary and segmental post-natal presentation of these lesions led Waner *et al* (2003) to suggest that developmental detriments may be influential in the pathogenesis of haemangioma.

To put the findings of this investigation into context, the developmental origins of the face are briefly described.

The face begins to form during the 4th week of embryological development with the formation of the mesenchyme. The head mesenchyme is distinctive in that it is derived from neural crest cells rather than mesoderm (Risau & Flamme, 1995). The neural crest cells that form the face migrate dorsolaterally from the neural tube to

produce five craniomesenchyme prominences consisting of a single frontonasal prominence, paired maxillary and paired mandibular prominences (Mitchell & Sharma, 2005). These cranial neural crest cells are encoded with specific morphogenetic information prior to their migration (Gilbert, 1994). These prominences then differentiate into cartilage, bone, cranial neurons, glia and the connective tissues of the face including the corneal endothelium and stroma and the dermis and adipose tissue of the skin. However, the fate of the neural crest cells depends to a large degree upon where the cells migrate and settle and to the local environmental cues (Le Douarin *et al.*, 2004).

Neural crest-derived mesenchyme unlike mesoderm-derived mesenchyme, do not possess endogenous angioblasts and must rely on in-migrating angioblasts and vascular sprouts for vascularisation (Noden, 1988). Angioblasts are the EC precursors that form the primitive vascular channels during embryonic development (Risau *et al.*, 1988; Risau, 1997). Angioblast migration is particularly prominent in the head region implying the presence of increased levels of motility or chemotactic factors (Noden, 1991). Therefore, Waner *et al* (2003) proposed that haemangioma preferentially occurred in regions where neural crest-associated angiogenic mechanisms were elevated.

The association between segmental haemangioma and PHACES syndrome deserves special consideration. Several of the associated anomalies, such as lingual thyroid and sternal clefting, have their origin between 6 and 8 weeks of gestational age, suggesting a so-called developmental-field defect (Frieden *et al.*, 1996). Furthermore, the parotid glands are frequently involved by overlying plaque-like haemangiomas. These glands also begin their development at about 6 weeks of intra-uterine life. Waner *et al* (2003) suggested that the precursors of parotid hemangiomas may have reached the region during this early time frame, with proliferation held in check until latter in gestation or after birth. The authors also suggested that the differential patterns of tissue involvement seen in focal and segmental haemangioma may reflect the timing of

deposition of haemangioma precursor cells, with earlier events resulting in segmental haemangioma and later events resulting in focal haemangioma. These ideas, although speculative, suggests that haemangioma is not just a tumour but may be a developmental problem associated with abnormal angiogenesis.

In order to explain the fascinating relationship between haemangioma and the placenta, Waner *et al* (2003) suggested that the regions of growing neuromesenchyma represented the 'fertile ground' for the implantation and proliferation of embolised placental cells or aberrant angioblasts by virtue of their inherent need to attract invading vascular precursors.

In view of these ideas and the findings from our investigation, we propose a model for the involvement of neural crest cells in the pathogenesis of haemangioma (Figure 7.1). In this model, some migrating neural crest cells possess abnormalities that may be caused by mutations (Figure 7.1-A). While normal neural crest-derived mesenchyma recruit angioblasts as part of the normal developmental process, the presence of abnormal neural crest cells leads to excessive recruitment of these cells resulting in the presence of excessive EPCs in the region of the haemangioma (Figure 7.1-B). We propose that these EPCs are recruited either from the developing vasculature or embolised from the placenta. A placental origin for these EPCs could explain the expression of various placental antigens by the ECs in haemangioma.

We hypothesise that EPC proliferation may be inhibited at this stage by passive immunity within the intra-uterine environment afforded by maternal circulation. During gestation, the maternal immune response against the foetus occurs in part by secretion of immuno-suppressors through the placenta (Jauniaux *et al.*, 1995). Circulating maternal angiogenesis inhibitors have also been described during pregnancy (Klauber *et al.*, 1997; Levine *et al.*, 2004). Thus, birth removes the intra-uterine suppression of angiogenesis and allows the proliferation of a pre-established lesion. This could explain the observed post-natal growth of these lesions. Alternatively, in the immediate post-

natal period, an unknown factor could stimulate excessive angiogenesis in the region of the ‘nascent’ haemangioma. The idea that hypoxia may initiate and maintain haemangioma growth is supported by a number of clinical observations, although direct experimental data is not yet been available. For example, a blanched area of skin is usually noticed before the actual growth of the lesion. This may be an area of local ischaemia in the skin, caused by an unknown event that creates a hypoxic environment and thus, triggers growth factor expression. Studies have shown increased expression of IGF-2 and VEGF in response to hypoxia (Yu *et al.*, 2004_b; Levy *et al.*, 1998; Stein *et al.*, 1998; Tong *et al.*, 2006). This could explain the high levels of these growth factors seen during the proliferating phase of haemangioma. Also observed on haemangioma ECs is GLUT-1, another hypoxia-inducible molecule (Loike *et al.*, 1992).

We hypothesise that the high levels of VEGF and IGF-2, in turn, induces proliferation of the EPCs and results in the recruitment of support cells including MCs, pericytes and macrophages. At this stage the EPCs are protected from apoptosis due to OPG expression. All these events ultimately result in increased blood vessel formation that characterises the proliferative phase of haemangioma (Figure 7.1-C). Preliminary results suggest that STAT-5 is activated in response to VEGF.

However, in the early stages of the involuting phase, an unknown angiogenic inhibitory factor is induced which results in the down-regulation of growth factor levels within the lesion. The consequence of low levels of VEGF (and other growth factors) results in the differentiation of EPCs into MSCs. These MSCs, in turn, differentiate into adipocytes that form the fibro-fatty tissue seen in involuting and involuted lesions (Figure 7.1-D). The signal for MSC differentiation into adipocytes *in vivo* is unclear. We propose a role for insulin and Dexamethasone in the adipogenesis of haemangioma, as these factors are involved in adipocytic differentiation *in vitro*.

The trigger for involution has also been suggested to be due to the ‘loss of protection’ of haemangioma cells from immune surveillance mechanisms (Ritter *et al.*,

2003; Friedlander *et al.*, 2005). Recent research has suggested that an immune response may be involved in the proliferation and involution of haemangioma. Ritter *et al* (2003) reported the expression of peripheral T cell surface markers, CD3 and CD8, and suggested a cytotoxic T cell phenotype in haemangioma. These authors also identified high levels of indoleamine 2, 3 dioxygenase (IDO) in proliferating haemangioma which decreased during involution. IDO is an enzyme involved in the tryptophan degradation. Availability of tryptophan is critical to the function of T cells in promoting apoptosis (Frumento *et al.*, 2002). Ritter *et al* (2003) therefore suggested that the high levels of IDO and concomitant low tryptophan concentrations in the proliferative phase delays the onset of involution. In retrospect, the trigger for involution could occur as a consequence of low IDO levels, high tryptophan concentrations and therefore increased T cell cyto-toxicity within the lesion.

Although a number of aspects in the model shown in Figure 7.1 are speculative, the possible link between neural crest cell development and the non-random facial presentation of haemangioma is an exciting notion that warrants further investigation.

7.1.1.5 THE OVERALL SIGNIFICANCE OF THIS RESEARCH

This investigation has resulted in an improved understanding of the cellular and molecular mechanisms regulating haemangioma proliferation and involution. The identification of an EPC to a MSC differentiation which may be responsible for governing the biology of haemangioma progression and the identification of TRAIL as potential therapy for haemangioma represent novel findings. From a clinician's perspective, the ultimate aim of haemangioma research is to acquire substantial understanding of haemangiogenesis that would allow the development of effective treatments for haemangioma. The results presented in this thesis have led to a better understanding of the pathogenesis of haemangioma with the potential to develop novel treatment strategies for this common tumour of infancy.

Figure 7.1: A proposed model for the development of haemangioma based on the speculative ideas and findings from this investigation. (see section 7.1.1.4 for details).

C

AFTER BIRTH

Proliferating phase

Hypoxia?

Loss of inhibitory factors

e.g., Loss of maternal immunity?

Low IFN- β in the overlying dermis?

Expression of other angiogenic factors?

Excessive Angiogenesis

VEGF
+ IGF-2
+ Other
growth
factors

STAT-5
signalling ?

EPCs
survival
and
proliferation

Recruitment of support cells,
e.g., MCs, macrophages,
pericytes

Excessive blood vessel
formation

■ OPG

Figure 7.1: A proposed model for the development of haemangioma based on the speculative ideas and findings from this investigation. *contd* (see section 7.1.1.4 for details).

D

AFTER BIRTH

Figure 7.1: A proposed model for the development of haemangioma based on the speculative ideas and findings from this investigation. (contd) (see section 7.1.1.4 for details).

APPENDIX 1: CHAPTER 3

1.1 PRELIMINARY EXPERIMENTS

1.1.1. METHODS

1.1.1.1 QUANTITATIVE REAL TIME PCR

In order to perform qRT-PCR accurately, a number of preliminary experiments were performed. These included (i) optimisation of PCR parameters for each primer set to yield PCR products of the predicted sizes without spurious non-specific bands and primer dimer, (ii) testing the efficiency of each primer set and (iii) identification of an appropriate house keeping gene to use as the internal reference gene. The importance of the house keeping gene and a brief introduction to qRT-PCR is discussed in section 2.1.1.1 (page 55).

1) *Testing the PCR Efficiencies of Each Primer Pair:* RNA purified from snap frozen normal skin was reverse transcribed into cDNA as described in sections 2.1.1.2.1 and 2.1.2 (pages 58 and 59). qRT-PCR was carried out as outlined in section 2.1.4 (page 65). PCR products were then separated by electrophoresis on 2.5% Agarose gels (See Appendix 4, section 4.1.4, page 295). PCR products were excised and purified from the gels as described in Appendix 4, section 4.1.5 (page 296).

Efficiencies for all the primer pairs used in this study were determined from diminishing dilutions of the respective purified PCR products. An initial 500-fold dilution of the purified PCR product was prepared from which eight-fold serial dilutions were prepared. 1 µl of each dilution was then used as template and amplified in duplicate by qRT-PCR for each primer set. The threshold Ct was plotted against the Log of template concentrations and the slope of the line generated was used to calculate the Efficiency (E) according to the equation:

$$E = 10^{[-1/\text{slope}]} - 1 \text{ (Rasmussen, 2001).}$$

Efficiencies for all the primer pairs were calculated to be approximately 2 which is the theoretical maximum (Rasmussen, 2001).

2) *Validation of an Appropriate Internal Reference Gene*: Two approaches were used to identify an appropriate internal reference gene for this study.

A) RNA was purified from six proliferating, eight involuting and seven involuted haemangioma biopsies and cDNA was synthesised using random hexamers as described in section 2.1.2 (page 59). GAPDH, CycA and 18SrRNA were amplified by qRT-PCR and the ΔC_t values of GAPDH and CycA mRNA were calculated relative to 18SrRNA using the formula :

$$\Delta C_t = C_{t(\text{GAPDH or CycA})} - C_{t(18\text{SrRNA})}$$

B) cDNA was also synthesised using Oligo(dT)₂₀. qRT-PCR was performed for GAPDH and CycA and the absolute C_t values were calculated when 500 ng of total RNA was used in the cDNA synthesis reaction.

1.1.1.2 IMMUNOHISTOCHEMICAL OPTIMISATION

HeLa cells, HUVECs, EOMA cells and placenta tissue sections were initially used to optimise the IHC staining procedures for the various antibodies used in this study. Cultured cells were grown onto 13 mm diameter borosilicate glass coverslips and cultured in their respective culture medium as described in section 2.1.6.2 (page 67). Immunostaining for STAT-3, p-STAT-3 (Tyr 705) and STAT-1 was performed on cultured HeLa cells fixed with 4% PFA and immunostaining for VEGFR-2 was done on HUVECs and EOMA cells fixed with a mixture of acetone:methanol, as described in section 2.1.8.2.2 (page 74). Fixed cells were incubated with primary antibodies against STAT-3, p-STAT-3 (Tyr 705), STAT-1 and VEGFR-2 followed by incubation with either an anti-rabbit DIG conjugate or an anti-mouse DIG conjugate followed by incubation with an anti-DIG-Rhodamine conjugate. Coverslips were stained in DAPI

(see section 2.1.8.3, page 75) and mounted in Antifade. Control coverslips were included in each experiment in which the primary antibody was omitted.

Formalin fixed paraffin embedded placenta sections were deparaffinised, rehydrated and subjected to a sodium citrate antigen retrieval step as outlined in section 2.1.8.2.1 (page 74). Sections were then blocked and incubated overnight at 4°C with rabbit anti- GLUT-1 primary antibodies. Bound GLUT-1 antibody was detected with an anti-rabbit Cy3 conjugate. Slides were then stained in DAPI and mounted in Antifade.

1.1.1.3 STATISTICS

The non-parametric Kruskal Wallis by ranks test was used to identify changes in CycA and GAPDH mRNA expression with 500 ng of RNA. This was followed by the multiple comparisons test to determine where the significant changes occurred. The R package was used to determine changes in mRNA expression relative to 18SrRNA. The linear mixed effects method of analysis was used to allow for repeated measures on subjects and for each transcript, a sequential bonferroni adjustment was made for multiple testing. Data is represented as relative fold changes in mRNA expression $\pm$ s.e.m.

1.1.2 RESULTS

1.1.2.1 PCR QUALITY CONTROL

PCR efficiencies were calculated as described in section 1.1.1.1 (page 267). All qRT-PCR assays were linear over a 10,000-fold range with Ct values ranging between 12.5 and 37 cycles (Figure 1.1). The correlation coefficients were consistently > 0.98 in each assay and each PCR amplification displayed an efficiency close to 2 (Figure 1.3). Melt-cure analyses were performed after every amplification to ensure the reactions were free from contaminating DNA. A typically melt-curve plot is shown in Figure 1.2.

Figure 1.1: An example of the cycling numbers obtained during a typical real-time PCR amplification of serially diluted PCR template. The Ct values changed proportionally according to the serial dilution of the sample. The figure above shows the PCR curves obtained when serially diluted STAT-1 PCR product was amplified by qRT-PCR. Samples that had Ct values below 30 were considered positive when there was no evidence of amplification in the no-template negative controls. A maximum of 1 Ct difference between duplicates was accepted.

Figure 1.2: The melt-curve obtained at the end of a PCR amplification with serially diluted STAT-1 template. As the amplified product have the same length and are of the same nucleotide ratio, they have the same melting temperature. This can be observed by a series of peaks one on top of each other on the melt-curve. Absence of other peaks indicates the absence of spurious non-specific product formation.

Figure 1.3: qRT-PCR primer pair efficiencies. The PCR amplification efficiencies using primers for STAT-1 (A), STAT-3 (B) and VEGFR-2 (C) were calculated as described in section 1.1.1.1. Serially diluted PCR product was amplified by qRT-PCR in duplicate. The slope of the line obtained by plotting the C_t value against $\text{Log [Concentration]}$ was then used to calculate the efficiency of the PCR reaction.

1.1.2.2 VALIDATION OF AN APPROPRIATE INTERNAL REFERENCE GENE

Primer pairs for four commonly used house keeping genes, 18SrRNA, GAPDH, CycA and HPRT, were designed for use in this investigation. An ideal reference gene would be one that is not differentially regulated across the three phases of haemangioma (proliferating, involuting and involuted) and is expressed at relatively similar levels as the gene of interest (Roche LC technical note 15/2002; Vandesompele *et al.*, 2002).

Preliminary experiments showed that a neat cDNA sample (from normal skin) amplifies 18SrRNA at cycle 10 ($C_t = 10$) whereas the test genes were not amplified until cycle 25 or later, representing an approximately 500,000-fold difference in abundance. Because the 18SrRNA transcript is far more abundant than all the test genes, it was not considered a suitable internal reference gene for this study. The use of HPRT as an internal reference was also discounted because of its late amplification in haemangioma samples.

To validate the use of GAPDH and CycA as internal reference genes, two different approaches were used. Absolute C_t values were calculated from proliferating, involuting and involuted haemangioma biopsies (eight per phase) for both genes when 500 ng of total RNA was used for the cDNA synthesis reaction. CycA mRNA expression changed between phases (Figure 1.4-A), with significant differences between the proliferative and involuting phase ($p < 0.01$) and also between the proliferative and involuted phase ($p < 0.01$). However, no significant change in GAPDH mRNA expression between the phases was identified ($p < 0.34$, Figure 1.4-B).

Figure 1.4: Validation of an appropriate internal house-keeping gene (Experiment 1). The Ct values obtained when qRT-PCR was performed with 500 ng of starting mRNA from each sample, were used to detect differences in CycA (**A**) and GAPDH (**B**) mRNA expression across phases of haemangioma. (**A**) Significant changes in CycA mRNA levels was detected between the proliferative and involuting phase and between the proliferative and involuted phase (** $p < 0.01$). (**B**) GAPDH mRNA levels did not vary between phases.

For the second approach, qRT-PCR was performed for GAPDH and CycA using 18SrRNA as the internal reference gene. The use of 18SrRNA was accepted in this case as it was found to be expressed in the same dynamic range as GAPDH and CycA. The Δ Ct values for GAPDH and CycA mRNA were calculated relative to 18SrRNA using the formula Δ Ct = Ct_(GAPDH or CycA) – Ct_(18SrRNA). Relative to 18SrRNA, GAPDH and

CycA mRNA levels did not change between phases ($p < 0.1229$ and $p < 0.054$, respectively) (Figure 1.5). However, since the p value obtained for CycA expression ($p < 0.054$) was so close to the chosen threshold for significance, GAPDH was chosen as a more suitable reference gene for this study.

Figure 1.5: Validation of an appropriate internal house-keeping gene (Experiment 2). To detect changes in CycA (A) and GAPDH (B) mRNA between proliferating, involuting and involuted lesions, ΔC_t values for CycA and GAPDH mRNA relative to 18SrRNA were analysed. Statistical analysis was performed using the R package as described in section 1.1.1.3. Relative to 18SrRNA, no statistically significant change in CycA or GAPDH mRNA expression was identified across the phases.

1.1.2.3 IMMUNOHISTOCHEMICAL OPTIMISATION

Paraffin embedded placenta sections, HeLa cells and EOMA cells were used at the start of this study to optimise the IHC staining procedures and to determine antibody specificity. Control slides in which the primary antibody was omitted showed no staining. Paraffin embedded tissue sections exhibited some background fluorescence, thought to be due to the autofluorescence of collagen fibres. A tissue bleaching step was therefore introduced into the immunostaining protocol using NaBH_4 which effectively reduced the problem, allowing specific signal to be detected.

HeLa cells in culture expressed STAT-3 (Figure 1.6-A). However, STAT-1 and p-STAT-3 (Tyr 705) expression was low in these cells (Figure 1.6-C and -E, respectively). Treatment with $\text{IFN}\alpha$ -2b for 10 min induced STAT-1 and p-STAT-3 (Tyr 705) expression (Figure 1.6-D and -F, respectively).

As expected, positive immunoreactivity was detected for VEGFR-2 and GLUT-1 when IHC staining was performed on EOMA cells and placenta tissue sections respectively (Figure 1.7). Previous studies have shown that VEGFR-2 is expressed by EOMA cells (Ilan *et al.*, 2003), and GLUT-1 is expressed in placenta (Hahn *et al.*, 1998).

Figure 1.6: Immunohistochemical localisation of STAT-3, p-STAT-3 (Tyr 705) and STAT-1 on HeLa cells. Cells were fixed and stained with rabbit anti-STAT-3 (**A**), rabbit anti-p-STAT-3 (Tyr 705) (**C&D**) or mouse anti-STAT-1 (**E&F**) antibodies. Bound antibodies were detected with either an anti-rabbit DIG conjugate or an anti-mouse DIG conjugate followed by incubation with an anti-DIG-Rhodamine conjugate (red). Cell nuclei were counterstained with DAPI (blue). (**A**) STAT-3 was expressed by unstimulated HeLa cells growing in culture. IFN α -2b (10,000 U/ml) stimulation for 10 min induced both p-STAT-3 (Tyr 705) (**D**) and STAT-1 (**F**) expression as compared to unstimulated cells (**C&E**, respectively). No staining was detected when the primary antibodies were omitted (**B**). Scale bars: 500 μ m (**A&B**), 100 μ m (**C&D**), 50 μ m (**E&F**).

Figure 1.7: Immunohistochemical localisation of VEGFR-2 on EOMA cells and GLUT-1 on placenta tissue sections. EOMA cells were fixed and stained with rabbit anti-VEGFR-2 antibodies. Bound antibodies were detected with an anti-rabbit-DIG conjugate followed by an anti-DIG-Rhodamine conjugate (Panel A, red staining). Placenta sections were incubated with rabbit anti-GLUT-1 antibodies followed by an anti-rabbit Cy3 conjugate (Panel C, red staining) for visualization. Cell nuclei were counter stained with DAPI (blue). No staining was detected in the negative controls (Panels B&D, respectively). The red staining seen in Panel D are the autofluorescent blood cells. Scale bars: 50 μ m.

1.1.2.4 WESTERN BLOTTING

1.1.2.4.1 Lysate preparation, transfer conditions and antibody specificity

In preliminary experiments, snap frozen normal skin and HeLa cells were used to optimise the Western blotting technique. Three lysis buffers were tested, (i) RIPA buffer, (ii) 50 mM Tris-HCL, pH 6.8, 5 mM EDTA and 2%SDS, and (iii) RIPA buffer lysis buffer containing 2% SDS and additional protease and phosphatase inhibitors. This modified RIPA buffer was chosen because it was highly reducing and effectively prevented the action of proteases and phosphatases from degrading the proteins within the samples. This allowed for long-term storage of the samples at -80°C for several

months. Proliferating haemangioma tissues had a higher concentration of proteins (570 µg/mg) than involuting and involuted haemangioma tissues (114 µg/mg and 27 µg/mg, respectively).

Skin was crushed to a powder in liquid nitrogen before the addition of the lysis buffer in order to maximise protein output as the skin tissue became tough and rubbery when solid pieces were put directly into the lysis buffers.

Complete transfer of proteins onto the PVDF membranes was a problem in this study. Therefore, a number of different combinations of the basic Western blotting transfer buffer components and transfer conditions were tested to ensure 90-95% of the protein bands were transferred onto the membranes. Increasing transfer time and adding SDS along with methanol to the transfer buffer resulted in the transfer of 90-95% of the protein onto the PVDF membranes. Following transfer, gels were stained with Comassie Blue-R (See Appendix 4, section 4.2.5, page 298). In initial experiments, prior to the blocking step, the membranes were also stained with Ponceau S to detect the presence of the fractionated proteins. The recipe for the Ponceau S stain is given in Appendix 4, section 4.2.22 (page 301).

1.1.2.4.2 Antibody quantitation

In preliminary experiments, lysates prepared from normal infant skin and Hela cells, treated and untreated with IFN α -2b (10,000 U/ml), for 10 min were used to show the staining patterns for STAT-3, p-STAT-3 (Tyr 705), STAT-1, CycA and GAPDH antibodies. All antibodies showed immunoreactive bands at the appropriate sizes and also stained in a quantitative manner i.e., when increasing amounts of protein lysates were run on the NuPage MES gels, there was an increase in band intensity of the correct size on the membrane (Figure 1.8). Figure 1.8-B and -C show decreasing band intensities with a decrease in protein concentration as identified by densitometry analysis using the Image J software. These results indicate that this method of quantification can be used to measure protein changes in haemangioma.

Figure 1.8: Antibody specificity by Western blotting. Decreasing amounts of total protein from HeLa cell lysates showed concomitant decrease in band intensity for STAT-3 (**A**). This decrease was quantifiable using the Image J program. Panel **B** and **C** show the decrease in STAT-3 (**B**) and p-STAT-3 (Tyr 705) (**C**) band densities obtained with decreasing amounts of total protein.

1.2 GENE EXPRESSION CHANGES IN HAEMANGIOMA

Figure 1.9: Correlation between STAT-3, STAT-1 and VEGFR-2 mRNA expression in proliferating, involuting and involuted haemangiomas. The mRNA levels for STAT-3, STAT-1 and VEGFR-2 were determined in haemangioma biopsies from the different clinical phases using qRT-PCR. The threshold value (Ct) was determined for each gene and then expressed as a difference (Δ Ct) between that for the target gene and that for internal house-keeping gene, GAPDH. Low Δ Ct values reflect greater mRNA expression. Proliferating haemangiomas ($\triangle$) had greatest STAT-3 and VEGFR-2 expression compared with involuting (+) and involuted ($\diamond$) lesions while involuting lesions expressed slightly higher STAT-1 mRNA than proliferating and involuted lesions. **(A)** STAT-3 and STAT-1 mRNA expression were correlated (Spearman rho $r = 0.692$; $p < 0.001$). **(B)** STAT-1 and VEGFR-2 mRNA expression were correlated (Spearman rho $r = 0.750$; $p < 0.001$).

Figure 1.10: STAT-3 expression correlates with proliferation in proliferating haemangioma. Black columns show STAT-3 positive cells, white columns show PCNA positive cells and grey columns show number of cells that stain positive for STAT-3 and PCNA. ‘Proliferating’ areas within proliferating lesions contained the most number of STAT-3 positive cells which decreased in the ‘involuting’ and ‘involved’ areas. ‘Involved’ areas contained few STAT-3 and PCNA positive cells. Percentage of cells that stained positive for STAT-3, PCNA and those that expressed both STAT-3 and PCNA (STAT-3+PCNA) are given in the corresponding table below each graph.

Figure 1.11: STAT-3 expression correlates with proliferation in involuting haemangioma. Black columns show STAT-3 positive cells, white columns show PCNA positive cells and grey columns show number of cells that stain positive for STAT-3 and PCNA. ‘Proliferating’ areas within an involuting lesion contained the most number of STAT-3 positive cells which decreased in the ‘involuting’ and ‘involuting’ areas. There were also fewer PCNA positive cells in the ‘involuting’ area as compared to the ‘proliferative’ area. The ‘involuting’ areas were devoid of any PCNA expression. ‘Involuting’ areas contained very few STAT-3 positive cells. Percentage of cells that stained positive for STAT-3, PCNA and those that expressed both STAT-3 and PCNA (STAT-3+PCNA) are given in the corresponding table below each graph.

APPENDIX 2: CHAPTER 4

Figure 2.1: Haemangioma explant model (1). Tissue taken from a proliferative haemangioma was embedded in fibrin gel and cultured as described in sections 2.3.2 and 2.3.3. Photographs were taken on day 7 in culture. **(A)** Phase contrast image showing the entire tissue piece with capillary-like outgrowths emanating from the tissue perimeter. Panel **B** and **C** are the enlargements of the areas outlined in Panels **A** and **B**, respectively. Scale bars: (A) 500 μm , (B) 200 μm and (C) 100 μm .

Figure 2.2: Haemangioma explant model (2). Proliferating haemangioma biopsies were cultured as described in sections 2.3.2 and 2.3.3. Panels **A** to **F** are the phase contrast images showing that the capillary-like structures follow a circular pattern of growth around the original tissue piece. Photographs were taken on day 14 in culture. Scale bars: 500 μm .

Figure 2.3: Phase contrast images showing the growth of a different cell type from haemangioma explants. 4-5% of cultured proliferating haemangioma explants gave rise to another cell type emanating from its surface. These cells exhibited the morphology similar to that of ECs. Photographs were taken on day 16 in culture. Scale bars: (A) 1000 μm and (B) 500 μm .

Figure 2.4: qRT-PCR results showing mRNA expression patterns for Collagen I, -II, -III, -VIII, Laminin-V, IGF-2 and VEGF-2 in HaemDMSCs and their original biopsy of origin. (A) Black columns show mRNA expression in HaemDMSCs and white columns show expression in the tissue piece. HaemDMSCs expressed similar levels of mRNA for Collagen I (Col-I), -II, -III and IGF-2 as the original biopsy from which they originated. They expressed more mRNA for Col-VIII but lower levels of VEGF mRNA than the tissue. mRNA for Laminin V (Lam-V) was not detected in the HaemDMSCs. (B) An ethidium bromide stained 1% agarose gel confirming that the amplified products were of the expected size.

Phase	Patient	Passage	Collagen-I	Collagen-II	Collagen-III	Collagen-VIII	Laminin-V	VEGF	IGF-2
Prolif	HaemDCs-A	3	1.3	9.8	1.9	2.6	-	7.5	12.8
Prolif	HaemDCs-B	3	6.2	13.3	4	4.4	-	10.1	-
Prolif	HaemDCs-C	3	7	12.8	4.5	8.5	-	7.1	14.4
Prolif	HaemDCs-D	3	6.5	13	5.4	5.7	-	-	11.3
Prolif	Tissue-A	NA	5.1	12.9	2.9	10.4	12.5	2.6	10
Prolif	Tissue-B	NA	3.7	9.9	1.8	-	9.3	2.9	9.6
	HUVECS	12	-	-	12.2	6.6	13.6	10.6	13.1
	3T3 Fibroblasts		-	-	-	-	-	-	-

Table 2.1: mRNA characterisation of HaemDCs. qRT-PCR was used to detect mRNA transcripts for various markers in HaemDCs. The table shows the raw Δ Ct values (relative to GAPDH) obtained from HaemDC isolates designated HaemDCs-A, HaemDCs-B, HaemDCs-C and HaemDCs-D. The original tissue pieces of HaemDCs-A and HaemDCs-B, HUVECs and 3T3 fibroblasts were also analysed in parallel. Key: ‘-’ indicates no amplification showing absence of mRNA transcripts; NA: Not applicable. See section 4.3.3.3 for details.

Figure 2.5: Adipocytic differentiation conditions does not induce the nuclear translocation of p-STAT proteins in HaemDMSCs. Immunolocalisation for STAT and p-STAT proteins was performed as described in section 2.3.14.1. Panel **A**: Immunolocalisation of p-STAT-3 (Tyr 705) (red) and STAT-3 (green) in HaemDMSCs before (**A&B**, respectively) and after stimulation with adipocytic differentiation media for 24 hrs (**B&D**, respectively). Cell nuclei were counterstained with DAPI (blue). Panel **B**: Immunolocalisation of p-STAT-5 (Tyr 695) (red) and STAT-5 (green) in HaemDMSCs before (**A&B**, respectively) and after stimulation with adipocytic differentiation media for 24 hrs (**B&D**, respectively). Scale bars: 20 μm .

2.1 PURIFICATION OF HAEMANGIOMA DERIVED ENDOTHELIAL CELLS

Boye *et al* (2001) have purified ECs from fresh haemangioma biopsies using Ulex-lectin coated magnetic beads. In this study, a similar protocol was used except that magnetic beads coated with antibodies to the EC specific marker, CD31 was used. However, in this study, purification of ECs from fresh haemangioma biopsies using this technique was unsuccessful. This may be due to several reasons, (i) low quantities of starting material. Boye *et al* (2001) did not specify the weight or volume of starting material that allowed successful isolation of ECs in their lab. (ii) different culture media and growth supplements. During this study, the quantity of haemangioma tissue available was a limiting factor. Cell recovery was low after the collagenase digestion and magnetic bead separation steps performed as described in section 2.3.16 (page 99). Similar to the conditions described by Boye *et al* (2001), this study utilised both fibronectin and gelatin coated dishes for cell culture. In addition, most of the additional supplements were added to the culture media with the exception of FGF-2. Another difference was that MCDB 131 minimal medium was used instead of the endothelial basal medium (EBM) medium described previously (Boye *et al.*, 2001). Unfortunately, the few cells that survived the purification procedure did not survive passed the first passage to allow for immunohistochemical characterisation. Figure 2.7 shows the isolated cells in culture following bead purification from two haemangioma biopsies.

A

B

Figure 2.6: CD31⁺ cells isolated from two proliferating haemangioma biopsies. Photographs were taken on day 7 in culture. The beads used for purification remained attached to some of the cells in culture. Scale bars: (A) 500 μm , (B) 200 μm .

APPENDIX 3: CHAPTER 5

	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5		Patient 6	
	T	C	T	C	T	C	T	C	T	C	T	C
TRAIL	3.9	>16	6.6	>16	5.1	>16	4.9	>16	4.9	15.7	4.9	>16
DR4	5.9	9.5	11.5	9.4	7.1	11.5	7.6	12.4	7.9	13.5	6.8	11.8
DR5	8.6	13.5	15.1	14.8	9.8	15.1	10.8	13	21	16.3	10.6	15.8
DcR1	5.6	9	11.5	9.7	6.3	11.5	8.4	11.2	10.7	12.1	7.9	12.3
DcR2	8.5	4.9	6.5	5.5	10.9	5.8	8.3	6.4	9.9	4.4	8.3	7.4
OPG	11.9	1.2	11.6	2.1	10.1	9.9	11.9	4.5	9.7	3.7	9.1	4.7

Table 3.1: Differences in mRNA levels for TRAIL and TRAIL receptors in haemangioma tissue and HaemDMSCs. qRT-PCR was used to analyse mRNA expression levels of TRAIL and TRAIL receptors in proliferating haemangioma biopsies and in their respective HaemDMSC isolates. Values in the table are the raw Δ Ct values obtained for each gene from the haemangioma tissue pieces (T) and the HaemDMSCs isolates (C).

Figure 3.1: OPG protein expression in the media of HaemDMSCs conditioned over a 72-hour period. Lane 1: 40 µg of total protein from conditioned media, Lane 2: 5 µg of total protein from conditioned media Lane 3: contains an equivalent amount of concentrated unconditioned cell culture media. Following electrophoresis and transfer onto a PVDF membrane, the membrane was blocked with the mouse anti-OPG MAB8051 primary antibody. Lane 1 shows the presence of an immunoreactive band at 60 kDa from the conditioned media of HaemDMSCs (black arrow). In addition, a band at 220 kDa was also detected (red arrow) in Lanes 1 and 2. Both bands were not present in unconditioned media (Lane 3).

APPENDIX 4

4.1 GENERAL MOLECULAR BIOLOGY TECHNIQUES

4.1.1 RNA Precipitation

Where necessary, RNA was precipitated by adding 2.5 times the volume 100% ethanol and 1/10 sodium acetate (pH 5.2). This was left at -20°C for a minimum of 20 min and centrifuged at 13,000 X g for 15 min at 4°C. The pellet was air dried in a laminar flow hood for 10 min and resuspended in 10-15 µl of nuclease-free, sterile dH₂O.

4.1.2 PCR Amplification

During PCR set-up, all reagents were kept on ice. Latex gloves, dedicated pipettors and barrier tips were used in a designated PCR product free room to prevent contamination by previously amplified material. Reactions were performed using thin-walled microamp (Invitrogen) PCR tubes in Perkin Elmer GeneAmp PCR system 2700 (version 2.07 software). All reagents were purchased from Invitrogen. Reactions followed the general protocol of using a cocktail mix of buffer, deoxynucleotide triphosphates (dNTPs) and dH₂O. The use of a cocktail of common reagents ensures that reactions contain identical reagent concentrations and delineating larger volumes increase pipetting accuracy. All PCR reactions were performed alongside a no template (water) control and amplification from an equivalent amount of RNA that had not been reverse transcribed. The control was necessary to ensure contamination was not present. Amplification was verified by agarose gel electrophoresis of 5 µl of PCR product and staining with ethidium bromide.

4.1.3 PCR Reaction Using *Taq* DNA Polymerase

cDNA synthesised from normal skin was used as template to optimize annealing conditions and primer concentrations for all primer sets used in this study. Optimum

reaction conditions were obtained in 20 µl final volume with 1X PCR Buffer (20 mM Tris-HCL (pH 8.4), 50 mM KCL), 1.5 mM MgCl₂, 200 µM dGTP, dCTP, dATP and dTTP, 1 U Taq polymerase (Invitrogen), 1µl cDNA template with varying primer concentrations. A range of annealing temperatures was examined for each gene. The thermal cycling conditions used for the PCR amplification involved the following steps: an initial denaturation at 95°C for 2 min, followed by 35 cycles of a 3-cycle amplification: denaturation at 95°C for 15 sec, annealing of primers to the template DNA initially carried out at 5°C below the T_m of the primer with the lowest T_m for 20 sec and extension at 72°C for 30 sec. This 35 cycle sequence was then followed by 72°C for 5 min, and the reaction was then held at 4°C. T_m of a given primer was calculated using the formula:

$$T_m = [3 \times G/C] + [2 \times A/T]$$

The resulting PCR fragments were visualised by agarose gel electrophoresis as outlined in section 4.1.4. For each gene, primer concentration titrated to 0.1 µM with an annealing temperature of 60°C produced a clean PCR fragment of the expected size with no visible non-specific products or primer dimers. Therefore, these conditions were used for all qRT-PCR amplifications (see section 2.1.4, page 65).

4.1.4 Agarose Gel Electrophoresis

DNA samples were mixed with 1 µl of 6 x agarose loading buffer (30% glycerol (v/v) in dH₂O, 0.025% bromophenol blue) and loaded into 1% agarose gels. The agarose gels were prepared by dissolving the appropriate amount of low melting point agarose (Sigma) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The solution was heated in a microwave to facilitate dissolution of the agarose and then cooled by running the flask under cold water. The solution was then supplemented with 1 µg/ml of ethidium bromide. The molten agarose was transferred into a cassette and a plastic comb inserted to form the lanes for loading the DNA samples. After polymerisation, the gel was immersed in TAE and the comb carefully removed. A 100

bp ladder (Invitrogen) was used as a standard. Electrophoresis was performed at 100 V for 45 min, or until the dye front was two-thirds of the way down the gel. Bands were observed under UV light and polaroid photographs taken.

4.1.5 Purification of DNA Fragments from Agarose Gels.

PCR products of the appropriate sizes were cut out of the agarose gel and purified using the QIAquick® gel extraction kit (Qiagen, GmbH, Germany). The slice was weighed, and 300 µl of QG buffer (pH 7.5) was added for every 100 mg of gel. The sample was incubated at 50°C until the gel slice had dissolved. Isopropanol (100 µl of 100%) was added per 100 mg of gel slice, and the sample was mixed. The sample was then loaded to the upper reservoir of a QIAquick® spin column provided in the kit placed in a 2 ml collection tube and centrifuged in a table top centrifuge for 1 min at 10,000 X g. Flow-through was discarded and 750 µl of PE buffer added. The solution was incubated for up to 5 min at room temperature before centrifugation for 1 min at 10,000 X g. After discarding the flow-through again, the column was centrifuged once again to elute the residual flow-through. The column was then placed in a clean 1.5 ml microcentrifuge tube, and 50 µl of elution buffer (10 mM Tris-HCL, pH 8.5) was added to the centre of the QIAquick® membrane. The sample was left to stand for 5 min to increase the DNA yield and then eluted by centrifugation at 13,000 X g for 2 min. Purified DNA was stored at -20°C until required.

4.1.6 Acetone Protein Precipitation

To each sample, four times the volume of ice cold acetone was added. The tubes were vortexed and incubated overnight at -20°C. The tubes were centrifuged at 13,000 X g for 10 min at 4°C and the supernatant was removed without disturbing the pellet. The uncapped tubes were airdried for 10-15 min on the bench top. The protein pellets were then resuspended in freshly prepared Western blotting lysis buffer.

4.2 STOCK SOLUTIONS, BUFFERS AND OTHER RECIPES

4.2.1 Phosphate buffered saline (PBS) 0.15 M pH 7.4

<u>Reagents</u>	<u>g/L</u>
NaCl	0.8 g
KCL	0.2 g
Na ₂ HPO ₄	1.15 g
KHPO ₄	0.2 g

PBS was made by dissolving the salts in dH₂O. The pH was adjusted to 7.4 using concentrated HCL or NaOH

4.2.2 Tris buffered saline (TBS)

<u>Reagents</u>	<u>g/L</u>
Tris	2.42 g
NaCL	8 g

TBS was made by dissolving the salts in dH₂O. The pH was adjusted to 7.6 using concentrated HCL

4.2.3 Tris Buffered Saline-Tween-20

TBST was prepared by adding 1ml Tween-20 to 1 L of TBS buffer.

4.2.4 RIPA Buffer (100 ml)

790 mg Tris base and 900 mg NaCl was dissolved in 75 ml dH₂O and the pH adjusted to 7.4. 10 ml of NP-40 and 2.5 ml of 10% Na-Deoxycholate was added and the solution was stirred until it turned colourless. 1 ml of 100 mM EDTA was added and the final volume adjusted to 100 ml with dH₂O. The solution was aliquoted and stored at -20°C. Final concentration: 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-Deoxycholate, 150 mM NaCl, 1 mM EDTA.

4.2.5 For Coomassie R-250 staining

Coomassie staining solution : 0.2 g Coomassie R-250, 10 ml acetic acid, 100 ml methanol was dissolved in 90 ml dH₂O.

Destaining Solution: 10% acetic acid, 40% methanol and 50% dH₂O.

4.2.6 Western blot transfer buffer

<u>Reagents</u>	<u>g/L</u>
Tris	3.03 g
Glycine	14.4 g
SDS	0.38 g
Methanol	150 ml

The reagents were combined and the volume made upto 1 L with dH₂O.

4.2.7 Stripping Buffer

2 g SDS, 750 µl β-mercaptoethanol and 10 ml Tris-HCL (0.5 M, pH 6.8) was dissolved in 90 ml dH₂O. Final concentration: 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCL pH 6.8.

4.2.8 4% Paraformaldehyde

4 g of PFA was added to 100 ml PBS. A few drops of NaOH were added followed by gentle heat treatment in a microwave. Once dissolved the pH was adjusted to 7.1 using concentrated HCL.

4.2.9 10 mM Sodium Citrate (pH 6.0)

This solution was made up fresh for each use. 0.3 g of tri-sodium citrate was dissolved in 100 ml of dH₂O. The pH was adjusted to 6.0 using a 1M HCL solution.

4.2.10 Csaba Stain

Briefly, the alcian-blue-safranin solution (Csaba stain) was prepared by adding 900 mg alcian blue, 45 mg of safranin, 1.2 g of ferric ammonium sulphate to 250 ml of 0.2 M sodium acetate buffer, pH 7.42.

4.2.11 Coating culture dishes with 1% gelatin

1 g of commercially available gelatin was dissolved in 100 ml of PBS by gentle heating in a microwave. Once dissolved this solution was autoclaved and filter sterilised through a 0.2 μ M filter. For coating culture dishes, enough 1% gelatin was added to cover the entire surface and the dish was left for at least 20 min in the incubator. Immediately before use, the gelatin was removed and the culture dishes were rinsed once with sterile PBS.

4.2.12 AG490 Stock Solution

AG490 powder was dissolved in 100% DMSO to a final concentration of 340 mM. A stock solution of 1mM was prepared by diluting 15 μ l of AG490 (340 mM) into 5 ml cell culture media. This solution was passed through a 0.2 μ M filter, aliquoted and stored at -20°C.

4.2.13 FACS buffer

100 ml PBS was combined with 100 mg sodium azide.

4.2.14 Alizarin Red Staining Solution

2 g of Alizarin red solution was dissolved in 100 ml of dH₂O. The pH was adjusted to 4.1-4.3 with 10 % ammonium hydroxide. This solution was stored at room temperature.

4.2.15 (A) Oil Red O stock stain

0.5 g of Oil red O (sigma) was dissolved in 100 ml of isopropanol and was stored at room temperature.

(B) Oil Red O working solution

Oil red stock stain was dissolved in dH₂O at a ratio of 3:2 and allowed to stand for a minimum of 10 min. The solution was then passed through a 75 µM cell strainer and used immediately.

4.2.16 Glycerin Jelly Mounting Medium

1 g of Gelatin was dissolved in 6 ml of dH₂O to which 7 ml of glycerol was then added. The mounting medium was stored at 4°C

4.2.17 Endothelial Cell Growth Supplement - For 3 mg/ml Stock Solution

15 mg of ECGS powder was resuspended in 5 ml pre-warmed PBS. To ensure dissolution, the vial was left at 37°C for 15 min and mixed at 5 min intervals. The solution was then filter sterilized through a 0.2 µM filter, aliquoted and stored at -20°C until required. ECGS was used in cell culture media at a final concentration of 15 µg/ml unless otherwise stated.

4.2.18 10% SDS Polyacrylamide Gels

(2 gels-1.5 mm thick)

<u>Reagents</u>	<u>g/L</u>
Acrylamide/bis (30% stock)	6.66 ml
1.5 M Tris pH 8.8	5 ml
dH ₂ O	8 ml
10% SDS in dH ₂ O	200 µl
TEMED	10 µl
10% ammonium persulphate	100 µl

The solutions were added in order shown. TEMED and 10% ammonium persulphate were added last to initiate polymerisation. The gel solution was added to the

casting apparatus and overlaid with isopropanol. The gel was left to polymerise for 30 min. The isopropanol was removed and the stacking gel was then made and cast on top of the separating gel.

4.2.19 4% Stacking Gel

(2 gels 1.5 mm thick)

<u>Reagents</u>	<u>g/L</u>
Acrylamide/bis (30% stock)	1.33 ml
0.5 M Tris pH 6.8	2.5 ml
dH ₂ O	6.1 ml
10% SDS in dH ₂ O	100 µl
TEMED	10 µl
10% ammonium persulphate	50 µl

4.2.20 Protein Gel Loading Buffer (10X)

To make 20 ml loading buffer, 11.3 ml Glycerol, 2.3 g SDS and 10 mg bromophenol blue was dissolved in 8.76 ml of Tris-Hcl (0.5 M, pH 6.8). Buffer was stored in aliquots at -20°C.

4.2.21 1X SDS Page Running Buffer (For 1 Litre)

3.03 g Tris base, 14.4 g Glycine, 1g SDS was dissolved in 1L of dH₂O.

4.2.22 Ponceau S Stain

Ponceau S Staining Solution was made up with 0.1% (w/v) Ponceau S 5% (v/v) acetic acid dissolved in dH₂O.

4.2.23 Western Blotting Apparatus

Bottom	Grey Side of Cassette Fibre pad 3 MM Filter Paper Electrophoresed Gel PVDF Membrane 3 MM Filter Paper Fibre pad
Top	Clear Side of Cassette

4.2.24 Sodium Orthovanadate

A 200 mM solution of sodium orthovanadate was prepared in dH₂O and the pH was adjusted to 10.0 using either 1M NaOH or 1M HCl. The yellow solution was boiled in a microwave until it turned colourless after which it was allowed to cool to room temperature. The pH was adjusted to 10.0 and the solution was boiled. These steps were repeated until the solution remained colourless and the pH stabilised at pH 10.0. Activated sodium orthovanadate was stored in 20 µl aliquots at -20°C until required.

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