

**REGULATION OF LYMPHANGIOGENESIS
BY INTERLEUKIN-7 (IL-7) AND THE
ASSOCIATION WITH LYMPHATIC
METASTASIS IN HUMAN BREAST CANCER**

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DEDICATION

I dedicate this thesis to my parents, my wife; Nada and our son; Ali.

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SUMMARY OF THE THESIS

The lymphatic system constitutes a major route for cancer cells to spread to the regional lymph nodes. Lymph node metastasis is considered to be a key prognostic factor in determining patients' prognosis in many solid tumours including breast cancer. However, studies on the lymphatic system had been very limited mainly due to the lack of biological and molecular markers that distinguish between blood and lymphatic vessels. The last few years however, have witnessed the identification of several novel molecular markers that are considered to be specific to the lymphatic endothelium. The detection of these specific lymphatic markers has led to the identification of the existence of intra-tumoural lymphatics and lymphangiogenesis (formation and growth of new lymphatic vessels) within tumours. Lymphangiogenesis is now considered to play a major role in cancer lymphatic spread in many tumours including breast cancer. It is thought that the production of some vascular endothelial growth factors (VEGFs) like VEGF-D, by cells within tumours could stimulate the generation of new or existing lymphatic vessels thereby provide a direct conduit for cancer cells to spread to the regional lymph nodes. The current knowledge of the regulation of lymphangiogenesis and the production of the vascular endothelial growth factors within the tumour microenvironment is still very poor. To this end, we sought to identify a potential regulator of lymphangiogenesis.

The results presented here demonstrated that amongst the many cytokines and growth factors tested, Interleukin-7 (IL-7) could indeed regulate lymphangiogenesis either directly via stimulating endothelial cell growth or indirectly via upregulating the expression of VEGF-D in these cells. The specificity of this effect of IL-7 was demonstrated by using neutralising antibodies and ribozyme transgenes.

In addition to endothelial cells, IL-7 was also found to have a direct effect on breast cancer cells. The detection of a fully functional receptor for IL-7, IL-7R, in these cells had enabled us to study the effects of IL-7 on breast cancer cells as well. IL-7 induced the growth of breast cancer cells and also upregulated the

expression of VEGF-D in these cells. Dissection of the signalling pathways of IL-7 in both endothelial and breast cancer cells revealed that IL-7 exerts these effects mainly via a Wortmannin sensitive pathway.

Clinically, IL-7 and its downstream signalling pathway signalling molecules levels of expression were correlated with higher lymphatic metastatic rate in human breast cancer. Additionally, breast cancers with higher levels of IL-7 were found in patients with the poorer prognosis and worst survival.

Therefore, it is concluded from these data that IL-7 plays an adverse effect in breast cancer metastasis. This effect of IL-7 is mediated via two arms; acceleration of lymphangiogenesis and stimulating breast cancer cells growth.

Inhibition of IL-7 signalling by using monoclonal antibodies, blocking agents to IL-7 or to one of its main intermediates could indeed be used as a way of targeted therapy to tackle metastatic spread of tumours particularly by the lymphatic route.

CHAPTER ONE

INTRODUCTION

1.1. LYMPHANGIOGENESIS

Lymphangiogenesis is the growth of new lymphatic vessels that occurs in both normal developing tissues and in pathological processes such as inflammation, wound healing and lymphoedema. Tumour lymphangiogenesis has been long considered to constitute a major route for tumour metastasis. The dissemination of malignant cells to the regional lymph nodes is an early step in the progression of many solid tumours (Pepper, 2001) and is an important determinant of prognosis. Until recently, research in this important process has been very slow largely due to the lack of biological markers specific to the lymphatic endothelium.

Tumour cell dissemination is mediated by mechanisms including local tissue invasion, lymphatic and blood spread or direct seeding of body cavities. Regional lymph nodes are often the first sites to develop metastases (Alitalo and Carmeliet, 2002; Oliver and Detmar, 2002), either draining via pre-existing afferent lymphatic vessels and / or via newly formed lymphatic capillaries. This is indeed the basis of the sentinel lymph node biopsy and indicates its particular importance in surgical management of cancers including breast, melanoma and others. However, not all tumours metastasise to the regional lymph nodes first. Furthermore, the presence of a metastasis in a lymph node does not necessarily mean that the tumour cells have been arrived via the lymphatic vessels (Van Trappen and Pepper, 2001). Tumour cells may pass directly into the blood vascular system through veno-lymphatic communications. The mechanisms determining whether regional lymph nodes or other sites first develop metastases

remain poorly understood. In fact, most disseminated tumour cells have a limited life span in blood stream. While many surviving cancer cells remain dormant in the host tissues, only a few develop into clinically detectable metastases. However, identification of those occult tumours cells, and prevention of their re-growth would be of great biological and clinical significance.

Tumourigenesis in humans is a multi-step process, and these steps reflect the genetic alterations that drive the progressive transformation to cancer. Contrary to normal cells, cancer cells have defective regulatory circuits that control normal proliferation and homeostasis. While normal cells require mitogenic signals to proliferate, malignant cells are self-sufficient for the growth signals and insensitive to the growth-inhibitory signals. Therefore, tumour cells are independent in generating their own growth signals. It has been well established that a complex series of cellular interactions between several types of cells like fibroblasts, immune cells, and endothelial cells as well as malignant cells within the tumour tissues could lead to cancer cells growth and metastasis (Hanahan and Weinberg, 2000). In addition to the ability to synthesize their own growth factors leading to an autocrine stimulation, cancer cells could indeed induce the stimulation of other cells like endothelial cells via a paracrine mechanism, thus generating neovascularisation in the local tumour microenvironment.

1.1.1 The lymphatic system

The initial description of putative lymphatic structures is credited to the Hippocrates, who described “white blood vessels” and thought they could be

glands. Later, Aristotle described lymphatic vessels as fibres with colourless fluid arranged between nerves and blood vessels. However, the first systematic characterization of the lymphatic system was made by Gasper Asellius in 1627 at about the same time William Harvey described the blood circulation (Witte *et al*, 2001). Some years later, Loise Petit made the first description of lymphatic metastatic spread during observing breast cancer metastasis in axillary lymph nodes (Swartz and Skobe, 2001).

The lymphatic system comprises a one way, open-ended complex network of capillaries, collecting vessels, lymph nodes, trunks and ducts. It is involved in transport of tissue fluids, extravasated plasma proteins and cells back into the blood circulation. Lymphatics also make an important part of the body's immunological surveillance system.

Formation of lymphatic vessels occurs early during foetal development by sprouting of endothelial cells. There are two main theories behind the embryonic endothelial cell sprouting. Sabin proposed the “centrifugal sprouting” theory; that is the development of the peripheral lymphatic system from isolated primitive lymph sacs exclusively by sprouting of endothelial cells into the surrounding tissues and organs (Clark, 1912; Sabin, 1902; Sabin, 1904). Most recent data favours this theory, including expression studies of lymphatic specific markers (Kaipainen *et al*, 1995; Kukk *et al*, 1996). The second theory of lymphatic development, the “centripetal sprouting”, was proposed by Huntington and McClure (Huntington and McClure, 1910). Huntington and McClure

proposed a vasculogenic mechanism for the development of the peripheral lymphatic system. In this theory lymphatic spaces would arise independently from the veins, fusing into a primitive lymphatic network and subsequently spread centripetally and connects to the venous system. The centripetally sprouting lymphatics would either integrate or replace the embryonic lymph sacs.

The lymphatic system seems to be an excellent pathway for malignant cell dissemination, because the initial lymphatics are much larger than blood capillaries and have incomplete basement membrane. Additionally flow velocity of lymph is much slower than blood flow and has similar consistency to that of the interstitial fluid enabling cell viability (Pepper, 2001; Sleeman *et al*, 2001; Swartz and Skobe, 2001). Conversely bloodstream is a highly aggressive medium for neoplastic cells due to serum toxicity, high shear stresses and mechanical deformation (Swartz and Skobe, 2001; Weiss, 2000). Additionally haematogeneous metastasis has low efficiency because a significant number of neoplastic cells are either quiescent or apoptotic due to hostile factors present in the serum (Mehes *et al*, 2001; Naumov *et al*, 2001). Furthermore, cancer cells may pass to bloodstream via lympho-venous shunts, high endothelial venules inside lymph nodes or may be drained through the thoracic duct (Pepper, 2001; Swartz and Skobe, 2001).

One of the major limitations of research on lymphatic vessels was the lack of histological, ultrastructural and immunohistochemical markers to accurately

discriminate between the lymphatic and blood endothelial cells. The main differences between lymphatic and blood vascular endothelia are listed in table

1.1.

Table 1.1 Summary of the main differences between lymphatic and vascular endothelium. ICAM: Intracellular adhesion molecule. JAM: Junctional adhesion molecule. PAL-E: Pathologische Anatomie Leiden – Endothelium. VEGFR-3: Vascular endothelial growth factor receptor-3. Prox-1: Prospero related homeobox gene-1. LYVE-1: Lymphatic vessel endothelial receptor-1. SLC: Secondary lymphoid chemokine. IP-10: IFN- γ -inducible protein-10.

	<i>Blood vessel</i>	<i>Lymphatic vessel</i>
Cell surface molecules	Von Willebrand factor (Factor VIII), VE-cadherin, ICAM, CD31, JAM1/JAM2, PAL-E (absent from arterioles and some capillaries) and others	VEGFR-3, prox-1, podoplanin, LYVE-1
Basement membrane	Present and continuous	Absent or incomplete basement membrane
Junction types	Tight junctions / Adherens / gap junctions.	Overlapping Loose junctions readily permit the passage of macromolecules, pathogens and migrating cells (not for larger ducts)
Enzymes	Presence of alkaline phosphatase and lack of 5'-nucleotidase	Lack of alkaline phosphatase and presence of 5'-nucleotidase
Chemokines	SLC	IP-10, Eotaxin
Pericytes	Mostly present (unreliable)	Mostly absent

Lymphatic capillaries are identified by the fact that they are lined by a single layer of endothelial cells, which are characterized by having poorly developed junctions with frequent large gaps between cells. These loose junctions readily permit the passage of large biological macromolecules, pathogens and migrating cells. Because pressure within lymphatic capillaries is only slightly higher than the interstitium, lumen patency is maintained by anchoring filaments that connect the abluminal surfaces of endothelial cells to the peri-vessels extracellular matrix (Leak, 1968; Pepper, 2001). Unlike blood capillaries, lymphatic capillaries lack a continuous basement membrane, and they are devoid of pericytes (Aukland, 1993). However, it should be noted that the latter is not true for larger collecting lymphatic ducts, which are supported by a thin connective tissue coat and higher up the lymphatic drainage tree by an additional smooth muscle wall. Although the initial lymphatics have no valves, the larger collecting ducts do have (Aukland, 1993). However these anatomical differences do not provide a practical way in the differentiation between blood and lymphatic vessels, particularly in regards to studies involving lymphatics.

1.1.2 Specific lymphatic markers

The ideal lymphatic endothelial marker would have some characteristics. It would be exclusively found (positive marker) on or excluded from (negative marker) lymphatic endothelial cells, rather than depending on relative differences in expression levels between blood and lymphatic vessels (Sleeman *et al*, 2001). They should be highly stable, specific, and sensitive. The main markers for the lymphatic endothelium are listed in table 1.2.

Table 1.2 Specific markers for the lymphatic endothelium, their expression sites and main biological function. VEGFR-3: vascular endothelial growth factor receptor-3, VEGF: vascular endothelial growth factor. LYVE-1: lymphatic vessel endothelial receptor-1. HA: hyaluronic acid. Nrp-2: neurorepellant receptor-2.

<i>Lymphatic marker</i>	<i>Molecular type</i>	<i>Sites of expression</i>	<i>Biological activity</i>	<i>Reference</i>
Podoplanin	Glomerular podocyte mucoprotein	Co-expressed with VEGFR-3 in lymphatic capillaries, vascular tumours, osteoblasts, renal podocytes and lung alveolar type-1 cells	Involved in maintaining lamellar permeability and the shape of podocyte foot processes in the kidneys	(Breiteneder-Geleff <i>et al</i> , 1997; Breiteneder-Geleff <i>et al</i> , 1999)
Prox-1	Transcription factor	Lens, heart, liver, pancreas and nervous system	Homeobox gene involved in the development and differentiation of lymphatic vessels. Prox-1 deficient neonates have failure of lymphatic sprouting and differentiation	(Wigle and Oliver, 1999)
LYVE-1	HA receptor-1	Kidney, pancreas, adrenal glands and thyroid gland	Transport of hyaluronan from extracellular matrix to lymph nodes	(Banerji <i>et al</i> , 1999; Carreira <i>et al</i> , 2001)
VEGFR-3	Receptor tyrosine kinase	Mainly on lymphatic vessels, but also reactivated in blood vessels in pathological conditions	Receptor for VEGF-C and VEGF-D	(Jussila <i>et al</i> , 1998; Kaipainen <i>et al</i> , 1995; Partanen <i>et al</i> , 2000; Valtola <i>et al</i> , 1999)
Desmoplakin	Endothelial adhering junction (complexus adhaerentes)	Small lymphatic endothelium, but absent from large lymphatic vessels such as the thoracic duct	Provide gaps through which macromolecules and circulating cells pass	(Ryan, 1989; Schmelz <i>et al</i> , 1994b)
Nrp-2	VEGF-C receptor	Lymphatic vessels endothelium	Neurorepellant semaphorins on neural cells	(Karkkainen <i>et al</i> , 2001)

1.1.2.1 Podoplanin

Podoplanin, is a 43 kDa surface glycoprotein that was recently cloned as a cell surface protein expressed on normal rat kidney podocytes, but not on podocytes in kidneys with a puromycin aminonucleoside nephrosis (PAN), a model for human minimal change nephropathy (Breiteneder-Geleff *et al*, 1997). It consists of 163 amino acids and has a single membrane spanning domain, two phosphorylation sites and six O-glycosylation sites in the large ectodomain. Originally, podoplanin was first cloned as OTS-8 in TPA-treated osteoplastic cells (Nose *et al*, 1990) and as the antigen recognised by the E11 antibody, which binds to osteoblast and osteocytes and is a marker for cells of the late osteogenic lineage (Wetterwald *et al*, 1996). An identical sequence was reported by Rishi *et al* (Rishi *et al*, 1995) as T1 α , a protein expressed on alveolar epithelial type 1 cells. The lung is a major site of podoplanin expression in the adult (Rishi *et al*, 1995; Wetterwald *et al*, 1996). Intravenous injection of antibodies against podoplanin caused proteinuria and flattening of podocytes, typical of the pathology seen in PAN suggesting that podoplanin is involved in maintaining lamellar permeability and the shape of podocyte foot processes (Matsui *et al*, 1998; Matsui *et al*, 1999).

Podoplanin is also expressed on epithelial cells of the choroid plexus cells and on lymphatic endothelial cells (Wetterwald *et al*, 1996). Light and electron microscopic immunohistology demonstrates the specificity of podoplanin expression on lymphatic but not blood vasculature endothelia in the skin (Breiteneder-Geleff, 1999). Furthermore, podoplanin was found to be expressed

on PAL-E-negative vessels and to co-localize with VEGFR-3 (Breiteneder-Geleff, 1999; Weninger *et al*, 1999). These studies suggest that podoplanin is a very promising marker for differentiating between lymphatic and blood vascular endothelium. To-date, the exact function of podoplanin is still unknown. However podoplanin may be involved in regulating the permeability of lymphatic vessels, or perhaps in maintaining their integrity (Sleeman *et al*, 2001).

1.1.2.2 Prospero related homeobox gene –1 (Prox-1)

Prox-1, the homologue of the *Drosophila* homeobox gene *prospero*, is a marker for the sub-population of endothelial cells that bud and sprout to give rise to the lymphatic system during early development (Wigle, 1999). Prox-1 gene spans more than 40 kb, consists of at least 5 exons and 4 introns and encodes an 83 kDa protein. Prox-1 gene is mapped to human chromosome 1q32.2 – q32.3. Chicken Prox-1 is highly expressed in the developing lens, retina and pancreas (Tomarev *et al*, 1996). Mouse Prox-1 expression was detected in the young neurons of the subventricular region of the CNS as well as the developing lens and the pancreas (Oliver *et al*, 1993).

Targeted deletion of the Prox-1 gene does not affect development of the blood vascular system, but the budding and sprouting of the developing lymphatics is ablated, suggesting that prox-1 plays a key role in lymphatic system development (Wigle and Oliver, 1999). These studies point towards a possible exclusive expression of prox-1 in lymphatic endothelium.

1.1.2.3 Lymphatic Vessel Endothelial receptor - 1 (LYVE-1)

LYVE-1 receptor is a type I integral membrane polypeptide expressed on the cell surface as a 60 kDa protein, which is reduced to approximately 40 kDa by glycosidase treatment (Banerji *et al*, 1999). LYVE-1 is abundant in spleen, lymph node, heart, lung, and foetal liver, less abundant in appendix, bone marrow, placenta, muscle, and adult liver, and absent in peripheral blood lymphocytes, thymus, brain, kidney, and pancreas. Expression of LYVE-1 is largely restricted to endothelial cells lining lymphatic vessels and splenic sinusoidal endothelial cells (Banerji *et al*, 1999). LYVE-1 may be involved in hyaluronan metabolism in the lymphatic system (Fraser *et al*, 1988; Fraser and Laurent, 1989; Sleeman *et al*, 2001). The co-localisation of LYVE-1 and hyaluronan on the luminal surface of lymphatic vessels suggests that HA may coat the lumen of lymphatic vessels through binding to LYVE-1 allowing hyaluronan-binding cells to adhere and migrate (Banerji *et al*, 1999).

The central core of the LYVE-1 Link module (C2-C3) is 57% identical to that of the human CD44 HA receptor, the only other Link superfamily HA receptor described to date with the closest homologue to LYVE-1. Nevertheless, there are distinct differences between LYVE-1 and CD44 suggesting that the two homologues differ either in the mode of HA binding or in its regulation. LYVE-1 receptor is almost exclusively restricted to lymph vessel endothelial cells, while CD44 is almost completely absent (Banerji *et al*, 1999). The highest concentration of LYVE-1 expression was found in submucosal lymph vessels underlying smooth muscle in the colon and the lacteal vessels of intestinal villi

that transport dietary lipid absorbed from the small intestine. CD44 is expressed abundantly in blood vessels and largely absent from lymphatic vessels (Picker *et al*, 1989). However, LYVE-1 is also expressed on sinusoidal endothelial cells of the spleen and placental syncytiotrophoblasts (Sleeman *et al*, 2001).

The development of antibodies against LYVE-1 has made the detection of lymphatics within tumours possible. For example, proliferating intratumoural lymph vessels have been identified in head and neck cancer (Beasley *et al*, 2002). Studies on LYVE-1 as a lymphatic marker was also helped in detecting lymphatics in primary malignant melanoma (Oliver and Detmar, 2002). Furthermore, the presence of LYVE-1 in tumours can indeed promote lymph node metastasis. Overexpression of VEGF-C in orthotopically transplanted MDA-435 or MCF-7 breast carcinoma (Mattila *et al*, 2002; Skobe *et al*, 2001b) or RIP1/Tag2-RIP1/VEGF-C transgenic mice (Mandriota *et al*, 2001), promoted proliferation of LYVE-1-positive lymph vessels and increased subsequent metastasis of tumour to lymph nodes. These studies indicate that LYVE-1 is a unique and highly specific marker to the lymphatic endothelium.

1.2.3.4 The vascular endothelial growth factor receptor - 3 (VEGFR-3)

While VEGFR-1 and -2 are expressed almost exclusively on vascular endothelial cells, VEGFR-3 is restricted to lymphatic endothelium (Eriksson and Alitalo, 1999; Olofsson *et al*, 1999; Veikkola *et al*, 2000). However, VEGFR-3 can also be up-regulated on tumour blood vessels (Partanen *et al*, 2000; Valtola *et al*, 1999). VEGFR-3, a tyrosine kinase receptor, has been shown to control the

development and growth of the lymphatic system. The importance of VEGFR-3 for the development of the lymphatic vasculature has been further strengthened by the fact that early onset primary lymphoedema is linked to the VEGFR-3 locus in distal chromosome 5q (Evans *et al*, 1999; Ferrell *et al*, 1998; Witte *et al*, 1998). A mutation in VEGFR-3 has been linked to hereditary lymphoedema (Ferrell *et al*, 1998). The mutation, which converts proline 1114 to leucine, occurs in the VEGFR-3 tyrosine kinase domain, indicating that a disturbance in VEGFR-3 signalling may play a part in the development of this disease. However, in the early embryonic development, VEGFR-3 is also essential for the formation of the primary cardiovascular network before the emergence of the lymphatic vessels, as VEGFR-3 knockout embryos die early in development because of cardiovascular failure (Dumont *et al*, 1998).

In humans, two isoforms of the VEGFR-3 protein occur, VEGFR-3S (short) and VEGFR-3L (long). The difference between the two lies in their carboxyl termini as a result of alternative mRNA splicing (Galland *et al*, 1993; Pajusola *et al*, 1993). VEGFR-3L is the predominant isoform in the tissues and it contains three additional tyrosyl residues, of which Tyr1337 serves as an important autophosphorylation site in the receptor (Fournier *et al*, 1995; Pajusola *et al*, 1993). The long isoform was able to mediate anchorage independent growth in soft agar and tumourigenicity in nude mice (Borg *et al*, 1995; Fournier *et al*, 1995; Pajusola *et al*, 1994).

Stimulation of VEGFR-3, using the specific ligands (VEGF-C and VEGF-D), induces a rapid tyrosine phosphorylation of Shc (Src homology collagen) and activation of MAPK (mutagen activated protein kinase) pathway results in an increased cell motility, actin reorganization and proliferation (Cao *et al*, 1998; Joukov *et al*, 1998). In a human erythroleukaemia cell line which expresses high levels of the VEGFR-3, VEGF-C stimulation induced activation of the signalling molecules Shc, Grb2 and SOS which lead to cell growth response (Wang *et al*, 1997). In these cells VEGF-C also induced tyrosine phosphorylation of the cytoskeletal protein paxillin by RAFTK, a member of the focal adhesion kinase family. The binding of VEGFR-3 to Grb2 is mediated by the Grb2 SH2 domain. The PTB (polypyrimidine tract binding protein) domain of Shc is required for Shc tyrosine phosphorylation by VEGFR-3 (Fournier *et al*, 1999; Fournier *et al*, 1995; Pajusola *et al*, 1994). Mutations in Shc phosphorylation sites increased VEGFR-3 transforming activity in the soft agar assay, suggesting that Shc has an inhibitory role in VEGFR-3 mediated growth response. Recently, VEGFR-3 has been found to be a strong activator of the signal transducers and activators of transcription (Stat-3 and Stat-5) (Veikkola *et al*, 2001). Stat proteins were therefore identified as novel targets for the VEGFRs, suggesting that they may be involved in the regulation of endothelial function. Stat proteins are also involved in other cytokines signalling suggesting that the regulation of VEGFR-3 signalling might be controlled by other cytokines.

VEGFR-3 has been employed as a marker for lymphatic vessels in normal and pathological tissue samples (Lymboussaki *et al*, 1998) and has been used to

demonstrate an apparent lymphatic origin of Kaposi's sarcoma cells (Jussila L *et al*, 1998). However, although VEGFR-3 stains PAL-E-negative capillaries (Lymboussaki *et al*, 1998; Paavonen *et al*, 2000), recent data show that VEGFR-3 can also be expressed in blood vessel endothelia (Partanen, 2000 b). It is also expressed in blood capillaries during the neovascularisation of tumours and in chronic inflammatory wounds (Kubo H, 2000; Kubo *et al*, 2000; Paavonen *et al*, 2000; Partanen *et al*, 1999; Valtola *et al*, 1999).

1.1.2.5 Other less specific markers

1.1.2.5.1 5'-Nucleotidase

5'-nucleotidase is an enzyme that acts on nucleoside-5'-phosphates, such as AMP (adenosine monophosphate) and adenylic acid, releasing inorganic phosphate. It has been shown that 5'-nucleotidase activity is stronger in lymphatic than in blood vessels (Weber *et al*, 1994; Werner *et al*, 1987). Conversely, the activity of another enzyme called 5'-alkaline phosphatase (ALPase) is higher in blood vessels than that in the lymphatics (Kato *et al*, 1991; Werner *et al*, 1987). ALPase catalyses the hydrolysis of monophosphate esters at alkaline pH on the vascular endothelial cells (Zoellner and Hunter, 1989). Methods have been developed to differentiate between lymphatic and blood vessels, by using different enzyme activities to produce different coloured histochemical products (Kato *et al*, 1991; Werner *et al*, 1987). Vessels that are ALPase negative but 5'-nucleotidase positive are classified as lymphatic vessels. However, these methods rely on quantitative rather than on qualitative measurements and therefore they are considered to be subjective and non-specific.

1.1.2.5.2 Weibel-Palade bodies and their contents

Weibel-Palade bodies are electron-dense rod-like inclusions that are present in the cytoplasm of blood vascular endothelial cells. Although some investigators reported that lymphatics do not contain Weibel-Palade bodies (Erhard *et al*, 1996; Sauter *et al*, 1998), i.e. considered as negative marker, other groups claim that these bodies are present in both lymphatic and blood vessel endothelial cells (Harrison *et al*, 1986; Magari and Ito, 1988; Marchetti *et al*, 1992; Nagle *et al*, 1987; Otsuki *et al*, 1990). These and other studies indicate that Weibel-Palade bodies cannot differentiate reliably between blood and lymphatic endothelia.

1.1.2.5.3 Basement membrane components

As stated above, peripheral lymphatic capillaries are characterised by the absence of (or incomplete) basement membrane (Ryan, 1989). Antibodies against basement membrane components such as collagen type IV, fibronectin, vitronectin and laminin have therefore been suggested to be useful in distinguishing blood from lymphatic microcapillaries (Erhard *et al*, 1996; Sauter *et al*, 1998; Yoshizawa *et al*, 1994). However, in tumour angiogenesis, the basement membrane of blood capillaries that are newly developed may be also absent or incomplete and that large lymphatic vessels may also have basement membrane (Madri *et al*, 1996; Paku and Paweletz, 1991).

1.1.2.5.4 Pericytes

Pericytes are, as stated above, absent from the peripheral lymphatic capillaries (Aukland and Reed, 1993). Thus, lack of pericytes around vessels in histological sections has been considered a sign to differentiate between lymphatic and blood capillaries. However, during angiogenesis the immature endothelial network is also lacking pericytes (Benjamin *et al*, 1998). Therefore, lack of pericytes cannot be considered to be lymphatic endothelial specific marker.

1.1.2.5.5 Intercellular junctions

The intercellular junctions between lymphatic endothelial cells have distinguishing features, including overlapping, interdigitated and attenuated interconnections which are open to provide large gaps through which macromolecules and circulating cells can pass (Ryan, 1989). These junctions contain a protein called desmoplakin, which is absent from blood vessels gap junctions. Thus, desmoplakin has been suggested as a possible marker for small lymphatic capillaries (Sawa *et al*, 1999; Schmelz *et al*, 1994). However, it is not expressed in larger lymphatic collecting ducts such as the thoracic duct (Schmelz *et al*, 1994). Furthermore, desmoplakin can also be detected in the junctions between cultivated blood vessel endothelial cells (Kowalczyk *et al*, 1998; Valiron *et al*, 1996). Therefore, desmoplakin cannot be considered as a specific lymphatic marker.

1.1.2.5.6 Pathologische Anatomie Leiden – Endothelium (PAL-E)

PAL-E has been widely reported to be absent from lymphatics, i.e. a negative marker (Erhard *et al*, 1996; Lymboussaki *et al*, 1999; Ruiter *et al*, 1993; Sauter *et al*, 1998). Thus, a lack of PAL-E staining on a capillary in a histological section is a good indication of lymphatic origin. However, when interpreting negative PAL-E staining factors, it should be remembered that PAL-E is also absent from arterioles (Ruiter *et al*, 1993) and from blood capillaries located in anatomical sites with a patent blood-brain barrier (Schlingemann *et al*, 1997).

1.1.2.5.7 Monoclonal antibodies

Monoclonal antibodies have been raised against thoracic duct endothelial cells with the aim to be used as a lymphatic-specific marker (Ezaki *et al*, 1990; Sawa *et al*, 1999). Some antibodies have been shown to bind blood vessels as well. However, a double stain with collagen type-IV antibodies could be used to differentiate lymphatic from blood vessels (Ezaki *et al*, 1990).

1.1.3 The vascular endothelial growth factors and receptors

The last 20 years of angiogenesis research have largely been dominated by discoveries using molecular and cellular biology. The detection of the vascular endothelial growth factors (VEGFs) started with the discovery of VEGF in 1989 (Keck *et al*, 1989; Leung *et al*, 1989). Since then, other vascular growth factors were identified and the VEGF family is currently consists mainly of VEGFs -A, -B, -C, -D, -E and PlGF (placental growth factor) (Figure 1.1) (Joukov, 1996;

Lee, 1996; Olofsson *et al*, 1999; Orlandini *et al*, 1996). There are three VEGF tyrosine kinase receptors identified so far, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1, KDR) and VEGFR-3 (Flt-4). VEGF-B and PlGF bind to VEGFR-1, whereas VEGF-A interacts with both VEGFR-1 and VEGFR-2. VEGF-E binds VEGFR-2 and both VEGF-C and VEGF-D bind VEGFR-3 (Figure 1.1). It has been recently revealed that VEGF family members are expressed in a variety of human tumours in different ways and tumour cells have been reported to be able to secrete VEGFs (Achen *et al*, 2001; Ferrara and Davis-Smyth, 1997; Salven *et al*, 1998). However, the angiogenic switch is thought to be carefully regulated, and at least some specific genetic events in tumour progression correlate with lymphatic metastasis, suggesting that a “lymphangiogenic switch” mechanism is also a distinct possibility (Jussila and Alitalo, 2002).

1.1.3.1 The lymphangiogenic factors: VEGF-C and VEGF-D

VEGF-C and VEGF-D differ from other VEGF family members by the presence of long N- and C-terminal propeptides flanking the VEGF homology domain (Achen *et al*, 1998; Joukov *et al*, 1996; Joukov V, 1996; Lee *et al*, 1996; Orlandini *et al*, 1996). The fully processed or mature forms of VEGF-C and VEGF-D consist of the VHD (VEGF Homology Domain), which acts as a ligand not only for VEGFR-3, but also for VEGFR-2 (Achen *et al*, 1998a; Joukov *et al*, 1997).

In midgestation embryos, VEGF-C is prominently expressed in regions where the lymphatic vessels undergo sprouting from embryonic veins, such as in the

perimetanepric, axillary and jugular areas, and in the developing mesenterium (Kukk *et al*, 1996). In adults, VEGF-C is expressed in the heart, small intestine, placenta, ovary and the thyroid gland. VEGF-C stimulates mitosis and migration of endothelial cells and increases vascular permeability. VEGF-C has been shown to induce lymphangiogenesis in transgenic mouse skin and in mature chick chorioallantoic membrane (Jeltsch *et al*, 1997; Oh *et al*, 1997). However, recombinant VEGF-C also promotes angiogenesis when applied to early chorioallantoic membrane of chicks, to mouse cornea or to ischaemic hindlimbs of rabbits (Cao *et al*, 1998; Witzembichler *et al*, 1998). Therefore, VEGF-C is likely to play a dual role both as an angiogenic and a lymphangiogenic growth factor.

If VEGF-C induces lymphangiogenesis, is it sufficient enough to increase the rate of metastasis to the lymph nodes? It has recently been reported that lymphatics surrounding a VEGF-C overexpressing tumour are enlarged, and it has been suggested that the increase in lymphatic diameter may be sufficient to increase metastasis (Pepper, 2001). Clinical studies correlating the levels of VEGF-C in tumours and their metastatic potential however remains controversial.

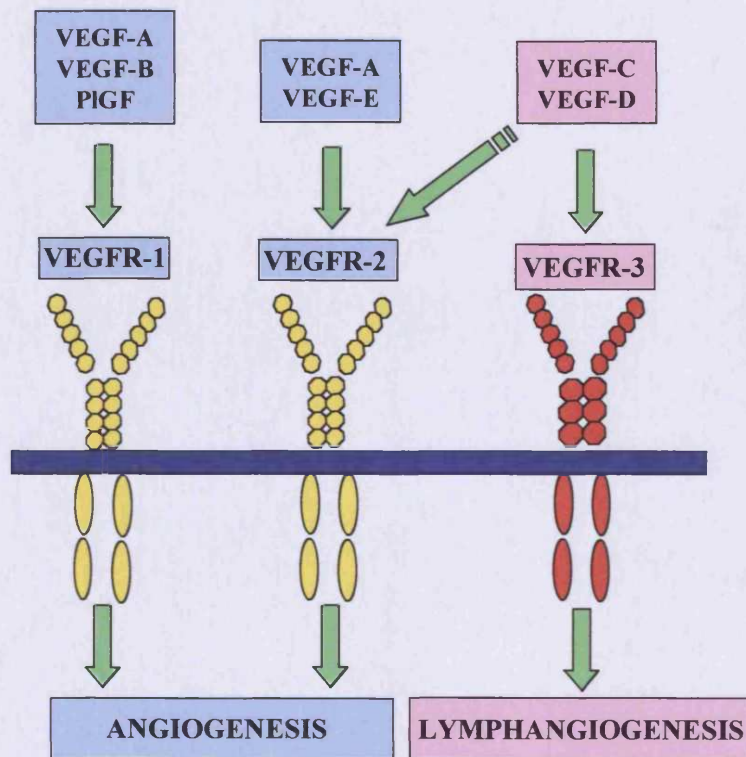


Figure 1.1 The currently known VEGFs and their receptors. VEGFR-1 (Flt-1) and VEGFR-2 (KDR) have seven extracellular immunoglobulin homology domains, but in VEGFR-3 (Flt-4), the fifth immunoglobulin domain is cleaved on receptor processing into disulfide-linked subunits. VEGFR-1 and VEGFR-2 mediate angiogenesis, whereas VEGFR-3 is involved mainly in lymphangiogenesis.

However, a significant correlation between VEGF-C expression and lymph node metastasis has been observed in a variety of carcinomas including breast (Kurebayashi *et al*, 1999), oesophageal (Kitadai *et al*, 2001a), gastric (Ichikura *et al*, 2001; Yonemura *et al*, 1999), colorectal (Akagi *et al*, 2000), thyroid (Bunone *et al*, 1999; Fellmer *et al*, 1999), head and neck (O-charoenrat *et al*, 2001), prostate (Tsurusaki *et al*, 1999), and lung (Niki *et al*, 2000a; Ohta *et al*, 2000).

VEGF-D is 48% identical to VEGF-C (Achen *et al*, 1998; Yamada *et al*, 1997b). It contains the eight conserved cysteine residues characteristic of the VEGF family and has a cysteine-rich COOH terminal extension similar to VEGF-C. In midgestation mouse embryos, VEGF-D expression is particularly abundant in the developing lung. VEGF-D is expressed in many adult tissues including the vascular endothelium, heart, skeletal muscle, lung, small and large bowel.

VEGF-D is known to have mitogenic effects in endothelial cells. Like VEGF-C, VEGF-D is proteolytically processed after secretion, and it binds to and activates both VEGFR-2 and -3 (Achen *et al*, 1998a; Orlandini *et al*, 1996; Yamada *et al*, 1997b). The fact that VEGF-D binds also VEGFR-2 has made it to be possibly angiogenic. However, the controversy remains as it has been shown that transgenic overexpression of VEGF-D led to lymphatic hyperplasia but not angiogenesis (Marconcini *et al*, 1999).

At present, little is known whether factors such as hypoxia, growth factors, cytokines and hormones regulate expression of VEGF-C and VEGF-D. It has

been recently shown that IL- β could up-regulate VEGF-C in colonic cancer (Akagi *et al*, 1999).

1.1.4 Molecular mechanisms in lymphangiogenesis

1.1.4.1 Does lymphangiogenesis occur in tumours?

Although the significance of pre-existing peritumoural lymphatics as conduits for tumour cell dissemination has been well recognised (Fisher, 1968), lymphatic vessels have been thought to be absent from tumours themselves (Folkman, 1996). This was largely due to the fact that conventional histological examination is not adequate enough to identify intratumoural lymphatics or to distinguish lymphatic from blood vessels. Until recently, it has remained unclear whether tumours can stimulate lymphangiogenesis or tumour metastasis stimulates molecular activation of the lymphatic system. Previous studies have failed to detect intra-tumoural functional lymphatics and therefore it was thought that lymphangiogenesis might not play a role in tumour metastasis as one might suspect (Carmeliet and Jain, 2000; Jain, 1987; Tanigawa *et al*, 1981). The initial concept of lymphatic spread of tumours was that tumour cells metastasise solely by the invasion of pre-existing lymphatics surrounding the tumour margin, i.e., tumours are not lymphangiogenic. However, the absence of intratumoural lymphatics may simply reflect the collapse of lymphatics within tumours due to the increased pressure and mechanical stress generated by the proliferating cancer cells (Leu *et al*, 2000). The detection of dilated and engorged lymphatics

in the peritumoural stroma was not sufficient evidence to claim that they are newly formed, although they were linked to the growth factors produced by tumour cells (Leu *et al*, 2000). Therefore the existence of intra-tumoural lymphatic vessels was rather a disputable issue (Baxter and Jain, 1990; Folkman, 1996; Jackson *et al*, 2001; Jain, 1987; Leu *et al*, 2000; Pepper, 2001; Tanigawa *et al*, 1981; Witte and Witte, 1997). However, most of these studies are indirect and performed using tracers or perfusion models, in which no lymphatics could be observed inside tumours. However, recent studies have strongly supported the existence of intra-tumoural lymphangiogenesis and some of these studies are summarised below.

1.1.4.2 Lymphangiogenesis and human solid cancers

During the last 2 years, several studies have demonstrated the existence of intra-tumoural lymphatics using experimental xenotransplanted tumour models, and aided by the newly discovered markers (Karpanen *et al*, 2001; Mandriota *et al*, 2001; Skobe *et al*, 2001a; Skobe *et al*, 2001b; Stacker *et al*, 2001).

1.1.4.2.1 Lymphatic markers and receptors in pancreatic adenocarcinoma

It has been recently found that transgenic mice overexpressing VEGF-C in β -cells of the endocrine pancreas (Rip-VEGF-C with a rat insulin promoter) developed extensive lymphangiogenesis around the endocrine islets of Langerhans (Mandriota *et al*, 2001). Furthermore, when tumours were induced in these VEGF-C overexpressing islets, by mating the mice with transgenic mice expressing the simian virus 40 T-antigen oncogene in the β -cells (Rip1-Tag2),

metastatic tumour cell aggregates of β -cell origin were observed in the surrounding lymphatic vessels. These mice also frequently developed metastases in the lymph nodes, which drain the pancreas, whereas tumours in mice lacking the VEGF-C transgene never metastasised, nor were tumour cells observed inside the lymphatic vessels (Mandriota *et al*, 2001). This indicates that tumours could indeed stimulate lymphatic vessels formation.

1.1.4.2.2 Lymphatic markers and receptors in oesophageal carcinoma

VEGF-C expression is associated with neoplastic progression in the oesophageal mucosa (Auvinen *et al*, 2002). There is an increase in VEGF-C expression in Barrett's epithelium as it progresses through dysplasia to adenocarcinoma. This is consistent with a similar increase in VEGFR-3 expression on lymphatic vessels in these tissues (Auvinen *et al*, 2002). Furthermore, VEGF-C expression was correlated with depth of tumour invasion, tumour stage, lymphatic invasion and lymph node metastasis in oesophageal cancer (Kitadai *et al*, 2001). However, a similar study did not find a significant correlation between VEGF-C expression and lymphatic invasion or lymph node metastases, although the expression was related to histopathological grade and hence prognosis (Noguchi *et al*, 2002).

1.1.4.2.3 Lymphatic markers and receptors in gastric cancer

It has been recently demonstrated that VEGF-C expression in gastric cancer cells was significantly related to depth of invasion, lymphatic invasion and lymph node metastases (Amioka *et al*, 2002; Ichikura *et al*, 2001; Kabashima *et al*,

2001; Takahashi *et al*, 2002; Yonemura *et al*, 1999). However, it seems that there is no correlation between VEGF-C expression and the degree of differentiation in gastric adenocarcinoma (Amioka *et al*, 2002). The clinical impact of the association between VEGF-C expression and prognosis is not fully understood. Nevertheless, there exists a relationship between the expression of VEGF-C in tumour tissues and poor prognosis as well as reduced survival in gastric cancers (Ichikura *et al*, 2001; Takahashi *et al*, 2002; Yonemura *et al*, 1999). A positive correlation between VEGFR-3 and VEGF-C mRNA expression was found in gastric cancer tissues and the majority of VEGFR-3 positive vessels are indeed considered as lymphatics (Yonemura *et al*, 2001). Furthermore, there is higher number of VEGFR-3 positive vessels in gastric cancers that are lymph node positive, with lymphatic invasion or are poorly differentiated (Yonemura *et al*, 2001). The expression of VEGFR-3 was significantly higher in the poorly differentiated gastric adenocarcinomas and in cancers with higher lymph node metastasis rate (Yonemura *et al*, 2001). The role of VEGF-D in gastric carcinomas is yet to be explored.

1.1.4.2.4 Lymphatic markers and receptors in colorectal cancer

There are several reports suggesting a correlation between VEGF-C expression and poor clinicopathological outcome in colorectal cancer (Akagi *et al*, 2000; Furudo *et al*, 2002; Parr and Jiang, 2003). VEGF-C expression was correlated with lymphatic and venous invasion, lymph node status, Dukes' stage, liver metastasis, depth of invasion, poorer histological grade and microvessel density (Akagi *et al*, 2000; Furudo *et al*, 2002). It has been recently revealed that a

positive correlation exists between the levels of VEGF-C expression and lymphatic metastasis in colorectal cancer and that both were linked to the 5 year survival rate (Furudoi *et al*, 2002). However, other studies have not demonstrated such a relationship between VEGF-C levels of expression and lymph node status in colorectal cancer (George *et al*, 2001; Parr and Jiang, 2003). VEGF-D expression was found to be higher in colorectal cancer tissues and is associated with lymph node involvement and reduced overall and disease-free survival (Parr and Jiang, 2003; White *et al*, 2002). However, in another study, colorectal tumour expression of VEGF-D mRNA was less than in normal tissue (George *et al*, 2001). In the latter study, it was suggested that a reduction in VEGF-D levels in the adenoma–carcinoma sequence allowed the more potent angiogenic cytokines VEGF-A and VEGF-C to bind more readily to the signalling receptors VEGFR-2 and VEGFR-3. Furthermore up-regulation of VEGFR-3 protein expression in colorectal cancer tissues and increased expression was associated with poorer overall survival (White *et al*, 2002). This demonstrates the potent paracrine nature of the interaction between VEGFR-3 on the vascular endothelium and its ligands, VEGFs -C and -D in the tumour microenvironment. Additionally, levels of lymphatic markers (Prox-1 and 5'-nucleotidase) were found to be significantly higher in colonic cancer tissues compared to normal tissues and levels of podoplanin mRNA was also higher in colonic cancer tissues although was not statistically significant (Parr and Jiang, 2003).

1.1.4.2.5 Lymphatic markers and receptors in prostate cancer

It has been demonstrated that the expression of VEGF-C in human prostatic carcinoma cells was significantly associated with the presence of lymph nodes metastasis (Tsurusaki *et al*, 1999). Furthermore, there was a positive correlation between the expression of VEGFR-3 and VEGF-C (Tsurusaki *et al*, 1999), suggesting the possible presence of a paracrine loop between prostatic cancer cell and the lymphatic endothelium within the tumour stroma.

1.1.4.2.6 Lymphatic markers and receptors in malignant melanoma

Metastatic melanomas had significantly more and larger tumour-associated lymphatic vessels and a relative lymphatic vessel area of >1.5% was significantly associated with poor disease-free and overall survival (Dadras *et al*, 2003). VEGF-D expression was shown to be up-regulated in human melanomas compared with melanocytes (Achen *et al*, 2001). VEGF-D was detected in melanoma cells and in vessels adjacent to immunopositive tumour cells, but not in vessels distant from the tumours. This suggests that VEGF-D binds to the endothelial cells of nearby vessels and contributes in a paracrine manner to the regulation of tumour lymphangiogenesis. The incidence of intratumoural lymphatics (assessed using LYVE-1 as a marker) was found to be significantly higher in metastatic melanomas and correlated with poor disease-free survival (Dadras *et al*, 2003).

1.1.4.2.7 Lymphatic markers and receptors in other solid tumours

Bronchogenic carcinoma: It has been indicated that a low ratio of VEGF-D:VEGF-C (low VEGF-D and high VEGF-C) is associated with lymph node metastasis and lymphatic invasion in lung adenocarcinoma (Niki *et al*, 2000). Proliferating intratumoural lymph vessels have been identified in these carcinomas (Beasley *et al*, 2002).

Head and neck carcinomas: Quantification of VEGF-C by real-time RT-PCR and immunohistochemistry in head and neck carcinomas revealed higher levels of mRNA in tumour tissue than in normal samples (Beasley *et al*, 2002). Furthermore, intra-tumoural LYVE-1 positive lymphatic vessels were found to be associated with a higher risk for local relapse as well as with poor disease-specific prognosis in Head and Neck squamous cell carcinomas (Maula *et al*, 2003). However, the same study had found that a high density of peritumoural LYVE-1 positive vessels unexpectedly was a sign of favourable survival (Maula *et al*, 2003).

1.1.5 Lymphatic microvessel density (LMVD)

Microvascular density has been used as a measure of tumour angiogenesis which is correlated to prognosis (Folkman and Shing, 1992; Fox, 1997; Marinho *et al*, 1997; Weidner, 1993; Weidner, 1995; Weidner *et al*, 1991). However, LMVD was rarely assessed because of the lack of a reliable lymphatic marker that is suitable for paraffin sections. Recently, antibodies against VEGFR-3 (Clarijs *et*

al, 2001; Kitadai *et al*, 2001a) and LYVE-1 (Carreira *et al*, 2001) that work on paraffin embedded tissue sections were used to evaluate the presence of intratumoural lymphatics and LMVD as a prognostic factor in several neoplasms. So far, most studies on LMVD have used VEGFR-3 as a lymphatic marker (Clarijs *et al*, 2001; Kitadai *et al*, 2001; Partanen and Paavonen, 2001; Sleeman *et al*, 2001). Although VEGFR-3 is highly specific marker for normal adult lymphatic vessels, its upregulation in angiogenesis in some tumours has made its role as a LMVD marker questionable. Therefore, there is currently little conclusive evidence to link LMVD with patients' survival. In ovarian cancer for example, the LMVD has no influence on the progression of the disease and in cervical cancer an increased amount of LMVD may even be associated with a better prognosis (Birner *et al*, 2001; Birner *et al*, 2000).

1.1.6 Lymphatic vessels as possible targets of anti-cancer therapy

With new advances in targeting technologies it is now possible to deliver drugs into a targeted tissue or organ, thereby increasing the potency of the drug at the intended target tissue while reducing side effects elsewhere in the body. Inhibition of angiogenesis for example, is already considered a promising area in cancer therapy.

As stated above, tumours with a higher incidence of lymph node positivity express high levels of VEGF-C and VEGF-D. Inhibition of their receptor, VEGFR-3 signalling activation might be an attractive approach to inhibit cancer lymphatic metastasis. In transgenic mice with targeted expression of a soluble

form of VEGFR-3 in the skin, lymphatic vessels initially formed normally, but the onset of the transgene expression led to regression of lymphatic vessels in embryos (Makinen *et al*, 2001). Furthermore, a soluble VEGFR-3 protein produced via an adenovirus vector could inhibit lymphangiogenesis in a transplantable human breast carcinoma model using MCF-7 cell line in SCID (severe combined immunodeficiency) mice (Karpanen and Alitalo, 2001). In another study, microhaemorrhage and the subsequent collapse of large tumour vessels was also reported in mice injected with blocking monoclonal antibodies against VEGFR-3 (Kubo *et al*, 2000). Primary lymphoedema, a rare autosomal dominant disorder of the lymphatic system, was recently linked to mutations in the VEGFR-3 tyrosine kinase domain (Karkkainen *et al*, 2000). Interruption of VEGFR-3 signalling results in lymphatic hypoplasia, underlining the importance of VEGFR-3 in the maintenance of lymphatic function during embryonic development (Karkkainen *et al*, 2000; Makinen *et al*, 2001). It was recently revealed that the use of neutralizing antibodies against VEGF-D decreases the number of lymphatic metastases of the VEGF-D-293 tumours in the mammary fat pads of SCID/NOD mice (Stacker *et al*, 2001).

1.2 BREAST CANCER AND LYMPHANGIOGENESIS

Breast cancer is the most common form of malignant neoplasms in females throughout the industrialized world. The United Kingdom has the highest incidence, with 1:10 women affected by the disease (Forbes, 1997). Women die from breast cancer in the UK more than any other form of cancer (HMSO, 1995). Each year there are approximately 25,000 new cases of breast cancer and 15,000 deaths from the disease in United Kingdom (HMSO, 1995).

1.2.1 Metastasis of breast cancer

The metastatic spread of tumour cells is responsible for the majority of cancer deaths. Thus studying the mechanisms of metastasis is crucial in order to prevent or minimize deaths from cancer. The pathogenesis of cancer metastasis in general is a cascade of multi-step and complex process. In order for the cancer to establish metastasis, it has to succeed to overcome several barriers such as basement membranes, surrounding stroma and enter the circulation, followed by colonisation of the target tissues. More than 90% of malignancies are of carcinomas i.e. of epithelial cell origin. Epithelial cells are surrounded by stromal tissues, which consist of endothelial cells, lymphocytes, fibroblasts and fat cells. The epithelial cell – stromal interaction is known to play a role in epithelial cancer cells metastasis. The apparently normal stroma surrounding the cancer plays an important role in the growth and metastasis of cancer. Some “normal” stromal cells like fibroblasts are morphologically and phenotypically different from fibroblasts in other normal tissues. The surrounding stroma can help cancer cells progression and spread by secreting growth and angiogenic /

lymphangiogenic factors to promote cancer cells growth, angiogenesis and lymphangiogenesis. Hepatocyte Growth Factor / Scatter Factor (HGF/SF) for example, is a cytokine that is synthesized and secreted by the mesenchymal stroma (predominantly fibroblasts). It helps dissociation (Stoker *et al*, 1987), motility of epithelial cells (Li *et al*, 1994) as well as invasion through the extracellular matrix (Rosen *et al*, 1994).

In order for cancer cells to leave the primary tumour and disseminate, there must be a reduction in their normal adhesion to other cells, and a coordinated changes in their adhesion to the extracellular matrix (ECM) (Jiang and Mansel, 2000). Degradation of the ECM is an essential step in the metastasis cascade. This degradation is achieved by several groups of proteases and collagenases. An important group of proteases called Matrix Metalloproteinases (MMPs) (Stetler-Stevenson *et al*, 1993). Increased MMPs activity is associated with increase in breast cancer cell invasiveness (Liotta *et al*, 1980). ECM metabolism is also regulated by another group of proteins called Tissue Inhibitors of Metalloproteinases (TIMPs). They are inhibitors of MMPs (Woessner, 1991). Lack of balance between MMPs and TIMPs activities can lead to cancer metastasis.

Metastasis of breast cancer is common as about 7% of patients with breast cancer present with widespread metastases at the initial presentation (Mansel *et al*, 2000). The most common sites of metastasis are lymph nodes, bones, lungs, liver, chest wall and central nervous system. Less common sites are the adrenals,

ovaries, pericardium, thyroid and bone marrow (Valagussa *et al*, 1978). Invasive ductal carcinoma is more likely to spread to liver, lung and bone. Invasive lobular carcinoma is more likely to spread to peritoneum, pleura, adrenal glands, uterus and ovaries (Dixon *et al*, 1991; Lamovec and Bracko, 1991). About one third of patients develop bone metastases during the course of their disease and over half of patients at the time of autopsy will have bone disease (Ciatto *et al*, 1988). Pulmonary metastases are found in 6% of patients at the time of initial diagnosis, 20% during the course of the disease and in up to 60% at the time of autopsy (Bunting *et al*, 1976). About 20-30% of patients with negative axillary nodes and 50-60% with positive axillary nodes will develop distant metastases (Wingo *et al*, 1995). This may suggest the presence of micrometastases at the time of initial presentation.

1.2.2 Angiogenesis in breast cancer

Angiogenesis is the formation of new blood vessels. It occurs normally during foetal development and in pathological conditions such as inflammation, wound healing and cancer. Due to deprivation of oxygen, nutrients and growth factors inside tumours, cells are able to expand and proliferate only to certain limit without new blood supply. Tumours of more than 1-2 mm are dependent on angiogenesis (Folkman and Shing, 1992). Endothelial cells undergo a transition from a quiescent to a proliferative state, losing adhesion within the vascular wall, detach and migrate directionally. The process of angiogenesis is a complex cascade of events and divided into 3 stages (Roses, 1999). Stage one, the initiation stage, is characterised by the secretion of the angiogenic factors by the

tumour cells and / or the extracellular matrix (ECM). These angiogenic factors stimulate the proliferation of endothelial cells as well as promote the production of proteases that may lead to the existing endothelial cell basement membrane and degradation of the ECM, the so called stage two or proliferation-invasion stage. This is followed by stage 3, the maturation-differentiation stage, where there is a morphological change in the endothelial cells that elongate and form new blood vessels to establish blood supply to the tumour cells. Angiogenic factors that are highly expressed in human breast cancers (Relf *et al*, 1997; Smith *et al*, 1999), together with their receptors expressed predominantly on vascular endothelium (Gunningham *et al*, 2000) orchestrate the process of tumour angiogenesis. As stated above, there are many reports indicating a statistically significant correlation between incidence of metastasis and microvessels density in breast cancer (Gasparini *et al*, 1997; Weidner, 1995; Weidner *et al*, 1992). Because the tumour is angiogenesis-dependent and tumour vasculature morphology is different from the normal blood vessels, alternative methods to treat cancer may be possible. VEGFs and their respective receptors may constitute a promising target for antiangiogenic therapy.

Extravasation of cancer cells into the circulation is not the end. Cancer cells have to survive inside the circulation as some of them die during the process. However, many cancer cells survive in the circulation and succeed in extravasating (Koop *et al*, 1995). Furthermore, in order to establish distant macrometastasis, cancer cells have to be able to induce angiogenesis in the new host tissue.

1.2.3 Lymphangiogenesis in breast cancer

As mentioned earlier, early metastasis to lymph nodes is a frequent complication in human breast cancer. However, the extent to which this depends on lymphangiogenesis or on invasion of existing lymph vessels remains ill defined. It has been suggested that breast carcinomas invade and destroy lymph vessels rather than promoting their proliferation and nodal metastasis can proceed via pre-existing lymphatics and that lymphangiogenesis does not appear to be a feature of invasive breast carcinomas (Williams *et al*, 2003). However, the same study revealed that a proportion of the peritumoural lymphatics contained tumour emboli associated with hyaluronan, indicating a possible role for LYVE-1 / hyaluronan interactions in lymphatic invasion or metastasis (Williams *et al*, 2003). Intra-tumoural lymphatic vessels have been demonstrated immunohistochemically in breast cancer (Witte, 1997). Lymphangiogenesis has indeed been recently quantified using real-time quantitative PCR (Cunnick *et al*, 2001). LYVE-1 level of expression was found to be higher in tumours that had spread to the regional lymph nodes. Other studies interpreted this merely as the presence of pre-existing lymphatic vessels by invading tumour cells, and has been proposed that lymphatic vessels are absent from most tumours (Carmeliet and Jain, 2000; Folkman, 1996; Leu *et al*, 2000; Tanigawa *et al*, 1981).

Increased lymphangiogenesis was correlated to VEGF-C over-expression in metastatic breast cancer (Skobe *et al*, 2001b). This was associated with profound lung metastasis and enlargement of the peritumoural lymphatics (Makinen *et al*,

2001; Skobe *et al*, 2001b). The rate of lung metastases was directly correlated with the extent of lymphatic microvascular density inside the tumour mass (Skobe *et al*, 2001b). A recent study found that VEGF-C expression was only detectable in node positive breast cancers, whereas expression of VEGF-A detected in both node positive and node negative tumours (Kurebayashi *et al*, 1999). However, other studies claim that although VEGF-C is present, it is not always sufficient to induce the formation of functional lymphatic vessels (Leu *et al*, 2000). These conflicting studies indicate the complex nature of lymphangiogenesis and the immature technologies involved in its investigations. Nonetheless, most studies have proven the existence of lymphangiogenesis in and around breast cancer tissues and the rate of lymphangiogenesis has been correlated to tumour lymphatic metastasis.

1.2.4 Cytokines and breast cancer

Cytokines are pleiotropic immune regulatory proteins predominately produced by immune cells such as T-cells, natural killer cells, monocytes and stromal or non-haematopoietic cells. Cytokines are polypeptides of low molecular weight; their structures are often stabilized by N- and / or O-glycosylation and also by intra-molecular disulphide bridges. These molecules are important modulators and regulators of many immune cell functions. Cytokines act on many different target cells (pleiotropism) and frequently affect the action of other cytokines in synergistic or antagonistic ways. Their actions can be auto-, para- or endocrine depending on cell and tissue types via specific receptors on their target cells. Most cytokines are difficult to detect in serum because their producer cells are

often adjacent to the target cells and usually only small amounts of cytokines are released.

Cytokines play a key role in breast carcinogenesis and subsequent proliferative behaviour. The concept of autocrine and/or paracrine pathways in breast carcinogenesis is now well established (Freiss *et al*, 1993; Lippman *et al*, 1986). There is an increasing importance of cytokines in regulating breast cell function and growth. Cytokines are secreted by many cell types including those of the breast, e.g., tumour necrosis factor- α (TNF- α) (Bebok *et al*, 1994), IL-1 β (Duncan *et al*, 1994a), IL-3 (Speirs *et al*, 1995a) and IL-6 (Adams *et al*, 1991a), where they have pleiotropic actions.

Paracrine effects of cytokines on breast cancer have been demonstrated *in vitro*; incubation of breast cancer cell lines with conditioned medium derived from fibroblasts results in growth promoter/inhibitor effects (Adams *et al*, 1991a; Ryan *et al*, 1993; Speirs *et al*, 1995; Vanroozendaal *et al*, 1992) Furthermore, cytokines including IL-1 β , IL-6 and tumour necrosis factor (TNF)- α have been shown to influence biosynthesis of 17 β -estradiol (E2) in breast epithelial cells *in vitro* by stimulating the reductive pathway of the enzyme 17 β -hydroxysteroid dehydrogenase (Adams *et al*, 1991; Duncan *et al*, 1994; Speirs *et al*, 1993). This enzyme is responsible for the reversible conversion of oestrone, a weak oestrogen, to the biologically more potent oestradiol (E2) (Bonney *et al*, 1986; Milewich *et al*, 1985).

The interactions between cytokines and malignant cells are thought to regulate proliferation and / or the metastatic ability of some cancers. Some cytokines, such as IL-8 (Hu *et al*, 1993; Koch *et al*, 1992; Strieter *et al*, 1992) and IL-1 β are angiogenic / lymphangiogenic mainly via the activation of VEGF-A and VEGF-C (Akagi *et al*, 2000; Akagi *et al*, 1999). It is known that several types of tumour cells are capable of producing cytokines such as IL-1 (Okuno *et al*, 1991), IL-4 (Mann *et al*, 1992), IL-6 (Miki *et al*, 1989) and IL-8 and TNF- α (Takeyama *et al*, 1991).

The downstream signalling pathways are common to many cytokines and growth factors including the VEGF family members and interleukins. For example, MAPK (mitogen activated protein kinase) pathway is involved in VEGFR-3 signalling and also in IL-7 signalling (Crawley *et al*, 1997). Furthermore, VEGFR-3 is known to be a strong activator of another molecules involved in many cytokines signalling such as the signal transducers and activators of transcription -5 (Stat-5) and also induce tyrosine phosphorylation and activation of phosphoinositol-3 kinase (PI3-K) in endothelial cells (Xia *et al*, 1996). These molecules are thought to be involved in the regulation of endothelial function after activation by the vascular endothelial growth factors. PI3-K activation is implicated in VEGFs-induced endothelial cell survival via activation of its downstream target serine kinase Akt/PKB (protein kinase B) (Gerber *et al*, 1998).

During the work summarised below in this thesis, the effects of cytokines on the process of lymphangiogenesis were studied. Amongst the cytokines studied, IL-7

was found to be particularly interesting and has not been previously implicated in lymphangiogenesis studies. Therefore, a brief literature review of this important cytokine is outlined below.

1.3 INTERLEUKIN-7 (IL-7)

1.3.1 Discovery of IL-7 and historical consideration

Interleukin-7 (previously called lymphopoietin-1) was discovered at Immunex Research and Development Corporation in 1988 (Namen *et al*, 1988b). It was originally isolated as a B-cell precursor growth factor from a stromal cell line derived from Whitlock-Witte cultures. Human cDNA was first cloned in 1989, one year after cloning murine IL-7 cDNA from Whitlock-Witte cells (Namen *et al*, 1988a). IL-7 stimulates the growth of foetal and adult thymocytes and thymic progenitor CD4⁺ CD8⁻ and CD3⁻ cells *in vitro* (Conlon *et al*, 1989). *In vivo*, IL-7 could accelerate lymphoid re-population and generation and lymphadenopathy in lymphopenic mice (Morrissey *et al*, 1991a; Morrissey *et al*, 1991b), suggesting a role for IL-7 in lymphoid generation. These studies indicated that IL-7 has proliferative effects on the lymphoid populations and indeed it can lead to the development of lymphoproliferative disease and lymphomas, peripheral and in the skin, suggesting a potential role of IL-7 in tumourigenesis. In 1994, the first IL-7 / IL-7R null mice were generated enabling *in vivo* studies on this cytokine (Peschon *et al*, 1994).

1.3.2 IL-7 molecular structure

Interleukin-7 (Figure 1.2) is a single chain 25kDa glycoprotein predicted to contain 4 α helices and to be internally disulfide cross-linked. Unlike a number of other cytokines and growth factors, IL-7 does not exhibit a species specificity as both murine and human IL-7 are cross reactive with comparable specific activities (Goodwin and Namen, 1992). Human IL-7 gene spans greater than 33Kbp. It is located at 8q12-q13 chromosome. The gene contains 6 exons and 5 introns and yields RNA transcripts of 1.8 and 2.4 kb. The gene encodes the IL-7 protein of molecular weight 2.5×10^3 D. IL-7 molecule displays very high stability even with extreme pH variation (from 2.1 to 8.0) (Costello *et al*, 1993). However, it loses its biological activity after the addition of 2-mercaptoethanol, pointing to the importance of its disulfide bonds (Appasamy, 1999).

IL-7 protein has been placed in the haematopoietin family. Up to 4 isoforms have been described as products of alternative splicing, and their structure have been predicted (Goodwin *et al*, 1989; Kroemer *et al*, 1998). IL-7 protein associates with the extra cellular matrix in the thymus (Kitazawa *et al*, 1997). It has also been shown to bind fibronectin *in vitro* (Ariel *et al*, 1997).

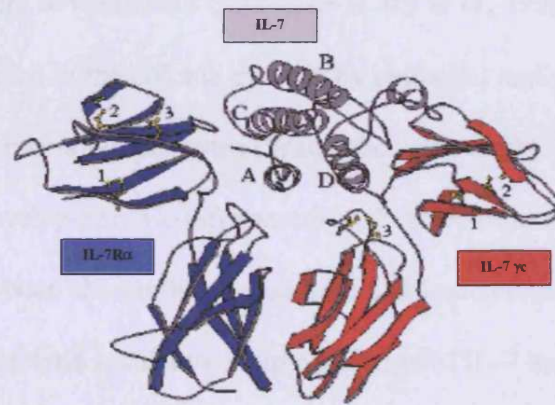


Figure 1.2 Prediction of the 3-D structure of IL-7. IL-7 binds to the extracellular parts of its transmembrane receptor (IL-7R). IL-7R is a heterodimer consists of the IL-7R α (blue) and the IL-7R γ_c chain (red). (www.chem.qmw.ac.uk)

1.3.3 Sites of IL-7 production

The thymus has been reported as the anatomical site of the highest IL-7 production (Namen *et al*, 1988a; Wiles *et al*, 1992). Other sites of production of IL-7 are bone marrow (Funk *et al*, 1995), intestinal mucosa where it appears to support extra-thymic development of $\gamma\delta$ cells (Laky *et al*, 1998; Watanabe *et al*, 1995). IL-7 produced in the colonic mucosa by epithelial and goblet cells, could act on the lamina propria lymphocytes (Watanabe *et al*, 1995). Furthermore, IL-7 transgenic mice develop chronic inflammation of the colonic mucosa (Watanabe *et al*, 1998). It has been shown that infection of a colonic epithelial cell line with different types of bacteria results in an up-regulation of IL-7 and its receptor (IL-7R) (Yamada *et al*, 1997a). Thus epithelial cell derived IL-7 may serve as a regulator for the proliferation of the intestinal lymphocytes. Follicular dendritic cells produce IL-7, suggesting possible roles in germinal centre formation (Kroncke *et al*, 1996). Keratinocytes also produce IL-7 (Heufler *et al*, 1993), presumably promoting $\gamma\delta$ T-cell survival in the skin. Cutaneous IL-7 levels are increased in psoriatic blocks (Bonifati *et al*, 1997) and after schistosoma *mansoni* infection (Roye *et al*, 1998). Cutaneous IL-7 may act as a trophic factor for Sezary lymphoma cells in the skin (Dalloul *et al*, 1992a). IL-7 can also be produced by chronic lymphoblastic leukaemia cells (Frishman *et al*, 1993a) and Burkett's lymphoma cells (Benjamin *et al*, 1994) and has been shown to be elevated in the serum of Hodgkin's patients (Trumper *et al*, 1994).

1.3.4 Main biological activities of IL-7

IL-7 functions primarily as a growth and anti-apoptotic factor for B and T cells. It promotes the growth of B-cell progenitors (Namen *et al*, 1988b) and is required for B and T cell lymphopoiesis (Appasamy, 1993; Costello *et al*, 1993). It enhances the growth of natural killer (NK) cells and promotes growth and differentiation of T cells (Rich *et al*, 1993; Silva *et al*, 1994). It also enhances the generation of cytotoxic T Cells (Alderson *et al*, 1990). It stimulates the lytic activity of peripheral blood monocytes (Alderson *et al*, 1991). IL-7 is also thought to promote the development of intestinal lymphocytes (Fujihashi *et al*, 1997). Thus, epithelial cell-derived IL-7 may serve as a regulator for the proliferation of the intestinal lymphocytes.

1.3.5 IL-7 Receptor (IL-7R)

IL-7 mediates its actions via engagement to and activation of its receptor, IL-7R (Figure 1.3), which is a heterodimer of an IL-7 binding chain, IL-7R α (Goodwin *et al*, 1990; Kondo *et al*, 1994; Noguchi *et al*, 1993) and the γ_c chain, which is shared by the cytokine receptors for IL-2, IL-4, IL-9 and IL-15. Both IL-7R α and γ_c are required for high affinity binding of IL-7 (Kondo *et al*, 1994). Human IL-7R gene is located on the human chromosome 5p13 (Venkitaraman and Cowling, 1992). The location of IL-7R gene is nearer to the location of growth hormone receptor, prolactin and leukaemia inhibition gene that have similar signalling characteristics to IL-7R α (Gearing *et al*, 1993).

The molecular weight of IL-7R is 80 kDa. Both IL-7R α and γ_c have a pair of conserved extra-cellular cysteine residues and an extra-cellular Trp-Ser-X-Trp-Ser motif and lack intrinsic tyrosine kinase activity (Goodwin *et al*, 1990) and two partially conserved regions in the membrane-proximal part of their intracellular domains (Murakami *et al*, 1991) characterizing them as members of the cytokine receptor super-family (Bazan, 1990). The extracellular domain of the IL-7R binding unit consists of an N-terminal region of about 100 amino acids with no clear sequence homology to other proteins, followed by FNIII domain containing the WSXWS motif. The γ_c chain is the functional component and augments IL-7 binding to IL-7R α chain (Noguchi *et al*, 1993). The IL-7R α protein exists in two forms: membrane-bound and soluble forms. The membrane-bound form has been shown by immunoprecipitation to associate with IL-2R γ_c (P64 isoform) (Goodwin *et al*, 1990; Noguchi *et al*, 1993). Subsequent work identified other IL-7R isoforms, p75 and p90 on activated T-lymphocytes (Armitage *et al*, 1992; Page *et al*, 1993). Ligand binding assays have demonstrated a range of IL-7 receptor affinities. There are two classes of IL-7 binding sites; a high (1 nm) and a low (35-40 pm) affinity (Park *et al*, 1990). In addition to IL-7, another cytokine, thymic stromal-derived lymphopoietin (TSLP) appears to be able to bind to IL-7R α as well (Ray *et al*, 1996). TSLP was originally described to promote the development of surface IgM⁺ cells (Friend *et al*, 1994).

1.3.6 Signalling through IL-7 R

The intracellular signalling pathways through which IL-7 exerts its various effects are still not clearly established. However, engagement of IL-7R with its ligand, IL-7, leads to series of intracellular phosphorylation events mediated by signalling molecules including the Janus kinases (Jak-1 and Jak-3), Src kinases and Stat (signal transducers and activators of transcription) 5a/b (Foxwell *et al*, 1995). IL-7R α has no intrinsic tyrosine kinase activity that is necessary for the signalling pathway (Goodwin *et al*, 1990). The γ_c does have an Src homology (SH2) domain in its cytoplasmic end that could play a role in the protein-protein interactions during signalling (Taniguchi and Minami, 1993). However, additional work showed that the cytoplasmic tyrosines of the γ_c chain were not essential for IL-7 triggering of intracellular kinases (Lai *et al*, 1997). Therefore, IL-7 tyrosine phosphorylation is completely mediated by other intracellular kinases (Hofmeister *et al*, 1999). The homodimerization of chimeric IL-7R α chains alone was not sufficient for signalling (Ziegler *et al*, 1995). Experiments in which the extracellular domain of the EpoR (erythropoietin receptor) was fused to the intracellular domains of IL-7R α and γ_c showed that the dimerization of the intracellular components of IL-7R α and γ_c was sufficient for Stat-5a/b phosphorylation (Lai *et al*, 1997).

Binding IL-7 to its receptor is followed by phosphorylation of Jak-1 and Jak-3 and Stat-5a/b (Foxwell *et al*, 1995). Therefore, it is the partially conserved regions in the membrane-proximal part of the intracellular domain of the IL-7R α

that plays the key role in the association with the Jak-1 and Jak-3 (Hofmeister *et al*, 1999).

The specific binding of IL-7 to IL-7R α chain triggers heterodimerization the latter with γ_c that in turn juxtaposes Jak-3 (pre-associated with γ_c) to IL-7R α . Jak-3 phosphorylates Jak-1 and IL-7R α (Hofmeister *et al*, 1999). Furthermore, IL-7R α will actively bind phosphatidylinositide-3'-Oh kinase (PI3-kinase). This interaction is ascribed to a ligand-induced phosphorylation of Tyr residue 449 of IL-7R α chain (Venkitaraman and Cowling, 1994).

As stated earlier, TSLP (Thymic Stromal Derived Lymphopoietin) is another molecule that actively interacts with the IL-7R α chain. *In vitro* TSLP can replace IL-7 in activating B-cell development (Ray *et al*, 1996). In studies with antibodies against IL-7R α and γ_c it was demonstrated that TSLP did not use the γ_c chain to stimulate B-cell proliferation. Analysis of downstream signalling events clearly distinguished TSLP from IL-7 in that, while both activated Stat-5a/b isoforms, TSLP did it independent of Jak-1 and Jak-3 (Hofmeister *et al*, 1999).

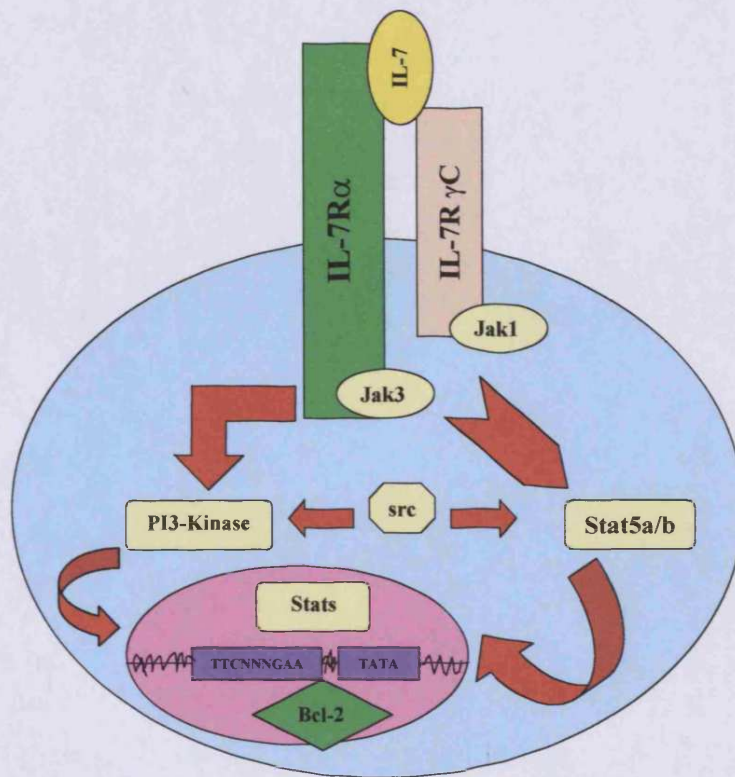


Figure 1.3 Overview of IL-7 signal transduction. IL-7 is associated with IL-7R α and γ_c and activates Janus kinases (Jaks). The Jaks in turn phosphorylate tyrosine-based docking sites on the receptor. Stats then bind via their Src homology (SH)2 domains. Stats are then phosphorylated by the Jaks, form homo-hetero-dimers and then translocate into the nucleus, where they bind target sequences like G-activated sequence (GAS) motif. Transcriptional activation of genes typically requires the coordinated function of multiple accessory transcription factors. Additionally, serine phosphorylation of some Stats may be important for maximal transcription of target genes.

1.3.6.1 The Jak-Stat Pathway

1.3.6.1.1 *Janus kinases*

The Janus kinase family of proteins is comprised of Jak-1, Jak-2, Jak-3, and Tyk-2. These proteins bind to cytokine receptors and play an essential role in cytokine signalling (Chen *et al*, 1997). Jak-1 and Jak-3 are associated with signalling pathway of IL-7. There is a physical pre-association of Jak-3 with the IL-2R α and Jak-1 with the γ_c chain (Noguchi *et al*, 1993).

Jak-1 has a wide range of expression profile in tissues (Ortmann *et al*, 2000). Jak-1 null mice have diverse abnormalities and immunodeficiency though Jak-1 deficient mice die peri-natally as a result of an incompletely defined neurologic defect (Rodig *et al*, 1998). In addition, they fail to IL-7 signalling via the γ_c . On the other hand, Jak-3 has a much more regulated and specific tissue expression. It is expressed at high levels in natural killer cells, thymocytes, T and B cells and myeloid cells (Gurniak and Berg, 1996) in both breast cancer cell lines and primary breast cancer tissues (Cance and Liu, 1995). In addition to IL-7, Jak-3 is also involved in IL-2, IL-4, IL-9, and IL-15 signalling (Johnston *et al*, 1995; Zeng *et al*, 1994).

The three-dimensional structure of the Jaks is still unknown. Jaks are large kinases of more than 1100 amino acids with molecular weights of 120–130 kDa (Figure 1.4).

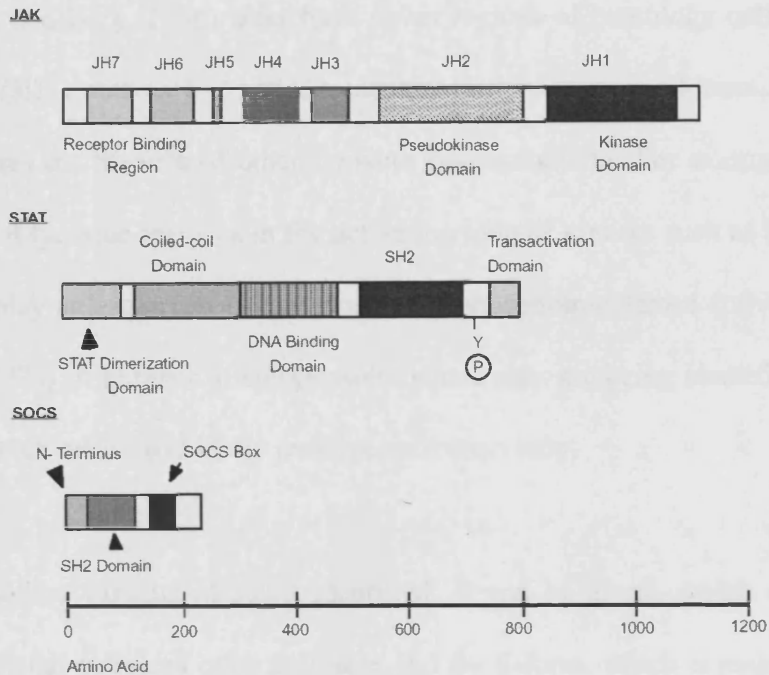


Figure 1.4 Structure of Janus kinases (Jaks), signal transducers and activators of transcription (Stats), and suppressors of cytokine signalling (SOCS). Regions of homology shared by Jaks have been termed Jak homology (JH) domains. JH1 is a kinase domain and JH2 is a pseudo-kinase domain. The amino-terminus of the Jaks appears to be important for association with cytokine receptors subunits. Stats have a conserved tyrosine residue, phosphorylation of which allows Stat dimerization; a Src homology (SH2) domain that mediates the dimerization; and an amino-terminal region that is known to play a role in the dimerization of Stats dimer. The amino-terminal, carboxy-terminal and coiled-coil regions of Stats can interact with other transcription factors. SOCS proteins share a similar structure with a central SH2 domain, a region at the amino-terminus that is variable in both length and in amino acid sequence, and a region of homology at the carboxy-terminus termed the 'SOCS box'.

Their mRNA transcripts range from 4.4 to 5.4 kb. Multiple spliced forms of Jak-3 have been identified, including a variant that lacks a segment of the catalytic domain (Gurniak and Berg, 1996). Jaks have seven regions of homology called Janus homology (JH) domains 1–7 and the carboxy-terminal tyrosine kinase, or JH1 domain, shares the features of other tyrosine kinase domains. For example, phosphorylation of tyrosine residues in the activation loop of kinases such as the insulin receptor play an important role in regulating phosphotransferase activity (Hubbard *et al*, 1994). A number of autophosphorylated sites are being identified in Jaks, two of which reside within the putative activation loop.

There are three splice variants of Jak-3 identified: B and M forms, which are expressed in epithelial cells and other cell lines and the S-form, which is mainly expressed in haematopoietic cells and has been implicated in IL-2R signal transduction (Lai *et al*, 1995). Jak-3 is expressed in breast, colon and lung cancers (predominantly Jak-3B). In HUT-78 cells, JAK-3B was found to be immunoprecipitated with the IL-7Rc. Jak-3B is a kinase-deficient form of Jak and needs to be associated with another isoform, Jak-3S (Lai *et al*, 1995). Furthermore, Jak-3B was significantly autophosphorylated in a breast cell line, BT-474, in the absence of Jak-3S, indicating that some other molecules could be involved in Jak phosphorylation in epithelial cells.

The critical function of Jak-3 was established when a form of human severe combined immunodeficiency (SCID) was found to result from Jak-3 mutations (Russell *et al*, 1995). Mice lacking Jak-3 also displayed a dramatic immune deficiency (Baird *et al*, 1998; Nosaka *et al*, 1995; Thomis *et al*, 1995), a

phenotype that resembles the γ_c deficient mice phenotype (Cao *et al*, 1995), as well as the IL-7 and IL-7R knockout mice phenotype. A recent study has indicated that lacking Jak-1 does not alter activation of Jak-3 and Stat-5, (Higuchi *et al*, 1996), indicating that Jak-1 may not be essential in IL-7R signalling.

1.3.6.1.2 Signal transducers and activators of transcription (Stats)

Stats are latent cytosolic transcription factors bind to the phosphorylated cytokine receptors via their SH2 domains (Greenlund *et al*, 1995). Seven Stat proteins have been identified in mammalian cells (Leonard and O'Shea, 1998). The Stats are localized as clusters on chromosomes: Stat-1 and Stat4 on chromosome 1, Stat-2 and Stat6 on chromosome 10, and Stat-3, Stat-5a and Stat-5b on chromosome 11 in murine system (Copeland *et al*, 1995). Stat-5a and Stat-5b have also been shown to co-localize on human chromosome 17 (Lin *et al*, 1996). All Stats share specific functional domains. Differences in the SH2 domains of different Stats determines the selectivity of Stat binding to various cytokine receptors. The SH2 domain in Stats plays an important role in association between Stats and IL-7R. A conserved tyrosine approximately 700 residues from the N-terminus is rapidly phosphorylated by activated Jaks, allowing Stat proteins to form dimers, based on the interaction between the phosphorylated tyrosines of the SH2 domain of each Stat. These dimers are stabilized by bivalent interactions with Jaks and translocate into the nucleus, where they modulate the expression of their target genes. Stat-5 is encoded by

two genes, Stat-5a and Stat-5b share 96% identity at the protein level (Liu *et al*, 1995), and are both activated by IL-7 as well as prolactin, growth hormone, erythropoietin, thrombopoietin, and IL-2 (Ortmann *et al*, 2000).

Binding of prolactin to its receptor leads to phosphorylation and activation of the Stat proteins, which in turn promote the expression of specific genes. The activity pattern of Stat-5a and Stat-5b in breast tissue suggests their active role in epithelial cell differentiation and milk protein gene expression (Liu *et al*, 1997). Stat-5a knockout mice develop impaired mammary gland development (Liu *et al*, 1995; Liu *et al*, 1997), whereas Stat-5b deficient mice are defective in both sexually dimorphic growth and in growth hormone dependent regulation of liver gene expression (Udy *et al*, 1997). Stat-5a/Stat-5b double knockouts were created (Teglund *et al*, 1998); one-third of these mice died within 48 h of birth, with the surviving mice developing a smaller than normal body size, which was apparently due to aberrant growth hormone signalling. Furthermore, nuclear extracts from invasive breast cancers display significantly higher levels of Stat than those from benign and normal breast tissues (Watson and Miller, 1995).

IL-7 induces Stat-5a/Stat-5b heterodimerization. Stat-3 is associated constitutively with Stat-5 isoform (Rosenthal *et al*, 1997). Stimulation of progenitor B cells by IL-7 also activates Stat-1 (van der Plas *et al*, 1996). Furthermore, IL-7 and IL-2 activate the same Stat proteins due to the existence of similar tyrosine phosphorylated motifs in the cytoplasmic domains of IL-2 and IL-7 receptors that constitute docking sites for Stat activation (Lin *et al*, 1995).

There are other types of proteins that were reported to interact with Jaks and mediate IL-7 signalling including Shc, Grb2, SHP-2, Vav, IRS, PI3-kinase, STAM, Pyk2 and others (Hofmeister *et al*, 1999). STAM (Signal Transducing Adaptor Molecule) is an adaptor molecule and was originally identified as a tyrosine phosphorylated protein that was induced by IL-7, IL-2, IL-4, IL-3 and GM-CSF (Takeshita *et al*, 1996). STAM contains an SH3 domain and an ITAM that is known to interact with the Zap-70 family tyrosine kinases. STAM has been shown to be associated with and phosphorylated by Jak-3 (Takeshita *et al*, 1996; Takeshita *et al*, 1997). A mutation in the SH3 domain of STAM affects c-myc promoter gene activity suggesting that STAM may be the component downstream of Jak-3 that is involved in the regulation of c-myc gene expression. Pyk2, a member of focal adhesion kinase (FAK) family PTK is physically associated with Jak-3. It mediates IL-2 induced cell proliferation without affecting Stat-5 pathway. Therefore, Pyk2 is an important component in Jak-3 signalling (Miyazaki *et al*, 1998). The adaptor protein SLP-76 is also of importance as mice lacking SLP-76 showed a profound block in thymic development and expansion of double positives (DP) thymocytes, thus suggesting that SLP-76 is crucial for T cell development (Pivniouk *et al*, 1998).

In summary, IL-7 signal transduction starts with the specific binding of IL-7 to the IL-7R α chain triggers heterodimerization with the γ_c chain. The dimer induces tyrosine residues phosphorylation in the cytoplasmic tail of the receptor providing docking sites for proteins with SH2 domains (Stats). Stats themselves

are phosphorylated, dissociated from the receptor, from homo- or hetero-dimers, and rapidly trans-locate to the nucleus where they can specifically bind to DNA sequences and regulate target gene transcription (see below).

1.3.6.1.3 Negative regulation (attenuation) of Jak-Stat pathway

Degradation of Stats is one way by which the Jak-Stat pathway can be negatively regulated. There are several proposed mechanisms to do so; dephosphorylation by phosphatases, cytokine-inducible inhibitor molecules, transcriptional repressors, and Stat degradation (Heinrich *et al*, 1998; Ortmann *et al*, 2000) (Figure 1.5).

It is well known that the IL-7 induced phosphorylation of cytokine receptors, Jaks and Stats, is transient. SHP-1 has been proposed as a negative regulator that can downregulate these phosphorylations (Haque *et al*, 1998; Klingmuller *et al*, 1995). SHP-1 can either dephosphorylate Jaks or activated receptor subunits, depending upon the pathway activated (Ortmann *et al*, 2000). The inhibitor molecules, SOCS-1 (Suppressor Of Cytokine Signalling) and CIS-1 (Cytokine Inducible Src homology-2 protein) have been also identified as suppressors of cytokine signalling (Yoshimura *et al*, 1995). Subsequently, several other members of inhibitors have been identified (CIS2-CIS7/ SOCS2-SOCS7) (Hilton *et al*, 1998).

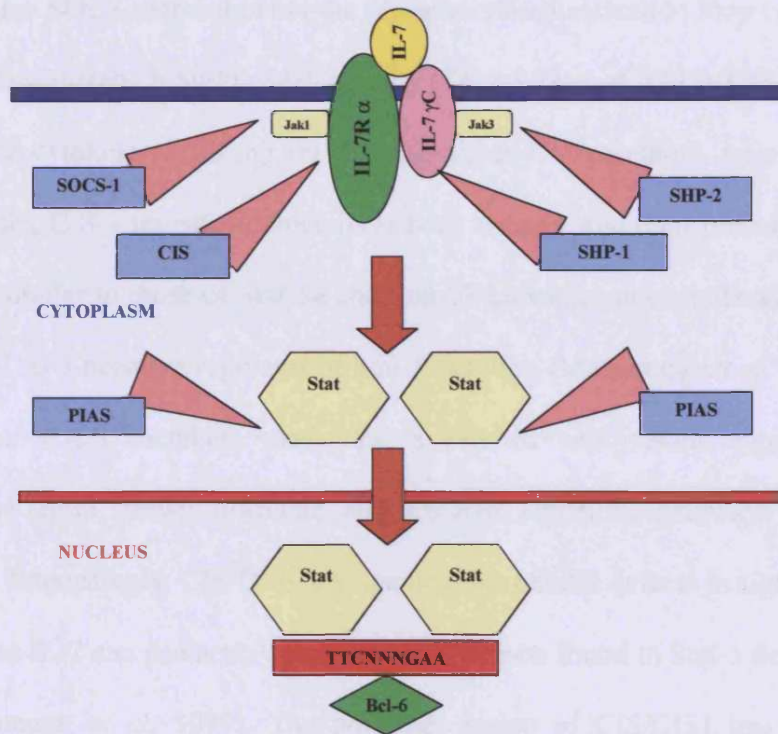


Figure 1.5 Model of attenuation of IL-7 signalling. Suppressor of cytokine signalling proteins (SOCS) can inhibit the Jaks. Cytokine inducible Src homology-2 protein (CIS) binds to activated IL-7R. SHP-1 can either inactivate IL-7R or the Jaks. SHP-2 can either inactivate IL-7R or the Jaks. Protein inhibitors of activated Stats (PIAS) are proposed to inactivate Stats. Stats themselves are degraded in the cytoplasm and / or nucleus by an unknown mechanism. Bcl-6 can bind to consensus Stat binding and represses the signalling pathway.

These molecules are characterized by the presence of central SH2 domain and carboxy-terminal region of homology called the SOCS box. Via their SH2 domains, some SOCS members bind the phosphorylated activation loop tyrosine residue in Jaks, thereby inhibiting Jak activity (Takeshita *et al*, 1997). CIS-1 may downregulate cytokine signalling by binding directly to receptors, rather than Jaks. Recently, CIS-1 transgenic mice have been created, and their phenotype is remarkably similar to those of Stat-5a and Stat-5b knockout mice, indicating the role of CIS-1 as a negative regulator of Stat-5 function (Matsumoto *et al*, 1999). Furthermore, SOCS members may play a role in noncytokine signalling, including the leptin, growth hormone, and prolactin signalling pathways (Pezet *et al*, 1999). Interestingly, CIS/CIS1 transgenic mice exhibit defects in signalling in response to IL-2 and prolactin quite similar to defects found in Stat-5 deficient mice (Matsumoto *et al*, 1999). The promoter region of CIS/CIS1 has Stat-5 responsive elements, leading to Stat-5- dependent expression of this gene (Matsumoto *et al*, 1997). Some SOCS proteins bind directly to Jaks and inhibit their catalytic activity, whereas CIS can bind to activated receptors and prevent docking by Stats (Ortmann *et al*, 2000). Thus, this family appears to serve in a classical negative feedback loop to regulate the Jak-Stat pathway.

Another family of Stat inhibitors called Protein inhibitors of activated Stats (PIAS), PIAS1 and PIAS3. PIAS1 and PIAS3 bind to Stat-1 and Stat-3, respectively and suppress Stats by an as yet unknown mechanism (Chung *et al*, 1997). They inhibit transcriptional activity of the Stats, but do not affect phosphorylation. Their specificity regarding cytokine signalling regulation has

not been determined. The accumulation of Stats in the nucleus can be regulated by these molecules at the level of nuclear import, export, or a combination of the two, the mechanisms that control these processes are not well characterized (Ortmann *et al*, 2000).

Bcl-6, a zinc-finger protein expressed in B cells and CD4⁺ T cells and frequently associated with non-Hodgkin's lymphoma, has also been recently shown to downregulate Stat function. It has been noticed that Bcl-6 deficient mice develop a severe systemic inflammatory disease, which is characterized by infiltrates of immunoglobulin E-bearing B cells and eosinophils (Ye *et al*, 1997).

1.3.6.2 *Src Kinases*

Src kinases are membrane-associated non-receptor protein tyrosine kinases. Each kinase has a unique N-terminal specific, a conserved SH1, SH2 and SH3 domains. The SH2 domains interact with phosphorylated tyrosine residues, and the SH3 domains interact with proline rich regions (Hofmeister *et al*, 1999). IL-7 activates the Src family kinases p59^{fyn} and p53^{lyn} in progenitor B and myeloid cells line (Seckinger and Fougereau, 1994; Venkitaraman and Cowling, 1992). p56^{lck} is also activated by IL-7 and the IL-7R is physically associated with both p59^{fyn} and p56^{lck} in human T cells (Page *et al*, 1995). In one study has indicated that mice deficient in p56^{lck} expression had severely reduced thymus cell numbers and demonstrated impaired development of the DP subset (Wallace *et al*, 1995). Mice deficient in p59^{fyn} do not show defects in T and B cell growth, thus suggesting signalling through p59^{fyn} is unlikely to mediate all of the

responses generated by IL-7 (Grabstein *et al*, 1993). However, another study revealed that p59^{fyn} and p56^{lck} activity induced by IL-7 does not correlate with proliferation in T cells induced by IL-7 (Page *et al*, 1995). Therefore, IL-7 may be able to utilize pathway(s) other than Src signalling.

1.3.6.3 PI3-Kinase Pathway

IL-7 induces the activation of PI3-kinase, that is mediated by tyrosine phosphorylation of the PI3-kinase subunit p85 (Dadi *et al*, 1994b). However, the trophic action of IL-7 on T cell progenitors may be not dependent on PI3-kinase based on experiments with the inhibitor Wortmannin (Kim *et al*, 1998). On the other hand, Jak-3 is associated with the p85 subunit of PI3-kinase in IL-7 stimulated T cells and seems to regulate PI3-kinase activation by mediating tyrosine phosphorylation of the p85 subunit (Sharfe *et al*, 1995). However, the IL-7-induced PI3-K activation might occur in the absence of Src family kinase activity (Sharfe *et al*, 1995).

IL-7 stimulation of human thymocytes resulted in the rapid tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-2 proteins (Hofmeister *et al*, 1999). Jak-1 and Jak-3 kinases are associated with both IRS-1 and IRS-2 in thymocytes (Hofmeister *et al*, 1999). IL-7 specifically induced the association of IRS-1 with Jak-3. The phosphorylation level of Jak-3-associated IRS-2 is much higher than IRS-1 (Sharfe and Roifman, 1997). Furthermore, the 160 - 185 kDa IRS proteins are associated with the p85 regulatory subunit of the

PI3-kinase, suggesting a close regulation between PI3-kinase and IRS proteins after IL-7 stimulation (Sharfe and Roifman, 1997).

PI3-K is considered as a critical signalling molecule, which regulates several cellular processes including survival and proliferation in different systems. PI3-K is consisting of a regulatory p85 subunit and a catalytic subunit which phosphorylate the 3-ring position of PI-4,5-bisphosphate to generate PI-3,4,5-triphosphate (PIP₃) (Coffer *et al*, 1998; Toker and Cantley, 1997). Other known activators of PI3-K are PDGF, NGF, IGF-1 and PMA that act as survival factors suppressing apoptosis induced by a number of agents (Coffer *et al*, 1998; Toker and Cantley, 1997). Transfection of cells with constitutively active PI3-K results in inhibition of apoptosis induced by c-Myc, UV radiation, TGF- β , Fas and TNF (Burow *et al*, 2000; Eves *et al*, 1998; Hausler *et al*, 1998; Kennedy *et al*, 1997). PI3-K activation of Akt/PKB and the subsequent phosphorylation of Bad may underlie the mechanism by which PI3-K signalling inhibits apoptosis. For example, hepatocyte growth factor / Scatter factor (HGF/SF) has been shown to exert cytoprotective function by PI3-K (Fan *et al*, 2000; Jin *et al*, 1997; Yamashita *et al*, 1994). PI3-K has been proposed to mediate events such as mitogenesis, cell adhesion, motility and cellular differentiation as well as providing protection against apoptosis (Toker and Cantley, 1997; Vanhaesebroeck *et al*, 1997). It has also been shown recently that ER α binds to the p85 α subunit of PI3-K in epithelial cells and activates the PI3-K/AKT2 pathway in an oestrogen-independent manner (Sun *et al*, 2001).

1.3.7 Cellular response to IL-7 signalling

Two types of cellular responses to IL-7 have been identified in lymphoid progenitors; a trophic effect and an effect supporting V(D)J recombination (V: versatility, D: diversity and J: joining).

1.3.7.1 The trophic response

The trophic effect of IL-7 is clearly demonstrated in the early development of T lymphocytes. Thymic precursors require IL-7 for survival, as shown by the significant repression of T cell development in the IL-7^{-/-} and IL-7R^{-/-} knockout mice (von Freeden-Jeffry *et al*, 1995). Cell maturation and differentiation induced by IL-7 are probably due to its trophic action by inhibiting apoptosis. Studies evaluating the functional competence of T cells from IL-7R^{-/-} mice revealed that such T cells failed to proliferate in response to alloantigen or phorbol myristate acetate and ionomycin, and the majority of these cells instead underwent apoptosis (Maraskovsky *et al*, 1996). The Bcl-2 family of proteins are possible mediators of IL-7 induced cell survival. These proteins have multiple homology domains and function as either anti-apoptotic members (i.e., Bcl-2, Bcl-XL) or pro-apoptotic members (Bax, Bak, Bad, Bid) (Chao and Korsmeyer, 1998). Bcl-2, is known to inhibit apoptosis, maintaining cellular viability during cell death (Reed, 1997). Bcl-2 can act on mitochondria, stabilizing membrane integrity, preventing the loss of permeability, thereby inhibiting the release of factors like cytochrome c (Kluck *et al*, 1997). Studies in IL-7^{-/-} mice indicated that Bcl-2 expression is dependent on IL-7 during early development of T cells

(von Freeden-Jeffry *et al*, 1997). However, IL-7 was also shown to promote the survival of Bcl-2 deficient T cells, suggesting that the anti-apoptotic action of IL-7 is independent of Bcl-2 (Nakayama *et al*, 1995).

IL-7 withdrawal leads to cell death may also be due to the action of a death-promoting factor. Apoptosis as a result of IL-7 withdrawal in pro-T cells does not seem to be dependent on the p53 pathway or the Fas/Fas ligand pathway, as blocking these leads to apoptotic death (Kim *et al*, 1998). However, IL-7 withdrawal did affect the level of Bcl-2, indicating a balance between anti-apoptotic and pro-apoptotic proteins (Kim *et al*, 1998). It is therefore possible that IL-7 could prevent cell death by the inhibition of a pro-apoptotic protein like Bid, Bad or Bax. Bid is a cytosolic protein that is cleaved by caspase-8 upon engagement of the Fas death pathway. Bad, is activated by PI3-kinase, possibly induced by IL-7 leads to the triggering of Akt, a serine-threonine kinase which phosphorylates Bad and promoting survival (Franke *et al*, 1997). It has been shown that Bcl-2 could interfere with the Bax induced release of cytochrome C inside mitochondria (Rosse *et al*, 1998), suggesting that IL-7 play a role in regulating the functions of anti-apoptotic and pro-apoptotic factors.

1.3.7.2 The role of IL-7 in V(D)J recombination

IL-7 could act as a co-factor for V(D)J recombination *in vitro* in murine lymphoid precursors. Experiments in knockout mice indicate a role of IL-7 in the control of locus accessibility for V(D)J recombination. Mice with targeted deletion of the IL-7R chain showed an impaired V(D)J rearrangement of the immunoglobulin heavy chain locus (Hofmeister *et al*, 1999). Mice lacking IL-7R

also showed a severe reduction of gene rearrangement of the TCR locus (Maki *et al*, 1996). Furthermore, mice lacking other components of the IL-7 signal transduction pathway, such as the γ_c chain or Jak-3, also showed a reduction of recombination at the TCR cluster1 (Malissen *et al*, 1997). Collectively, these studies suggest that IL-7 plays an important role in the V(D)J recombination.

1.3.8 Target genes in IL-7 signalling

As stated above, IL-7 induces the transcription of different genes in order to exert its various trophic cellular responses. IL-7 regulates apoptosis through the induction of the anti-apoptotic Bcl-2 (Akbar *et al*, 1996). IL-7 may also regulate the nuclear factor of activated T cells (NFAT). This factor regulates a novel Rho/PI3-K/PKC pathway as demonstrated by the IL-2-mediated Bcl-2 expression (Gomez *et al*, 1998). The nuclear import of cytoplasmic NFAT is dephosphorylated by serine / threonine phosphatase calcineurin and by rapid nuclear rephosphorylation and shuttling to the cytoplasm (Clipstone and Crabtree, 1992; Shibasaki *et al*, 1996). This interaction inhibits the phosphorylation dependent translocation of NFAT4/NFATx to the nucleus but keeps the enzymatic activity of the phosphatase. Bax protein also interrupts the calcineurin / Bcl-2 interaction (Shibasaki *et al*, 1997). Therefore, NFAT activity is negatively regulated by Bcl-2, while Bax has an opposing effect (Shibasaki *et al*, 1997). Furthermore, Bcl-2 could interact with Raf-1 and directs the protein kinase to intracellular membranes (Wang *et al*, 1996). Thus resulting in an increased protection against apoptosis and reflects the connection between the

Ras / Raf pathway and the Bcl-2-mediated regulation of cell death (Hofmeister *et al*, 1999).

It has been shown that IL-7 enhances IL-2 mRNA accumulation in lymphocytes by increasing the DNA binding activity of both NFAT and AP-1 indicating that IL-7 signalling may involve the activation of the c-Fos / c-Jun proteins (Gringhuis *et al*, 1997). The transcriptional activation of c-Fos was shown to require Stat-5 163 which is activated by IL-7 through heterodimerization of Stat-5 isoforms (Mui *et al*, 1996; Rosenthal *et al*, 1997).

IL-7 also regulates the transcription of c-myc and induces its expression in lymphocytes and neurons (Crawley *et al*, 1996; Michaelson *et al*, 1996; Morrow *et al*, 1992). c-Myb is another transcription factor essential for the maintenance of early haematopoietic cells, in the regulation of the survival of thymoma and IL-2- dependent T cells via bcl-2 gene induction (Salomoni *et al*, 1997). c-Myb binding site is required for the activation of V(D)J recombination in both the TCR γ and TCR δ enhancers (Salomoni *et al*, 1997).

1.3.9 IL-7 and VEGF signalling pathways

Although signalling via VEGFR-3 (receptor for VEGF-C and VEGF-D) involves complex molecular pathways, but it mainly involves the MAPK and PI3-K pathways. Recent studies have indicated the presence of cross-talks between the MAPK and the PI3-K pathways as phosphorylation of Raf by Akt resulted in inhibition of the Raf-MEK (MAP kinase) – ERK (extracellular signal regulated kinase) pathway (Zimmermann and Moelling, 1999). PI3-Kinase activation

mediates IL-7 transduction and is involved mainly in proliferative signals. The cross talk between MAPK and PI3-K pathways leads to increased cell survival by stimulating the transcription of the pro-survival gene(s) and by post-translational modification and inactivation of components of the cell death machinery. VEGFR-3 can also strongly activate Stat-5 (Korpelainen *et al*, 1999), also known to be activated and phosphorylated by IL-7 (Foxwell *et al*, 1995) suggesting that Stat-5 activation is probably involved in the regulation of lymphatic endothelium. As mentioned above, the PI3-K pathway is linked to mitogenesis and cell survival by the activation of the serine-threonine kinase Akt (protein kinase B). PI3-K, activated in both IL-7 and VEGFR-3 signalling pathways, is a critical signalling molecule that regulates several cellular processes including mitogenesis, cell adhesion, motility, differentiation and anti-apoptotic activities.

1.3.10 IL-7 and malignancies

Malignant cells including breast cancer cells are surrounded by stroma and extracellular matrix which, as mentioned above, is composed of various cells like macrophages, lymphocytes, neurophils, fibroblasts, vascular endothelial cells as well as natural killer cells (Leek *et al*, 1994). All these cells including the malignant cells interact with each other via a complex network of extra-cellular signals, such as cytokines and growth factors and others. These interactions are thought to regulate the proliferation and metastatic activity of malignant cells as well as modulate the host immune system towards the tumour cells (Hasday *et al*, 1990).

1.3.10.1 IL-7 and haematopoietic malignancies

IL-7 is known to enhance the progression of some types of lymphomas and leukaemias. Studies on such malignancies indicate that IL-7 could stimulate the growth of both B and T acute lymphoblastic leukaemia (ALL) cells *in vitro* (Eder *et al*, 1992; Touw *et al*, 1990) and chronic lymphoblastic leukaemia (CLL) (Frishman *et al*, 1993). It has also been shown that IL-7 increases DNA synthesis in acute (AML) and chronic (CML) myeloid leukaemia cells (Digel *et al*, 1991). Other studies have demonstrated that IL-7 is also able to stimulate proliferation of cutaneous T cell lymphoma (Sezary syndrome) (Dalloul *et al*, 1992; Qin *et al*, 2001) as well as Hodgkin's disease (Foss *et al*, 1995). IL-7 is produced by chronic lymphoblastic leukaemia cells (Frishman *et al*, 1993) and Burkett's lymphoma cells (Benjamin *et al*, 1994). IL-7 levels have been found to be elevated in the serum of Hodgkin's lymphomas patients (Trumper *et al*, 1994). IL-7 transgenic mice develop a progressive cutaneous lymphoproliferative disorder as well as generalised lymphoproliferation that progress to malignancy making IL-7 as an oncogene in the living organism (Rich *et al*, 1993). Furthermore, a recent study has suggested a pathogenic role for IL-7 and IL-7R in the development of thyroid lymphoma (Takakuwa *et al*, 2000). Activation of specific Jaks and Stats has been demonstrated in several haematological malignancies including lymphomas and leukaemias (Gouilleux-Gruart *et al*, 1996; Migone *et al*, 1995; Weber-Nordt *et al*, 1996; Zhang *et al*, 1996).

1.3.10.2 IL-7 signalling and human solid tumours

Although the relationship between IL-7 and haematopoietic malignancies is well established, little is known about its interactions with solid tumours. IL-7, IL-7R and their signalling intermediates have been shown to have an involvement in solid human cancers. An outline of this is given below.

1.3.10.2.1 Expression of IL-7 in tumours

IL-7 mRNA is expressed in colorectal (Maeurer *et al*, 1997; Watanabe *et al*, 1995), oesophageal (Oka *et al*, 1995), renal (Trinder *et al*, 1999), head and neck squamous cell carcinoma (Paleri *et al*, 2001) as well as Warthin's tumour of parotid gland (Takeuchi *et al*, 1998). IL-7R mRNA is expressed in lung, colon, renal and CNS cancer cell lines (Cosenza *et al*, 2002). Recently, it has been revealed that IL-7 can stimulate the proliferation of prostatic cells in benign prostatic hyperplasia, a benign prostatic enlargement, by increasing growth patterns of the fibromuscular tissues in the prostate similar to that of wound healing (Kramer *et al*, 2001). In melanomas, IL-7 mRNA was detected in more than 60% of tumours (Mattei *et al*, 1994). Transfection of the IL-7 gene in human melanoma cell lines using a retroviral vector showed an unaltered expression of MAGE-1 and MAGE-3 antigens and a retarded growth in T cell deficient mice (Miller *et al*, 1993).

1.3.10.2.2 *Production of IL-7 by tumour cells*

As stated above, IL-7 is produced *in vivo* by normal human intestinal epithelial mucosa and stimulates the proliferation of lamina propria and intra-epithelial lymphocytes (Watanabe *et al*, 1995). IL-7 is also produced by colorectal cancer tissues and promote the expansion of tumour infiltrating lymphocytes (TIL) in these cancers (Maeurer *et al*, 1997). The activity of the tumour infiltrating lymphocytes (TIL) is thought to be regulated by cytokines. TIL is composed of helper T CD4⁺ cells, B lymphocytes as well as natural killer cells.

1.3.10.2.3 *Expression of IL-7R in tumour cells*

It has been shown that intestinal epithelial lymphocytes are completely absent from IL-7R α knockout mice, but only partially absent from IL-7 knockout mice (Fujihashi *et al*, 1997), suggesting a possible more significant role for IL-7R in intestinal lymphocyte proliferation. As stated above IL-7R is expressed in breast, lung, colon, renal and CNS cancer cell lines (Cosenza *et al*, 2002).

1.3.10.3 *IL-7 and breast cancer*

There is very limited data relating IL-7 to breast cancer. It has been shown that nuclear extracts from breast cancers display significantly higher levels of Stat-5a, a molecule involved in the downstream signalling pathway of IL-7, in invasive breast cancers, than those from benign and normal breast tissue (Watson and Miller, 1995), suggesting a possible enhanced IL-7 signalling in invasive breast cancer.

1.3.11 Therapeutic value of IL-7

There are conflicting reports about the therapeutic value of IL-7. While some studies directed to block its signalling pathway to treat certain malignancies, others directed to use it as an immune stimulating factor against cancer.

1.3.11.1 IL-7 as immune stimulating agent against cancer

It has been shown that intravenous IL-7 administration (5µg twice daily for 20 days) to mice with metastatic breast cancer had no therapeutic effect, however it has prolonged survival if chemotherapy was given to these mice, but this could be due to more rapid lymphoid reconstitution with IL-7 treatment (Talmadge *et al*, 1993). In another study, mammary adenocarcinoma tumour cells co-transfected with IL-7 and B7.1 resulted in a high level of protective immunity compared with IL-7 or B7.1 transfection alone or corynebacterium parvum (Cayeux *et al*, 1995).

It has been shown that IL-7 transduced tumour cells administered in conjunction with dendritic cells creates potent anti-tumour responses to lung cancer in a murine model (Sharma *et al*, 1997). IL-7 gene transfer in non-small cell lung cancer *in vitro* significantly augments T lymphocyte activities, inhibits tumour cell proliferation and modifies tumour cell surface phenotype, suggesting that IL-7 gene therapy may be effective in modifying host anti-tumour responses in non-small cell lung cancer (Sharma *et al*, 1996). IL-7 could also enhance TIL in renal cancer (Sica *et al*, 1993). In addition, IL-7 could enhance the growth and production of interferon γ from human renal cell carcinoma reactive TIL and to a

lesser extent enhance IL-2 induced lymphokine activated killer activity of TIL in renal carcinoma (Sica *et al*, 1993). Further *in vivo* studies in mice have revealed that administration of recombinant IL-7 (10µg twice daily for 7 days intraperitoneally) resulted in a 75% reduction in pulmonary metastases from renal carcinoma (Komschlies *et al*, 1994). However, most of these studies are now considered old and mostly used immature technologies.

In one *in vivo* study, administration of IL-7 (5ng twice daily) in mice with melanoma has been shown to decrease primary tumour size and metastatic lesions, but did not affect survival unless administered in combination with hyperthermia (Wu *et al*, 1993). However, in sarcomas, IL-7 can stimulate the growth of cytotoxic T lymphocytes that can be utilized in adoptive immunotherapy. IL-7 activation of CD8⁺ from draining lymph nodes of mice injected with syngeneic fibrosarcomas enhances anti-tumour responsiveness when these lymphocytes were injected to mice with fibrosarcoma (Lynch *et al*, 1991). Furthermore, IL-7 producing glioma cells were not rejected in mice depleted of CD8⁺ cells but were rejected in mice depleted of CD4⁺ or NK1.1⁺ cells, suggesting that CD8⁺ T cells may play an important role in IL-7 induced tumour rejection (Aoki *et al*, 1992; Jicha *et al*, 1991).

However, to-date the extent of IL-7 and its receptor level of expression in solid tumours has not been fully elucidated. IL-7 R activation and the downstream signalling pathway in relation to solid tumour biology has not been fully explained. Furthermore, there has been no clear correlation between the level of

expression of IL-7 / IL-7R or their downstream signalling intermediates with tumour behaviour, size, grade of differentiation, rate of metastasis, nodal involvement as well as survival and prognosis in these cancers.

1.3.11.2 Blocking IL-7 signalling in cancer

The fact that IL-7 is a proliferative and growth factor for some haematopoietic malignancies has led to some attempts to selectively block IL-7. Sweeney *et al* for example, have recently demonstrated that DAB₃₈₉IL-7 (diphtheria toxin, a recombinant fusion protein) is selectively cytotoxic for only those cells bearing the IL-7R, which may serve a potential therapeutic agent against IL-7R bearing malignancies (Sweeney *et al*, 1998). The expression of IL-7R can also be further explored as a target for directed therapy such as IL-7R immunoconjugates. However, it has been demonstrated that IL-7 can induce signal transduction events in cells that do not express IL-7R because of its capacity to engage other surface receptors such as Flt-3 and c-kit (Cosenza *et al*, 2002).

Constitutive Stats activation have been detected in other several solid tumours including prostate (Dhir *et al*, 2002), renal (Dhir *et al*, 2002; Horiguchi *et al*, 2002), lung (Fernandes *et al*, 1999), ovarian (Huang *et al*, 2000), pancreatic (Coppola, 2000) cancers as well as melanomas (Pansky *et al*, 2000). The development of assays to detect activated Stats in these tumours may be of benefit in terms of diagnosis, treatment as well as assessing prognosis. Assays developed to detect activated Stats might include DNA binding assays and the development of antibodies designed to detect the activated phosphotyrosine

forms of specific Stats could be used to compare normal and tumour tissues by immunohistochemistry (Bowman *et al*, 2000). Tumours with constitutive activation of Stat-3 and Stat-5 are predicted to be resistant to chemotherapeutic modalities, therefore, the development of selective inhibitors of Stats activation to be used in combination with more conventional chemotherapy may be a promising anti-cancer therapeutic modality (Bowman *et al*, 2000). Better understanding of the mechanisms underlying aberrant stats signalling during oncogenesis may lead to the development of novel cancer therapies based on interrupting key steps in this pathway.

IL-7 is well established as a proliferative and trophic cytokine that induces the development and proliferation of haematopoietic cells and malignancies. Recently, several publications have demonstrated the expression of IL-7 receptor in non-haematopoietic neoplasms (Saito *et al*, 1997; Yamada *et al*, 1997a). It is proposed that IL-7 might have some indirect effects on tumourigenesis via regulating some intracellular mechanisms, which might sensitise the cells to the effects of other cytokines and / or proliferative agents. The expression of IL-7 mRNA in some non-haematopoietic malignancies suggest the possibility for an autocrine growth pathway for IL-7. The production of IL-7 by some human solid tumours including colon and other cancers suggest a possible impact on the process of tumourigenesis. Furthermore, the detection of a functional IL-7 receptor in human solid malignancies also supports this concept. While the exact effects of IL-7 signalling activation is still unclear, the downstream signalling intermediates are upregulated in several human solid tumours including lung,

prostate, renal, ovarian, melanomas as well as head and neck tumours. This could be explained by the possibility of the occurrence of some changes in tumour microenvironment leading to changes in tumour development, behaviour and progression. Aberrant Jak – Stat pathways could indeed result in oncogenesis in some solid tumours. Understanding of the mechanisms underlying these aberrant pathways in IL-7 signalling may lead to the development of novel cancer therapies based on interrupting key steps in these pathways. The relationship of IL-7 and solid tumours are still far from clear. Better understanding of the effects of IL-7 on endothelial and epithelial cell development, growth and differentiation as well as the mechanisms that control the activation of IL-7 signalling might have an important impact on oncogenesis. Therefore more studies are required to address this important issue.

CHAPTER TWO

AIMS OF THE THESIS

Cancer cells metastasise via blood and lymphatic vessels as well as locally. Amongst all the other prognostic factors, the status of lymph nodes is the most important indicator of the prognosis. Although angiogenesis has been extensively explored in the past decade, research and knowledge on lymphangiogenesis is however limited. This was due to the lack of molecular markers specific to the lymphatic endothelium. During the last few years there has been successful identification of markers that appear to be lymphatic specific, including LYVE-1, prox-1 and podoplanin. A major breakthrough in this field was the discovery of VEGFR-3, a tyrosine kinase receptor. As stated in the introduction chapter, the ligands of VEGFR-3, VEGF-C and VEGF-D, are the only currently known lymphangiogenic factors. The study presented here has been conducted to understand the regulation of lymphangiogenesis and the importance of targeting it in cancer therapy.

The main aims of this thesis were therefore:

- Study the effects of cytokines, including IL-7, on the expression of the specific lymphatic markers in endothelial cells.

- Investigate the mechanism(s) via which IL-7 exerts its effects on the lymphatic characteristics of endothelial cells.

- Explore IL-7 signalling pathway(s) in endothelial cells.

- Develop an *in vivo* lymphangiogenesis model.

- Assess the effects of IL-7 on endothelial cells growth and migration.

- Investigate the role of IL-7 in breast cancer:
 1. Dissection of IL-7 signalling pathways in breast cancer cells.
 2. Investigating the effects of IL-7 on breast cancer cells growth.
 3. Assess the relation of IL-7 to different clinicopathological characteristics in patients with breast cancer.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 GENERAL MATERIALS

3.1.1 Cell lines

The following cell lines have been used during this study:

HECV	Human endothelial cell line
MDA-MB-231	Human breast cancer cell line
MCF-7	Human breast cancer cell line
HL-60	Human leukaemia cell line

The above cells were obtained from the European Collection of Animal Cell Culture (ECACC). Human umbilical vein endothelial cells (HUVEC) were obtained from TCS, Biologicals (TCS, Biologicals Ltd, Buckingham, UK). HECV cell line was obtained from Interlab Cell Line Collection (ICLC), Naples, Italy.

3.1.2 Antibodies

Primary antibodies

The following is a list of primary antibodies used during this study. The antibodies were obtained from Santa Cruz Biotechnology, California, USA.

Anti-IL-7*	Mouse monoclonal
Anti-IL-7R	Rabbit polyclonal
Anti-Jak-1	Rabbit polyclonal
Anti-Jak-3	Mouse polyclonal
Anti-PI3-K	Rabbit polyclonal

Anti-Stat-5	Mouse Monoclonal
Anti-VEGF-A	Rabbit polyclonal
Anti-VEGF-B	Rabbit polyclonal
Anti-VEGF-C	Rabbit polyclonal
Anti-VEGF-D*	Mouse monoclonal
Anti-LYVE-1	Rabbit polyclonal
Anti-VEGFR-1 (Flt-1)	Rabbit polyclonal
Anti-VEGFR-2 (Flk-1/KDR)	Mouse monoclonal
Anti-VEGFR-3 (Flt-4)	Rabbit polyclonal
Anti-Actin	Mouse monoclonal
Anti-Factor VIII (von Willebrand)	Rabbit polyclonal
PY-20	Mouse monoclonal
PY-99	Mouse monoclonal

*These primary antibodies were purchased from Research and Development Systems.

Secondary antibodies

Horse Radish Peroxidase (HRP) conjugated anti-goat IgG, anti-rabbit IgG and anti-mouse IgG antibodies were obtained from Sigma (Poole, Dorset, UK). A/G protein agarose beads used during immunoprecipitation were obtained from Santa-Cruz Biotechnology (Santa-Cruz, California, USA).

3.1.3 General solutions

The following list of solutions was used routinely during this study. All reagents were supplied by Sigma (Poole, Dorset, UK):

Tris-Boric acid-EDTA (TBE)

A stock solution of TBE was made at 10X normal concentration and contained 0.89M Tris, pH 8.3, 0.89M Boric acid and 31mM EDTA. The solution was made by dissolving 108g Tris, 55g Boric acid and 9.3g EDTA in 1 litre dH₂O.

Tris Buffered Saline (TBS)

A 10x stock solution was made up by dissolving 24.22g Tris and 80.06g NaCl in 1 litre dH₂O and adjusting the pH to 7.4 with concentrated HCl. This solution contained 200mM Tris and 1.37M NaCl.

Balanced Salt Solution (BSS)

A 5 litre stock solution of 1xBSS consisted of 137mM NaCl, 2.6mM KCl, 1.7mM Na₂HPO₄ and 8.0mM KH₂PO₄ and was made by dissolving 40g NaCl, 1g KCL, 5.75g Na₂HPO₄ and 1g KH₂PO₄ in dH₂O and adjusting the pH to 7.4 with NaOH.

Tris-EDTA (TE) buffer

A 1 litre stock solution at 1x concentration contained 10mM Tris, pH 7.6 and 1mM EDTA and was made by dissolving 1.211g Tris and 0.292g EDTA in 1 litre dH₂O.

3.1.4 Other reagents and chemicals

- Recombinant human IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, interferon- γ (IFN- γ), HGF/SF, tumour necrosis factor alpha (TNF- α) and VEGF-D were purchased from National Biology Standard Bureau - NBSB (Salisbury, England, UK) and Chemicon International (Temecula, California, USA).
- MTT reagent used to assess cell growth was obtained from Sigma (Pool, Dorset, UK).
- PicoGreen[®] dsDNA quantitation Kit was obtained from Molecular Probe (Oregon, USA)
- Downstream IL-7 signalling molecules inhibitors were purchased from Calbiochem-Novabiochem Corp. (San Diego, USA):
 - Jak-3 inhibitor: Jak-3 Inhibitor 1 {4-(4'-Hydroxyphenyl)amino-6,7-dimethoxyquinazoline; WHI-P131}.
 - Jak-Stat pathway inhibitor: AG 490 {Tyrophstin B42; α -Cyano-(3,4-dihydroxy)-N-benzylcinnamide; N-Benzyl-3,4-dihydroxy- α -cyanocinnamide}.
 - PI3-Kinase inhibitor: Wortmannin {KY 12420}.
 - Stat-5 inhibitor: Piceatanol.

- The chemiluminescent substrate kit KPL (Kirkegaard and Perry Laboratories, Maryland, USA) LumiGLO™ was distributed by Insight Biotechnology (Middlesex, UK).

3.2 GENERAL METHODS

3.2.1 Cell culture and storage

3.2.1.1 Preparation of Growth Medium and Maintenance of Cells

Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) (pH 7.3) containing; 2mM-glutamine, 15mMHEPES and 4.5mMNaHCO₃, DMEM was then supplemented with 10% heat inactivated foetal calf serum (PAA Laboratories, Austria), 50 units/ml of benzylpenicillin (Britannia, Pharmaceuticals, Ltd) and 50 µg/ml of streptomycin (Gibco BRC, Paisley, Scotland). The cell lines were cultured in monolayers either 25cm² or 80cm² culture flasks (Cell Star, Germany) at cell densities of 1x10⁵ cells/ml. Culture flasks were loosely capped and placed in an incubator at 37°C with a 98% humidification (water tray in the incubator) with 5% CO₂ in air. The flasks were then left until sub-confluent (2-3 days) for experimental work or fully confluent (7 days) for subculture.

3.2.1.2 Trypsinization and Counting of cell lines

- All handling of cells performed under sterile conditions using class II hoods and autoclaved instruments to keep conditions sterile as much as possible.

- Following removal of medium, the flasks were rinsed once with 5ml of HBSS (Hanks Balanced Salt Solution) (137mM NaCL; 8mM Na₂HPO₄; 3mM KCl; 1.5mM KH₂PO₄) buffer to remove all possible traces of serum, which would inhibit the enzymatic action of trypsin.
- 1-2ml of trypsin / EDTA (trypsin 0.01% (w/v) and EDTA 0.05% (w/v) in HBSS buffer) was added to the flask and it was incubated for 5 minutes at 37°C to allow cell detachment.
- Once the cells had detached from the surface of the flask, the effect of trypsin was neutralised by the addition of 5ml of DMEM and placed in a universal container.
- The cells were centrifuged at 15000 g for 5 minutes.
- The excess medium aspirated and the pellet re-suspended in 5mls of DMEM.
- The cells were then re-cultured in flasks, counted for immediate experimental work or stored by freezing in liquid nitrogen (see below).
- Cell counts were performed using an improved Neubauer haemocytometer counting chamber with an inverted microscope (Reichert, Austria) at 10x10 magnification.

3.2.1.3 Storage of cell lines by freezing in nitrogen

The cell lines were stored in liquid nitrogen by resuspending at a cell density of 1×10^6 cells/ml in DMEM containing 10% (v/v) dimethylsulphoxide (DMSO; Fisons, UK). 1ml aliquots of cell suspension were transferred into cryopreserve

tubes, transferred to -80°C for 24 hours before storage in liquid nitrogen (-196°C) until required.

3.2.1.4 Resuscitation of cell lines

After removal from liquid nitrogen, the cells were allowed to thaw rapidly to 37°C and the cell suspension was transferred to a universal container, with 2ml of DMEM. The cells were then incubated at 37°C for 10 minutes, centrifuged at 1600 g for 5 minutes and the excess medium was removed. The cell pellet was re-suspended in DMEM and washed twice to remove any possible trace of DMSO. After the final wash, the cell pellet was re-suspended in 5ml of DMEM and the cell suspension transferred to a 25cm^2 tissue culture flask. The cells were incubated at 37°C , 98% humidification and 5% CO_2 in air.

3.2.2 Generation of cDNA templates for application in PCR

3.2.2.1 Total RNA isolation using the guanidine thiocyanate method

RNA is susceptible to degradation by RNases and therefore special care must be taken to minimise this during its isolation. All methods involving RNA isolation rely on the use of strong denaturants to inhibit the action of endogenous RNases. The use of guanidine, thiocyanate and chlorine are amongst the most effective protein denaturants and inhibitors of ribonucleases. The guanidine thiocyanate method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) involved a rapid procedure for combining acid guanidine thiocyanate – phenol-

chloroform, in a single step RNA extraction. Using this method, the extraction of RNA is set under acidic conditions, so that the DNA is selectively partitioned into the organic phase whilst the RNA remains in the aqueous phase. The quality and concentration of the RNA isolated can then be detected using a spectrophotometer at a wavelength of $A_{260\text{nm}}/A_{280\text{nm}}$.

Cell lines were grown until 80-90% confluent in 25cm² tissue culture. The culture medium was aspirated and the monolayer of cells was detached from the surface of the flask by using 1ml of RNA isolation reagent (Advanced Biotechnologies Ltd, UK), aided by a cell scraper, 1ml aliquots of resultant homogenate were transferred to polypropylene tubes and incubated on ice for 5 minutes to permit the complete dissociation of nucleoprotein complexes. One fifth of the volume of chloroform (200µl per sample) was added to the homogenate, followed by vigorous shaking for 15 seconds, incubation on ice for 5 minutes and centrifugation at 15000 rpm for 15 minutes at 4⁰C. The homogenate will be separated into two distinct phases: the lower organic phase containing DNA and protein and the upper aqueous phase containing RNA. The majority of the aqueous phase (40 – 50% of the total volume of the homogenate) was carefully aspirated (without disturbing the interphase) and transferred to a polypropylene tube. An equal volume (about 500 - 600µl per sample) of isopropanol (Sigma) was added to the aqueous phase and incubated on ice for 10 minutes.

This was followed by centrifugation at 12000 rpm for 10 minutes and the supernatant fraction was subsequently removed. The resultant RNA pellets were washed once using 1ml of 75% ethanol in diethyl pyrocarbonate (DEPC; Sigma) treated H₂O at 12000 rpm for 5 minutes. The RNA pellets were briefly dried at 55°C for 2 - 5 minutes in order to evaporate the remaining ethanol and dissolved in 50 - 75µl of DEPC treated water by vortexing for 1 minute. The purity of the resultant RNA was determined by measuring its absorbance at wavelength $A_{260\text{nm}}/A_{280\text{nm}}$ (WPA UV 1101, Biotech Photometer). $A_{260\text{nm}}/A_{280\text{nm}}$ ratio gives an estimate of the purity of the RNA. Pure RNA solutions have an optical density ratio of 2.0. Optical density values less than 1.5 indicates ethanol or protein contamination. The samples were either stored at -80°C until used later or ready for reverse transcriptase (RT-PCR), (see below).

3.2.2.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is a simple, versatile and sensitive technique, which has greatly enhanced the study of genes and how they are controlled. It provides an alternative and more sensitive approach for the analysis of mRNA, compared to other procedures such as; Northern blots and RNA dot blots. This technique has the advantage over more traditional methods in that it requires a small amount of RNA and yet more sensitive and rapid. In this study *Reverse-iT™* First Strand Synthesis Kit was used. According to the manufacturer's instructions the following components were mixed together in a polypropylene tube (Appendoff):

RNA template (volume depends on concentration)

PCR H₂O (volume depends on RNA concentration)

[RNA template and PCR H₂O mix provides 13 µl volume)

1µl of anchored oligo dT at a concentration of 0.5µg/µl.

4µl of 5 x 1st strand synthesis buffer

2µl of 5mM dNTP mix

The resultant total volume in each Appendoff tube is 20µl. The samples were heated at 47°C for 50 – 60 minutes followed by incubation at 75°C for 10 minutes in order to inactivate any RTase. The cDNA samples were then diluted to 1:2 by adding 20µl of PCR H₂O (distilled, deionised and UV-treated H₂O). The samples are ready for amplification using PCR or they were stored at -20°C until required.

3.2.2.3 Polymerase chain reaction (PCR)

The polymerase chain reaction is a very useful technique for enzymatically amplifying a nucleic acid target sequence. It was discovered by Karey Mullis in 1983, for which he was awarded the Nobel prize in 1994. Two short synthetic oligonucleotides that hybridise to complementary strands flanking the target sequence are used as primer for DNA synthesis; the primers are aligned with 3' ends directed towards each other. Thus repeating cycles of denaturation of template, annealing of primers and extension by DNA polymerase results in amplification. A key development in PCR technology was the use of Taq polymerase, a thermostable enzyme from *Thermus aquaticus*, a bacterium native

to hot springs. The use of this enzyme means that fresh polymerase does not need to be added after each denaturation step, facilitating automation and recycling costs.

Amplification of cDNA templates previously prepared was performed using PCR. The following method briefly outlines a typical PCR procedure used for cDNA amplification in this study:

1µl of 1:2 diluted cDNA template

1µl of forward primer (at a working concentration of 10 picomoles)

1µl of reverse primer (at a working concentration of 10 picomoles)

The above components were added to the pre-aliquoted (23µl) PCR Ready-Load Master Mix™ (0.625U of *Taq* DNA Polymerase from *Thermus aquaticus*; 75mM Tris-HCl (pH 8.8 at 25°C); 20mM (NH₄)₂SO₄; 1.5mM MgCl₂; 0.01% (v/v) Tween 20; 0.2mM from each of the following: dATP, dCTP, dGTP and dTTP, supplied by Advanced Technologies Ltd, UK) in a thin walled PCR tube to make the volume to 26µl. alternatively, the reaction volume can vary (see separate experimental chapters), for example 12µl reaction (6µl Master Mix, 3µl PCR H₂O, 2µl of primers (F & R) & 1µl cDNA) or 16µl reaction (8µl Master Mix, 5µl PCR H₂O, 2µl of primers (F & R) & 1µl cDNA. PCR reactions were performed using a Gene Amp PCR thermal cycler.

PCR conditions were variable between reactions (see relevant experimental chapters), but generally consisted of 35-40 cycles of denaturation (94⁰C),

annealing (55⁰C) and extension (72⁰C), with a final extension phase at 72⁰C for 10 minutes.

3.2.2.4 Agarose gel electrophoresis

The generated PCR products were separated on agarose gel at either 0.8% or 2% depending on the size of the PCR product. 0.8g or 2g dissolved in 100ml of TBE (Tris Borate Electrophoresis) buffer (0.45M Tris-HCl; 0.44M boric acid; 12.5mM EDTA)). A low percentage gel (large pore size) would be used if one were to separate PCR products with a high number of base pairs and conversely, high percentage gel (small pore size) would be used to separate PCR products with a low number of base pairs (<500 bp). In the present study, 20mls of agarose gel solution at the appropriate concentration was poured into a universal container and cooled down before casting the gel in the mould in the electrophoresis tank. A comb was then inserted into the gel mould and the gel was allowed to set at room temperature for about 30-40 minutes.

Once the gel had set, TBE buffer was carefully poured into the electrophoresis tank until it reached a level of about 5mm from the surface of the gel. The PCR products were loaded into the wells (5µl per well depending on the comb type). A 1 kb pair ladder was prepared according to the manufacturers instructions (Pharmacia Biotech, USA) and delivered into the first well (5µl per well) in the same manner as for the PCR products.

A power pack (Gibco BRL, Life Technologies Inc) was connected to the electrophoresis apparatus and the gel was run at a constant voltage of 100 Volts. Electrophoresis was continued for about 35 – 55 minutes or until the samples had migrated about two-thirds down the agarose gel (depending on the PCR product size). The PCR products were then stained using ethidium bromide (10mg/ml) for 5 minutes with continuous agitation to ensure even staining of the agarose gel. This was sometimes followed by de-staining in tap water for about 1-2 hours, if preferred, to reduce the background staining on the agarose gel from the fluorescent dye. PCR products were then visualised on the agarose gel using an UV transilluminator (UVP Inc) and connected to a thermal printer and image scanning unit for permanently and electronically storing images.

3.2.3 Real time quantitative RT- PCR

Quantitative real-time polymerase chain reaction (PCR) is a relatively new technology that provides a broad dynamic range for detecting specific gene sequences with excellent sensitivity and precision. DNA and RNA can be quantified using this method without laborious post-PCR processing. It is based on the detection of a fluorescent signal produced proportionally during amplification of a PCR product.

The chemistry is the key to the detection system. Figure 2.1 shows a typical Taqman reaction. A probe (TaqMan) is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labelled at the 5' end with a reporter fluorochrome (usually 6-carboxyfluorescein [6-FAM]) and

a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine) added at any T position or at the 3' end. The probe is designed to have a higher T_m (melting temperature) than the primers, and during the extension phase, the probe must be 100% hybridized for the assay to be successful.

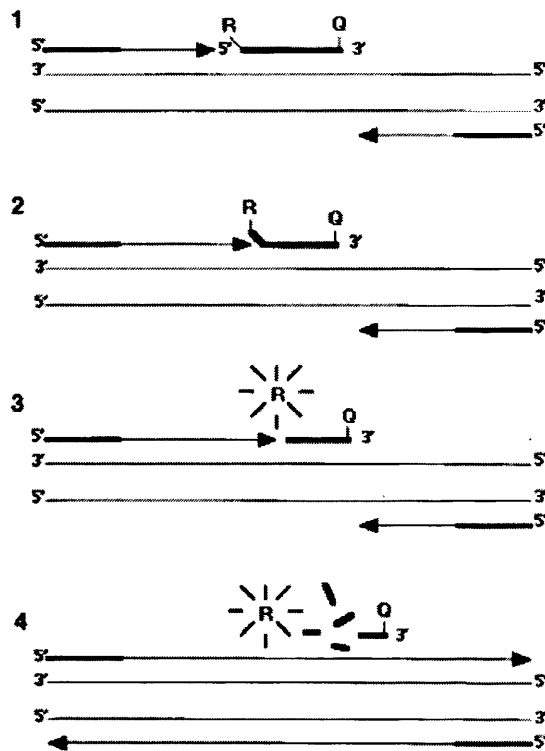


Fig 2.1 Fluorogenic 5' nuclease chemistry used in Taqman based analysis. (1) Forward and reverse primers are extended with *Taq* polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes attached anneals to the gene sequence between the two primers. (2) As the polymerase extends the primer, the probe is displaced. (3) An inherent nuclease activity in the polymerase cleaves the reporter dye from the probe. (4) After release of the reporter dye from the quencher, a fluorescent signal is generated.

As long as both fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as *Taq* polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of *Taq* degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle.

The system used was iCycler iQ™ detection system that consists of a 96-well thermal cycler module connected to a laser and charge-coupled device (CCD) optics system (Biorad). An optical fibre inserted through a lens is positioned over each well, and laser light is directed through the fibre to excite the fluorochrome in the PCR solution. Emissions are sent through the fibre to the CCD camera, where they are analysed by the software's algorithms. Collected data are subsequently sent to the computer. Emissions are measured constantly.

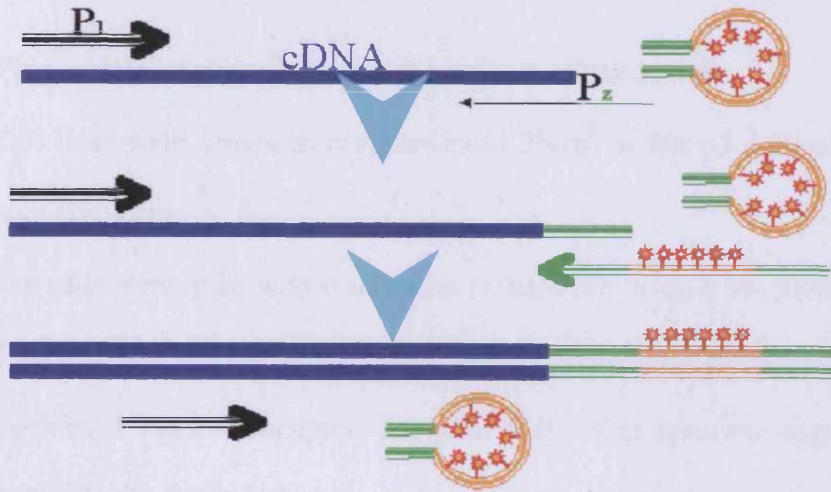
In the Ampliflor system, the quantitation used here include hot start *Taq* polymerase which require minimum 12 minutes to activate at the beginning of the reaction, dNTPs, salts, DNA template. A target specific forward primer (P1), a target specific reverse Z primer (Pz) which incorporate a unique Z sequence at the 3' end of the primer (green part of Pz), Figure 2.2A, and a universal primer that is linked to the fluorophore FAM. The probe also has a stem sequence that is complementary to the Z primer sequence in Pz.

A typical Amplifor based Q-PCR is comprised of 8µl hot-start Q master mix, 1µl P1 primer at 10 pmol/µl, 1µl Pz primer (one tenth of P1 and the probe), and 1µl probe (10pmol/µl). The typical reaction is: 95°C for 15 minutes to activate hot-

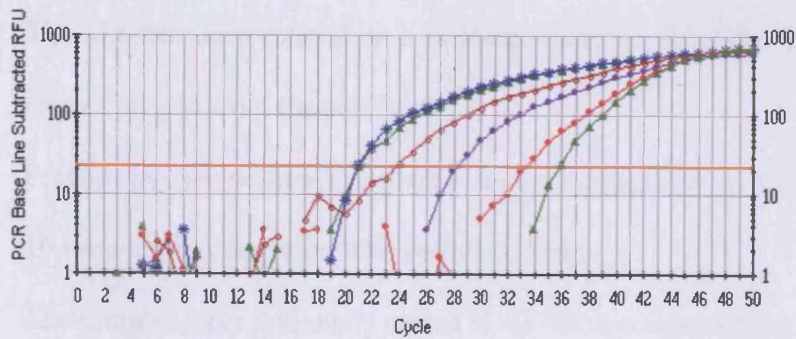
start Taq polymerase, followed by 50 cycles at: 95°C for 12 seconds, 55°C for 40 seconds, and 72°C for 15 seconds. Detection of fluorescence signal was carried out at the annealing step. The above reaction will allow the P1 and Pz to first amplify the specific target. Pz will soon be consumed due to its low amount added (normally take 12-15 cycles). This will then allow the probe stepping in as a specific primer/probe to amplify the P1/Pz amplicon, with resultant fluorescence DNA products. In all the reactions, an internal standard with known number of copies was included, for calculation of the known samples (Figure 2.2B)

The information analysed by the computer and two graphs are plotted; to show the threshold cycle against the log starting quantity, copy number to show the correlation coefficient and the slope. In addition a table is generated to show the number of copies in each well of the plate. More specific conditions will be explained separately in the relevant experimental chapters.

A:



B:



PCR Amplification vs Cycle: bcsqbrca2.opd

Figure 2.2 Quantitative PCR analysis using the Ampliflor system. The analysis is comprised of four main components, in addition to the routine enzymes and chemicals, the target specific forward primer (P₁), target specific reverse primer (P_z) with a unique Z sequence (green part of P_z), which is complementary to the stem of a universal probe (green stem in the orange probe). The probe is conjugated with the fluorophore, FAM.

3.2.4 Western Blotting

3.2.4.1 Preparation of protein from cell lysates for SDS-PAGE

- Cell lines were grown as monolayers in 25cm² or 80cm² culture flasks until confluent.
- The cells were lysed with lysis buffer (10mM NA₃VO₄, 0.5% SDS, 0.5% Triton X-100, 2 mM CaCl₂, 100 µg/ml (1mM) phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml (10 U/ml) aprotinin and 0.1% IGEPAL CA-630) and the resultant lysates were transferred to 1ml Appendoff tubes.
- The samples were rotated on a rotating wheel for 40 minutes in order to extract protein from the cell lysate.
- Protein was subsequently collected by centrifugation at 16,000 rpm for 10 minutes in order to remove cellular debris.
- The samples were optionally stored at -20⁰C to continue later.

3.3.4.2 Determination of total cellular protein concentration

In order to detect the differences in protein levels in cells following various treatments, equal amounts of protein from each sample were used. The initial cell counts were the same and cells undergone same culturing conditions. The method used in this study was based on that described by Bohlen *et al* (Bolen *et al*, 1972) using the reagent, 4-phenylspiro[furan-2(3H),1'-phthalan]-3,3' -dione (fluorescamine). Fluorescamine reacts with protein amino groups to yield a

highly fluorescent product at an alkaline pH and can be detected with a fluorescent plate reader. A brief summary of this method is outlined below:

40 μ l of protein from cell lysates and 40 μ l serial dilutions of a 100 mg/ml bovine serum albumin (range of 50 – 0.79 mg/ml, diluted with cell lysis buffer) were placed in a 96-well microtiter plate. Into each well, 60 μ l of phosphate buffer (pH 8.8) was pipetted, before a 25 μ l of fluorescamine solution (0.3 mg/ml in acetone) was added into each well. The plate was then placed into the fluorescent plate reader (Denly, Sussex, UK) and the fluorescence measured at $\lambda_{\text{excitation}}=540$ nm and $\lambda_{\text{emission}}=590$ nm. A standard curve for the BSA was constructed and the protein concentrations from various cell samples was determined accordingly.

3.3.4.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The method used in this study was the one based on the method used by Laemmli (Laemmli, 1970). The technique works on the basis that when a protein sample is heated in the presence of a reducing agent such as 2-Mercaptoethanol (2-ME; Sigma) and sample buffer containing an ionic detergent SDS, the protein becomes denatured with subsequent protein coating from SDS. This coating gives the protein a high negative charge, which is proportional to the length of the polypeptide chain.

The samples are then loaded onto a polyacrylamide gel and a high voltage is applied. This causes the protein components to migrate in the direction of the

positively charged anode. The ionic detergent SDS binds to proteins in proportion to their molecular weights and this binding is independent of their sequence. The resultant protein-SDS complexes are then separated on the basis of their molecular mass, through the molecular sieving properties of the gel. The size of the separated proteins can then be determined by comparing the gel mobility of a particular band, with that of a known protein standard (Ladder). Alternatively, a plot of molecular weight from known protein standards can be plotted against their mobility, in order to determine the size of unknown protein bands. Following electrophoresis, bands on the membrane may be visualised by staining with Coomassie Blue, or the proteins are electroblotted onto nitrocellulose membranes for subsequent probing with relevant antibodies.

3.2.4.4 Gel preparation

Polyacrylamide gels (30% acrylamide mix, crosslinker ratio 29:1; Bio-Rad) are formed by polymerising acrylamide with a cross-linking agent (methylene-bis-acrylamide) in the presence of a catalyst / chain initiator mixture, producing a cross-linked matrix with a particular pore size. The most commonly used catalyst is TEMED (N,N,N,'N'-tetramethylethylenediamine; Sigma) and the initiator is the persulphate ion ($S_2O_8^{2-}$), obtained in the form of ammonium persulphate (Sigma). The rate at which gels set may be controlled by varying the concentrations of persulphate and TEMED within the acrylamide mixture. The porosity of the gel is determined by the relative proportion of the acrylamide monomer to the cross-linking agent.

Gels are usually referred to in terms of the total percentage of acrylamide, monomer and bis present. Most protein separations are carried out using gels within the range of 5-15%. The appropriate choice of acrylamide concentration is based upon the separation range required for the protein. Therefore, a low percentage gel (large pore size) would be used if one were to separate proteins of a high molecular weight and conversely, a high percentage gel (small pore size) would be used to separate low molecular weight proteins. Table 3.1 outlines the ingredients, which were used for preparing resolving and stacking gels used in this study.

Table 2.1 SDS-PAGE resolving and stacking gel components. The volumes shown are for 10ml (resolving gels) and 5ml (stacking gels) respectively.

<i>Stock Solutions</i>	<i>15ml Resolving Gel (8%)</i>	<i>15ml Resolving Gel (10%)</i>	<i>15ml Resolving Gel (12%)</i>	<i>5ml Stacking Gel</i>
DH₂O	6.9ml	5.9ml	4.9ml	3.4ml
30% acrylamide mix	4.0ml	5.0ml	6.0ml	0.83ml
1.5M Tris (pH 8.8)	3.8ml	3.8ml	3.8ml	N/A
1.0M Tris (pH 6.8)	N/A	N/A	N/A	0.63ml
10% SDS	0.15ml	0.15ml	0.15ml	0.05ml
10% ammonium persulphate	0.15ml	0.15ml	0.15ml	0.05ml
TEMED	0.009ml	0.006ml	0.006ml	0.005ml

SDS-PAGE was carried out by assembling the gel (Mini Protean II gel system) apparatus according to the manufacturers instructions (Bio-Rad Laboratories, Richmond Ca, USA). A brief description of the procedure is outlined below:

- Using 1ml transfer pipette the resolving gel mixture was added to the gel system, by carefully running the solution down one side between the glass plates. This solution was added until it reached a position of 0.5cm away from the lower end of the gel plate.

- To ensure that the gel set with a smooth surface a 0.1% solution of SDS was run down the side between the glass plates until a layer of about 2mm formed on top of the gel solution (the great difference in densities between the SDS solution and the gel, resulted in the SDS solution resting on the surface of the gel mixture without causing serious mixing of the solutions), the gel was allowed to set at room temperature for about 30 –50 minutes. When the gel had set a very clear refractive index change was observed between the polymerised gel and the overlaying SDS solution.

- The SDS solution was removed with some filter paper and the gel rinsed once using distilled water. The stacking gel was then prepared as outlined in Table 2.1.

- The stacking gel was added to the gel cassette in the same manner as the resolving gel until it reached the top end of the gel plate.
- A well forming comb was gently inserted between the glass plates until it reached within 1mm from the top of the resolving gel and left to set at room temperature. The refractive changes around the comb indicated that the gel had set.
- The comb was carefully removed from the stacking gel and the gel cassette was assembled in the electrophoresis tank as outlined in the manufacturer's instructions (Bio-Rad Laboratories, Richmond, USA).
- The central reservoir was then filled with the running buffer (25mM Tris-HCl; 3.5mM SDS; 192mM glycine) so that it flooded over and filled the wells.
- The standard protein (detection range 14-66 kDa) or high molecular weight markers (SDS6H Sigma) were prepared according to the manufacturer's instructions (detection range 29-205kDa) and loaded (10µl/well) first.
- The protein samples prepared previously were loaded into the wells (10-15µl per well). The protein samples were pre-mixed with a dense loading buffer (10% glycerol; 5% 2-mercaptoethanol; 3% SDS; 80mM Tris-HCl

(pH 6.8); 0.012% bromophenol blue), which ensured that the sample settled at the bottom of the well.

- The power pack was connected to the apparatus and the gel was run at a constant current of 15mA. Electrophoresis was continued until the leading edge had reached the bottom of the gel (as indicated by the bromophenol blue present in the loading buffer).

- Following SDS-PAGE the gel was removed from the gel cassette, rinsed in transfer buffer (25mM Tris-HCl; 192mM glycine; 20% (v/v) methanol) to facilitate the removal of electrophoresis buffer, salts and detergents. The gel was then left to equilibrate in transfer buffer for 20 minutes.

- **Electroblotting:** Proteins are transferred onto an inert membrane support (e.g. nitrocellulose or nylon) and once attached, the protein of interest can be detected by specific monoclonal or polyclonal antibody against it. A nitrocellulose membrane (Amersham International Plc) was cut to the dimensions of the gel (9 x 6cm) and immersed in transfer buffer for about 10-20 minutes to ensure proper binding of the protein to the membrane. Similarly, filter paper was cut to the dimensions of the gel (9 x 6cm) and soaked in transfer buffer for 10-20 minutes. Two pieces of wetted filter paper were placed on the bottom electrode (cathode) and on top of this was placed one piece of pre-wetted nitrocellulose membrane and this was

then covered with two further pieces of pre-wetted filter paper forming a sandwich. The surface of this sandwich was carefully smoothed out to remove the formation of air bubbles, which may interfere with protein transfer. The top electrode was placed on top (anode) of this sandwich and a current was applied. The proteins were then transferred for 40 minutes using the following settings: 5 Volts, 500mA and 8 Watts.

- Following electroblotting, the nitrocellulose membranes were removed and stained in Ponceau S (40ml of 2% w/v Ponceau S concentrate; containing 30% w/v trichloroacetic acid and 30% w/v sulphosalicyclic acid (Sigma) was diluted with 360ml of distilled water to provide a 1:10 working solution) for one minute at room temperature. The staining of proteins on nitrocellulose membranes using Ponceau S has two functions; firstly, it verifies that the proteins have been transferred to the membranes and secondly, it aids in the visualisation of the molecular weight markers. In addition the Ponceau S stain is a reversible protein stain and does not interfere with subsequent immunoprobng steps. Excess staining was then removed by rinsing the membranes in distilled water and the position of the molecular weight markers were indicated on the membranes using a pencil.
- The membranes were coated with 10% milk solution (10g of semi-skimmed milk powder (Marvel) dissolved in 100ml of TBS (Tris Buffered Solution) buffer (20mM Tris-HCl; 150mM NaCl (adjusted to

pH 7.5)), containing 100µl (0.1%) of Tween 20 (Sigma) detergent to block non-specific protein sites for 40 – 60 minutes.

- The membranes were transferred to 50ml polypropylene tubes (Falcon) and incubated in 5ml of 3% milk solution in TBS and 0.1% Tween 20 containing the relevant primary antibodies in the appropriate concentrations for 1 hour at room temperature on a rotating wheel to allow for an even antibody coating on the membrane.
- Excess primary antibody was removed by washing the membranes three times, for 10 minutes per wash, using 5 – 10ml of 3% milk with 1x TBS and 0.1% Tween 20 solution per wash.
- The secondary horseradish peroxidase-conjugated antibodies were prepared in a similar manner to that of the primary antibodies. The membranes were incubated with 5ml (for each membrane) of secondary peroxidase-conjugated antibody at room temperature on a rotating wheel for 1 hour.
- Excess secondary peroxidase-conjugated antibody was removed by washing the membranes in the same manner described above for the primary antibody.

- This was followed by two 15-minute washes in 5 – 10ml of TTBS buffer (Tween 20 Tris Buffered Solution) containing 100ml of TBS buffer and 200µl (0.2%) and Tween 20 (Sigma) detergent. Finally the membranes were washed with 10 ml of TBS buffer only.

- **Detection using chemiluminescence:** In the presence of hydrogen peroxide (H₂O₂) and the chemiluminescent substrate luminol, Horse Radish Peroxidase (HRP) oxidises the luminol with concomitant production of light, the intensity of which is increased 1000 fold by the presence of a chemical enhancer such as phenol. The light emission can be detected by exposing the nitrocellulose membrane to a photographic film

- 10ml of KPL chemiluminescent reagent A was mixed with 10ml of KPL chemiluminescent reagent B in a universal container and poured into a weighing boat. The nitrocellulose membranes were removed from the TBS solution and excess buffer were allowed to drain onto a tissue. The membranes were immersed in the KPL chemiluminescent solution for about 1 minute and excess solution was allowed to drain off the membranes using tissue paper.

- The membranes were covered in Saran Wrap, placed in an autoradiography film cassette and transferred into a dark room. Using safe darkroom lighting conditions, a piece of autoradiography film

(Kodak XOMAT-AR) was placed on top of the membranes and the cassette closed. Exposure of the film to the membranes was conducted for 1 and 5 minutes in the first instance, in order to ascertain the strength of the signal. The exposure times for subsequent membranes was adjusted to give the best possible resolution of the antibody signal on the film. Following exposure, the film was developed using an automated film developer (X-ograph Imaging Systems, Malmesbury, Wiltshire, UK).

- After all the films had been developed, the membranes were stained using Amido black stain (100mg of Naphthalene black dissolved in 100ml of 10% v/v acetic acid and 25% v/v propan-2-ol) by immersing them for 15 seconds. The membranes were then placed in an Amido black de-stain (10% v/v acetic acid and 25% v/v propan-2-ol) solution in order to bring up the protein bands on the membrane. The Amido black stain provides a permanent record of the membrane, which can then be compared to corresponding autoradiography film, to aid in the orientation of the membrane.

3.2.4.5 Immunoprecipitation

Immunoprecipitation provides a powerful technique for the detection and quantitation of specific target antigens within cellular lysates. Using this technique, very small amounts of protein (around 100pg) can be detected. Immunoprecipitation is an invaluable technique used to analyse intracellular

phosphorylation events occurring following extracellular stimulation. The process of immunoprecipitation involves cell lysis, followed by the addition of a specific antibody directed against the target protein present within the cell lysate sample. The resultant antigen-antibody complexes are then collected by the addition of staphylococcal protein A, protein G or their mixture, which is covalently attached to sepharose or agarose. These immune complexes are precipitated by centrifugation, separated by SDS-PAGE and analysed by immunoprobng. A brief description of the immunoprecipitation method used in this study is outlined in the following paragraph.

- The primary antibody was added to each sample at the appropriate concentrations, in order to precipitate out the desired protein for subsequent detection by immunoprobng.
- The samples were rotated on a rotating wheel for 1 hour.
- Conjugated A/G protein agarose beads at the appropriate concentrations were then added.
- The samples were rotated on a rotating wheel for one hour to allow to the antibody – antigen complex to get attached to the beads.
- Centrifugation at 7000 – 8000 rpm for 5 minutes, to remove unbound protein complexes from the supernatant fraction.
- The protein pellets were washed twice using 500µl of lysis buffer (10mM NA_3VO_4 , 0.5% SDS, 0.5% Triton X-100, 2 mM CaCl_2 , 100 µg/ml (1mM) phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml (10 U/ml) aprotinin and 0.1% IGEPAL CA-630).

- The samples were then re-suspended in 40-60µl of sample buffer (10% glycerol; 5% 2-mercaptoethanol; 3% SDS; 80mM Tris-HCl (pH 6.8); 0.012% bromophenol blue).
- The samples were denatured under reducing conditions by boiling at 100°C for 5 minutes.
- The samples were then stored at -20°C until ready for use for SDS-PAGE.

3.2.5 Cell growth assays

To determine changes in cell growth upon treatment with various cytokines and growth factors. Four types of growth assays were employed during this study and summarised below.

3.2.5.1 MTT assay

This assay is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into an insoluble formazan precipitate that is dissolved and quantified by spectrophotometry (Alley *et al*, 1988). A 96 well culture cell culture plate was used. Cells were counted with a haemocytometer counting chamber and a specific number of cells were seeded to each well with culture medium (DMEM). The cells were then treated with the cytokine / growth factor of interest. The culture plate was then incubated for 72 hours at 37°C. The cells were washed twice with BSS. A sterile solution of MTT in 0.5mg/ml in BSS was added into each well. The culture plate was then incubated at 37°C for 4 hours.

MTT solution and medium were then removed by aspiration. The crystals produced by MTT reagent within the cells were then extracted by the addition of 100µl of Triton X100 (10% in dH₂O). The cells were incubated at 4⁰C for 24 hours. The absorbance of the colourimetric products was then measured at a wavelength of 540nm using a spectrophotometer (Titertecek).

3.2.5.2 Crystal violet assay

This is a simple assay useful for obtaining quantitative information of relative density of cells adhering to multi-well cluster dishes. The dye in this assay, crystal violet, stains DNA. Upon solubilization, the amount of dye taken up by the monolayer can be quantified in a spectrophotometer or plate reader. The final concentration of crystal violet was 0.2% mixed with 2% ethanol and dH₂O. Cells were counted first and before treated with the cytokine / growth factor of interest and cultured in DMEM in a 96-well culture plate for 72 hours. The medium was discarded. The cells were then fixed with 100µl of 10% formaldehyde added into each well for more than 30 minutes. 100µl of crystal violet extraction solution (10% w/w acetic acid) was then added into each well. The absorbance of the colourimetric products was then measured at a wavelength of 540nm using a spectrophotometer (Titertecek).

3.2.5.3 Hoeschet 33258 assay

Hoeschet 33258 assay was based on a technique described in 1990 (Rago *et al*, 1990) using the chemical bisbenzimidazole (Hoeschet 33258), a fluorescent marker that binds to double strand DNA and can thus be quantified by a fluorescence plate reader. The process relies on the lysis of the cells allowing mixing of the fluorochrome and the cellular DNA. Hoescht 33258 is made up at a concentration of 1mg/ml in dH₂O. Cells were cultured in a 96 culture plate after with the cytokine / growth factor of interest at 37 °C, 98% humidity and 5% CO₂ for 72 hours. 100µl of SDS (0.05% in SSC) was added to each well and the plate was placed in the incubator at 37 °C for about an hour (or unlimited time) for complete lysis of the cells and release of their DNA. A 100µl of Hoescht 33258 (1µg/ml in 1 x SSC) was then added. The plate was read in microplate fluorescence reader (Denley Wellfluor™) at $\lambda_{\text{excitation}}=356\text{nm}$ and $\lambda_{\text{emission}}=458\text{nm}$.

3.2.5.4 Assessment of cell growth using PicoGreen® analysis

PicoGreen® dsDNA quantitation reagent is an ultrasensitive fluorescent nucleic acid stain for quantifying double stranded DNA in solution. The cells were then treated with the cytokine / growth factor of interest and cultured in DMEM at 37°C, 98% humidity and 5% CO₂ for 72 hours, as above. The medium discarded. 100µl of SDS (0.05% in SSC) was added to each well and the plate was placed in the incubator at 37°C for about an hour (or unlimited time) for complete lysis of the cells and release of their DNA.

The PicoGreen reagent was prepared according to the manufacturer's instructions by a 200-fold dilution of PicoGreen reagent in a buffer containing 10 mM Tris-HCL and 1mM EDTA at pH 7.5. 100 μ l of the prepared solution was added into each well. The plate was then read in microplate fluorescence reader (Denley Wellfluor™) at $\lambda_{\text{excitation}}=485\text{nm}$ and $\lambda_{\text{emission}}=520\text{nm}$.

3.2.6 Cloning and expression of PCR products in mammalian cells

The method by which *Taq* polymerase amplified PCR products are inserted into plasmid vectors for constitutive or induced expression in selected mammalian cell line is described below.

3.2.6.1 Production of PCR products

The design of primer sets is critical for correct expression of PCR products in a vector. Primer sets are designed to amplify up the desired target region DNA of interest and may require modifications to create suitable PCR products. Depending on the vector, the products to be cloned may have to incorporate a Kozak codon and / or a stop codon for proper translational termination. Some vectors require PCR to produce amplified products that can be cloned directly in frame so as to ensure the correct codon arrangement.

Following PCR, the PCR products should reveal a single discrete band and if so may be used directly in the cloning reaction. Alternatively, if multiple bands and / or smearing are produced, the PCR reaction may require modification to optimise conditions, before progressing to the cloning reaction.

The cloning reaction involves the ligation of the PCR products (insert) into the reading frame of the plasmid vector. This ligation requires the proper vector-insert molar ratio (1:1-1:3 depending on vector), and may occur spontaneously if the PCR primers were designed to allow PCR products directly inserted into the reading frame of the vector, which are suitably prepared as follows:

3.2.6.2 TOPO cloning

T-A Topo cloning vector was extensively used in previous studies. The vector is linearized with each end having a T overhang. Following *Taq* polymerized based PCR, an additional A was added to each 3' end of the PCR product. This T- and A- overhang in vector and PCR product respectively, allows direct ligation in the presence of a suitable ligase. Spontaneous ligation between vector and appropriate insert occurs when approximately 10 ng of insert (0.5-0.2 µl of a typical PCR sample) is mixed gently with the vector at correct ratio (dependant upon vector), and the reaction is made up to 5 µl with sterile water, then left for five minutes at room temperature. Following ligation, the cloning reaction should be transformed immediately into *E. coli*, otherwise the transformation efficiency may decrease.

3.2.6.3 Construction of non-TA based expression vectors

Alliteratively, for vector and inserts that will not TA ligate into a vector, a digestion of the DNA strands may be required, with specific restriction enzymes prior to a ligation reaction. This ligation requires the presence of an enzyme known as T4 DNA ligase (Promega Corporation, Madison, USA), which catalyses the joining of two strands of DNA between the 5'- phosphate and 3'-hydroxyl groups of adjacent nucleotides resulting in either a cohesive-ended or blunt-ended configuration. This ligation reaction requires incubation at room temperature for three hours, followed by precipitation of DNA prior to transformation into *E. coli*.

3.2.6.4 Transformation into *E. coli*

The cloning reaction was transformed into the competent *E. coli* cells (JM109, Promega U.K. Ltd.) or Oneshot™ for T-A based ligation as follows: a 100 µl aliquot of the chemically competent bacteria (stored at -70°C) was thawed by immersion in an ice-bath for 5 minutes. Following this, the cloning reaction mixture was added and gently mixed with a pipette, this suspension was then placed on ice for 30 minutes. Cells were then heat shocked, in a water bath, at exactly 42°C for 2 minutes, then immediately placed back on ice for 2 minutes. Following which, 900 µl of SOC medium or LB medium (as appropriate) was added. These cells were then incubated for 60 minutes at 37°C with shaking at approximately 225 rpm. The resultant transformation mix was then plated out (at multiple volumes) onto pre-warmed LB-agar plates containing the appropriate

antibiotic e.g. G418 or hygromycin (approx. 50 µg/ml) to the plasmid vector resistance and incubated at 37°C overnight.

LB (Luria-Bertani) medium and agar plates (1 liter)

Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water. Then adjust the pH to 7.0 with NaOH, and made up to 1 liter. The solution was autoclaved, before allowed to cool and then can be stored at room temperature. To create LB plates, add 15g of agar to the one liter of LB medium before autoclaving. Autoclave, then add the desired antibiotic when the solution cooled to < 50 °C, pour into plates. Allow to set, then store inverted at 4°C.

3.2.6.5 Selection and analysis of colonies

Selection occurs in the presence of the antibiotic, as cells without the plasmid vector will not survive. Following overnight incubation, the plates should reveal a moderate number of bacterial colonies containing the plasmid vector. These colonies would have incorporated the plasmid vector, which encodes a gene to enable resistance to specific antibiotics. The next step is to establish which of these surviving cells containing the plasmid vector also has the insert incorporated into the vector, arranged in the correct direction. Some cloning reactions, particularly during blunt end ligations, result in the PCR products being inserted into the plasmid back to front. Screening of the colonies is required to determine which of the colonies grown on the plate, contains the vector plus insert in the correct arrangement, prior to amplification.

Screening in this study involved PCR of approximately 10-20 colonies or as appropriate, to amplify the target sequence of insert DNA at the correct direction within the plasmid vector. Individual labeled colonies are picked by using a pipette tip and then placed into the PCR reaction cocktail ready for specific amplification of the desired sequence. This is achieved through use of the forward primer for the plasmid and the reverse primer specific for the inserted PCR products. This ensures that amplified products, at the expected size, are that of the plasmid and insert in the correct position. These colonies are then ready for amplification.

3.2.6.6 Amplification and purification of plasmid DNA

Following identification of positive desired colonies with the appropriate vector and insert positioning, few single positive colonies were transferred, aseptically, to inoculate 2ml of LB medium, containing 100 µg/ml of an appropriate antibiotic, and incubated until culture grows to mid-log phase at 37°C in a rotary shaker. At which stage, the resultant culture was then added into 100 ml LB medium (with the appropriate antibiotic), and incubated overnight at 37°C under rotation.

To harvest the culture, centrifuge the cells at 6000g for 15 minutes at 4°C, then remove the medium so that only the cell pellet remains. This pellet is then resuspended in 10 ml of resuspension buffer from a plasmid extraction kit (Qiagen), which contains RNase inhibitors (available in the pack). To lyse the cells, 10 ml of cell lysis buffer is added and the solution is mixed gently to avoid

shearing of genomic DNA. Following a 5-minute incubation at room temperature, 10 ml of neutralization buffer is added and mixed, and then transferred to the QIAGEN filter cartridge. After 10 minutes at room temperature, the cell lysate is filtered into the barrel of the QIAGEN tip, which is then allowed to enter the resin by gravity flow. The plasmid DNA binds to the anion-exchange resin.

To remove all contaminants from the plasmid preparation, the resin is washed through with wash buffer. RNA, proteins, dyes, and low molecular weight impurities are removed by this medium-salt wash. The DNA was eluted from the resin through the addition of a high salt elution buffer, and was collected in a 50 ml universal tube. Plasmid DNA was then concentrated and desalted by isopropanol (10.5ml) precipitation, followed by a series of centrifugation and washing steps. The pellet was then dried and resuspended in a suitable volume of water or TE buffer. The yield of DNA was determined through DNA quantitation using spectrophotometer at 260 nm. Following which, small amount of plasmid DNA was separated on agarose gel (0.8%) to check both plasmid purity and size. After that, the DNA inserts were isolated by restriction digestion, if required.

3.2.6.7 Restriction enzyme digestion

Restriction enzymes cut specific DNA sites, dependant on enzyme, when a particular DNA sequence is recognized. Therefore, specific restriction enzymes can be used to cut open the plasmid DNA at specific and desired sites, which

result in the release of the fragment of insert DNA. This procedure is described here.

To 1-2 µg of purified plasmid, the followings were added: 2 µl of 10x appropriate restriction buffer, 10-20 units of each type of appropriate restriction enzyme, and made up to a volume of 20 µl with sterile water. This reaction was then incubated for 2-4 hours or as appropriate in a water bath at 37⁰C. After digestion was completed, 2 µl of the reaction was mixed with a loading buffer and run on agarose gel (0.8%) to determine the size of the restriction products. After, confirmation of successful digestion of the plasmid, the total digest reaction was separated on a large agarose gel (0.8%) and the DNA bands, corresponding to the insert DNA, excised from the gel. The insert DNA was then isolated and purified from the agarose using a DNA column purification system (DNA mini-prep system, Promega, UK). The resultant DNA was re-suspended in 20µl sterile water and its concentration was determined by a spectrophotometer. The DNA was then stored at -20°C until needed, largely for sequencing purpose.

3.2.6.8 DNA sequencing

The sequencing of a DNA fragment detects and produces the specific nucleotide sequence of the DNA sample under examination, for identification and analysis purposes. This sensitive technique allows the identification and confirmation of gene and ensures protein expression in good manner from DNA samples. DNA sequencing has been employed to confirm the nature of PCR products, cloned fragments of DNA, and any nucleotide mutations. The sequencing technique

used here was the “BigDye Terminator Cycle Sequencing Ready Reaction Kit” (PE Applied Biosystems, California, USA).

Each sequencing reaction requires 4.0 μ l of Terminator ready reaction mix, 200-500ng of DNA, 0.5 μ l of specific sequencing primer and deionised water to the volume of 20 μ l. These reagents are added to thin walled PCR tube and undergo PCR for 25 cycles (96⁰C for 10 seconds; 50⁰C for 5 seconds; 60⁰C for 4 minutes). Prior to sequencing, the products are precipitated, washed and dried as previously described. The nucleotide sequence was then determined using an automated DNA sequencer (ABI PRISM, Version 3). Resulting nucleotide sequences were submitted to the Gene Bank database for alignment and analysis. Confirmation of gene presence in a DNA sample is enabled through this method.

3.2.6.9 Transfection via electroporation of mammalian cells

Once the plasmid DNA has been isolated, purified and quantified, it is ready to be introduced into cultured mammalian cells. The method employed during this study, utilized electroporation of cultured cells to allow plasmid DNA to be incorporated into the cells. This electroporation technique used the Easy Jet Plus system (Flowgen, Staffordshire, UK), which passed a voltage of 310 volts across the cells to produce small perforations in the cell wall integrity, thus allowing passage of plasmid DNA across cell membranes to be integrated into the cells. This procedure is described here: 3 μ g of plasmid DNA was added to resuspended ($\sim 1 \times 10^6$) cells and mixed. The mixture was left to stand at room temperature for 2-5 minutes. The mixture was then transferred into an electroporation cuvette (Euro Gentech, Southampton, UK) ready for

electroporation. The cuvette was loaded into the electroporator and a pulse of electricity (310 volts) was passed through the cuvette. The mixture was then immediately (within 10 seconds) transferred into 10 ml of pre-warmed culture medium (must be within 30 seconds). This reaction was then cultured under the usual incubation conditions.

3.2.6.10 Establishing a stable expression mammalian cell line

To create a stable cell line that expresses the gene of interest, the culture must first be selected to yield only a population of cells expressing the plasmid-insert construct, which either constitutively induce or express the molecule of interest.

The above electroporated cells were allowed to growing to semi-confluence. Selection of plasmid positive cells relies on the presence of an additional drug (antibiotic) resistance gene in the plasmid. Plasmids used in this study have dual resistance genes, i.e. one for prokaryotic selection (as already mentioned earlier) and one for mammalian selection. For the latter, modified antibiotics that are capable of entering mammalian cells are used, such as G418, Zeocin or Hygromycin B. Thus antibiotics (dependant upon plasmid) are added to cultured cells at a concentration of around 100 µg/ml to kill any plasmid absent cells. This antibiotic selection period may continue for a number of weeks, at which point the cells remaining should all contain the plasmid and the inserted DNA fragment. Cells are routinely tested for the presence of the plasmid and insert, using RT-PCR.

Once the plasmid has been incorporated into the mammalian cells, the inserted DNA fragment may be expressed consecutively, or may require an inducing agent to stimulate and promote the expression of the desired gene, depending on the expression vector used. The regulated expression system used required the addition of inducing agents, such as doxycycline (Clontech Laboratories, California, USA). Following induction, this cell line may then be employed in a series of *in vitro* studies, to examine for the presence and influence of the expressed gene.

3.2.7 Immunohistochemical staining

Immunohistochemical staining was performed using fixed tissue sections in 50% methanol and 50% acetone for 15 minutes. The sections were then dried on air for 10 minutes and stored at -20°C in foil-rapped slide trays. Specimens were then placed in PBS (Optimax wash buffer) for 5 minutes. The slides were incubated with the appropriate primary antibodies at specific dilution for 1 hour. After 4 washes with PBS, the slides were placed in universal multi-link biotinylated secondary antibody at a specific concentration and incubated for 30 minutes. This was followed by 4 washes with PBS. Slides were then placed in avidin biotin complex (ABC – Vector Labs) for 30 minutes.

The bound antibody was detected with diaminobenzidine tetrahydrochloride (3,3'-diaminobenzidine) - DAB (Sigma). DAB was added for 5 minutes. The slides were washed with H_2O for 5 minutes and placed in Mayer's haematoxyline for 1 minute. Further wash with H_2O for 10 minutes (nuclei

become blue). This was followed by dehydration in methanol (3 times) and clearing in 2 changes xylene before mounting under a cover slip and read the slides. Negative controls (using PBS buffer or an irrelevant antibody instead of the primary antibody) were used in this study. The concentrations of antibodies were different to each staining, details of which will be explained separately in the relevant experimental chapters.

CHAPTER FOUR

CYTOKINES AND HUMAN ENDOTHELIAL CELLS: THE EFFECTS OF INTERLEUKIN-7 ON THE EXPRESSION OF SPECIFIC LYMPHATIC MARKERS

4.1 INTRODUCTION

Malignant cells are surrounded by stroma and extracellular matrix which is composed of various cells like macrophages, lymphocytes, neutrophils, fibroblasts, vascular endothelial cells as well as natural killer cells (Leek *et al*, 1994). All these cells including the malignant cells interact with each other via a complex network of extra-cellular signals, such as cytokines and growth factors and other protein molecules. These interactions are thought to regulate the proliferation and metastatic activity of malignant cells as well as modulate the host immune system towards the tumour cells (Hasday *et al*, 1990).

Cytokines play an essential role in carcinogenesis and subsequent proliferative behavior. Cytokine pathways in breast carcinogenesis is now well established (Freiss *et al*, 1993). Some cytokines including tumour necrosis factor alpha (TNF- α) (Bebok *et al*, 1994), interleukin-1 β (Duncan *et al*, 1994b), IL-3 (Speirs *et al*, 1995b) and IL-6 (Adams *et al*, 1991b) are secreted by several cell types such as breast cancer cells where they are known to exert pleiotropic actions. Paracrine effects of these factors especially IL-6 have been indicated in some *in vitro* studies; incubation of breast cancer cell line with conditioned medium derived from fibroblasts results in growth promoter / inhibitor effects (Adams *et al*, 1991a; Ryan *et al*, 1993; Speirs *et al*, 1995a; Vanroozendaal *et al*, 1992). Furthermore, cytokines including IL-1 β , IL-6 and TNF-a (Adams *et al*, 1991a; Duncan *et al*, 1994a; Speirs *et al*, 1993) have been shown to influence the biosynthesis of 17 β -estradiol (E2) in breast epithelial cells *in vitro* by stimulating the reductive pathway of the enzyme 17 β -hydroxysteroid dehydrogenase (17-

HSD). This enzyme is responsible for the reversible conversion of oestrone, a weak oestrogen, to the biologically more potent oestradiol (Bonney *et al*, 1986; Milewich *et al*, 1985). These studies demonstrate the importance of cytokines in regulating breast cell function and growth.

As stated in the general introduction chapter, the last few years have witnessed the identification of specific markers to the lymphatic endothelium including Podoplanin, a glomerular podocyte membrane mucoprotein (Breiteneder-Geleff *et al*, 1999; Weninger *et al*, 1999). Prox-1, a homeobox gene product that is involved in regulating development of the lymphatic system (Oliver *et al*, 1993; Wigle and Oliver, 1999). Most recently, a novel hyaluronan receptor, LYVE-1 has been shown to be restricted to lymphatic vessels in normal tissues (Banerji *et al*, 1999; Mandriota *et al*, 2001) and associated with the tumours (Mandriota *et al*, 2001; Skobe *et al*, 2001b; Stacker SA, 2001). 5'-nucleotidase (an enzyme that acts on nucleoside-5'-phosphates, such as AMP and adenylic acid, releasing inorganic phosphate) activity has been shown to be stronger in lymphatic than in blood vessels (Weber *et al*, 1994; Werner *et al*, 1987a) although it is not entirely specific.

The regulation of these genes in endothelial cells is to-date not very clear. The effects of factors like hypoxia or cytokine regulation on their expression in the lymphatic endothelium have not been explored. In addition, as there is currently no established cell line of lymphatic origin, we developed a new method to evaluate the effects of cytokines on lymphatic development. The effects of

several cytokines on the expression of lymphatic markers in endothelial cells (with lymphatic characteristics) were studied.

4.2 MATERIALS AND METHODS

4.2.1 Materials

HECV cell line, which possesses both vascular and lymphatic characteristics (Ye *et al*, 2003), was purchased from Interlab Cell Line Collection (ICLC), Naples, Italy. Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) (pH 7.3) containing; 2mM L-glutamine, 15mM HEPES and 4.5mM NaHCO₃, DMEM was then supplemented with 10% heat inactivated foetal calf serum (PAA Laboratories, Austria), 50 units/ml of benzylpenicillin (Britannia, Pharmaceuticals, Ltd) and 50 µg/ml of streptomycin (Gibco BRC, Paisley, Scotland).

Human recombinant IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-11, IL-12, Interferon- γ (IFN- γ) and Tumour necrosis factor- α (TNF- α) were purchased from National Biology Standard Bureau - NBSB (Salisbury, England, UK). Human recombinant IL-7 and hepatocyte growth factor / scatter factor (HGF/SF) were purchased from Chemicon International (Temecula, California). Pairs of forward and reverse primers were purchased from Invitrogen (Paisley, Scotland, UK).

4.2.2 Methodology

4.2.2.1 Generation of cDNA from cell lines and RT PCR

HECV cells were cultured in DMEM as above in 12 x 25cm² flasks. The cells were treated with the above cytokines at the following concentrations (incubated for 24 hours 37°C and 5% CO₂): IL-1B (50U/ml), IL-2 (60U/ml), IL-4 (100ng/ml), IL-5 (20ng/ml), IL-6 (10000U/ml), IL-7 (40ng/ml), IL-8 (60ng/ml), IL-10 (40ng/ml), IL-11 (400ng/ml), IL-12 (50ng/ml), IFN-γ (1μg/ml), TNF α (10ng/ml) and HGF/SF (10ng/ml). The concentrations of these cytokines were decided either in accordance to the manufacturers recommended usage or after performing several toxicity assays to determine the optimum concentration. Negative controls were used throughout the experiments.

RNA extraction and reverse transcription kits and PCR mix were purchased from ABgene (Surrey, England, UK). RNA was extracted from HECV cultured cells (in RNA extraction buffer) using the standard guanidine isothiocyanate method by following the manufacturer's protocol. The concentration of RNA was measured with a spectrophotometer. Reverse transcription was performed from 1μg of total RNA using oligo dt primer according to the manufacturer's instructions. Conventional PCR primers were designed using Beacon Designer software (Palo Alto, California), to allow amplification of regions that have no overlap with other known genes and span at least one intron. Primers were synthesized by Life Technologies (Paisley, Scotland, UK) (Table 4.1). Conventional PCR was performed using cDNA from cells together with the PCR master mix using respective primers. The reaction conditions were: 94°C for 40

seconds, 55°C for 50 seconds, 72°C for 45 seconds and a final extension phase of 10 minutes for 38 cycles. The PCR products were separated on a 2% agarose gel and stained with 10ml ethidium bromide prior to examination under UV light and a photograph taken.

Table 4.1 Sequence and size of pairs of primers used in the RT-PCR.

<i>Primer</i>	<i>Forward (5' – 3')</i>	<i>Reverse (3' – 5')</i>	<i>Size</i>
β -ACTIN	atgatatgccgcgctcg	cgctcggtgaggatctca	520bp
PODOPLANIN	tgggtggaatcatggtgtggttatgg	acaagtgaacctcctgcctcctc	365bp
PODOPLANIN (Q-PCR)	cctggtggaatcatagtgg	tcacatgggtgtaagctattac	86bp
PROX-1	gcgaactcgtatgaagatg	gcagatgacctgtatatgg	1995bp
PROX-1 (Q-PCR) - <i>Taq</i>	cgtgcaggaaaagtctaccaa	gagcgcgatgtgtcttcaga	90bp
LYVE-1	gtgcttcagcctggtgttg	gcttgactcttgactcttc	920bp
Factor VIII	ggagagatgggacactaaca	actgaacctgacctacagtcatatggaccgactgaggt	125bp

4.2.2.2 Real Time quantitative RT-PCR

Real time quantitative PCR was carried out using the iCycler iQTM system (Bio Rad) was used to determine levels of expression of podoplanin and prox-1 amplification. The iCycler iQTM system incorporates a gradient thermocycler and a 96-channel optical unit. AmplifluorTM detection system was used in the current study (Intergen, England, UK), which included the use of specific sense primer, a universal FAM-labelled probe and a specific anti-sense primer that incorporate a Z sequence that is complementary to the probe. The internal standards used in the study were specific plasmids generated using a pCR2.1 cloning vector (Invitrogen, Paisley, Scotland, UK) as described in Chapter 3. Quantitative PCR was carried out in 96-well plate with 10pmol sense primer, 1pmol anti-sense-Z primer (Table 4.1), and 10pmol FAM-probe, using a customer hot-start Q-PCR master mix, with the following conditions: 95°C for 15 minutes, followed by 50

cycles at 95°C for 15 seconds, 55°C for 40 seconds and 72°C for 15 seconds. The copy number of each transcript was calculated from the internal standards and shown here as copies/50ng RNA.

4.3 RESULTS

4.3.1 The effects of cytokines on the expression of specific lymphatic markers in human endothelial cells

Amongst the cytokines tested, IL-7 has increased the expression of podoplanin and prox-1 in HECV cells. This effect was persistent with repeating experiments (Figure 4.1). Although both IL-1 β and IL-12 had also an effect on the expression of podoplanin, this was not consistent on repeating the experiment. Therefore, only IL-7 was used in subsequent experiments.

4.3.2 The effects of IL-7 on the expression of lymphatic markers in HECV cells

HECV cells were treated with 40ng/ml of IL-7 and cultured as above for 1, 2, 4 and 24 hours. Messages of LYVE-1 were increased when HECV treated with IL-7 (Figure 4.2). This effect of IL-7 was maximal in the period between 2-4 hours incubation and declined after 24 hours. IL-7 significantly increased the expression of prox-1 in HECV and similar to LYVE-1, the maximal expression was noted between 2-4 hours incubation with IL-7. Similarly the expression of podoplanin was significantly increased by IL-7, but the expression was higher between 1-4 hours.

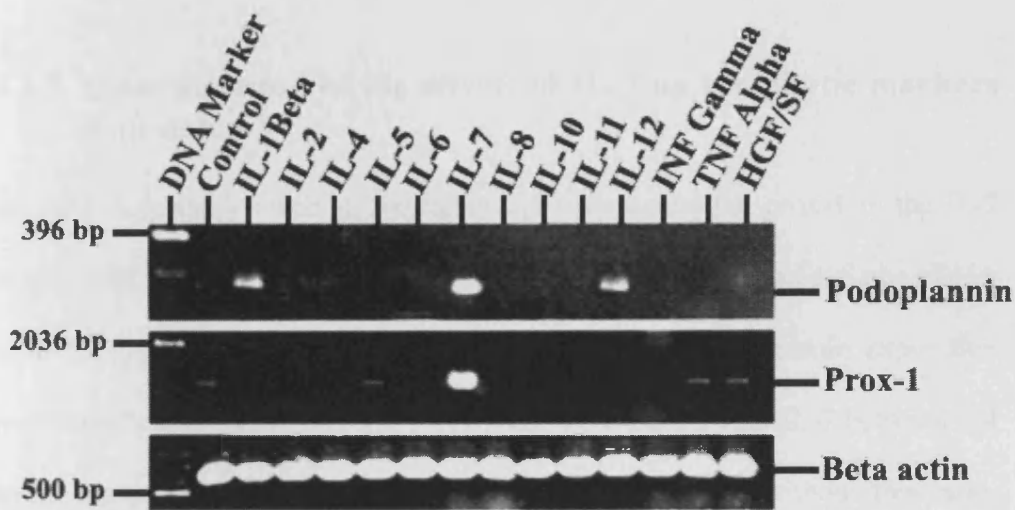


Figure 4.1 IL-7 increases the expression of both podoplanin and prox-1 in HECV cells treated with IL-7. Treatment with other cytokines does not have any persistent effect on the expression of these markers. Initially, IL-1 β and IL-12 appeared to have increased the expression of podoplanin in HECV cells, but this was not consistent with repeating the RT-PCR (upper panel). The control means HECV cells not treated with any cytokine. β -actin was used as an internal housekeeping gene.

IL-7 did not have any effect on the expression of the angiogenic marker, Factor VIII (von Willebrand factor) in these cells (Figure 4.2).

4.3.3 Quantification of the effects of IL-7 on lymphatic markers expression

In order to quantify levels of expression of podoplanin and prox-1 in the IL-7 treated HECV cells, real time quantitative RT-PCR was performed. Same cDNA from the time course experiment was used. Levels of podoplanin expression were significantly higher in HECV cells that were treated with IL-7 between 1-4 hours compared to podoplanin expression in HECV cells without treatment. Similarly levels of expression of prox-1 in HECV treated with IL-7 were significantly higher than in those without treatment with IL-7. The mean levels of expression of podoplanin and prox-1 are summarised in table 4.2. The data are also represented graphically in Figures 4.3 and 4.4.

Table 4.2 Mean number of transcript copies/50ng mRNA of podoplanin and prox-1 in HECV as assessed by RT quantitative PCR. ^a p=0.012, ^b p=0.009, ^c p=0.005, ^d p=0.011, ^e p=0.001, ^f p=0.003, ^g p=0.005, ^h p=0.023 vs respective controls (Statistics calculated using the two sample student's *t* test and results considered to be significant if p value was less than 0.05).

<i>Lymphatic marker</i>	<i>Control</i>	<i>+ IL-7 (1 hr)</i>	<i>+ IL-7 (2 hrs)</i>	<i>+ IL-7 (4 hrs)</i>	<i>+IL-7 (24 hrs)</i>
Podoplanin	125±17	341±52 ^a	386±82 ^b	318±68 ^c	216±73 ^d
Prox-1	649±87	1780±210 ^e	1660±310 ^f	1367±206 ^g	973±156 ^h

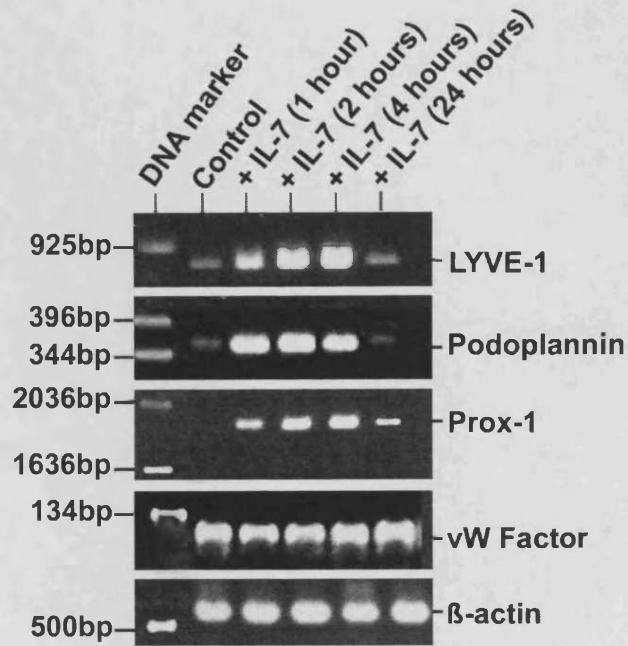


Figure 4.2 IL-7 increases the expression of the lymphatic markers, LYVE-1, podoplanin and prox-1 in HECV. The maximum effects are within 1-4 hours treatment with IL-7. IL-7 does not have any effect on the expression of the highly specific angiogenic marker, Factor VIII (von Willebrand factor). β -actin was used as the internal housekeeping gene.

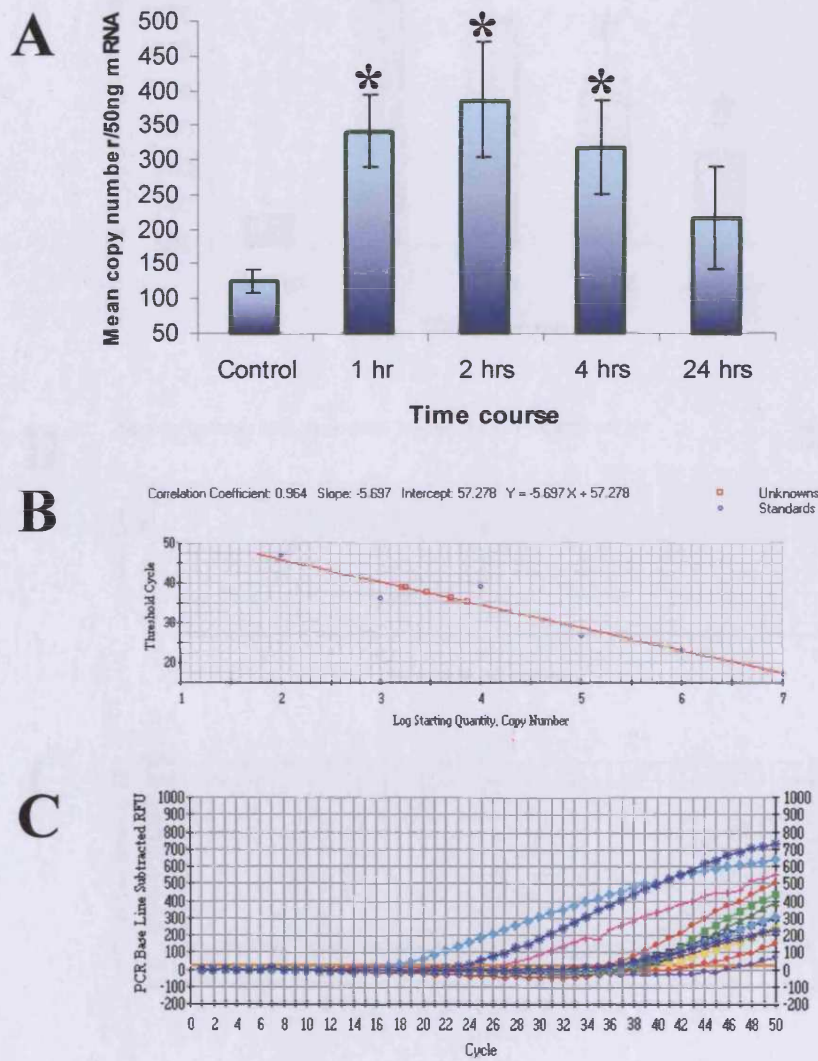


Figure 4.3 Real Time quantitative RT-PCR. Mean copy number / 50ng mRNA podoplanin is significantly higher in HECV cells treated with IL-7. The maximum effects are within 1-4 hours. * $p < 0.05$ using student's t test (A). The correlation coefficient (B), shows an index of 0.964 comparing podoplanin levels with those of a standard. (C) shows the amplification of copy numbers in relation to the cycle number.

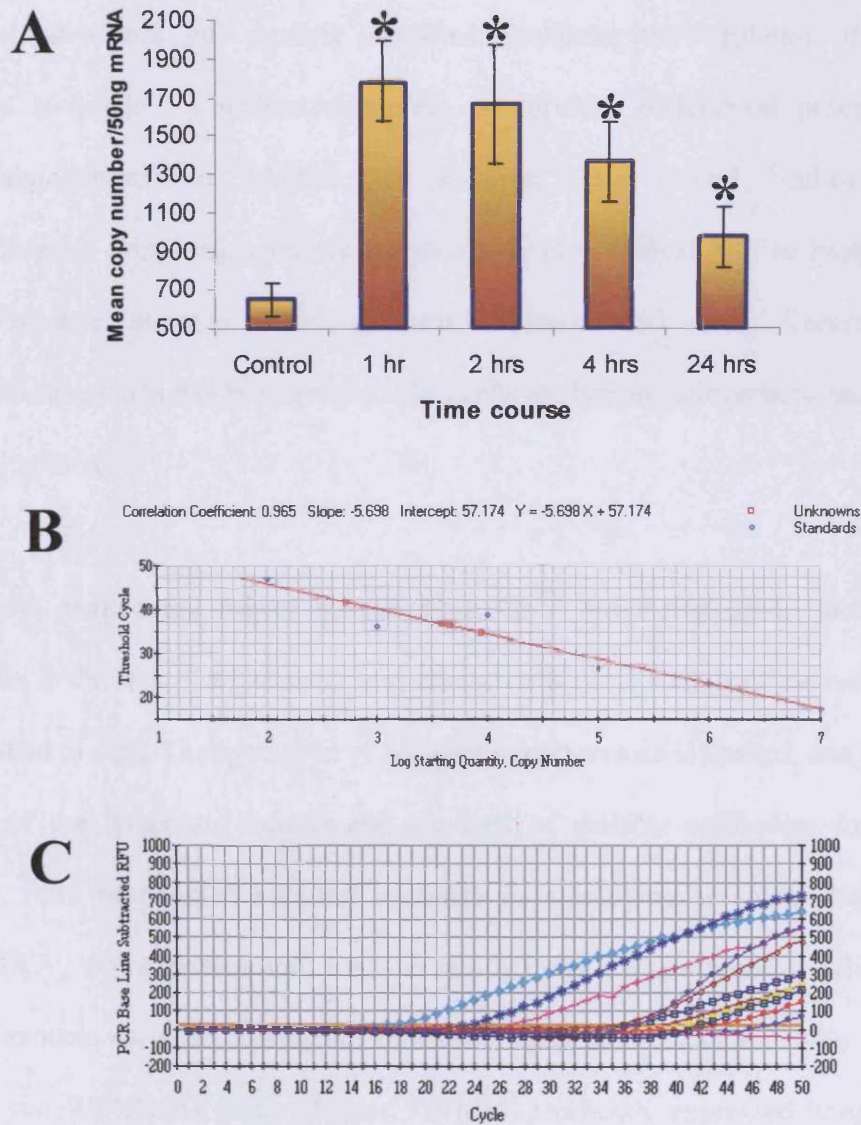


Figure 4.4 Real Time quantitative RT-PCR. Mean copy number / 50ng mRNA prox-1 is significantly higher in HECV cells treated with IL-7. The maximum effects are within 1-4 hours. * $p < 0.05$ using student's t test (A). The correlation coefficient (B), shows an index of 0.965 comparing prox-1 levels with those of a standard. (C) shows the amplification of copy numbers in relation to the cycle number.

4.4 DISCUSSION

Specific markers to the lymphatic endothelium including podoplanin, prox-1 and LYVE-1 have been only recently identified. Exploring the regulation of these markers is crucial in understanding the so far less understood process of lymphangiogenesis. In addition to processes like wound healing and lymphoedema, lymphangiogenesis is thought to play critical role in lymphatic spread of solid tumours including breast, colorectal and others. Contrary to angiogenesis, which has been extensively explored, lymphangiogenesis has been poorly explored.

The most challenging aspect in searching for a lymphangiogenic factor or regulator, is the lack of a lymphatic endothelial cells as no such cell line has been established to-date. The extraction of such cells from tissues is limited, due to the nature of the lymphatic vessels and the lack of suitable antibodies for cell sorting. Here we adopted a unique approach by employing an established cell line, HECV, which posses both vascular and lymphatic characteristics. The cell which express vascular endothelial molecules such as VE-cadherin (Ye *et al*, 2003), von Willebrand factor (Factor VIII), it specifically expressed lymphatic markers including podoplanin, prox-1 and LYVE-1. Using this cell as a model, we were able to differentiate the response of a cell to a cytokine either towards a lymphatic or vascular or both properties. IL-7 increased the expression of lymphatic markers but not that of the blood vascular markers.

Regulation of lymphangiogenesis by cytokines is an attractive starting point as they are known to be involved in many intercellular autocrine and / or paracrine mechanisms (Adams *et al*, 1991a; Duncan *et al*, 1994; Freiss *et al*, 1993; Hasday *et al*, 1990; Ryan *et al*, 1993; Speirs *et al*, 1995a; Vanroozendaal *et al*, 1992). It has been found recently for example that IL-8 could mediate angiogenesis *in vivo* (Koch *et al*, 1992). Although the cytokines used here were genetically recombinant, we believe that they could induce a similar *in vitro* effect like the natural ones. Obtaining natural cytokines is not easy and can be very expensive. However, of course inducing an *in vitro* effect by these cytokines by no means reflects exactly of what is happening in tumour tissues *in vivo*.

In this study, IL-7 increased the expression of the markers specific to the lymphatic endothelium. This might be the beginning of understanding part of the regulation of expression of these markers. Low levels of LYVE-1 expression in HECV cells were significantly increased by IL-7. Untreated with IL-7, these cells did not express podoplanin or prox-1. However, HECV without IL-7 treatment did express weakly LYVE-1, a highly specific lymphatic marker. Although, HECV cells are known to have lymphatic characteristics (Ye *et al*, 2003), they also have blood vascular properties. Here, HECV cells did express Factor VIII (von Willebrand factor), a highly specific angiogenic marker, regardless to treatment with IL-7 (Figure 4.2).

IL-7 induced its maximum effects on the expression of these markers within 1 – 4 hours incubation. It is unclear however, why does the expression of these markers decrease at 24 hours. HECV are rapidly dividing transformed cells and

probably related to instability of their gene transcription or probably due to the consumption of the cytokine in the culture medium. The real time quantitative RT-PCR however, showed that the levels of expression of both podoplanin and prox-1 are significantly higher compared to their expression in the untreated cells.

Although the expression of podoplanin (not shown here) and prox-1 (Figure 4.1) in HECV cells treated with IL-1 β and IL-12 seemed to be different from the control, this was not consistent. IL-7 did not have any effect on the expression of the highly specific angiogenic factor, Factor VIII (von Willebrand factor). Therefore, it is concluded from this initial study that IL-7 increases the expression of podoplanin, prox-1 and LYVE-1 at mRNA level in HECV cells. This suggests that IL-7 is probably lymphangiogenic rather than angiogenic in endothelial cells.

CHAPTER FIVE

THE ROLE OF THE VASCULAR ENDOTHELIAL GROWTH FACTORS IN THE IL-7 INDUCED INCREASED LYMPHATIC MARKERS EXPRESSION

5.1 INTRODUCTION

The VEGF family is comprised of VEGFs -A, -B, -C, -D, -E as well as PlGF (Joukov V, 1996; Lee J, 1996; Olofsson *et al*, 1999; Orlandini *et al*, 1996). These growth factors are ligands for the three VEGF tyrosine kinase receptors identified so far, VEGFR-1, -2, and -3. While, VEGFs -A and -B, -E and PlGF are ligands for VEGF receptors -1 and -2 (VEGFR-1 and VEGFR-2) and considered to play an important role in tumour angiogenesis (Shweiki *et al*, 1995), VEGFs -C and -D activates VEGFR-3 signalling that mediates mainly lymphangiogenesis (Chapter 1) . It has been recently revealed that VEGF family members are expressed in a variety of human tumours and in fact tumour cells are capable to secrete them at various levels (Achen *et al*, 2001; Ferrara and Davis-Smyth, 1997; Salven *et al*, 1998). However, the angiogenic switch is thought to be carefully regulated, and at least some specific genetic events in tumour progression correlate with lymphatic metastasis, suggesting that a “lymphangiogenic switch” mechanism may also be a distinct possibility (Jussila and Alitalo, 2002).

It has recently been demonstrated that primary solid tumours expressing VEGF-C and VEGF-D induce the *de-novo* formation of new lymphatic capillaries (lymphangiogenesis), thereby providing a direct conduit for the dissemination of tumour cells to regional lymph nodes (Karpanen *et al*, 2001; Mandriota *et al*, 2001; Pepper, 2001; Skobe *et al*, 2001b; Stacker *et al*, 2001). VEGF-C is mainly expressed in the heart, small intestine, placenta, ovary and the thyroid gland. VEGF-C induces mitosis and migration of endothelial cells and it increases

vascular permeability. VEGF-C has been shown to induce lymphangiogenesis in transgenic mouse skin and in mature chick chorioallantoic membrane (Jeltsch *et al*, 1997; Oh *et al*, 1997). The association of VEGF-C overexpression, lymphatic vessel density and lymph node metastases has been described in a variety of carcinomas including thyroid, prostate, gastric, colorectal, and lung (Akagi *et al*, 2000; Bunone *et al*, 1999; Niki *et al*, 2000b; Ohta *et al*, 2000; Ohta *et al*, 1999; Skobe *et al*, 2001b; Yonemura *et al*, 1999). VEGF-D is mainly expressed in many adult tissues including the vascular endothelium, heart, skeletal muscle, lung, small and large bowel. VEGF-D is mitogenic for endothelial cells and binds to and activates mainly VEGFR-3 (Achen *et al*, 1998a; Orlandini *et al*, 1996; Yamada *et al*, 1997b).

At present, it unknown what are the factors that regulate the expression of these lymphatic vascular endothelial growth factors and receptors namely VEGF-C, VEGF-D and VEGFR-3 in endothelial cells. IL- β has been identified to up-regulate VEGF-C expression (Akagi *et al*, 1999). The current knowledge about the existence of cross talks and interactions between various cytokines and growth factors has made cytokines particularly interleukins attractive targets for investigating lymphangiogenesis. For example, MAPK (mutagene activated protein kinase) pathway that is involved in VEGFR-3 signalling is also activated in IL-7 signalling (Crawley *et al*, 1997). PI3-K, a molecule involved in IL-7 signalling is also implicated in the VEGFs-induced endothelial cell survival via activation of its downstream target serine kinase Akt/PKB (Gerber *et al*, 1998).

Stimulation of VEGFR-3, using the specific ligand, induces a rapid tyrosine phosphorylation of Shc and activation of MAPK pathway results in an increased cell motility, actin reorganization and proliferation (Cao *et al*, 1998; Joukov *et al*, 1998). VEGFR-3 has also been found to be a strong activator of Stat-5 (Veikkola *et al*, 2001), a molecule involved in other cytokine signalling, including IL-7, suggesting that the regulation of VEGFR-3 signalling might be controlled by other cytokines. Here, the effects of IL-7 on the expression of the vascular endothelial growth factors and receptors in endothelial cells were explored.

5.1 MATERIALS AND METHODS

5.1.1 Materials

Human endothelial (HECV) cells were cultured as described in Chapter 1. Anti-VEGF-A (Rabbit polyclonal), Anti-VEGF-B (Rabbit polyclonal), Anti-VEGF-C (Rabbit polyclonal), Anti-Flt-4 (Anti-VEGF-R3) (Rabbit polyclonal), Anti-VEGFR-1 (Rabbit polyclonal), Anti-Flk-1 (Mouse monoclonal), Anti-Factor VIII (von Willebrand factor) (Rabbit polyclonal), Anti-Actin (Mouse monoclonal) and was purchased from Santa Cruz Biotechnology (California, USA).

Anti-LYVE-1 antibody was prepared as follows:

The LYVE-1 peptides were generated from the C-terminal region and a region in N-terminal of the protein and synthesised by MWG gmbh

(Milton Keynes, England). These peptides were attached to the KLH and injected into rabbit with complete Freund's solution, following a standard procedure. The antibodies were obtained following a series of purification steps. Anti-sera were obtained after series injection and booster injections. Immunoglobulin was purified using a protein-A sepharose affinity column (Sigma, Poole, Dorset, England). Briefly, the rabbit serum underwent delipidation by mixing 1,1,2-trichlorotrifluoroethane with the rabbit serum in a 3:2 ratio and underwent agitation for 30 minutes. Then the mix was spun down at 5000rpm for 10 mins, and the top layer kept for purification. Delipidated anti-sera was diluted with PBS buffer and loaded onto the sepharose-column (equilibrated with 10 column volumes of 10mM PBS (pH to 7.4). The column was then washed with 7 column volumes of PBS. Elution of the bound antibodies was achieved with 5 column volumes of elution buffer (11.1g Glycine-HCl brought to volume of 1 litre with dH₂O then pH to 2.7). 1ml fractions were collected into tubes containing neutralisation buffer (12.1g Tris, 4.2ml HCl, brought to 100mls and pH to 8.0), and mixed immediately. Purified LYVE-1 antibodies were then stored in 50% glycerol at -20°C. The specificity of the antibodies was verified using the synthetic peptides and cell lysates. The cell lines used were HECV, MCF-7, MDA-MB 231 and BT-483. Both peptides were blotted onto a nitrocellulose membrane, at four different concentrations, to confirm that the purified antibodies specifically recognised the peptide that

they were raised against. A variety of human normal and cancer cell line lysates were also examined with Western Blot analysis to confirm the presence of LYVE-1 proteins (Parr *et al* 2004).

Anti-IL-7 (Mouse monoclonal) and Anti-VEGF-D (Mouse monoclonal) neutralizing antibodies were purchased from Research and Development Systems (R & D Systems, Oxford, England, UK). HRP antibodies (Mouse monoclonal), HRP antibodies (Rabbit monoclonal), peroxidase conjugated secondary antibodies to rabbit IgG. Protein A/G agarose beads used during immunoprecipitation were obtained from Sigma (Poole, Dorset, England, UK).

IL-7 downstream signalling molecules inhibitors: Jak-3 Inhibitor 1 {4-(4'-Hydroxyphenyl) amino-6,7-dimethoxyquinazoline; WHI-P131}, Jak-Stat pathway inhibitors: AG 490 {Tyrophstin B42; α -Cyano-(3,4-dihydroxy)-N-benzylcinnamide; N-Benzyl-3,4-dihydroxy- α -cyanocinnamide} and piceatanol. PI3-Kinase inhibitor: Wortmannin {KY 12420} were purchased from Calbiochem (UK).

5.2.2 RNA extraction and RT-PCR

RNA extraction and reverse transcription kits and PCR mix were purchased from ABgene (Surrey, England, UK). RNA was extracted from HECV and breast cancer cells (in RNA extraction buffer) using the standard guanidine isothiocyanate method by following the manufacturer's protocol. The concentration of RNA was measured with a spectrophotometer. Reverse

transcription was performed from 1g of total RNA using oligo dt primer according to the manufacturer's instructions. Conventional PCR primers were designed using Beacon Designer software (Palo Alto, California), to allow amplification of regions that have no overlap with other known genes and span at least one intron. Primers were synthesized by Life Technologies (Paisley, Scotland, UK) (Table 5.1). Conventional PCR was performed using cDNA from cells together with the PCR master mix using respective primers. The reaction conditions were: 94°C for 30 seconds, 55°C for 40 seconds, 72°C for 45 seconds and a final extension phase of 10 minutes for 38 cycles. The PCR products were separated on a 2% agarose gel and stained with 10µl ethidium bromide prior to examination under UV light and a photograph taken.

Table 5.1 Primers sequences and sizes used in the RT-PCR.

<i>Primer</i>	<i>Forward (5' – 3')</i>	<i>Reverse (3' – 5')</i>	<i>Size</i>
β-Actin	atgatatgccgcgctcg	cgtcggtaggattctca	525bp
LYVE-1	gtgcttcagcctggtgtg	gcttgactcttgactctc	920bp
PECAM	gactggacggtgcaaat	actgaacctgacctacaactgcagggtca	80bp
VEGF-A	attggaggctgccttg	gctctatcttcttggc	466bp
VEGF-B	tggtgtcatggatagatgtgtatac	cttgcaacggaggaagc	535bp
VEGF-C	ggcttctcctggtgacatctg	ttgctgggacacattgacattc	660bp
VEGF-D	cgatcatctcagtcacattg	cttctggcaggcagcaggtctc	460bp
VEGF-R1	gaacgagaaggacggactc	tggtggaactgctgatgg	470bp
VEGF-R2	gcctctgtgggttgccctagtg	ccctctcctctcccactttgtg	495bp
VEGF-R3	ctgtgcctgcgactgtg	cagcgtggacaggttgag	515bp

5.2.3 Western Blotting

HECV cells were cultured in DMEM until 75% confluent with or without treatment with IL-7 (40 ng/ml), VEGF-D (20 ng/ml), anti-IL-7 or anti-VEGFD antibodies (1ng/ml). After 24 hour incubation with the test material at 37⁰C, cells were pelleted and lysed in HCMF buffer containing 0.5% SDS, 1% Triton X-100, 2 mM CaCl₂, 100 µg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin, and 10 mM sodium orthovanadate for 40 min. After adding samples buffer, the total cell lysates were boiled at 100°C for 5 min. Equal amounts of protein from each sample were added onto an 12% polyacrylamide gel. After electrophoresis, proteins were blotted onto nitrocellulose sheets and blocked in 10% for 30 min before probing with peroxidase- conjugated secondary species-specific antibodies. Protein products were visualized with an enhanced chemiluminescence system (Amersham International Plc).

5.3 RESULTS

5.3.1 The effects of IL-7 on the expression of the vascular endothelial factors

Both VEGF-A and VEGF-B are expressed in endothelial cells (Figure 5.1A). IL-7 did not affect the expression of these factors as shown by the RT-PCR. Low level of VEGF-C expression was increased in HECV although this was not consistent on repeating the RT-PCR. However, IL-7 significantly increased VEGF-D expression in these cells. The maximum effect was observed between

1-4 hours. Levels of expression of these genes were assessed with relative comparison to β -actin, an internal housekeeping gene.

5.3.2 The effects of IL-7 on the expression of the vascular endothelial growth factors receptors in endothelial cells

Treating HECV with IL-7 did not affect the expression of both VEGFR-1 and VEGFR-2 (Figure 5.1B). However, IL-7 increased the expression of VEGFR-3 in HECV. The increased expression was noted mainly within 1-4 hours incubation. This effect of IL-7 was consistent with its effects on the expression of both LYVE-1 and VEGF-D.

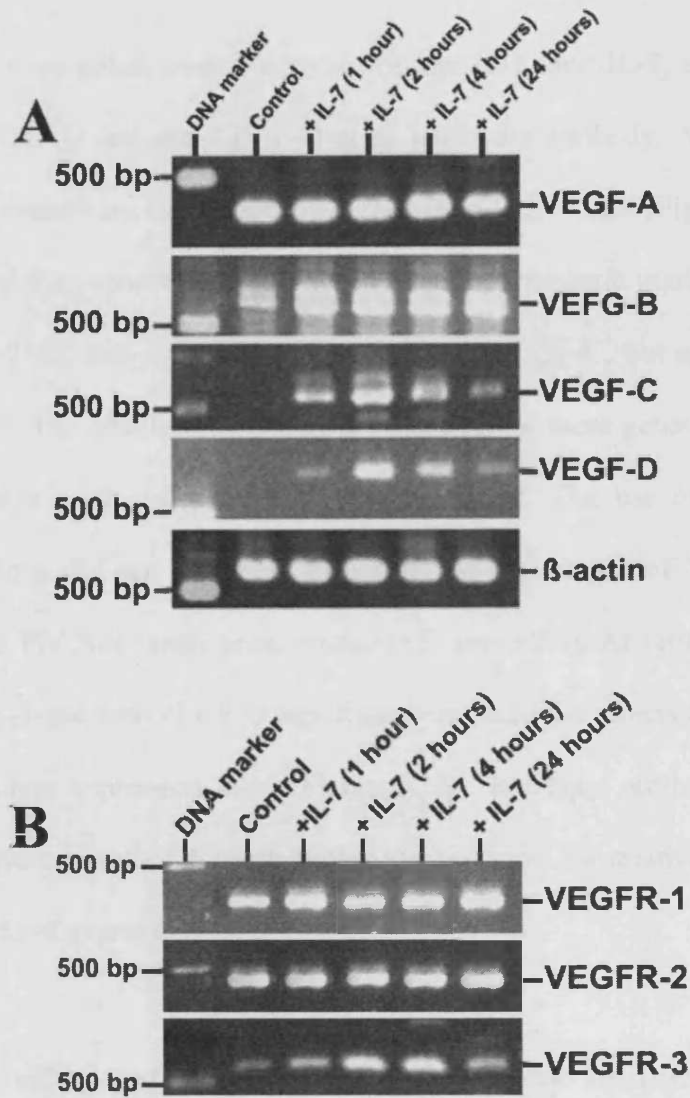


Figure 5.1 A time course RT-PCR. IL-7 increases the expression of both VEGF-C and VEGF-D in HECV cells. The maximal effects are within 1-4 hours. Low levels of VEGFR-3 expression were also increased in response to IL-7. IL-7 did not affect the expression of the angiogenic growth factors and receptors (VEGF-A, VEGF-B, VEGFR-1 and VEGFR-2 respectively). B-actin was used as the internal housekeeping gene.

5.3.3 The effects of anti-IL-7 and anti-VEGF-D neutralising antibodies on the IL-7 induced expression of VEGF-C, VEGF-D and lymphatic markers

HECV cells were either treated with or without IL-7, anti-IL-7, anti-VEGF-D, VEGF-D, VEGF-D and anti-VEGF-D or an irrelevant antibody. As with figure 5.1, IL-7 increased the expression of VEGF-D in these cells (Figure 5.2A). It also increased the expression of the highly specific lymphatic marker, LYVE-1. Although IL-7 did also increase the expression of VEGF-C, but again this was not consistent. The effects of IL-7 on the expression of these genes were similar to those effects produced when VEGF-D were used. The use of neutralising antibodies alone did not have any effect on the expression of VEGF-A and VEGF-B and PECAM (angiogenic marker) (Figure 5.2A). At protein level, the use of anti-IL-7 and anti-VEGF-D significantly reduced the effects of IL-7 on the VEGF-D protein expression level (Figure 5.2B). Irrelevant antibodies, β -actin (RT-PCR) and anti-actin (Western Blotting) were used for relative comparison between levels of expression.

5.3.4 The effects of IL-7 signalling pathway inhibitors on the expression of lymphatic markers and growth factors

The increased levels of LYVE-1 and VEGF-D expression in HECV cells in response to IL-7 was significantly diminished in the presence of the PI3-K irreversible inhibitor, Wortmannin at both mRNA and protein levels (Figure 5.3 A and B respectively). However, AG490, piceatanol and Jak-3 inhibitor-1 did not affect their expression. The expression of VEGF-C was also diminished with Wortmannin.

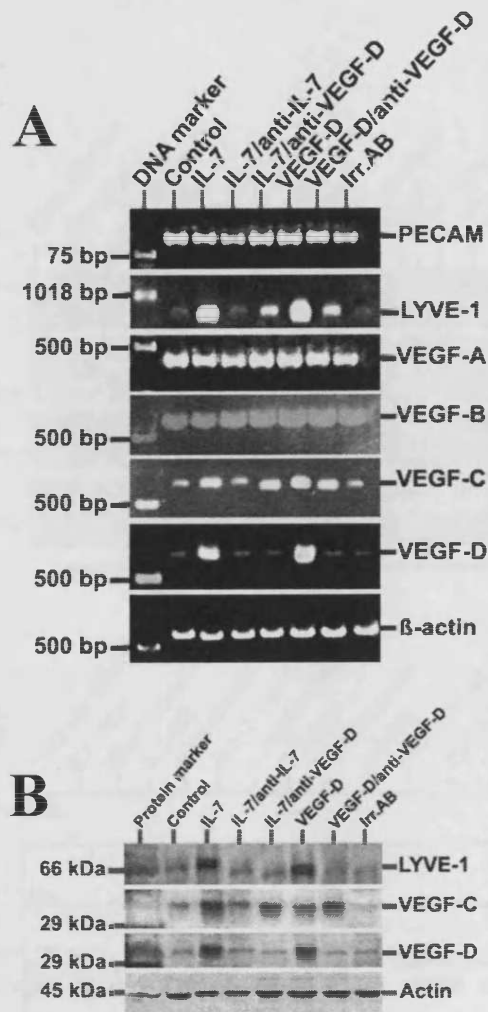


Figure 5.2 HECV treated with IL-7 shows increased levels of expression of LYVE-1 and VEGF-D as shown by the RT-PCR (A) and Western Blotting (B). IL-7 also had some effect on the expression of VEGF-C. The effect of IL-7 is similar to that exerted by VEGF-D (a known lymphangiogenic growth factor). The use of neutralising antibodies against IL-7 and VEGF-D reversed these effects of IL-7. The effect of using anti-VEGF-D antibody was similar to that produced by anti-IL-7 suggesting that IL-7 mediates these effects via a VEGF-D dependent mechanism. IL-7 did not affect the expression of the angiogenic marker PECAM or the angiogenic factors VEGF-A and VEGF-D.

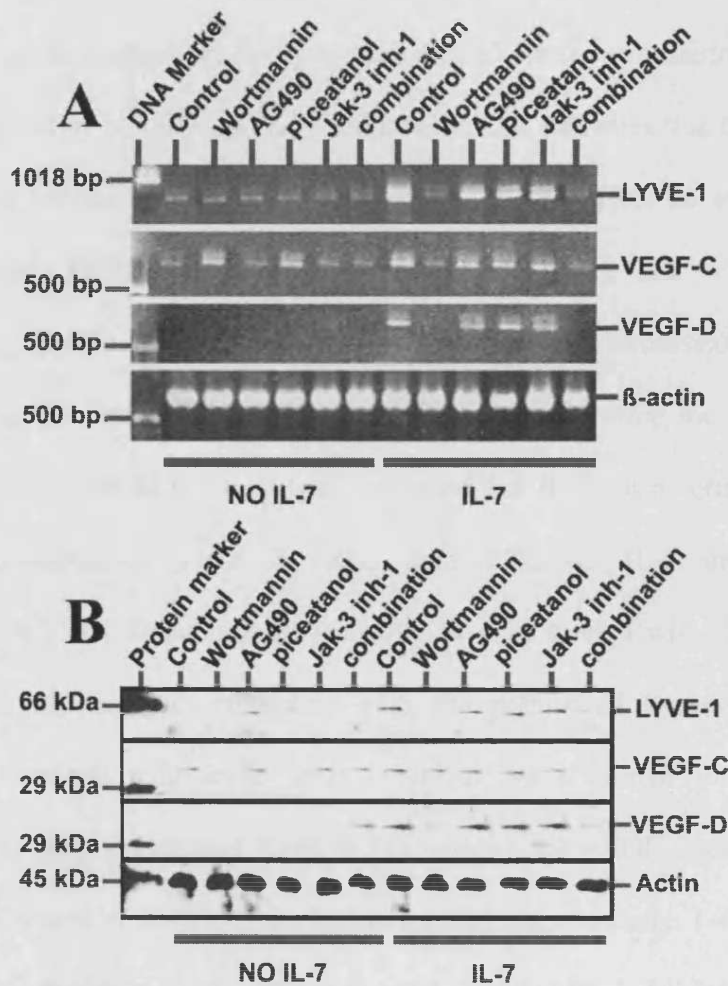


Figure 5.3 IL-7 increased the expression of LYVE-1, VEGF-C and VEGF-D in HECV as shown by the RT-PCR (A) and Western Blotting (B). The expression of VEGF-C was not consistent on repeating the experiment. The inclusion of Wortmannin reversed this effect of IL-7 at both gene and protein levels (A and B respectively). The other IL-7 downstream inhibitors; AG490, piceatanol, and Jak-3 inhibitor-1 did not affect the expression. However, if all 4 inhibitors used together (combination), the expression of these markers decreases to a level similar to that of the control. β -actin and actin were used as the internal housekeeping markers.

5.4 DISCUSSION

This chapter has provided evidence that the effects of IL-7 on the expression of lymphangiogenic markers are likely to be mediated via the stimulation of VEGF-D, and possibly by VEGF-C in endothelial cells. This indicates that the effects of IL-7 are indirect and via an autocrine route. IL-7 did not affect the expression of VEGF-A and VEGF-B nor their receptors, VEGFR-1 and VEGFR-2 in endothelial cells. IL-7, however, increased the expression of the lymphangiogenic factors, VEGF-C and VEGF-D. On repeating the experiments at both gene and protein levels, it was concluded that IL-7 has a consistent effect on the expression of VEGF-D rather than VEGF-C. IL-7 increased the expression of VEGF-D maximally when HECV cells treated with IL-7 between 1-4 hours. This is again consistent with the pattern of expression of the lymphatic markers, podoplanin, prox-1 and LYVE-1 shown in Chapter 4. Furthermore, the expression of VEGFR-3 (a receptor for VEGF-C and VEGF-D) was also increased by IL-7 with the best expression seen between 1-4 hours. IL-7 did not have any effect on the expression of both VEGFR-1 and VEGFR-2 that have mainly angiogenic signalling effects.

Signalling via VEGFR-3 (receptor for VEGF-C and VEGF-D) involves the MAPK and PI3-K pathways. As mentioned in the introduction chapter that both IL-7 and VEGFs signal via both MAPK and PI-3K pathways as well as cross talks do exist between the MAPK and the PI3-K pathways as phosphorylation of Raf by Akt results in inhibition of the Raf-MEK (MAP kinase) – ERK pathway (Zimmermann and Moelling, 1999). PI3-Kinase activation mediates IL-7

transduction and involved mainly in proliferative signals. Here, the effects of IL-7 on the expression of LYVE-1 and VEGF-D were shown to be mainly mediated via the PI3-K pathway as the inclusion of Wortmannin has significantly revert these effects in endothelial cells. It seems that the Jak-stat pathway is not involved in the lymphangiogenic effects of IL-7 as the use of Jak-3 inhibitor –1 did nit bear any effects on the IL-7 induced endothelial cells. However, the effects of IL-7 on the expression of LYVE-1 in endothelial cells are VEGF-D dependent. This is supported by first: the IL-7 induced expression of lymphatic markers was completely blocked by using anti-VEGF-D antibody, and second: the use of a human recombinant VEGF-D produced an effect that closely mimicked that of IL-7. It is noteworthy that HECV cells only expressed very low levels of VEGF-D (both at mRNA and protein levels). This suggests that IL-7 acts as a trigger or enhancer to VEGF-D production within tumour tissues. The next 2 chapters will unfold the impact of these effects of IL-7 on HECV cells growth, migration and their ability to generate lymphatic tubules.

CHAPTER SIX

IL-7 RECEPTOR ACTIVATION AND THE ROLE OF IL-7 IN ENDOTHELIAL CELL GROWTH AND MIGRATION

6.1 INTRODUCTION

Interleukin-7, a pleiotropic and anti-apoptotic cytokine is produced in thymus, bone marrow, intestinal epithelium and skin. It is mainly known for its immune regulatory functions in processes like inflammation, wound healing and malignancies. However, IL-7 also induces the proliferation and differentiation of some haematopoietic malignancies including certain forms of leukaemias and lymphomas. IL-7 is expressed in colorectal (Maeurer *et al*, 1997; Watanabe *et al*, 1995), oesophageal (Oka *et al*, 1995), renal (Trinder *et al*, 1999), head and neck squamous cell carcinoma (Paleri *et al*, 2001) as well as Warthin's tumour of parotid gland (Takeuchi *et al*, 1998). The intracellular mechanisms mediating signalling for the various effects of IL-7 are not clearly established. However, engagement of IL-7R with its ligand, IL-7, leads to a series of intracellular phosphorylation events mediated by signalling molecules including the Janus kinases (Jak-1 and Jak-3), Stat-5 (signal transducers and activators of transcription-5) (Foxwell *et al*, 1995) and the activation of PI3-Kinase (Dadi *et al*, 1994). This could have an impact in understanding the intercellular signal interactions between various types of cells within tissues like tumour stroma.

IL-7, its receptor and their downstream signalling intermediates expression in endothelial and breast cancer cells were not explored to-date. Furthermore, although IL-7 has trophic and proliferative effect in many cells like haematopoietic, its effects on endothelial cells are unknown. Here the expression of IL-7 and its signalling molecules was assessed in HECV and the effects of IL-

7 on HECV growth and migration were also explored, in order to understand its cellular role in these cells.

6.2 MATERIALS AND METHODS

6.2.1 RNA extraction and RT-PCR

Human breast cancer cell lines (MDA MB 231 and MCF-7), a leukaemia cell line, HL-60 and human endothelial cell, HECV were used here. Frozen human normal colonic mucosa and normal skin used to prepare control cDNA were collected from patients immediately after surgery.

RNA extraction was performed as in chapter 3. Primers (Table 6.1) were synthesized by Life Technologies (Paisley, Scotland, UK). Conventional PCR was performed using cDNA from cells or tissues together with the PCR master mix using respective primers. The reaction conditions were: 94°C for 40 seconds, 55°C for 50 seconds, 72°C for 40 seconds and a final extension phase of 10 minutes for 36 cycles. β -actin, a housekeeping gene, was used as an internal RT-PCR control. The PCR products were electrophoresed on a 2% agarose gel and stained with 10 μ l ethidium bromide prior to examination under UV light and a photograph taken.

Table 6.1 Primers size and sequence used in the RT-PCR

<i>Primer</i>	<i>Primer sequence (5'-3')</i>	<i>Size</i>
IL-7 F	attgtgatattgaaggtaaagatg	480 bp
IL-7 R	catcaaaattttattccaaca	
IL-7R F	tctggagaaagtggctatgc	440bp
IL-7R R	cctggcggttaagctacatc	

6.2.2 Immunoprecipitation

Antibodies: Anti-IL-7 Receptor (Rabbit polyclonal), Anti-Jak-1 (Rabbit polyclonal), Anti-Jak-3 (Mouse polyclonal), Anti-PI3-K (Rabbit polyclonal), Anti-Stat-5b (Mouse Monoclonal), and PY-99 antibodies (Mouse monoclonal), were purchased from Santa Cruz Biotechnology, California, USA. HRP antibodies (Rabbit monoclonal), peroxidase conjugated secondary antibodies to rabbit IgG. Protein A/G agarose beads used during immunoprecipitation were obtained from Sigma (Poole, Dorset, England, UK).

Immunoprecipitation was used to detect phosphorylation of IL-7R, Jak-1, Jak-3, PI3-K and Stat-5b in HECV cells. HECV were cultured and incubated with IL-7 for 24 hours at 37⁰C. After lysing the cells as above, primary antibodies (1:500 concentration) were added to each sample and incubated for 1 hour on a spinning wheel, followed by adding conjugated A/G protein agarose beads (5 µl) for another hour. The Ag/Ab complex was collected, washed and lysed using HCMF buffer. The complex was boiled in a sample buffer (10% glycerol; 5% 2-mercaptoethanol; 3% SDS; 80mM Tris-HCl (pH 6.8); 0.012% bromophenol blue). Samples were separated on 8% polyacrylamide. Immunoprobng achieved by using IL-7R, Jak-1, Jak-3, PI3-K, Stat-5b and PY-99 primary antibodies (1:1000) before detection with chemiluminescence and photograph taken.

6.2.3 MTT growth assay

HECV cells were cultured IN DMEM (5000 cells per well) in a 96 well culture cell culture plate. Various concentrations of IL-7 were used to treat HECV. A solution of MTT in 0.5mg/ml in BSS was added into each well. The culture plate was then incubated at 37⁰C for 4 hours. MTT was then removed by aspiration. The crystals produced by MTT reagent within the cells were then extracted by the addition of 100µl of Triton X100 (10% in dH₂O). The cells were incubated at 4⁰C for 24 hours. The absorbance of the colourimetric products was then measured at a wavelength of 540nm using a spectrophotometer (Titertecek).

6.2.4 PicoGreen[®] analysis

HECV cells were treated with IL-7, with or without the presence of signalling inhibitors at sub-toxic concentrations (prior determined over a wide range of concentrations): Wortmannin (25µl/ml), piceatanol (50µl/ml), Jak-3 inhibitor-1 (40µl/ml) and AG 490 (10µl/ml) over specified periods of 7 days (0, 1, 3, 5 & 7). HECV growth was measured using a Picogreen[®] technology and results are expressed as relative value of DNA concentration. At the specified periods, the cells were fixed with 100µl of 10% formaldeyde solution. At the end of 7 days, the cells were lysed with a solution of 0.1% Triton X100 in TE buffer (10 mM Tris-HCL and 1mM EDTA at pH 7.5) for about 60 minutes before quatitation the DNA with PicoGreen[®]. The PicoGreen[®] reagent was prepared according to the manufacturer's instructions by a 200-fold dilution of PicoGreen[®] reagent in a TE buffer. 100µl of the prepared solution was added into each well. The plate

was then read in microplate fluorescence reader (Denley Wellfluor™) at $\lambda_{\text{excitation}}=485\text{nm}$ and $\lambda_{\text{emission}}=520\text{nm}$.

6.2.5 Endothelial cell migration assay

This is based on an assay described previously (Jiang *et al*, 1999). Endothelial cells were seeded in a 6 well cell culture plate until fully confluent. The cells were washed with HEPES-buffered DMEM (10% FCS) and light mineral oil was added. The monolayer was wounded with a 28-gauge sterile needle to produce a wound of approximately 300 μm in width in the presence of either medium, IL-7, IL-7 and neutralising anti-IL-7, IL-7 and neutralising anti-VEGF-D, VEGF-D, VEGF-D and anti-VEGF-D or an irrelevant antibody. Images were recorded using a time-lapse video recorder coupled with a colour CCD camera for 90 minutes. Endothelial cell migration distance was analysed using Optimas 6.0 software package. Over 6 points were measured per frame. The distance the cells had migrated from the initial wound edge was given using the formula: (distance at time 0 – distance at time X) / 2.

6.3 RESULTS

6.3.1 Expression of IL-7 and IL-7R

IL-7 mRNA was expressed in normal skin, normal colonic mucosal tissues, normal breast and breast cancer tissues and in HL-60 cell line (Figure 6.1). IL-7 mRNA was not expressed in HECV cells and breast cancer cells (MCF-7 and MDA MB-231), however, IL-7R was expressed in all these cells and tissues (Figure 6.1). β -actin was used as the internal housekeeping gene.

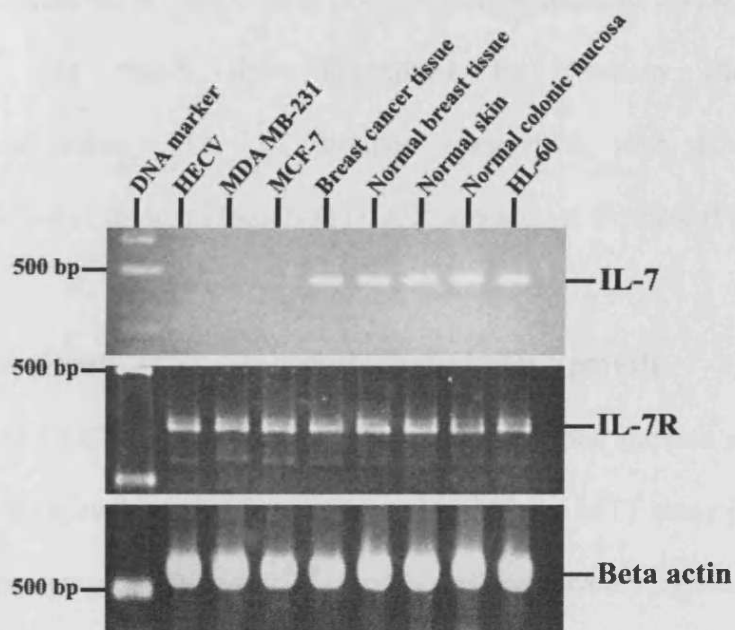


Figure 6.1 IL-7 expressed in normal breast tissues, breast cancer tissues, normal skin, normal colonic mucosal tissue and HL-60 cells, but not in HECV cells and breast cancer cell lines; MDA MB-231 and MCF-7 (Upper panel). However, IL-7R did express in all these cell lines and tissues (middle panel). β -actin was used as the internal housekeeping gene (lower panel).

6.3.2 IL-7R and its signalling pathway molecules activation in endothelial cells

Following treatment of HECV with IL-7, phosphorylation of IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 was determined by Western Blotting and immunoprecipitation. After 15 minutes stimulation with IL-7, tyrosine phosphorylation of these molecules in HECV was seen to increase (Figure 6.2).

6.3.3 The effects of IL-7 on endothelial cell growth

Treatment of HECV with a range of IL-7 concentrations showed a significant increase in the growth of HECV cells as assessed by the MTT assay (Figure 6.3). The mean percentage growth of HECV cells was significantly increased with IL-7. The optimum concentration of IL-7 used to induce this effect on HECV cells was seen between 0.63 ng/ml and 10 ng/ml. However, the growth of HECV cells was tailed off at higher IL-7 concentration (above 10 ng/ml), although the growth rate was still significantly higher than the untreated cells.

6.3.4 The pathways involved in the IL-7 induced endothelial cell growth

HECV cells were either treated with or without IL-7 together with the presence of signalling inhibitors; Wortmannin, piceatanol, Jak-3 inhibitor-1 and AG 490 for 48 hours. The IL-7 induced HECV growth was significantly inhibited by the use of Wortmannin (PI3-K inhibitor), but not with the use of other inhibitors (Figure 6.4). Table 6.2 summarizes the means of the relative fluorescence units (RFU) of HECV growth in relation to the use of IL-7 signalling inhibitors.

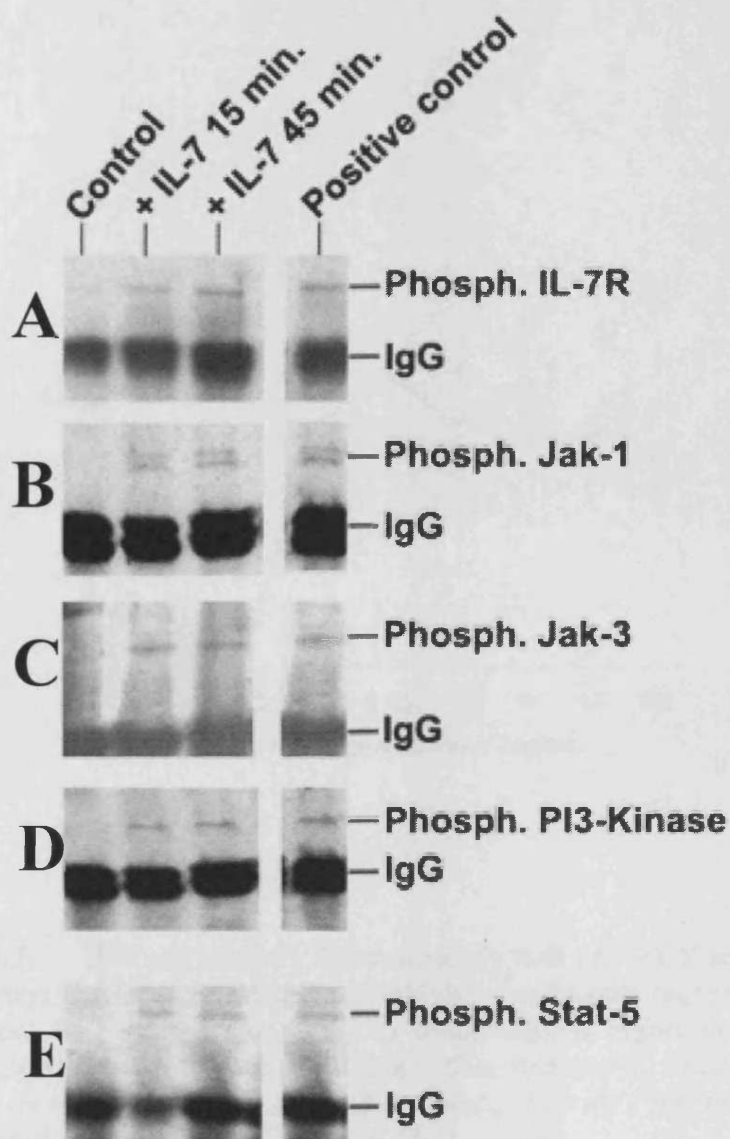


Figure 6.2 Phosphorylation of IL-7R and its downstream signalling molecules in endothelial cells. IL-7R (A), Jak-1 (B), Jak-3 (C), PI3-K (D) and Stat-5 (E) proteins were tyrosine phosphorylated after 15 minutes and were more phosphorylated after 45 minutes treatment with IL-7 and probed with PY-99. This indicates that a fully functional IL-7R is present in HECV cells that can stimulate the signalling pathway to induce gene transcription.

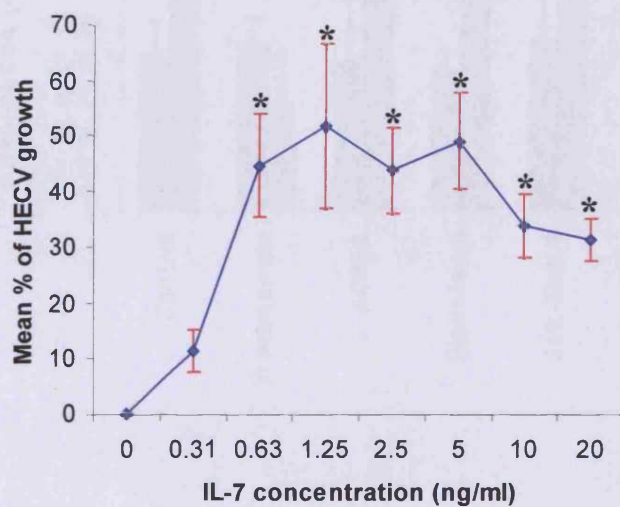


Figure 6.3 IL-7 significantly induced the growth of HECV as shown in the MTT assay. The mean percentage growth was significantly higher after 48 hours incubation. IL-7 induced this effect in concentrations higher than 0.63 ng/ml. HECV growth started to decline if higher concentrations of 10 ng/ml and above. * $p < 0.05$ vs control (no IL-7) (as assessed using students *t* test and P value was considered to be significant if less than 0.05).

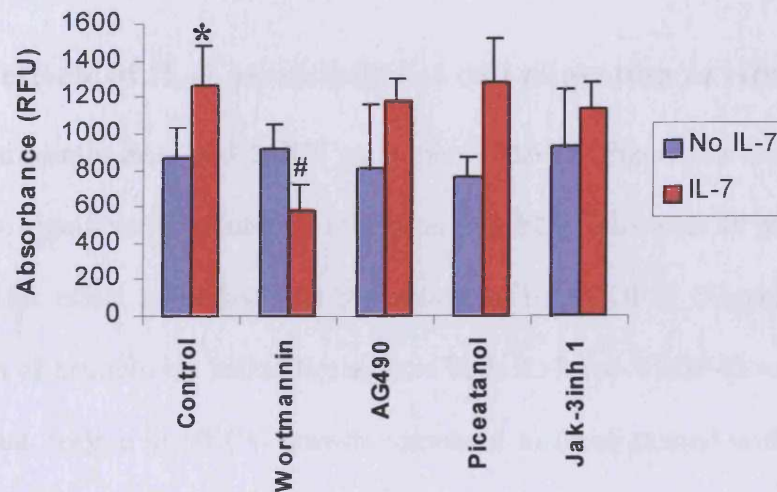


Figure 6.4 PicoGreen[®] analysis. IL-7 significantly increases HECV cells growth after 48 hours incubation (* $p = 0.005$ vs no IL-7). Wortmannin significantly reduces this effect of IL-7 on HECV growth (# $p = 0.015$ vs no Wortmannin). RFU: Relative Fluorescence Units, which represent the relative DNA concentration of HECV cells. Statistics used: student t test and P value considered to be significant if less than 0.05.

Table 6.2 HECV growth in relation to the use of selective IL-7 signalling pathway inhibitors as assessed by PicoGreen[®] analysis. Values represent means fluorescence unites \pm standard deviation.

	<i>Control</i>	<i>Wortmannin</i>	<i>AG490</i>	<i>Piceatanol</i>	<i>Jak-3 inh.</i>
No IL-7	865 \pm 214	914 \pm 142	808 \pm 113	756 \pm 243	924 \pm 153
IL-7	1264 \pm 166	573 \pm 127	1182 \pm 342	1272 \pm 113	1128 \pm 321

6.3.5 The effects of IL-7 on endothelial cell migration *in vitro*

IL-7 has significantly increased HECV migration distance (Figure 6.5 and 6.6). IL-7 started to significantly induce the migration of HECV cells after 40 minutes incubation. This effect mimicked that one produced by VEGF-D (Figure 6.7). The inclusion of neutralising antibodies against both IL-7 and VEGF-D resulted in a significant decline in HECV growth compared to those treated with IL-7 alone (Figures 6.5 and 6.6). The VEGF-D induced endothelial cell migration was also neutralised by anti-VEGF-D antibodies. The inclusion of an irrelevant antibody did not affect HECV cells growth and the migration distance was similar to that produced by the control cells. The migration distance as measured by pixel units are summarised in Table 6.3.

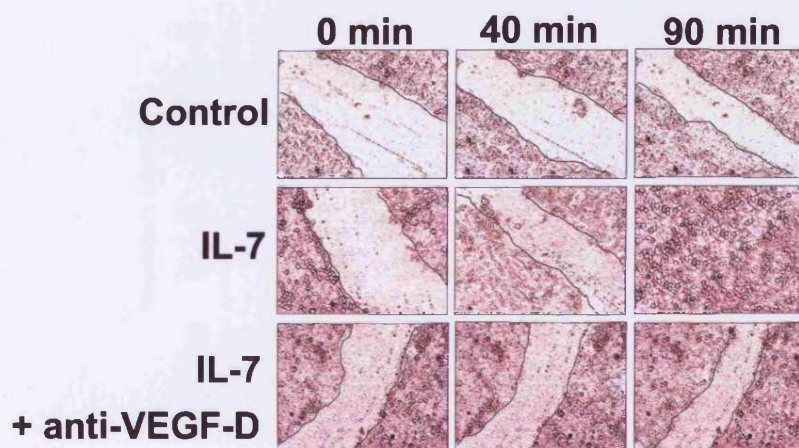


Figure 6.5 *In vitro* HECV migration assay. IL-7 speeds up the closure of the wound compared to cells not treated with IL-7 (The wound is completely closed – middle panel) after 90 minutes. The inclusion of anti-VEGF-D antibody has completely reversed this effect of IL-7, suggesting that IL-7 induces endothelial cell migration via a VEGF-D dependent mechanism (magnification x200).

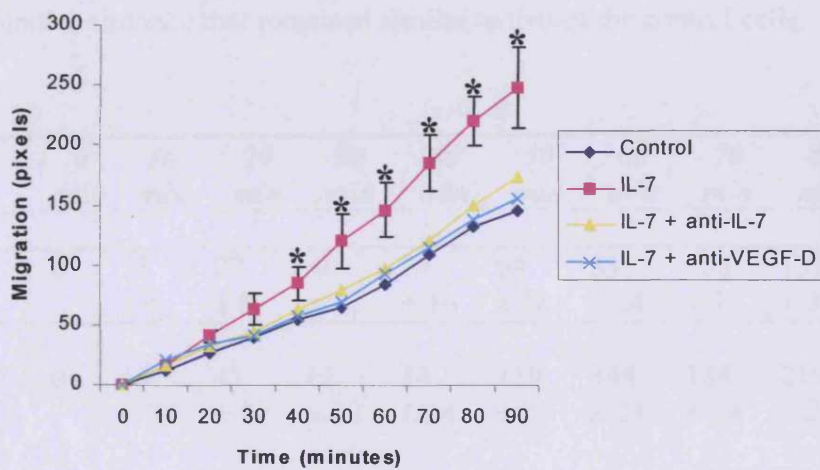


Figure 6.6 *In vitro* HECV migration assay. IL-7 significantly increased HECV migration compared to the untreated cells after 40 minutes incubation. The inclusion of anti-IL-7 or anti-VEGF-D completely neutralised this effect of IL-7, indicating that IL-7 induces this effect by a VEGF-D dependent mechanism. * $p < 0.05$ vs others using student's *t* test (P value is significant if less than 0.05).

Table 6.3 HECV migration assay. HECV treated with IL-7, IL-7 and anti-IL-7, IL-7 and anti-VEGF-D, VEGF-D only, VEGF-D and anti-VEGF-D or irrelevant antibody for 90 minutes. The mean migration distance was significantly higher in cells treated with either IL-7 or VEGF-D. This effect was abolished in the presence of anti-VEGF-D antibody. Values here represent the means \pm standard deviations. The inclusion of an irrelevant antibody did not affect the migration distance that remained similar to that of the control cells.

	<i>0</i>	<i>10</i>	<i>20</i>	<i>30</i>	<i>40</i>	<i>50</i>	<i>60</i>	<i>70</i>	<i>80</i>	<i>90</i>
	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>	<i>min</i>
Control	0	11 \pm 4	27 \pm 8	40 \pm 12	54 \pm 16	64 \pm 18	83 \pm 24	108 \pm 37	131 \pm 33	145 \pm 34
IL-7	0	16 \pm 3	41 \pm 5	63 \pm 13	84 \pm 14	119 \pm 23	145 \pm 23	184 \pm 24	219 \pm 21	247 \pm 34
IL-7 + anti-IL-7	0	16 \pm 5	33 \pm 11	44 \pm 15	63 \pm 17	78 \pm 24	97 \pm 26	120 \pm 39	149 \pm 38	173 \pm 41
IL-7 + anti-VEGF-D	0	21 \pm 8	34 \pm 10	41 \pm 13	57 \pm 19	69 \pm 23	92 \pm 12	114 \pm 31	138 \pm 38	155 \pm 47
VEGF-D	0	18 \pm 8	43 \pm 18	61 \pm 26	101 \pm 33	122 \pm 25	147 \pm 28	190 \pm 35	231 54	264 \pm 61
VEGF-D + anti-VEGF-D	0	14 \pm 5	30 \pm 13	44 \pm 15	63 \pm 23	93 \pm 34	121 \pm 25	141 \pm 24	161 \pm 51	181 \pm 38
Irrelevant antibody	0	11 \pm 7	27 \pm 9	39 \pm 17	59 \pm 15	93 \pm 35	114 \pm 37	133 \pm 45	158 \pm 49	171 \pm 68

6.4 DISCUSSION

IL-7 is known to be produced by cells of the thymus (Namen *et al*, 1988a; Wiles *et al*, 1992), bone marrow stromal cells (Funk *et al*, 1995), intestinal endothelium (Laky *et al*, 1998; Watanabe *et al*, 1995) from keratinocytes in the skin (Heufler *et al*, 1993). IL-7 mRNA is expressed in skin, the colonic mucosa, breast cancer and normal breast tissues as well as in HL-60 cell line. IL-7 was not expressed in endothelial cells or in the breast cancer cell lines. IL-7R was however, expressed in all these cell lines and tissues. Within tissues for example in the tumour microenvironment, among other types of cells, haematopoietic, inflammatory, immune, endothelial and malignant cells interact with each other via a complex network of extracellular signals, such as cytokines. Therefore, IL-7 produced by other cells of stromal origin might stimulate endothelial and / or malignant cells via a paracrine mechanism. This is supported by the ability of IL-7 to activate its receptor as well as its main downstream signalling molecules in endothelial cells as shown by the immunoprecipitation analysis (Figure 6.2).

IL-7 has accelerated significantly the growth of HECV cells as shown by the MTT assay. The range of concentrations within which the optimum effect was achieved was between 0.63ng/ml and 10ng/ml. The response to IL-7 tailed off at higher levels of concentrations. IL-7 functions primarily as a growth and anti-apoptotic factor for haematopoietic cells (Rich *et al*, 1993; Silva *et al*, 1994). Cell maturation and differentiation induced by IL-7 are probably due to its trophic action by inhibiting apoptosis. It has been shown that T lymphocytes for example have failed to proliferate in response to alloantigen or phorbol myristate

acetate and ionomycin in IL-7R^{-/-} mice and instead these cells underwent apoptosis (Maraskovsky *et al*, 1996). IL-7 may be maintaining the viability of cells by repressing a 'death-inducing' factor and / or activating a 'life-promoting' factor (Hofmeister *et al*, 1999). Furthermore, IL-7 could prevent cell death by inhibiting some pro-apoptotic proteins like Bid, Bad or Bax (Hofmeister *et al*, 1999).

The intracellular mechanisms mediating signalling of IL-7 are not yet clearly established. However, activation of IL-7R by IL-7 leads to series of intracellular phosphorylation events mediated by signalling molecules including the Janus kinases (Jak-1 and Jak-3) and Stat-5 (signal transducers and activators of transcription-5) (Dadi *et al*, 1994; Foxwell *et al*, 1995; Lin *et al*, 1995; Pernis *et al*, 1995). Here, the IL-7 induced HECV accelerated growth was significantly reduced by treating these cells with Wortmannin, a potent anti-fungal product that acts as a potent PI3-K inhibitor. Furthermore, Wortmannin significantly decreased the expression levels of both LYVE-1 and VEGF-D in HECV cells (Chapter 5, Figure 5.3).

PI3-K is a critical signalling molecule that regulates several cellular processes including survival and proliferation in different systems. PI3-K is involved in the suppression of TNF induced apoptosis as demonstrated by kinase dead mutant PI3-K construct that results in an enhancement of apoptosis (Burow *et al*, 2000). Although, Jak-3 inhibitor-1 did have a similar effect especially on days 1 and 3, this effect was not persistent after 3 days and was statistically insignificant.

Therefore, it seems that IL-7 exerts its effects on the endothelial cell growth mainly via the PI3-K pathways rather than other pathways.

This study also showed that IL-7 increased endothelial cell migration. This was similar to that effect produced by VEGF-D. This effect of IL-7 seems to be specific and VEGF-D dependent, as the use of anti-IL-7 and anti-VEGF-D antibodies completely abolished these effects of IL-7. This might support the assumption that IL-7 is lymphangiogenic to endothelial cells. Lymphatic vessels developed from endothelial cells after increased growth and migration. The only currently known lymphatic marker is the VEGF-D. The data presented here strongly support the assumption that IL-7 is lymphangiogenic in these cells and its effects are probably mediated by VEGF-D. This will be further demonstrated in the next chapter.

It has been shown here that IL-7R and the main intermediates involved in the IL-7 signalling pathway are present and can be activated in response to IL-7 in human endothelial cells. This could have an impact in understanding the intercellular signal interactions between various types of cells within tissues. The presence of IL-7 in the tumour stroma could act via a paracrine mechanism to affect the lymphangiogenic properties of endothelial cells in tissues like tumour stroma. This could have an impact on the process of lymphangiogenesis, particularly in terms of solid tumours dissemination and spread.

CHAPTER SEVEN

THE ROLE OF IL-7 IN INDUCING THE FORMATION OF LYMPHATIC VESSEL LIKE TUBULES

7.1 INTRODUCTION

The lymphatic system is involved in transport of tissue fluids, extravasated plasma proteins and cells back into the blood circulation. Lymphatics also make an important part of the body's immunological surveillance system. However, it is also a major route for spread of tumour cells. In fact the extent of lymph node involvement is a key prognostic factor and constitutes an integral part of staging in many human cancers including breast, gastric, colorectal and others.

While theories behind the embryonic lymphatic vessels development exist (Jussila and Alitalo, 2002; Sabin, 1902; Sabin, 1904), development of newly formed lymphatics particularly intra-tumourly is still far from clear. However, it is thought that lymphatics differentiate from lymphangioblasts, or via sprouting from pre-existing blood capillaries (Schneider *et al*, 1999; Wilting *et al*, 1999).

As stated in the introduction chapter, intratumoural lymphangiogenesis constitute a conduit for spread of tumour cells to the regional lymph nodes. The development of these lymphatic vessels inside tumours is still unclear and it is thought that many factors and intracellular signals contribute to this process. It has been shown in the previous two chapters that IL-7 could up-regulate LYVE-1 and VEGFR-3 signalling in endothelial cells *in vitro*. It also increased endothelial cell growth and migration via a VEGF-D dependent mechanism. Lymphangiogenesis starts by endothelial cell growth and migration before formation of lymphatic vessels and function in the transport of cells and tissue

fluids. Here, the role of IL-7 in inducing lymphatic like tubules formation *in vitro* and *in vivo* was explored.

7.2 MATERIALS AND METHODS

7.2.1 Endothelial tubule formation assay

The endothelial tubule assay was performed as described previously and modified in our laboratory (Trochon *et al*, 1996). Matrigel (200µg/ml: reconstituted basement membrane; Becton Dickinson) was added to wells in a 96-well culture plate and allow to air dry. HECV (in 100 µl DMEM) were seeded onto re-hydrated Matrigel at 10^5 cells / well and incubated at 37⁰C for about 2 hours in order to attach to Matrigel. The medium was then carefully aspirated, and a second layer of Matrigel was added to the cells. This second layer of Matrigel was mixed with specific reagents as follows: Matrigel only, Matrigel with IL-7, Matrigel with IL-7 and anti-IL-7, with IL-7 and anti-VEGF-D, VEGF-D only or VEGF-D and anti-VEGF-D using the appropriate concentrations. After setting of the second layer of Matrigel, 100 µl of DMEM mixed with the aforementioned reagents were added into the corresponding wells. The cells were then incubated at 37⁰C for 24 hours. Endothelial tubule formation was visualised microscopically. Lengths of these tubules are given in units (number of pixels was used) using Optimas 6.0 software package. The mean length was obtained from 3 independent experiments (8 – 10 frames per test). Statistical analysis was conducted using the student's *t* test and P value considered to be significant if less than 0.05.

7.2.2 Generation of IL-7R null endothelial cells via construction of expression vectors and ribozyme transgenes

The secondary structure of IL-7R (Figure 5.1) was generated using the Zucker's mFold programme. Two sites (150 and 409 of the coding region of human IL-7R mRNA) were found suitable for targeting by hammerhead ribozymes. The two respective ribozymes were generated using touch down PCR, with the following primers (the 5' end 6 bases on each end of each primer are for SpeI and PstI restriction sites, for forward and reverse primers respectively):

IL-7R-rib-1 F: 5'ctgcagttggtccttcccgatagatctgatgagtcctgagga'3

IL-7R-rib-1 R: 5'actagtaacctgaggctcctttgacctgagtgttcgttcctcacgga'3

IL-7R-rib-2 F: 5'ctgcagacaggtcagtgaaatgctgctgatgagtcctgagga'3

IL-7R-rib-2 R: 5'actagttagccagttggaagtgaatggatttcgttcctcacgga'3

The PCR products were T-A cloned into a pcDNA4.1-GFP cloning vector (Invitrogen), as given in the general methods chapter. Following amplification and selection in One-Shot E. coli, Plasmids were extracted, purified, and electroporated into HECV cells using an electroporator (Flowgen, Sussex, England, UK). The vector without ribozyme sequence was used as control. Positive colonies whose IL-7R was lost as the result of the ribozyme were selected, following amplification using RT-PCR of IL-7R and the plasmid sequence.

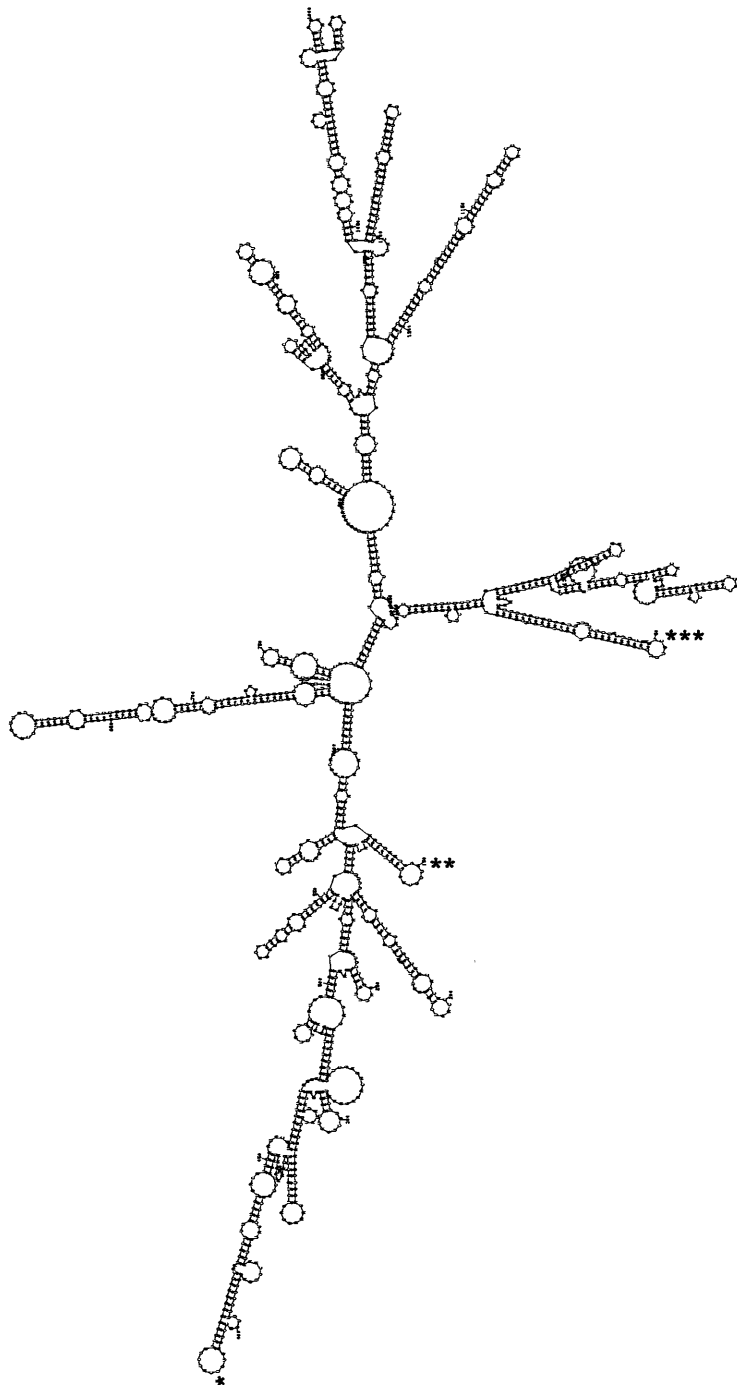


Figure 7.1 IL-7R (23-1400) The secondary structure of human IL-7R. The coding sequence was folded using the Zucker's mFold programme. * Indicates the suitable sites for the hammerhead ribozyme to target.

7.2.3 *In vivo* lymphangiogenesis model

This study conducted in accordance with UK Home Office Regulations. 20 of 7-week-old female athymic nude mice (CD-1; Charles River Laboratories, UK) were sub-grouped into 4 groups. Each group injected subcutaneously in two specific locations with either HECV cells (1.5×10^6) or MDA MB-231 (2×10^6) cells in Matrigel (0.5 mg/ml), mixed with either DMEM medium only or with IL-7 (20ng/ml). After 3 days, the nude mice were killed by terminal anaesthesia (halothane) and the injection sites (Matrigel pockets) were dissected. Blood and lymphatic vessels were stained immunohistochemically using anti-Factor VIII (von Willebrand) and anti-LYVE-1 antibodies respectively. The number of tubules and the strength of immuno-reactivity were measured. Statistical analysis was conducted using the Mann Whitney U test and P value considered to be significant if less than 0.05.

7.3 RESULTS

7.3.1 The effect of IL-7 on the ability of endothelial cells to generate microtubules *in vitro*

The ability of HECV cells to generate microtubules *in vitro* was significantly induced by IL-7 (20ng/ml) ($P = 0.004$ vs control) (Figure 7.2). IL-7 effects on endothelial cells were similar to that produced by VEGF-D ($P = 0.0013$ vs control and 0.12 vs IL-7). The IL-7 induced tubule formation was completely neutralised by the inclusion of either anti-IL-7 or anti-VEGF-D antibodies. Anti-VEGF-D antibodies also neutralised the effects of VEGF-D. The inclusion of an

irrelevant antibody did not have any effect on the ability of HECV to form microtubules (Table 7.1).

7.3.2 IL-7R knocked out endothelial cells

To create IL-7R null HECV, two ribozymes were used (IL-7R-rib-1 and IL-7R-rib-2). Following the procedure above, the desired colonies (contains the required inserts) were selected and verification of the plasmid extraction was performed (Figure 7.3 A and B respectively). IL7-R-rib-1 was successful in knocking out the receptor from most of the HECV cells grown in the selection medium (containing the antibiotic G418) at both mRNA and protein levels as shown by the RT-PCR and Western Blotting (Figure 7.3 C and D respectively). However, IL-7R-rib-2 was unable to knock out the IL-7R from HECV completely.

The transgenic HECV cells (using the IL-7R-rib-1 ribozyme) have lost their response to IL-7 regarding the ability to generate microtubules *in vitro* (Figure 7.4). VEGF-D however, continued to induce microtubules in these cells (Figure 7.4). The inclusion of antibodies against IL-7 and VEGF-D did not alter the ability of these cells to generate tubules as the inclusion of an irrelevant antibody. HECV transfected with the control plasmid did not show any difference in their ability to generate microvessels compared with the wild type HECV cells (Figure 7.5). The means and standard deviation of the wild HECV, IL-7R knocked out HECV and the control plasmid transfected HECV were given in Table 7.1.

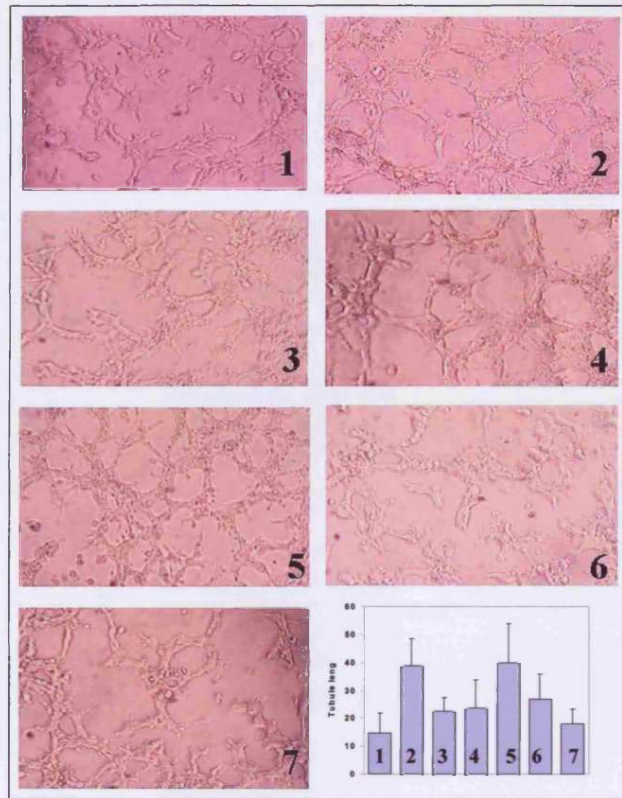


Figure 7.2 *In vitro* tubule formation assay. IL-7 significantly increased the ability of HECV cells to form tubules (1: no IL-7, 2: with IL-7). The inclusion of anti-IL-7 (3) and anti-VEGF-D (4) completely reversed this effect of IL-7. VEGF-D (5) has also significantly increased the ability of HECV to form tubules *in vitro* and its effect was neutralized with anti-VEGF-D (6). The inclusion of an irrelevant antibody (7) did not have any change from the control HECV cells. The bar graph represents the means of tubule length in each condition.

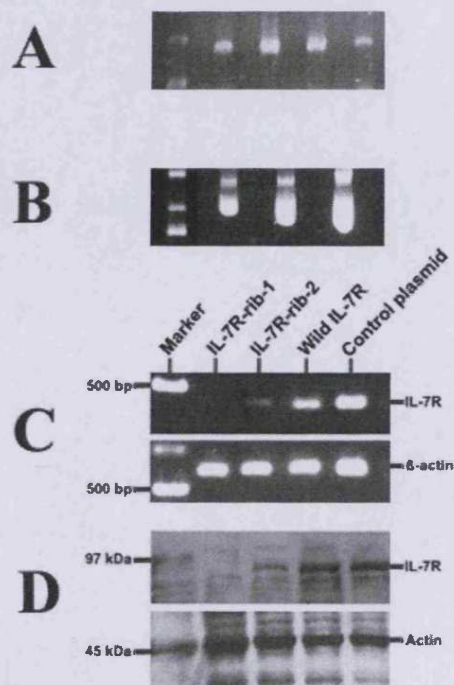


Figure 7.3 The desired colonies containing the inserts were selected (A). Plasmid insertion in the correct direction was verified by RT-PCR (B). IL-7R mRNA was completely knocked out from HECV cells using IL-7R-rib-1 ribozyme and partially using IL-7R-rib-2 ribozyme as shown by the RT-PCR (C). This was reflected on protein level as revealed by the Western Blotting (D). β -actin and anti-actin were used as the housekeeping markers.

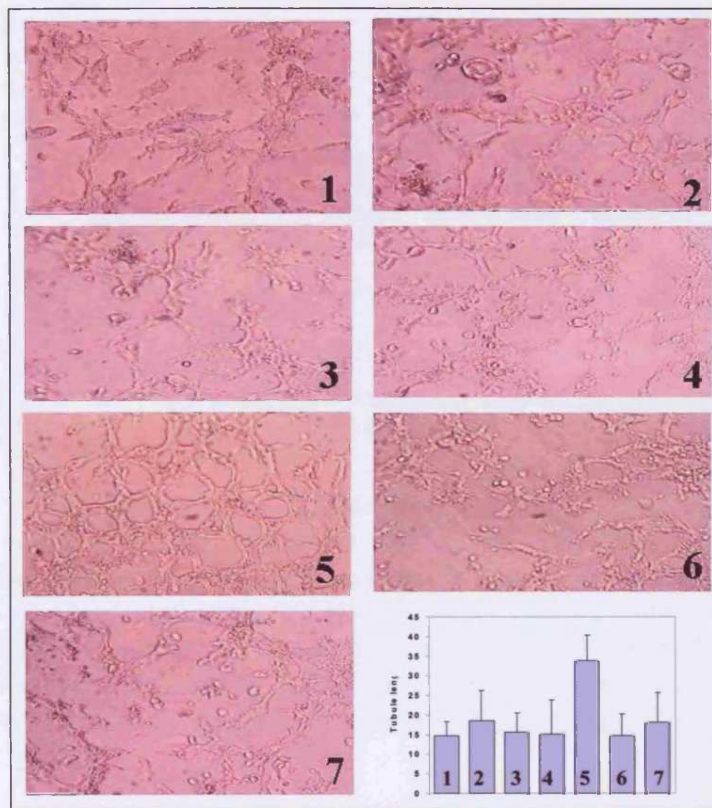


Figure 7.4 IL-7R null HECV cells lack the increased ability to generate microtubules *in vitro* in response to IL-7 (1: HECV without IL-7, 2: with IL-7). Inclusion of antibodies against IL-7 (3) and VEGF-D (4) did not have any effect on the ability of HECV to generate tubules. Treatment with VEGF-D (5) continued to induce these transgenic cells to generate tubules and this effect was neutralised by the inclusion of anti-VEGF-D (6). The use of an irrelevant antibody (7) did not change the behaviour of these cells. The bar graph represents the means of tubule length in each condition.

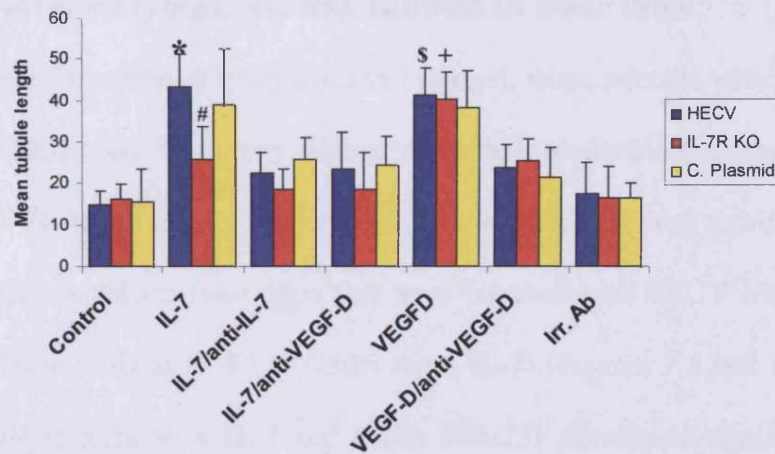


Figure 7.5 *In vitro* tubule formation assay. IL-7 significantly increases HECV to generate tubular structures (* $p=0.004$ vs control), This effect is similar to that produced by VEGF-D on HECV ($p=0.0013$ vs control and 0.12 vs IL-7) IL-7R knocked out HECV cells (IL-7R KO) did not show any difference in their ability to generate tubules with or without IL-7 (# $p=0.216$ vs control). VEGF-D however, continued to induce the IL-7R KO cells to form microtubules (+ $p=0.029$ vs control). Neutralising IL-7 antibody completely reverses the effects of IL-7 and VEGF-D.

Table 7.1 Means and standard deviations of the tubular length produced by HECV (wild type), IL-7R KO HECV and HECV transfected cells with the control plasmid.

	<i>Wild type</i>	<i>IL-7R KO</i>	<i>C. Plasmid</i>
Control	14.72±7.08	16.25±3.57	15.62±7.79
IL-7	43.34±9.79	25.84±7.68	38.74±13.79
IL-7/anti-IL-7	22.52±4.89	18.44±4.89	25.73±5.27
IL-7/anti-VEGF-D	23.51±10.23	18.62±8.65	24.43±6.87
VEGFD	41.36±13.78	40.21±6.57	38.24±8.79
VEGF-D/anti-VEGF-D	23.63±8.75	25.25±5.57	21.28±7.37
Irrelevant Antibody	17.53±5.15	16.49±7.54	16.35±3.62

7.3.3 IL-7 induces lymphatic like tubules in nude mice

IL-7 induced the formation of lymphatics in Matrigel, when injected with HECV and MDA MB-231 cells. The mean number of strongly stained vessels that were positive to LYVE-1, but not for Factor VIII (von Willebrand), was significantly higher in sections obtained from mice that were injected with HECV with IL-7 compared to those without IL-7 ($p= 0.026$ vs no IL-7) (Figures 7.6 and 7.8 A). Similarly, mice injected with IL-7 and MDA MB-231 developed significantly higher number of LYVE-1 stained vessels compared to those without IL-7 and to HECV injected pockets with IL-7 ($p= 0.026$ vs no IL-7 and 0.09 vs HECV and IL-7) (Figures 7.7 and 7.8 B).

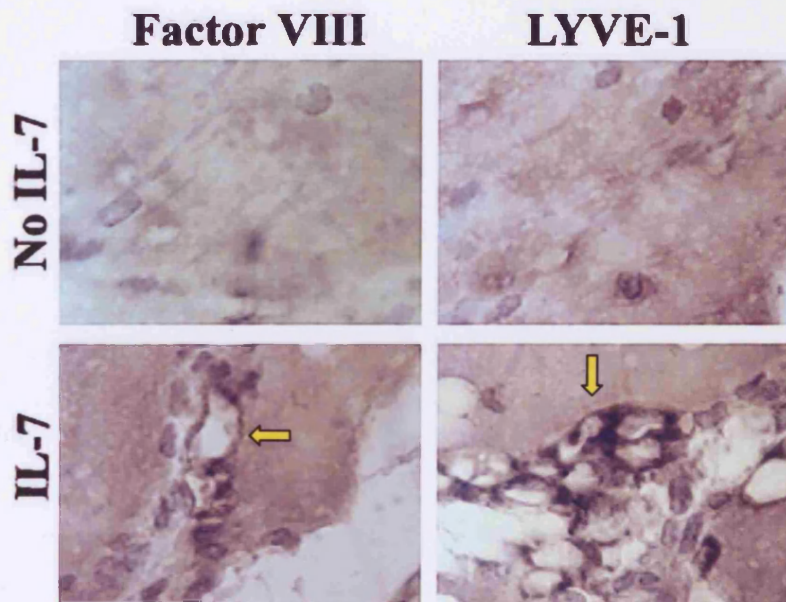


Figure 7.6 IL-7 induced the formation of lymphatics in Matrigel sections. HECV cells in Matrigel sections without IL-7 treatment failed to induce the formation of LYVE-1 positive tubules in these sections (top panel). However, HECV cells treated with IL-7 induced the formation of tubules that were stained negative to von Willebrand factor (Factor VIII) and strongly positive to LYVE-1 indicating the lymphatic characteristics of these tubules (lower panel) (magnification x400).

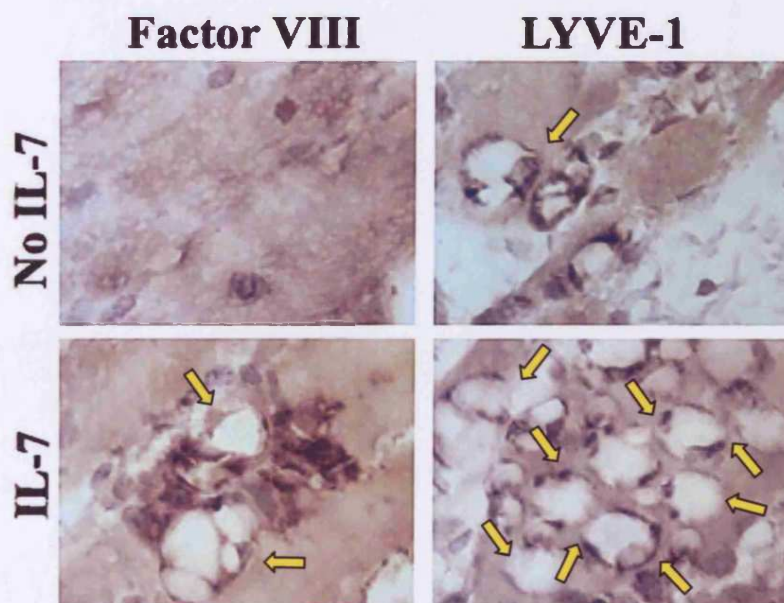


Figure 7.7 Matrigel sections injected with breast cancer cell line, MDA MB-231 with or without IL-7. The number of tubules that were stained strongly positive to LYVE-1 (but not to von Willebrand factor) was significantly higher in cells treated with IL-7 (lower panel) (magnification x400).

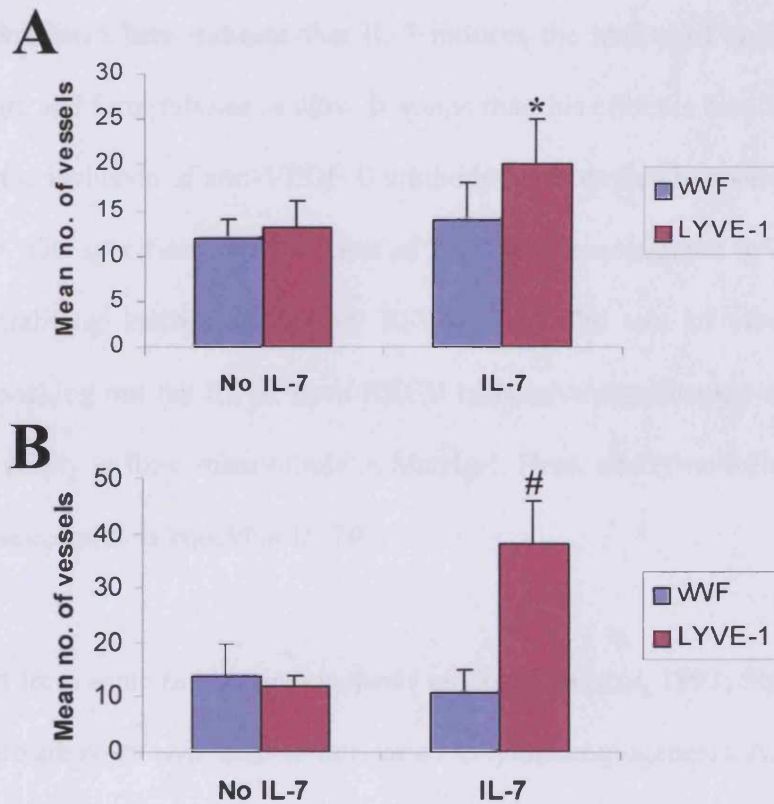


Figure 7.8 The number of strongly stained vessels that are positive to LYVE-1, but not for von Willebrand Factor, was significantly higher in sections obtained from mice that were injected with HECV with IL-7 compared to those without IL-7 (* $p=0.026$ vs no IL-7) (A). The number of LYVE-1 stained vessels was also significantly higher in sections obtained from Matrigel that had MDA MB-231 with IL-7 compared to those without IL-7 (# $p=0.021$ vs no IL-7 and 0.09 vs HECV cells) (B).

7.4 DISCUSSION

The results presented here indicate that IL-7 induces the ability of endothelial cells to migrate and form tubules *in vitro*. It seems that this effect is mediated via VEGF-D, as the inclusion of anti-VEGF-D antibody has completely reversed this effect of IL-7. The specificity of this effect of IL-7 was demonstrated by the use of both neutralizing antibodies against IL-7 and by the use of ribozymes transgenes. Knocking out the IL-7R from HECV cells have significantly reduced their *in vitro* ability to form microtubule in Matrigel. Here, ribozyme technology proved to be successful to knockout IL-7R.

To-date, apart from some *in vivo* angiogenesis models (Hu *et al*, 1993; Strieter *et al*, 1992), there are no *in vivo* studies in relation to lymphangiogenesis. As stated in the introduction chapter, this was due to the lack of lymphatic molecular markers. With the availability of antibody against LYVE-1, it was possible to develop an *in vivo* lymphangiogenesis assay. Using a nude mice model, IL-7 significantly increased the formation of microtubules in Matrigel pockets injected into these mice. It seems that lymphatic vessels have migrated into these pockets as proved by the immunohistochemistry analysis. Immunostaining of these tubules with anti-mouse anti-LYVE-1 antibody displayed a strong reactivity compared to negative staining with anti-von Willebrand (Factor VIII) antibody. Therefore, these vessels are of lymphatic rather than blood vascular origin. Furthermore, there was significantly higher number of LYVE-1 positive vessels in sections that contain MDA MB-231 cells (treated with IL-7) compared to those without IL-7 treatment or with those that contain HECV cells instead.

Human recombinant IL-7 is structurally similar to mouse IL-7 and therefore IL-7 might have stimulated the autocrine secretion of VEGF-D from mouse endothelial cells, although this cannot be proved in this setting. The increased mean number of LYVE-1 stained tubules with MDAMB-231 cells compared to HECV cells indicates that IL-7 might have also stimulated the production of VEGF-D from MDA MB-231 cells and hence the significant increase in the mean number of these tubules. The results shown in this experimental model might indicate that the production of VEGF-D from breast cancer cells exceeds that from HECV cells. As human VEGF-D produced from breast cancer cells is structurally similar to mouse VEGF-D, it might well be capable of activating its receptor, VEGFR-3 on the mouse endothelium to generate these lymphatic like tubules. Although this might provide an answer about the increased number of lymphatic vessels when MDA MB-231 cells over HECV cells, but this cannot be confirmed from the experiment in this model alone.

It has been well established that a complex series of cellular interactions between several types of cells like fibroblasts, immune cells, and endothelial cells as well as malignant cells within the tumour tissues could lead to cancer cells growth and metastasis (Hanahan and Weinberg, 2000). In addition to the ability to synthesize their own growth factors leading to an autocrine stimulation, cancer cells could indeed induce the stimulation of other cells like endothelial cells via a paracrine mechanism, thus generating neovascularisation in the local tumour microenvironment.

It is concluded that IL-7 induces the generation of lymphatic tubules both *in vitro* and *in vivo* probably via a VEGF-D dependent pathway. This was evident by the ability of anti-VEGF-D antibodies to neutralize IL-7 effects on HECV migration and by the higher number of LYVE-1 stained tubules. As tumours need neovascularisation to grow and metastasise, microvascular density has been used as a measure of tumour angiogenesis which is correlated to prognosis (Weidner, 1995b). The *in vivo* model demonstrated in this study proved to be useful to study lymphangiogenic factors and lymphangiogenesis.

CHAPTER EIGHT

THE EFFECTS OF IL-7 ON BREAST CANCER CELLS

8.1 INTRODUCTION

Cytokines play a key role in breast carcinogenesis and subsequent proliferative behaviour. Autocrine and paracrine pathways in breast carcinogenesis is well established (Freiss *et al*, 1993; Lippman *et al*, 1986) as there is now an increasing evidence that cytokines in general are involved in regulating breast cell function and growth. The paracrine effects of cytokines on breast cancer have been demonstrated *in vitro*; incubation of breast cancer cell lines with conditioned medium derived from fibroblasts results in growth promoter/inhibitor effects (Adams *et al*, 1991a; Ryan *et al*, 1993; Speirs *et al*, 1995a; Vanroozendaal *et al*, 1992). The interactions between cytokines and malignant cells are thought to regulate the proliferation and / or the metastatic ability of some cancers. Some cytokines, such as IL-8 (Hu *et al*, 1993; Koch *et al*, 1992; Strieter *et al*, 1992) and IL-1 β for example are thought to be angiogenic / lymphangiogenic via activating VEGF-A and VEGF-C respectively (Akagi *et al*, 2000; Akagi *et al*, 1999).

IL-7 is known to induce the growth and differentiation of some haematopoietic malignancies including some forms of leukaemias and lymphomas (Benjamin *et al*, 1994; Dalloul *et al*, 1992; Digel *et al*, 1991; Eder *et al*, 1992; Foss *et al*, 1995; Foxwell *et al*, 1995; Frishman *et al*, 1993; Qin *et al*, 2001; Rich *et al*, 1993; Takakuwa *et al*, 2000; Touw *et al*, 1990; Trumper *et al*, 1994; Yamada *et al*, 1997a). Some haematological malignancies are also capable of producing IL-7 such as chronic lymphoblastic leukaemia cells (Frishman *et al*, 1993) and Burkett's lymphoma cells (Benjamin *et al*, 1994). Some solid tumours like colonic cancer (Maeurer *et al*, 1997) are also known to produce this cytokine. IL-

7 has been linked to the host immune system against these tumours particularly in the regulation of tumour infiltrating lymphocyte (TIL) level as in colorectal (Maeurer *et al*, 1997; Watanabe *et al*, 1995) and renal (Ditonno *et al*, 1992; Sica *et al*, 1993) . However, the exact role of IL-7 in solid tumours is not clearly established yet.

It has been shown that inappropriate activation of some of its signalling pathway intermediates could lead to malignant transformation. PI3-K for example, known to be activated in IL-7 signalling, is involved in several cellular processes including mitogenesis, cell adhesion, motility, cellular differentiation and provide protection against apoptosis (Toker and Cantley, 1997; Vanhaesebroeck *et al*, 1997). Stat-5 (involved in the Jak-stat pathway of IL-7) was originally identified as a prolactin-responsive mammary gland factor has been reported to be inappropriately activated in breast tissues suggesting a role in epithelial cell differentiation, milk protein gene expression (Wakao *et al*, 1994). It has been shown that stat-5 knockout mice develop breast hypoplasia (Liu *et al*, 1995; Liu *et al*, 1997). Furthermore it has been demonstrated that invasive breast cancer tissues express significantly higher levels of stat-5 compared to benign and normal breast tissues (Watson and Miller, 1995).

Lymphatic spread of breast cancer was correlated with increased lymphangiogenesis (Skobe *et al*, 2001b). Although all VEGFs have detected in several breast cancer cells lines (Kurebayashi *et al*, 1999), their levels of expression varied consistently particularly in regards to the lymphangiogenic growth factors VEGF-C and VEGF-D.

The downstream signalling pathways are common to many cytokines and growth factors including the VEGF family members and interleukins. For example, VEGFR-3 is known to be a strong activator of Stat-5 and also induce tyrosine phosphorylation and activation of PI3-K in endothelial cells (Xia *et al*, 1996). PI3-K activation is implicated in VEGFs-induced endothelial cell survival via activation of its downstream target serine kinase Akt/PKB (Gerber *et al*, 1998). Therefore, here we have screened breast cancer cell lines to detect the expression of VEGFs and studied the effects of IL-7 on their expression in these cells. The effects of IL-7 on the growth of breast cancer cells as well as exploration of the main signalling pathways involved in such effects have been investigated in this chapter.

8.2 MATERIALS AND METHODS

8.2.1 Materials

Breast cancer cell lines, MDA MB-231, MCF-7 and BT-483 were used here. Cell lines were cultured in DMEM as described in Chapter 3. Human recombinant IL-7 was purchased from Chemicon International (Temecula, California). Anti-IL-7 Receptor (Rabbit polyclonal), Anti-Jak-1 (Rabbit polyclonal), Anti-Jak-3 (Mouse polyclonal), Anti-PI3-K (Rabbit polyclonal), Anti-stat-5 (Mouse monoclonal) and PY-99 antibodies (Mouse monoclonal), Anti-VEGF-C (Rabbit polyclonal) and Anti-VEGF-D (Mouse monoclonal) were used. Horse Radish Peroxidase (HRP) conjugated secondary antibodies and Protein A/G agarose beads used during immunoprecipitation were also used as

stated in Chapter 3. Specific IL-7 downstream signalling molecules inhibitors were used to dissect the IL-7 signalling pathways in breast cancer cells as in Chapter 6.

8.2.2 RT-PCR, Western Blotting and immunoprecipitation

Forward and reverse primers for VEGF-A, VEGF-B, VEGF-C, VEGF-D and β -actin were used and the size and sequences are given in Chapters 5 and 6. Conventional PCR was performed using cDNA of MDA MB-231, MCF-7 and BT-483 together with the PCR mix using forward and reverse primers. The reaction conditions were: 94°C for 50 seconds, 55°C for 45 seconds, 72°C for 40 seconds and a final extension phase of 10 minutes for 37 cycles. β -actin was used as an internal housekeeping gene. The PCR products were run on a 2% agarose gel and stained with 10ml ethidium bromide prior to examination under UV light and a photograph taken.

Western Blotting was performed as described in Chapter 3. Primary antibodies against IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 were in a concentration of 1:500 used. Intracellular phosphorylation of these proteins was detected by immunoprecipitation with the respective antibody. In immunoprecipitation, samples were probed with PY-99 antibodies.

8.2.3 Cell growth assays

To determine the effects of cytokines and growth factors on cellular growth after treatment with human recombinant interleukin-7, the four types of growth assays stated in the Chapter 3 were employed here.

8.2.3.1 MTT assay

A general description of this technique was described in Chapter 3. Here, the effect of IL-7 on MDA MB-231 and MCF-7 cell lines was analysed. These cell lines were cultured in DMEM and cultured in a 96-well cell culture plate (10^5 cells / well). The cells were treated with various concentrations of IL-7 (0.01ng/ml - 80ng/ml). The plates are then incubated for 72 hours at 37°C in the incubator (98% humidity and 5% CO_2). After washing the cells twice with BSS, 0.5mg/ml in BSS of MTT was added. The culture plates were then incubated at 37°C (98% humidity and 5% CO_2) for 4 hours. The crystals produced by MTT reagent within the cells were then extracted by the addition of 100 μl of Triton X100 (10% in dH_2O). The absorbance of MTT was then measured at a wavelength of 540nm using a spectrophotometer (Titertecek[®]) as in Chapters 3 and 6.

8.2.3.2 Crystal violet assay

This assay is used to further analyse and confirm the effects of IL-7 on MDA MB-231 and MCF-7 cell lines. MDA MB-231 and MCF-7 cells (10^5 cells / well) were cultured and treated with IL-7 in a similar way to the MTT assay. The cells were then fixed with 100 μ l of 10% formaldehyde added into each well. 100 μ l of crystal violet solution (0.2% mixed with 2% ethanol and dH₂O) was added into each well. The level of absorbance is measured at a wavelength of 540nm using a spectrophotometer.

8.2.3.3 Hoeschet 33258 assay

The cell counts per well and the range of IL-7 concentrations were similar to the above settings. 100 μ l of SDS (0.05% in SSC) was added to each well and the plate was placed in the incubator at 37 °C (98% humidity and 5% CO₂) for 2 hours. 100 μ l of Hoescht 33258 (1 μ g/ml in 1 x SSC) was added into each well. The plate was read in microplate fluorescence reader (Denley Wellfluor™) at $\lambda_{\text{excitation}}=356\text{nm}$ and $\lambda_{\text{emission}}=458\text{nm}$.

8.2.3.4 Assessment of cell growth using PicoGreen® analysis

Breast cancer cell lines, MDA MB-231 and MCF-7 were cultured in MDEM and treated with IL-7 in a similar way as above. A 100 μ l of the PicoGreen® reagent (200-fold diluted in a buffer containing 10 mM Tris-HCL and 1mM EDTA at pH 7.5) was added into each well. The plate was then read in microplate

fluorescence reader (Denley Wellfluor™) at $\lambda_{\text{excitation}}=485\text{nm}$ and $\lambda_{\text{emission}}=520\text{nm}$.

8.3 RESULTS

8.3.1 Detection and phosphorylation of IL-7R and its downstream signalling molecules in breast cancer cells

IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 proteins were all detected in both MDA MB-231 and MCF-7 cells (Figure 8.1). These proteins were also tyrosine phosphorylated at 15 minutes and increased by 45 minutes in MCF-7 cells in response to treatment with IL-7 (10 ng/ml) (Figure 8.2).

8.3.2 Expression of the vascular endothelial growth factors (VEGFs) in breast cancer cells

All VEGFs were expressed in breast cancer cells with variable levels. VEGF-A, VEGF-B and VEGF-C mRNA were consistently expressed in MDA MB-231, MCF-7 and BT-483 cells as shown by the RT-PCR (Figure 8.3). However, VEGF-D mRNA was expressed only in BT-483 cells and its expression in MDA MB-231 and MCF-7 cells was either weak or absent (Figure 8.3).

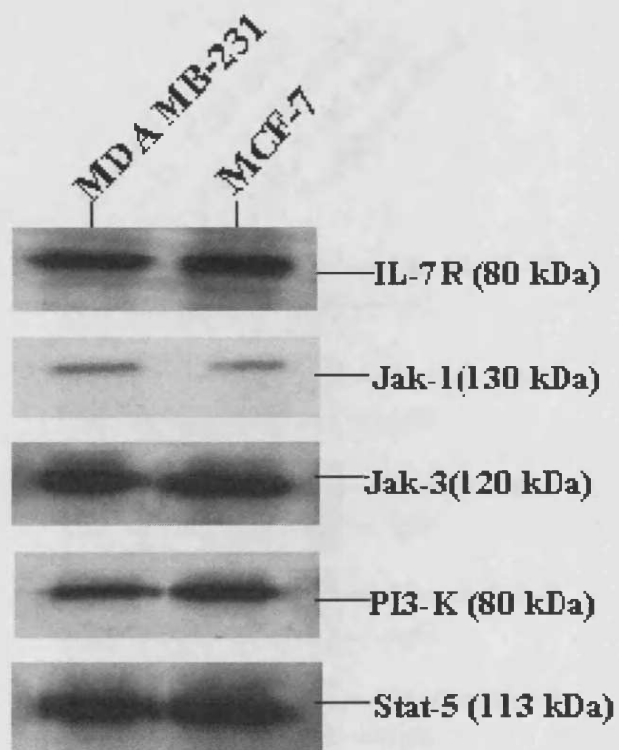


Figure 8.1 Western Blotting showing the detection of IL-7R protein and its downstream signalling intermediates (Jak-1, Jak-3, PI3-K and stat-5) in MDA MB-231 and MCF-7 cells.

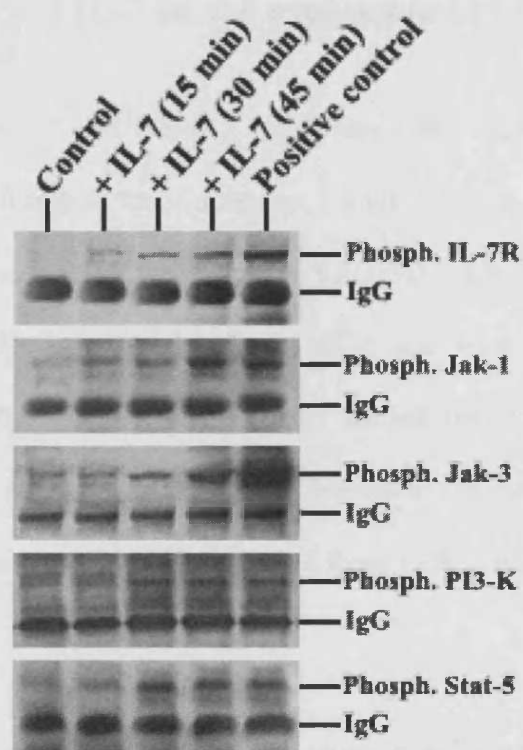


Figure 8.2 Immunoprecipitation showing the tyrosine phosphorylation of IL-7R, Jak-1, Jak-3, PI3-K and stat-5 in MCF-7 cells. The phosphorylation is noted at 15 minutes and increased by 45 minutes in all these molecules in response to treating MCF-7 cells with 10 ng/ml of IL-7. These molecules were probed with PY-99.

8.3.3 The effects of IL-7 on the expression of VEGF-D in breast cancer cells

MDA MB-231, MCF-7 and BT-483 cells were either not treated (control) or treated with IL-7 (40 ng/ml) for 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours and 24 hours. IL-7 increased the expression of VEGF-D mRNA in both MDA MB-231 and MCF-7 cells as shown by the RT-PCR and Western Blotting (Figures 8.4 and 8.5 respectively). The effect of IL-7 started after 1hour incubation and gradually increased to 24 hours incubation. However, the expression of VEGF-D in BT-483 cells was not affected by treating these cells with IL-7 (Figures 8.4 C and 8.5 C).

8.3.4 The effects of IL-7 on breast cancer cells growth

To assess the effects of IL-7 on breast cancer cells growth, MDA MB-231 and MCF-7 cells were used. Incubation of MDA MB-231 and MCF-7 with various concentrations of IL-7 for 72 hours has resulted in a significant growth acceleration of both these cell lines as demonstrated by the MTT, Picogreen[®] analysis (Figure 8.6), Hoescht 33258 and crystal violet growth assays (Figure 8.7). IL-7 has significantly increased the growth of both MDA MB-231 (at 0.02ng/ml) and MCF-7 (at 1.5ng/ml) (absorbance: 0.195±0.006 without IL-7 and 0.204±0.007 with IL-7, p=0.002, 0.205±0.005 without IL-7 and 0.265±0.012, p=0.01 respectively) as demonstrated by the MTT assay (Figure 8.6). In the Hoescht 33258 assay, IL-7 significantly induced MDA MB-231 and MCF-7 growth (325.9±10.9 without IL-7 and 499.9±10.5 with IL-7 p=0.043 and 449.9±13.9 with IL-7 vs 330.4±10.4 without IL-7 p=0.009). The effect of IL-7 on these cells growth was further confirmed by the crystal violet assay

(0.244 ± 0.009 with IL-7 vs 0.176 ± 0.011 without IL-7 $p=0.031$ for MDA-MB-231 and 0.154 ± 0.021 with IL-7 vs 0.069 ± 0.013 without IL-7 $p=0.026$ for MCF-7 cells). Similarly, IL-7 has significantly accelerated the growth of MDA MB-231 and MCF-7 cells (Fluorescence units: 330.25 ± 12.39 without IL-7 vs 401.66 ± 27.42 with IL-7, $p=0.004$) and MCF-7 cell (348.88 ± 21.05 without IL-7 vs 389.93 ± 6.37 with IL-7, $p=0.01$) as shown by the PicoGreen[®] analysis. Generally, the effects of IL-7 reached maximum and tailed off afterwards. The optimum concentration of IL-7 to induce maximal growth was $\sim 5\text{ng/ml}$ (Table 8.1).

8.3.5 Effects of signalling pathways inhibitors on IL-7 induced breast cancer cells growth

MDA MB-231 and MCF-7 growth was significantly inhibited by the addition of Wortmannin and Jak-3 inhibitor 1 (Figure 8.8). MDA MB-231 growth was significantly reduced by the inclusion of Wortmannin and Jak-3 inhibitor 1 ($p=0.001$ and $p=0.0001$ respectively vs no inhibitors). Similarly MCF-7 growth was significantly declined in the presence of Wortmannin and Jak-3 inhibitor 1 ($p=0.0029$ and $p=0.00016$ vs no inhibitors). The inhibitory effects of Wortmannin and Jak-3 inhibitor 1 on IL-7 were seen at non-toxic concentrations. However, the addition of piceatanol or AG 490 did not have any effects on the growth.

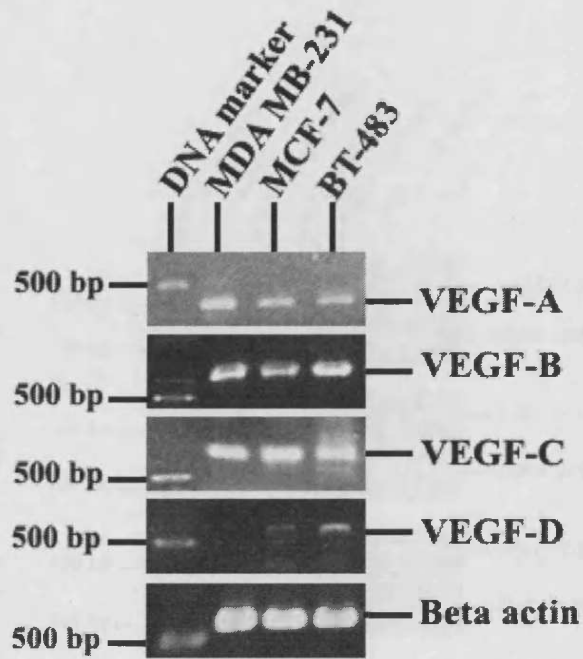


Figure 8.3 VEGFs -A, -B and -C were expressed in the three breast cancer cell lines (MDA MB-231, MCF-7 and BT-483) at mRNA level. However, the expression of VEGF-D mRNA was noted to be very weak in MCF-7 cells and absent in MDA MB-231 compared to BT-483.

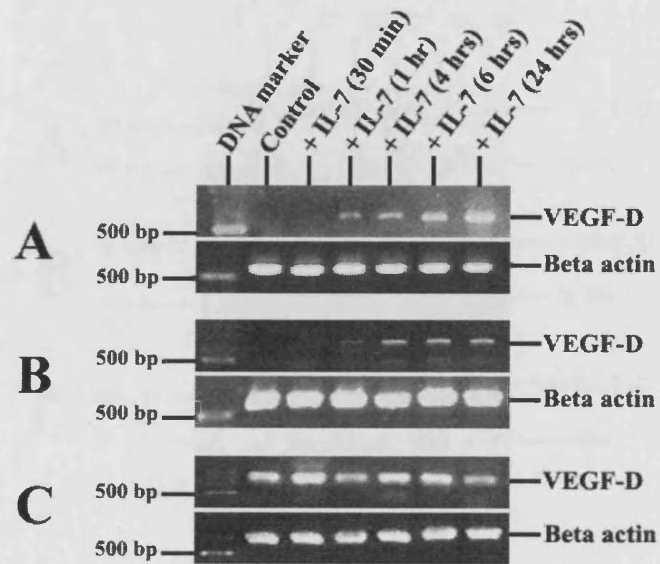


Figure 8.4 IL-7 increased the expression of VEGF-D in MDA MB-231 (A) and MCF-7 (B). This effect was noted if these cells were treated with IL-7 for an hour and the expression tends to increase gradually with time particularly clear with MDA MB-231 cells (A). However, IL-7 does not seem to affect the expression of VEGF-D in BT-483 cells (C).

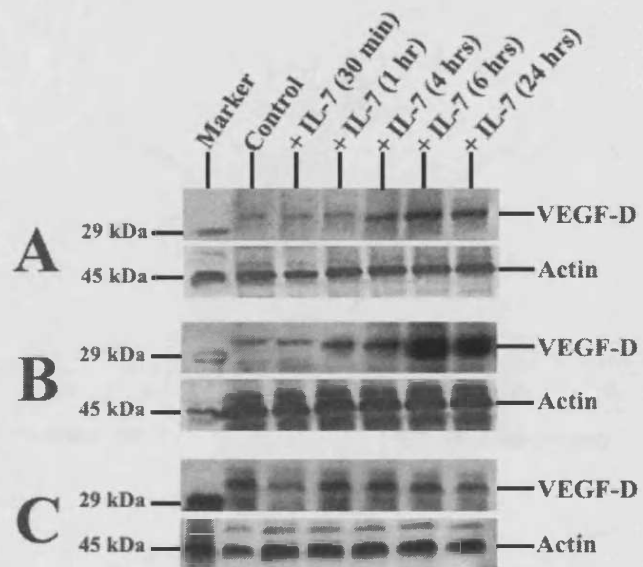


Figure 8.5 Western Blotting showing the effects of IL-7 on the expression of VEGF-D in MD MB-231 (A), MCF-7 (B) and BT-483 cells (C). Breast cancer cells treated with IL-7 produce more VEGF-D protein compared to those without treatment. However, protein levels in BT-483 cells (C) were unaffected by IL-7 treatment.

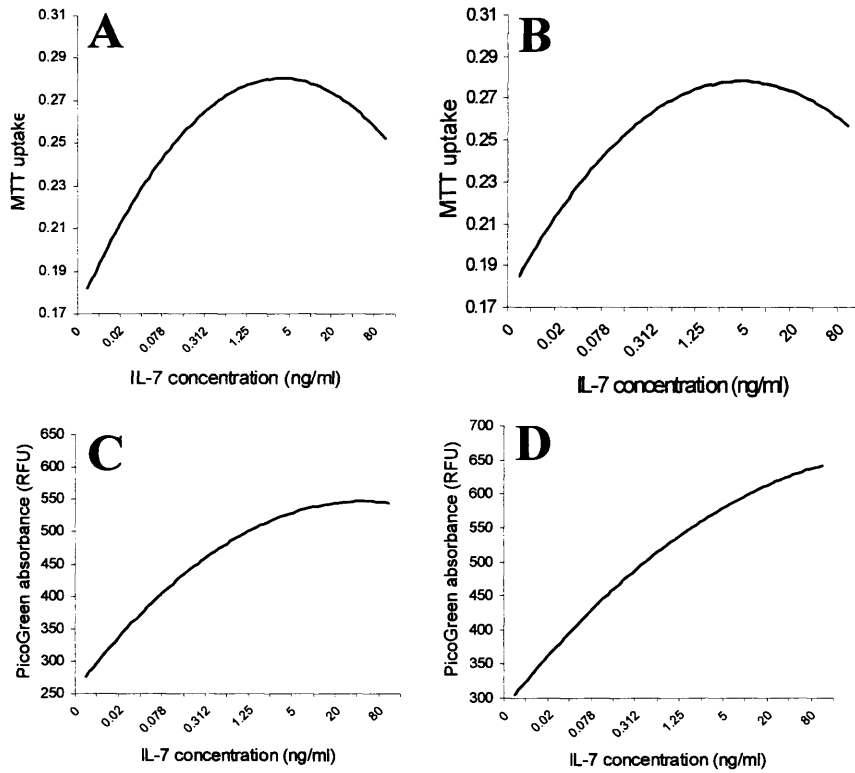


Figure 8.6 MTT assay (top, A: MDA MB-231, B: MCF-7) and PicoGreen[®] analysis (bottom, C: MDA MB-231, D: MCF-7): IL-7 significantly increased the growth of both MDA MB-231 and MCF-7. Statistical analysis using the student *t* test and P value considered significant if less than 0.05.

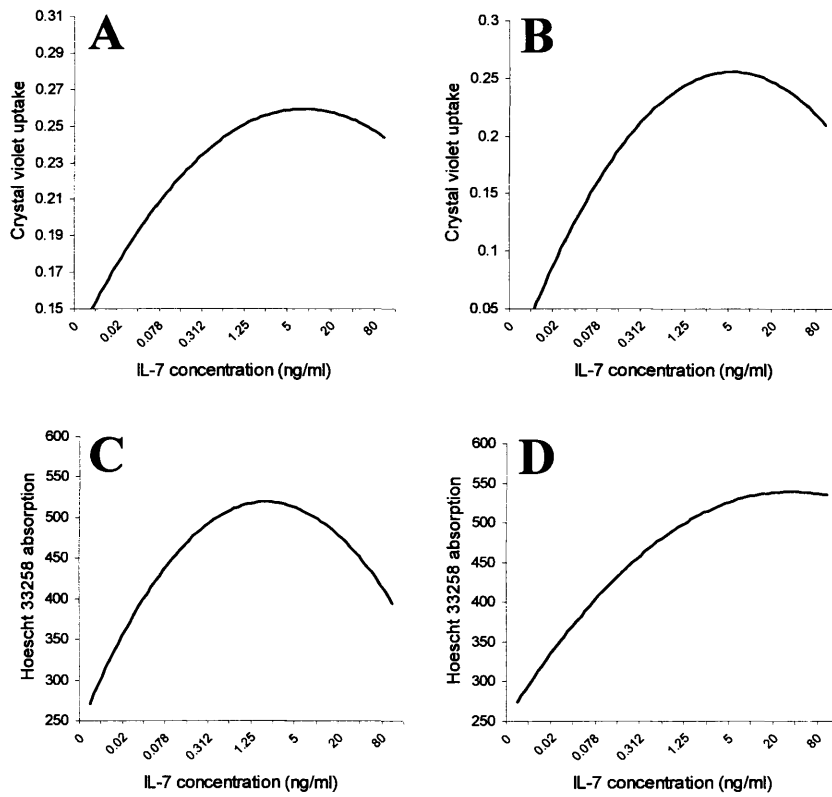


Figure 8.7 Crystal violet growth assay (top, A: MDA MB-231, B: MCF-7) and Hoescht 33258 assay (bottom, C: MDA MB-231, D: MCF-7): IL-7 significantly accelerated the growth of both types of cells.

Table 8.1 Means of MDA MB-231 and MCF-7 growth changes in response to treatment with IL-7 as assessed by PicoGreen®, MTT, Hoescht 33258 and crystal violet assays). *p<0.05 vs no IL-7 treatment (Statistics using student *t* test). R.F.U.: Relative Fluorescence Units; Abs.: Absorbance.

IL-7 concentra (ng/ml)	PicoGreen® (R.F.U.)		MTT (Abs.)	
	MDA MB-2.	MCF-7	MDA MB-23	MCF-7
0	330.3±12.4	348.9±21.1	0.195±0.007	0.205±0.005
0.01	333.8±10.8	365.9±11.7	0.204±0.008	0.216±0.017
0.02	328.9±17.6	372.3±16.2	0.224±0.015*	0.207±0.006
0.04	377.2±12.1	366.9±15.1	0.221±0.019*	0.213±0.008
0.08	338.9±17.0	389.9±15.1	0.247±0.014*	0.205±0.002
0.15	401.7±27.4*	449.1±6.4*	0.242±0.011*	0.265±0.012*
0.3	441.8±13.5*	470.3±13.7*	0.265±0.023*	0.272±0.017*
0.6	487.9±10.0*	562.3±20.7*	0.288±0.020*	0.290±0.006*
1.2	559.1±28.1*	524.9±44.7*	0.268±0.019*	0.283±0.016*
2.5	600.9±18.6*	599.5±23.7*	0.284±0.007*	0.284±0.004*
5	573.7±20.4*	646.8±22.8*	0.285±0.011*	0.292±0.003*
10	557.4±14.1*	641.2±20.2*	0.303±0.015*	0.269±0.008*
20	517.9±24.3*	649.7±21.1*	0.273±0.007*	0.263±0.045*
40	527.1±26.2*	609.9±20.0*	0.265±0.008*	0.275±0.010*
80	519.5±16.4*	586.9±15.2*	0.230±0.010*	0.243±0.017*

Hoescht 33258 (R.F.U.)		Crystal violet (Abs.)	
MDA MB-231	MCF-7	MDA MB-231	MCF-7
325.9±10.9	330.4±10.4	0.176±0.011	0.069±0.013
354.3±13.2	340.5±13.4	0.175±0.013	0.066±0.007
371.0±13.3	331.4±8.8	0.174±0.006	0.073±0.014
327.9±15.6	350.9±22.3	0.173±0.017	0.078±0.016
355.5±28.0	335.0±16.6*	0.173±0.013	0.154±0.021*
499.9±10.5*	416.1±14.7*	0.199±0.004	0.259±0.009*
512.7±22.4*	449.9±13.9*	0.244±0.009*	0.208±0.016*
575.3±15.9*	488.1±27.7*	0.267±0.010*	0.240±0.007*
545.1±27.1*	553.5±10.7*	0.272±0.013*	0.250±0.006*
517.1±23.9*	565.6±29.5*	0.284±0.003*	0.252±0.025*
503.9±33.1*	569.4±29.5*	0.282±0.017*	0.283±0.017*
551.5±32.6*	573.2±8.0*	0.272±0.016*	0.273±0.013*
439.5±10.1*	540.5±23.8*	0.230±0.027*	0.236±0.038*
411.3±12.3*	513.8±25.5*	0.246±0.012*	0.215±0.024*
378.5±10.9*	492.9±19.5*	0.231±0.027*	0.196±0.009*

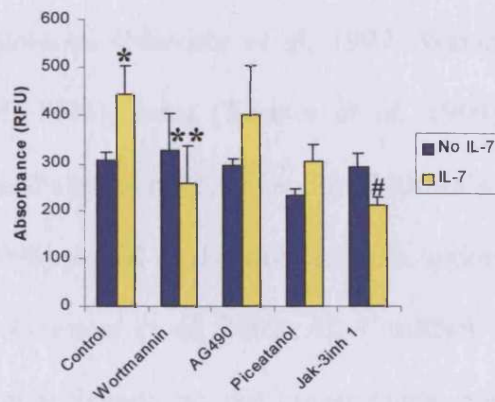
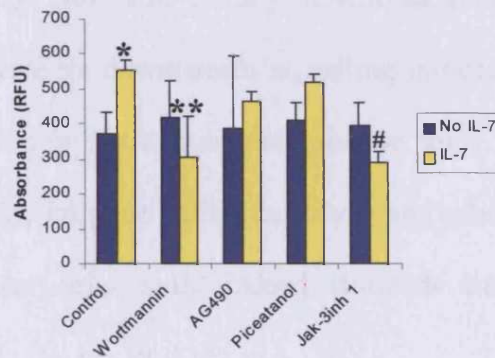
A**B**

Figure 8.8 PicoGreen[®] analysis. MDA MB-231 growth (A) was significantly reduced by the inclusion of Wortmannin and Jak-3 inhibitor 1 (** $p=0.001$ and # $p=0.0001$ respectively vs no inhibitors). Similarly MCF-7 growth (B) was significantly reduced in the presence of Wortmannin and Jak-3 inhibitor 1 (** $p=0.0029$ and # $P=0.00016$ vs no inhibitors). IL-7 has significantly increased both MDA MB-231 and MCF-7 growth (* $P<0.05$ vs no IL-7). Statistics were conducted using the student *t* test and P value considered to be significant if less than 0.05.

8.4 DISCUSSION

Cytokines, including IL-7, control a variety of important biological responses related to cell growth, differentiation and anti-apoptotic signals (Leonard, 1999). IL-7 is expressed in colorectal (Maeurer *et al*, 1997; Watanabe *et al*, 1995), oesophageal (Oka *et al*, 1995), renal (Trinder *et al*, 1999), head and neck squamous cell carcinoma (Paleri *et al*, 2001) and in Warthin's tumour of parotid gland (Takeuchi *et al*, 1998). IL-7R is also detectable in tumours including lung, colon, renal and CNS (Cosenza *et al*, 2002). IL-7 mRNA was expressed in normal breast, breast cancer tissues, but not breast cancer cells MDA MB-231 and MCF-7 (Chapter 6). However, a fully functional receptor, IL-7R was detected and could activate its downstream signalling molecules, Jak-1, Jak-3, PI3-K and Stat-5 as shown by the immunoprecipitation study. In the body, IL-7 from haematological cells, immune cells, fibroblasts and other stromal cells in and around breast cancer cells could indeed stimulate tumour cells via a paracrine mechanism.

The expression of the VEGF family members in breast cancer cells has been explored here. It seems that all VEGFs are expressed in the screened breast cancer cell lines, MDA MB-231, MCF-7 and BT-483 at different levels. This heterogeneity in the levels of expression might hypothetically determine the ultimate behaviour of cancer cells in relation to switching the angiogenic or the lymphangiogenic routes. For example, it has been previously reported that the expression of VEGF-C is mainly detectable in node positive breast cancers, whereas expression of VEGF-A is detected in both node positive and node

negative tumours (Kurebayashi *et al*, 1999). Other studies claim that although VEGF-C is present in breast cancer cells, it is not always sufficient to induce the formation of functional lymphatic vessels (Leu *et al*, 2000). Although, Kurebayashi *et al* have reported that VEGF-D is weakly expressed in some breast cancer cell lines, there are no current studies about the regulation of VEGFs expression in tumour cells including breast cancer. Here, IL-7 was found to increase the expression of VEGF-D in both MDA MB-231 and MCF-7 cells as demonstrated at both mRNA and protein levels. This effect seems to be very similar to that produced by IL-7 on the endothelial cells. This indicates that the signalling pathways of both cytokines might be shared in different cells within the tumour microenvironment for example. This effect of IL-7 was observed mainly after 1hour incubation with these cells. As, breast cancer cells are not very easy to grow and study compared to the fast growing HECV cells, further specificity tests like the use of neutralising antibodies or ribozymes were not employed here.

IL-7 has significantly accelerated the growth of breast cancer cells (MDA MB-231 and MCF-7) in this study and this effect was convincing as it is supported by 4 different assays and the findings were consistent, despite the different assay sensitivities and other variations related to cell counts and experimental conditions. The increased growth was mainly observed after 72 hours incubation with IL-7 and the concentration of IL-7 seems to be crucial to induce its trophic action on breast cancer cells. The mean concentration range that induced maximal growth was ~ 5ng/ml, which is within the reported range of its

maximum biological activity *in vitro* applications on other cells including T cells and leukaemia cells.

IL-7 induced breast cancer cell growth was significantly reversed back to the control levels by the inclusion of Wortmannin (PI3-K pathway inhibitor). PI3-K protein is expressed in breast cancer cells (Figure 8.1). It has been reported that PI3-K is frequently activated in breast cancer and ER α could bind its p85 α subunit and activate the Akt2 pathway (which is important for malignant transformation) in an oestrogen-independent manner (Sun *et al.*, 2001). Wortmannin, a natural fungal product, is a potent inhibitor of PI3-K inhibitor. There are several studies suggesting its applications as an anti-inflammatory and anti-neoplastic agent. It has been recently found that systemic Wortmannin administration significantly prolongs mean survival of SCID mice with human lung cancer (Boehle *et al.*, 2002). These data suggest that inhibition of the PI3-K pathway could be a potential target for cancer therapy. Jak-3 is a non-receptor tyrosine kinase that plays an important role in coordinating signals received through IL-7R. Although, inhibition of Jak-3 did not affect the IL-7 induced endothelial cells growth (Chapter 6), here however, interrupting the Jak-stat pathway, the growth of breast cancer cells was significantly reduced. Therefore, it seems that IL-7 activates different pathways in endothelial and breast cancer cells although it did activate PI3-K pathway in both cell types.

It is concluded that IL-7 could induce the growth of breast cancer cells *in vitro* and this effect is mediated via Jak-3 and a Wortmannin sensitive pathways. This may have an important impact on breast cancer development, progression and

aggressiveness and thus might have diagnostic and or prognostic significance. The development of IL-7R blocking molecules or the use of selective inhibitors to the downstream signalling pathway of IL-7 should be further explored as a potential therapeutic strategy in solid tumours including breast cancer.

CHAPTER NINE

IL-7 EXPRESSION IN HUMAN BREAST CARCINOMA AND THE ASSOCIATION WITH CLINICOPATHOLOGICAL CHARACTERISTICS

9.1 INTRODUCTION

It has been shown in the previous chapters that IL-7 is a pleiotropic cytokine that induces the growth of both endothelial and breast cancer cells *in vitro*. Furthermore, IL-7 was also shown to have lymphangiogenic potential via inducing VEGF-D / VEGFR-3 signalling on the vascular endothelium. IL-7 exerts these functions mainly via a Wortmannin sensitive pathway. As stated in Chapter one, it is known that IL-7 signalling pathway triggers its trophic and anti-apoptotic effects via the activation of its transmembrane receptor, IL-7R and other intracellular molecules including Jak-1, Jak-3, PI3-K and Stat-5.

IL-7, its receptor and the aforementioned molecules have been previously shown to be expressed in several human cancers (Cosenza *et al*, 2002; Maeurer *et al*, 1997; Paleri *et al*, 2001; Takeuchi *et al*, 1998; Trinder *et al*, 1999). Observations of increased levels of IL-7 signal transduction components in breast tumour samples suggest a role for IL-7 in breast tumour biology. The Jaks are large kinases that can be phosphorylated in some breast cancer cell lines (Cance and Liu, 1995b). It has been shown recently that there is frequent activation of PI3-K in breast cancer. ER α can bind to the p85 α subunit of PI3-K in epithelial cells and activates the PI3-K/Akt2 pathway in an oestrogen-independent manner (Sun *et al*, 2001). Stat-5 could be inappropriately activated in a wide variety of human cancers (Bowman *et al*, 2000). Activation of Stat-5 in breast tissue suggests their active role in epithelial cell differentiation and milk protein gene expression (Liu *et al*, 1997). Stat-5 knockout mice develop impaired mammary gland development (Liu *et al*, 1995; Liu *et al*, 1997). Furthermore, It has been shown

that nuclear extracts from breast cancers display significantly higher levels of Stat-5 in invasive breast cancers than those from benign and normal breast tissue (Watson and Miller, 1995), suggesting a possible enhanced IL-7 signalling in invasive breast cancer. Further studies suggest that activated Stats signalling could be involved in oncogenesis by stimulating cell proliferation and preventing apoptosis. Aberrant Stats signalling may contribute to malignant transformation by promoting cell cycle progression and / or cell survival. In contrast to normal signalling, in which Stat activation is quick and transient, constitutive signalling by Stats has been associated with malignant progression (Bowman *et al*, 2000). Further studies have demonstrated the activation of Jaks and Stats in several haematological malignancies including lymphomas, leukaemias (Gouilleux-Gruart *et al*, 1996; Migone *et al*, 1995; Weber-Nordt *et al*, 1996; Zhang *et al*, 1996) and myeloma (Catlett-Falcone *et al*, 1999) as well as solid tumours like human head and neck squamous cell carcinoma (Grandis *et al*, 1998), prostate cancer (Dhir *et al*, 2002), renal cancer (Dhir *et al*, 2002; Horiguchi *et al*, 2002), lung carcinoma (Fernandes *et al*, 1999), ovarian cancers (Huang *et al*, 2000), pancreatic cancer (Coppola, 2000) and melanomas (Pansky *et al*, 2000).

However, to-date there is no available data about the association of IL-7 (and its receptor or their downstream signalling intermediates expression) with tumour or patients clinicopathological characteristics in human breast cancer. In this chapter, the expression of IL-7, its receptor and their downstream signalling intermediates in relation to tumour histopathological grade, stage, nodal status and prognosis was studied in a cohort of patients with breast cancer.

9.2 MATERIALS AND METHODS

9.2.1 Materials

Breast tissue samples (n=163: tumour tissues n=122, normal tissues n=41) were obtained from patients (mean age = 62 years, range = 36-89) immediately after surgical resection. The samples were immediately snap frozen in liquid nitrogen and stored in a deep freezer until use. Clinicopathological parameters of the patients from whom these samples were taken are given in Table 9.1. The median follow up of these patients where the tissues obtained from was 72.2 months range 12-96).

Table 9.1 Clinicopathological parameters of patients from whom tissue samples were obtained.

	<i>Grade *</i> <i>(1-3)</i>	<i>TNM stage**</i> <i>(1-4)</i>	<i>NPI #</i> <i>(1-3)</i>	<i>Survival</i> <i>(5 years)</i>
Tumour tissues (n=122)	G1: 25	TNM1: 54	NPI1: 57	Disease free ^s : 55
	G2: 39	TNM2: 37	NPI2: 39	Died ^{ss} : 18
	G3: 58	TNM3: 18	NPI3: 26	Others ^{sss} : 49
		TNM4: 13		

- * Histological grade (Bloom Richardson classification): G1 = well differentiated, G2 = moderately differentiated and G3 = poorly differentiated / undifferentiated.
- ** TNM according to the Union Internationale Centre Le Cancer (UICC) staging system: TNM1 = Stage 0 and 1.

TNM2 = stages 2a and 2b.

TNM3 = stages 3a and 3b.

TNM4 = stage 4.

- # NPI: Nottingham Prognostic Index, $NPI = (0.2 \times \text{tumour diameter in cm}) + \text{lymph node stage} + \text{tumour grade}$ (Todd *et al*, 1987). NPI1 constitutes the excellent (≤ 2.4) and the good groups (2.41-3.4), NPI2 constitutes the moderate groups (3.41-4.4 and 4.41-5.4) and NPI3 constitutes the poor prognosis group (≥ 5.5).
- At 5 years follow up: § Disease free are patients who had cancer resection and remained disease free without local or distal metastasis. §§ Disease specific death (excluding deaths due to other causes). \$\$\$ Patients who were alive at 5 years, but with the disease (local recurrence and / or distant metastasis).

9.2.2 Quantitative analysis of gene expression

Real time quantitative PCR was carried out using the iCycler iQTM system (Bio Rad), which incorporates a gradient thermocycler and a 96-channel optical unit. AmplifluorTM detection system was used in the current study (Intergen, England, UK), which included the use of specific sense primer, a universal FAM-labeled probe and a specific anti-sense primer that incorporate a Z sequence that is complementary to the probe (Whitcombe *et al*, 1999a; Whitcombe *et al*, 1999b). The internal standards used in the study were specific plasmids generated using a

pCR2.1 cloning vector (Invitrogen, Paisley, Scotland, UK), as previously reported (Cunnick *et al*, 2001). Quantitative PCR was carried out in 96-well plate with 10pmol sense primer, 1pmol anti-sense-Z primer (Table 9.2), and 10pmol FAM-probe, using a customer hot-start Q-PCR master mix, with the following conditions: 95⁰C for 15 minutes, followed by 50 cycles at 95⁰C for 15 seconds, 55⁰C for 40 seconds and 72⁰C for 15 seconds. The copy number of each transcript was calculated from the internal standards and shown here as copies/50ng RNA. Statistical analysis was carried out using student *t* test.

Table 9.2 Primers sequences used in the quantitative RT-PCR

<i>Primer name</i>	<i>Primer sequence (5'-3')</i>
IL-7ZF	catcagatcaattattggaca
IL-7ZR	actgaacctgaccgtacagcacggaataaaaacatacc
IL-7R F	tctggagaaaagtgctatgc
IL-7R ZR	actgaacctgaccgtacaaaagcacaggtcagtgat
Jak-1 F	tacacagcagaggaactgtg
Jak-1 ZR	actgaacctgaccgtacataccagagcttggtgtctc
Jak-3 F	ctgtgtaccactccctctt
Jak-3 ZR	actgaacctgaccgtacaatcctgtacagcaggacttg
PI3-K ZF	actgaacctgaccgtacaactcaaaaattg
PI3-K R1	gcatttttctggaaattca
Stat-5 F1	tacaatgaacagaggctggt
Stat-5 ZR	actgaacctgaccgtacaatgtctggtgatctgaagg

9.3 RESULTS

9.3.1 Levels of transcripts of IL-7 and its signal transduction components

The expression of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 mRNA was analysed using real time quantitative RT-PCR in normal and tumour breast samples. The levels of transcripts were analysed using quantitative RT-PCR. The number of copies of IL-7 and PI3-K transcript was statistically larger in breast tumour samples compared to the normal tissues (Figure 9.1 and Table 9.3). Although Stat-5 levels of transcripts were higher in tumour tissues, this did not achieve statistical significance. Normal breast tissues expressed higher transcript copies of IL-7R, Jak-1 and Jak-3 compared to breast cancer tissues (Figure 9.1).

9.3.2 Expression of IL-7 and its signalling molecules in relation to tumour grade

Histopathological grading of breast cancer is defined in this study as: grade 1 (well differentiated), grade 2 (intermediate differentiation) and grade 3 (undifferentiated). The mean number of IL-7, IL-7R, Jak-3 and PI3-K transcripts was higher in grade 3 tumours compared to grades 2 and 1 (Figure 9.2 and Table 9.4), where as, for Jak-1 and Stat-5, grade 2 tumours had the highest mean number of copies.

9.3.3 Association of IL-7 and its signalling molecules expression with TNM staging

The levels of Jak-1 and PI3-K were significantly higher in TNM3 tumours compared with TNM1 tumours (Figure 9.3 and Table 9.5). Although the levels of IL-7, IL-7R and Stat-5 were generally higher in tumours with TNM3 and 4 compared to TNM1 and TNM2 stages, the difference was not statistically significant. However, there was no difference in the TNM classification regarding Jak-3 level of expression (Figure 9.3 and Table 9.5).

9.3.4 Expression of IL-7 and its signalling complex in relation to patients' prognosis

Nottingham Prognostic Index (NPI) was used as an indicator of prognosis here. IL-7 expression was significantly higher in the node positive group compared to the node negative patients ($P=0.03$) (Figure 9.4 and Table 9.6). Mean copy numbers of IL-7 were higher in patients who had the worst prognosis (NPI3) compared with those who are in the NPI1 group ($P=0.004$). IL-7R, Jak-3 and PI3-K levels of expression show a similar trend. However, both Jak-1 and Stat-5 levels of expression were higher in patients with NPI1 compared with those with NPI2 and NPI3.

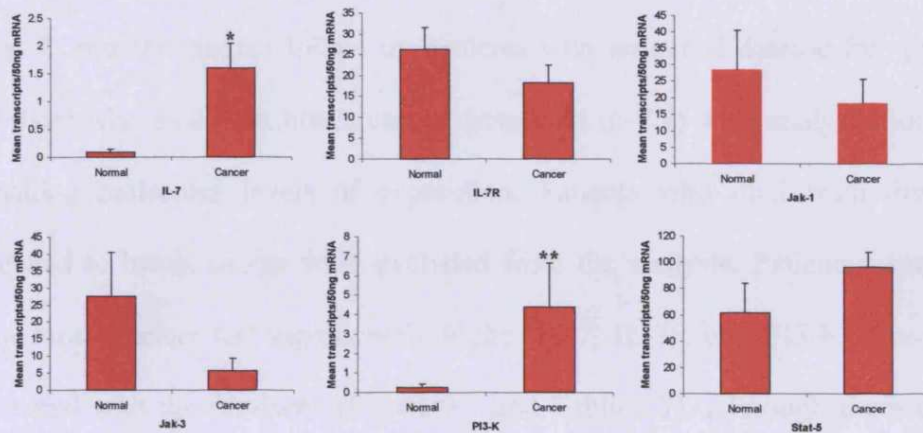


Figure 9.1 Quantitative RT-PCR analysis of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 in normal and breast cancer tissues. *p=0.023, **p=0.041 vs corresponding normal tissues.

Table 9.3 Means and standard deviation of the levels of expression of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 in normal and breast cancer tissues.

	<i>IL-7</i>	<i>IL-7R</i>	<i>Jak-1</i>	<i>Jak-3</i>	<i>PI3-K</i>	<i>Stat-5</i>
Normal	0.09±0.06	26.15±5.3	28.5±12.1	27.6±12.8	0.233±0.17	61.6±22.1
Cancer	1.6±0.45	18.26±4.3	18.4±7.2	5.7±3.6	4.37±2.3	96.4±11.1

9.3.5 IL-7, IL-7R and PI3-K and association with survival

After 72 months median follow up, patients who remained disease-free (n=55) and those who died from breast cancer metastasis (n=18) were analysed for IL-7 signalling molecules levels of expression. Patients who died from diseases unrelated to breast cancer were excluded from the analysis. Patients who died from breast cancer had significantly higher IL-7, IL-7R and PI3-K transcripts compared with the survivors (Figure 9.5 and Table 9.7). Although there was a similar trend in regards to Jak-1, Jak-3 and Stat-5, but the numbers were not statistically significant.

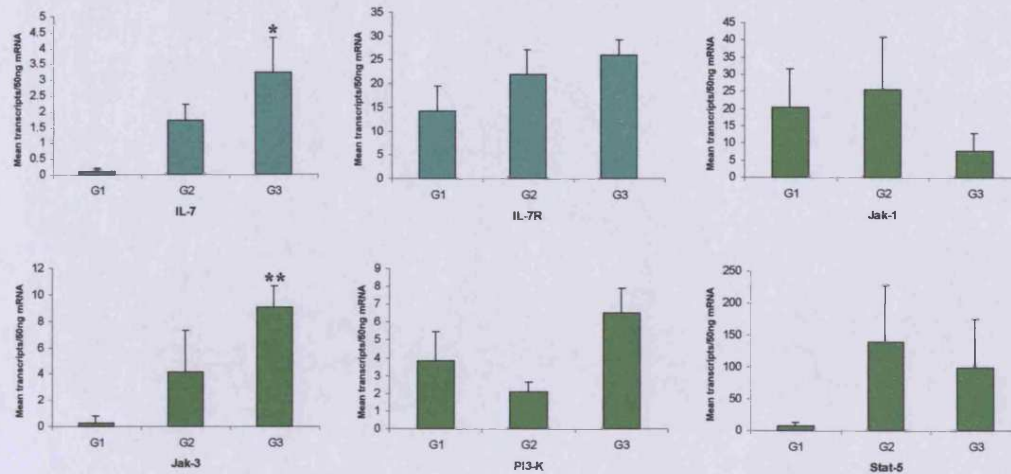


Figure 9.2 The levels of expression of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 in relation to tumour grade. The number of transcript copies/50 ng mRNA was higher in the highly undifferentiated tumours (grade 3) compared with those for grades 1 and 2 (except for Jak-1 and Stat-5). * $p=0.027$, ** $p=0.05$ vs grade 1.

Table 9.4 Means and standard deviations of the levels of expression of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 in relation to tumour grade.

	<i>IL-7</i>	<i>IL-7R</i>	<i>Jak-1</i>	<i>Jak-3</i>	<i>PI3-K</i>	<i>Stat-5</i>
G1	0.02±0.03	14.3±6.3	20.4±11.2	0.3±0.1	3.8±2.1	7.5±4.7
G2	0.2±0.5	21.9±9.4	25.5±15.3	4.2±1.9	2.1±1.3	138.6±88.5
G3	3.2±12.6	26±6.4	7.83±4	9±4.8	6.5±5.1	99.2±75.3

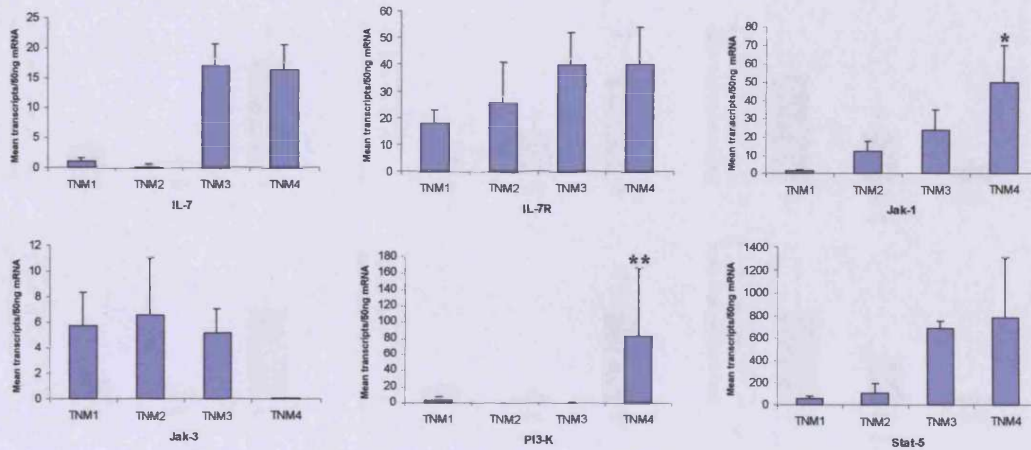


Figure 9.3 The levels of expression of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 in relation to tumour stage using the TNM classification. There was trend towards a higher expression in tumours of TNM3 and TNM4 stages. * $p=0.047$, ** $p=0.01$ vs TNM1 tumours.

Table 9.5 The levels of expression of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 in relation to tumour stage using the TNM classification.

	<i>IL-7</i>	<i>IL-7R</i>	<i>Jak-1</i>	<i>Jak-3</i>	<i>PI3-K</i>	<i>Stat-5</i>
TNM1	1±5.5	18.1±6.2	1.3±0.7	5.7±3.4	3.54±1.2	50.9±29.4
TNM2	0.21±0.4	25.92±8.4	12±5.4	6.6±4.5	0.17±0.07	98.6±87.6
TNM3	17.1±31.4	40.2±6.3	23.5±11	5.3±3.4	0.33±0.2	681±642
TNM4	0.06±0.04	40.2±13.7	49.3±20.1	0.06±0.02	82.7±82.7	772.2±531

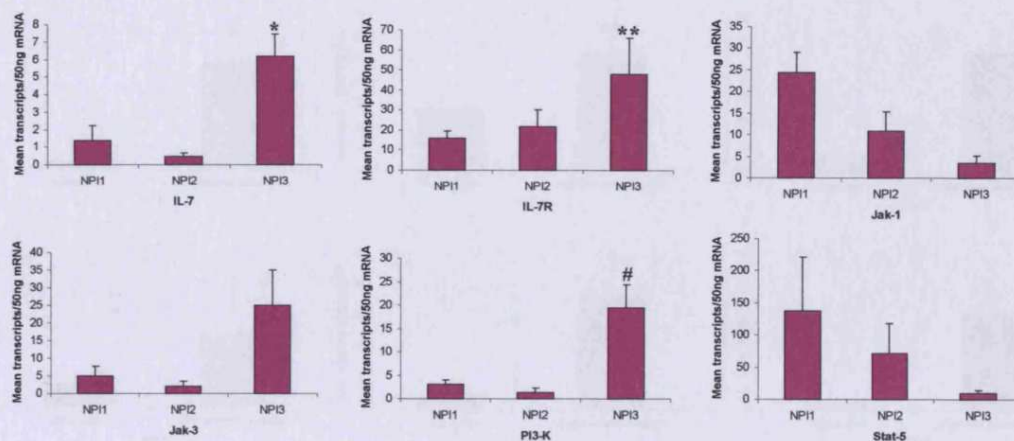


Figure 9.4 The levels of expression of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 in relation to the patient's prognosis using the Nottingham Prognostic Index (NPI). *p=0.033, **p=0.021, #p=0.003 vs NPI1.

Table 9.6 Means and standard deviations of the levels of expression of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 in relation to the patient's prognosis using the Nottingham Prognostic Index (NPI).

	<i>IL-7</i>	<i>IL-7R</i>	<i>Jak-1</i>	<i>Jak-3</i>	<i>PI3-K</i>	<i>Stat-5</i>
NPI1	1.363±0.88	16±3.31	24.3±4.8	5±2.77	2.94±1.11	137.6±84
NPI2	0.459±0.179	21.8±8.4	10.8±4.5	2.11±1.46	1.398±0.95	71.3±47
NPI3	6.16±1.26	48±18	3.52±1.58	25±10	19.4±5	9.67±3.8

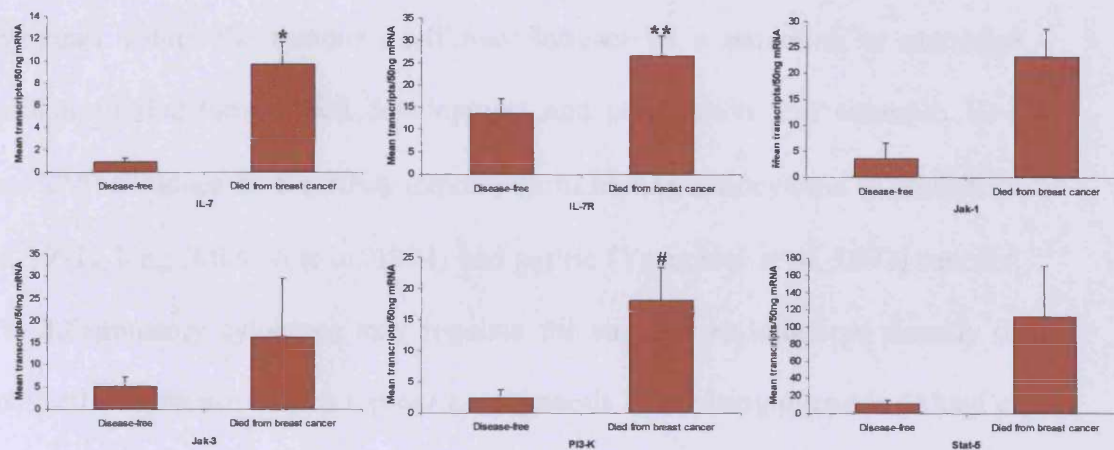


Figure 9.5 Quantitative RT-PCR analysis in relation to patients' survival. * $p=0.33$, ** $p=0.007$, # $p=0.015$ vs patients who remained disease free (patients who survived for 5 years, but with recurrence and / or metastasis are not shown here).

Table 9.7 Means and standard deviations of the levels of expression of IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5 in relation to patients' survival.

	<i>IL-7</i>	<i>IL-7R</i>	<i>Jak-1</i>	<i>Jak-3</i>	<i>PI3-K</i>	<i>Stat-5</i>
Dis. free	1.2±0.22	14.5±4.2	3.9±2.1	5.1±2.3	3.5±1.2	11.3±3.5
Died	9.7±2.1	26.9±6.1	23.7±6.1	16.7±13.2	18.1±7.3	115.2±47.3

9.4 DISCUSSION

This study has shown that aberrant expression of interleukin-7, its receptor and their signalling molecules in human breast cancer. It is well recognised that cytokines within the tumour itself may interact by a paracrine or autocrine fashion altering tumour cell development and progression. For example, IL-1 and TNF- α induce IL-8 mRNA expression in human astrocytoma (Kasahara *et al*, 1991), lung (Mizuno *et al*, 1994) and gastric (Yasumoto *et al*, 1992) cancers. Pro-inflammatory cytokines may regulate the vascular endothelium directly or indirectly and hence induce tumour angiogenesis / lymphangiogenesis (Akagi *et al*, 1999; Ristimaki *et al*, 1998). The data here suggest that the effects of IL-7 within breast cancer tissues are probably mediated via a paracrine pathway, as breast cancer cells express IL-7R, but not IL-7 itself (Chapter 6 Figure 6.1).

The observation that IL-7 expression is higher in grade 3 and TNM3 and TNM4 tumours is particularly interesting. Although IL-7 has been indicated as a haematopoietic growth factor and may regulate the cell-mediated immune system, previous studies have demonstrated that IL-7 failed to exert any significant immune enhancing effects in cancers including breast cancer (Cayeux *et al*, 1995; Talmadge *et al*, 1993). Additionally, it is known that patients with solid malignancies in general have indeed a profound suppression in their cellular immunity, especially in the tumour microenvironment (Oka *et al*, 1993; Wang *et al*, 1991). Furthermore, some cytokines could interact with each other within the tumour itself and suppress the activity of the infiltrating immune cells (Oka *et al*, 1995). Therefore, it is our view that over-expression of IL-7 and its

signalling intermediates in the highly undifferentiated and higher TNM tumours may not be desirable and may outweigh its presumed immune regulatory role in breast cancer. In fact, this over-expression may be detrimental to the patients, as we have shown that patients with poor prognosis (i.e. the group with NPI3) had significantly raised levels of IL-7, IL-7R, Jak-3 and PI3-K. The case is further strengthened by the data that patients who died from breast cancer also exhibited significantly higher levels of these molecules than those who remained disease free.

How IL-7 and its signalling complex are involved in the more aggressive tumours and poor clinical outcomes is presently unclear. However, the data shown in previous chapters indicate that IL-7 exerts specific growth promoting effects on endothelial and breast cancer cells. Furthermore, IL-7 increased the expression of VEGF-D in both endothelial and breast cancer cells and also shown to upregulate VEGFR-3 on the endothelial cells suggesting that increased levels of IL-7 and IL-7 receptor may be a trigger to the lymphangiogenesis in breast cancer. This point is supported by the data that node positive tumours (NPI2 and NPI3) exhibited higher levels of IL-7 and IL-7R, when compared with node negative tumours (NPI1). It has been recently shown that levels of VEGF-D expression are significantly higher in tumour compared to normal tissues in breast (Cunnick *et al*, 2003). Furthermore, the later study has also revealed that levels of VEGF-D expression were significantly higher in node positive compared to node negative disease (Cunnick *et al*, 2003).

The increased expression of VEGF-D in breast cancer and endothelial cells demonstrated in Chapter 5 might explain the higher expression of IL-7 in the more aggressive tumours and less favourable prognoses groups. In summary, this study has demonstrated for the first time that there is a quantitative association between IL-7 signalling complex and some clinicopathological parameters in breast cancer. There was a trend towards higher expression of these markers in Grade 3 and TNM3/4 tumours. IL-7 and its signalling molecules are highly expressed in the worst prognosis patients. This aberrant expression of IL-7 signalling complex might have a potential role in influencing development, growth and differentiation of breast cancer. Studies in previous chapters have demonstrated that IL-7 induces lymphangiogenesis in endothelial cells. This may explain the association of IL-7 over expression in the node positive tumours and in tumours belong to NPI3 patients. This may have diagnostic, prognostic and therapeutic importance.

CHAPTER TEN

LOCALIZATION OF IL-7 AND ITS DOWNSTREAM SIGNALLING MOLECULES IN BREAST CANCER TISSUES: AN IMMUNOHISTOCHEMICAL ANALYSIS

10.1 INTRODUCTION

IL-7 is considered to be an important modulator of the immune cell functions. However, dys-regulation of functions of some cytokines is well recognised in patients with malignant disease. These interactions are thought to regulate the proliferation and metastatic activity of malignant cells as well as modulate the host immune system towards the tumour cells (Hasday *et al*, 1990).

IL-7 mediates its actions via engagement to its specific receptor, IL-7R. After its activation by IL-7, IL-7R initiates a series of phosphorylation events mediated by signalling molecules including the Janus kinases (Jak-1 and Jak-3) and Stats (signal transducers and activators of transcription) mainly Stats-5 (Dadi *et al*, 1994b; Foxwell *et al*, 1995; Lin *et al*, 1995; Pernis *et al*, 1995). Stats are phosphorylated and rapidly dissociated from the receptor, form homo or hetero dimmers and rapidly translocate to the nucleus where they bind specific DNA and could regulate target gene transcription.

Jak-1 is physically associated with IL-7R α and Jak-3 with the γ_c chain (Noguchi *et al*, 1993). Jak-3 is expressed at high levels in natural killer cells, thymocytes, T and B cells and myeloid cells (Gurniak and Berg, 1996). It has been demonstrated that Jak-3 is expressed in breast cancer cell lines, primary breast cancer as well as colon and lung cancers (Cance and Liu, 1995a).

PI3-K is a critical signalling molecule that regulates several cellular processes including survival and proliferation in different systems. PI3-K has been proposed to mediate events such as mitogenesis, cell adhesion, motility and cellular differentiation as well as it provides protection against apoptosis (Toker and Cantley, 1997; Vanhaesebroeck *et al*, 1997). PI3-K activation of Akt / PKB and the subsequent phosphorylation of Bad could be the mechanism by which PI3-K signalling could inhibit apoptosis. PI3-K is also involved in the suppression of TNF induced apoptosis as demonstrated by kinase dead mutant PI3-K construct that results in an enhancement of apoptosis (Burow *et al*, 2000). In breast cancer, there is a frequent activation of PI3-K and ER α could bind its p85 α subunit and activate the PI3-K/AKT2 pathway in an oestrogen-independent manner (Sun *et al*, 2001).

Stats are latent cytosolic transcription factors that bind to the phosphorylated cytokine receptors via their SH2 domains (Greenlund *et al*, 1995). Stat-5 is encoded by two genes, Stat-5a and Stat-5b that share about 96% identity at the protein level (Liu *et al*, 1995). The activity pattern of Stat-5 in breast tissue suggests their active role in epithelial cell differentiation and milk protein gene expression (Liu *et al*, 1997). *Stat-5a* knockout mice develop impaired mammary gland development (Liu *et al*, 1995; Liu *et al*, 1997) and breast cancer tissues display significantly higher levels of *Stat-5a* in invasive breast cancers than those from benign and normal breast tissue (Watson and Miller, 1995), suggesting a possible enhanced IL-7 signalling in invasive breast cancer.

It has been revealed that high frequency of inappropriate activation of Stats in general occurs in a wide variety of human cancers. Recent studies suggest that activated Stats signalling could be involved in oncogenesis by stimulating cell proliferation and preventing apoptosis (Chapter 1). Aberrant Stats signalling may also contribute to malignant transformation by promoting cell cycle progression and / or cell survival. Furthermore, constitutive signalling by Stats has been increasingly associated with malignant progression (Bowman *et al*, 2000). Constitutive activation of Stats have been demonstrated in human head and neck squamous cell carcinomas (Grandis *et al*, 1998), Myeloma cells and bone marrow extracts (Catlett-Falcone *et al*, 1999), prostate (Dhir *et al*, 2002), renal (Dhir *et al*, 2002; Horiguchi *et al*, 2002), lung (Fernandes *et al*, 1999), ovarian (Huang *et al*, 2000), pancreatic (Coppola, 2000) cancers as well as melanomas (Pansky *et al*, 2000), but interestingly not in non-malignant mammary epithelial cell lines (Garcia *et al*, 1997; Sartor *et al*, 1997). Additionally, constitutive activation of Stats is also detected in metastatic lymph nodes suggesting a correlation with invasive tumours (Garcia and Jove, 1998).

To-date no studies have shown the expression of IL-7 or its downstream signalling molecules in breast cancer tissues. The level of expression of IL-7, IL-7 receptor (IL-7R) and their downstream signalling molecules have been analysed using real time quantitative RT-PCR in Chapter 9. However, the localisation of these molecules within the cells and tissues cannot be achieved by molecular quantification alone therefore the aim of this chapter is to analyse the

expression of these molecules immunohistochemically, using a cohort of breast cancer tissues

10.2 MATERIALS AND METHODS

10.2.1 Materials

Breast tissue samples were obtained from 16 breast cancer patients immediately after surgery. The mean age of patients was 61 years (range 38-89). Tumour tissues (n=16) and matched background breast tissues (n=16) were snap frozen and stored at -80°C . The normal tissues (background) were taken from sites away from the tumour site. Appropriate ethical approval was obtained from the local ethics committee. Tumour grade, TNM stage and patients prognoses (NPI) are defined as in Chapter 9 and are listed in table 10.1.

Table 10.1 Pathological characteristics of breast cancer tissues.

	<i>Grade (1-3)</i>	<i>TNM (1-4)</i>	<i>NPI (1-3)</i>
Tumour tissues (n=16)	G1: 5	TNM1: 4	NPI1: 4
	G2: 5	TNM2: 3	NPI2: 4
	G3: 6	TNM3: 5	NPI3: 8
		TNM4: 4	

10.2.2 Immunohistochemistry

Immunohistochemical staining was performed using paired frozen-sectioned tissues (cancer tissues paired with their normal tissues from same patients). Frozen sections were cut and fixed in 50% methanol and 50% acetone for 15 minutes. The sections were then dried on air for 10 minutes and stored at -20°C in foil-rapped slide trays. Specimens were then placed in PBS (Optimax wash buffer) for 5 minutes. The slides were incubated with primary rabbit polyclonal antibodies against IL-7R, Jak-1, Jak-3, PI3-K and stat-5 (Santa Cruz Biotechnology) at 1:50 dilution for 1 hour. After 4 washes with PBS, the slides were placed in universal multi-link biotinylated anti-rabbit secondary antibodies (Dako) at a concentration of 1:100 and the slides were incubated for 30 minutes. This was followed by 4 washes with PBS. Slides were then placed in avidin biotin complex (ABC – Vector Labs) for 30 minutes. The bound antibody was detected with diaminobenzidine tetrahydrochloride (3,3'-diaminobenzidine) - DAB (Sigma). DAB was added for 5 minutes. The slides were washed with H_2O for 5 minutes and placed in Mayer's haematoxyline for 1 minute. Further wash with H_2O for 10 minutes (nuclei become blue). This was followed by dehydration in methanol (3 times) and clearing in 2 changes xylene before mounting under a cover slip and read the slides. Negative controls (using PBS buffer instead of the primary antibody or irrelevant antibody) were used in this study. However, positive controls were not used here as obtaining tissues from organs like thymus gland was extremely difficult.

10.2.3 Quantification of IL-7 expression in breast cancer tissues

Quantification of IL-7 expression and immunohistochemical grading were performed as described previously (Paleri *et al*, 2001). The staining was assessed on random pattern fields and a four-point scale was employed for grading. Semi-quantitative scoring was achieved and grading was scored as 0 = negative, +1 = weakly positive, +2 = positive, +3 = strongly positive. Scoring was performed by two independent observers and any disagreement was resolved by discussion and re-evaluation of the slides. Chi square test was used to assess statistical significance and to detect the presence of correlation between IL-7 expression and various patient and tumour factors.

10.2 RESULTS

10.3.1 The distribution of the IL-7 signalling molecules in breast cancer and normal breast tissues

All molecules (IL-7, IL-7R, Jak-1, Jak-3, PI3-K and Stat-5) were identified in both normal and cancer tissues at different levels. The distribution of these molecules within the cells and tissues was also different. While IL-7 was mainly localised in stromal and infiltrative cells within the stroma, IL-7R molecule was localised on plasma membranes of most cells including malignant and endothelial cells (Figure 10.1). Jak-1, Jak-3 and PI3-K molecules were mainly found in the cytoplasm of most cells including cancer cells. Stat-5 protein was localised in the malignant cells in both the cytoplasm and nucleus. Semi-quantitative analysis of the 16 paired tissue samples revealed that cancer tissues

stained mainly strongly positive to IL-7, PI3-K and Stat-5 compared to normal tissues whereas IL-7R, Jak-1 and Jak-3 were generally stained either negative or weakly positive in cancer tissues (Figure 10.2 and Table 10.2).

10.3.2 Correlation of IL-7 and its signalling molecules to tumour grade

The levels of IL-7, IL-7R, PI3-K and Stat-5 expression was higher in the poorly differentiated cancer tissues (G3 tumours) compared to G1 and G2 tumours (Figure 10.3). The difference was statistically significant in relation to IL-7, IL-7R, PI3-K and Stat-5 between G3 and G1 tissues, both tumours and their matched background tissues ($p < 0.001$) (Table 10.4). Of the 12 G3 samples (normal background = 6 and tumour tissues = 6), the score was +3 in 10 samples for IL-7 and IL-7R, 9 samples for both PI3-K and Stat-5 and 8 for Jak-3. The rest of the 12, were either scored +1 or +2. However, G1 tumours were scored either negative (0) or weakly positive (+1) in relation to the aforementioned molecules (Table 10.3). There was no significant difference in the pattern of Jak-1 and Jak-3 levels of expression in relation to tumour grade.

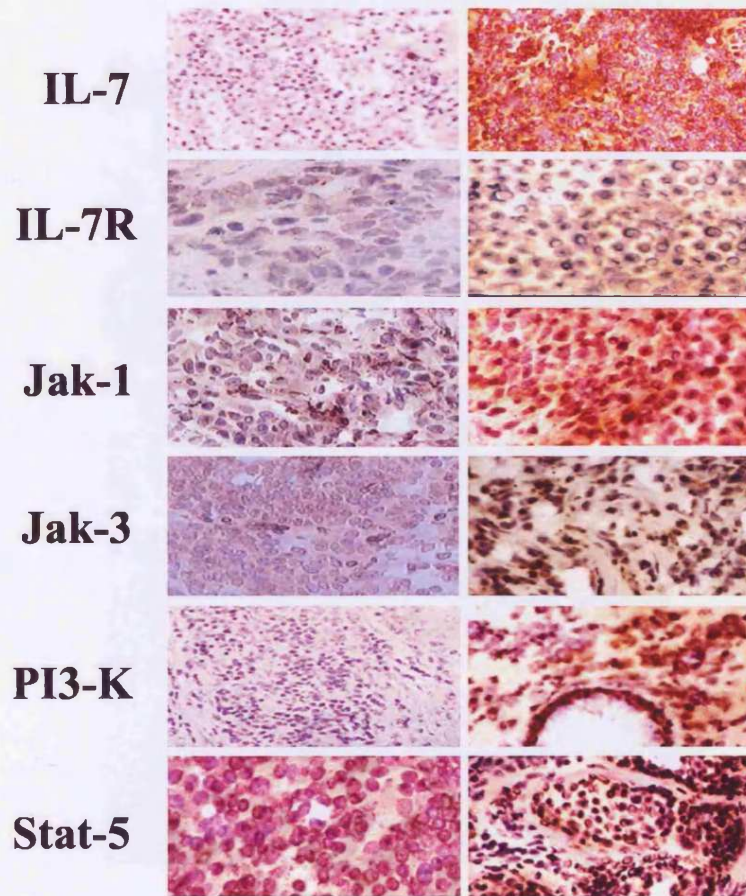


Figure 10.1 Immunohistochemistry of breast cancer tissues. The cellular distribution of IL-7 and its signalling molecules is different. IL-7 is mainly identified in the stroma surrounding the malignant cells within other epithelial or stromal cells like fibroblasts and other inflammatory cells. While, IL-7R is localised as a transmembrane molecule, the other molecules (Jak-1, Jak-3, PI3-K and Stat-5) have been mainly localised as cytoplasmic and nuclear molecules. The left panel: represents the negative controls of these molecules (magnification x200).

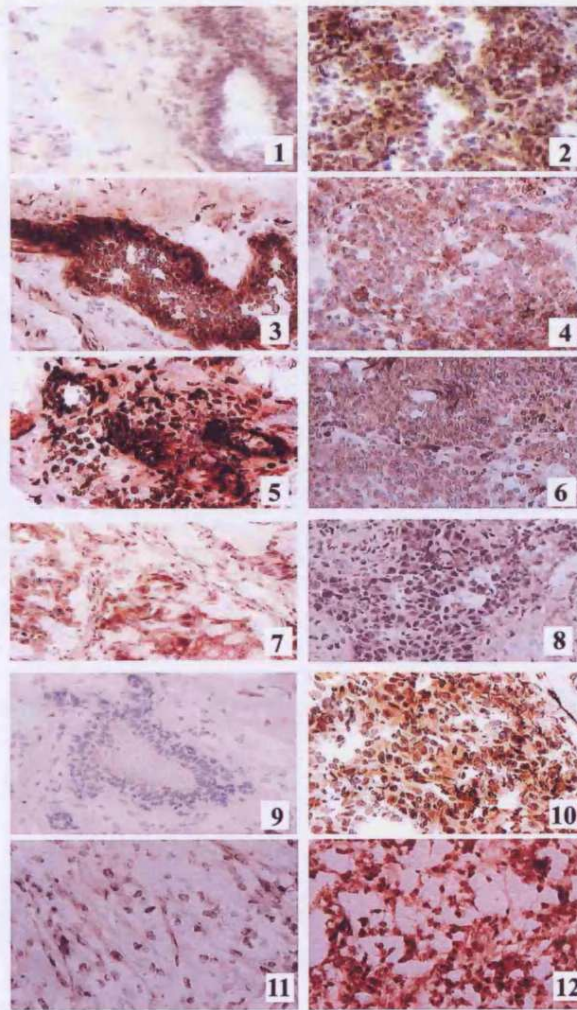


Figure 10.2 Immunohistochemical staining of a typical pair of normal (left panel) and cancerous (right panel) tissues. Semi-quantitative analysis revealed that the cancerous tissues stained strongly positive for IL-7, PI3-K and Stat-5 (2, 10 and 12) compared with the normal tissues (1, 9 and 11). However, the normal tissues stained strongly positive for IL-7R, Jak-1 and Jak-3 (3, 5 and 7) compared with the cancerous tissues (4, 6 and 8) (magnification x200).

Table 10.2 Immunohistochemistry staining scores for IL-7, IL-7R and Jak-1 (A) and Jak-3, PI3-K and Stat-5 (B) in normal (background) and cancer tissues.

A

<i>IHC score</i>	<i>IL-7</i>				<i>IL-7R</i>				<i>Jak-1</i>			
	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>
Background	8	5	3	0	1	1	5	9	2	2	6	6
Cancer	0	1	5	10	7	4	1	4	8	5	2	1

B

<i>IHC score</i>	<i>Jak-3</i>				<i>PI3-K</i>				<i>Stat-5</i>			
	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>
Background	1	1	6	8	12	1	2	1	9	6	2	0
Cancer	9	7	0	0	0	0	5	11	0	0	4	12

10.3.3 Detection of the IL-7 signalling molecules in relation to TNM classification

As with tumour grade, both tumour tissues and their normal matched background tissues exhibited same pattern of expression of IL-7 and its signalling molecules. TNM3 and TNM4 samples were stained strongly positive for the IL-7 and IL-7R compared to TNM1 samples (Figure 10.4). There was statistically significant difference between TNM4 and TNM1 immunoreactivity as the former scored +3 for IL-7, IL-7R, Jak-3, PI3-K and Stat-5 in 7 out of the 8 (background = 4 and tumour = 4) TNM4 samples compared to TNM1 samples which were mainly displayed either negative (0) or weakly positive (+1) for these molecules ($p < 0.001$) (Table 10.4). However, there was no difference in staining of Jak-1 in these tissues.

10.3.4 Immunostaining of the IL-7 signalling molecules in relation to patients' prognosis

IL-7, IL-7R, Jak-3 and PI3-K stained strongly positive in tumours tissues taken from patients with the poorer prognosis (NPI3) using the Nottingham Prognostic Index (Figure 10.5). Of the 16 NPI3 samples, 15 scored +3 for IL-7, 13 scored +3 for IL-7R and 11 scored +3 for both Jak-3 and PI3-K. The difference in immunoreactivity between NPI3 and NPI1 (for both background and tumour tissues) was statistically different ($p < 0.001$) for the aforementioned molecules. However, There was no difference in Jak-1 and Stat-5 immunostaining in relation to the NPI prognostic index (Table 10.5).

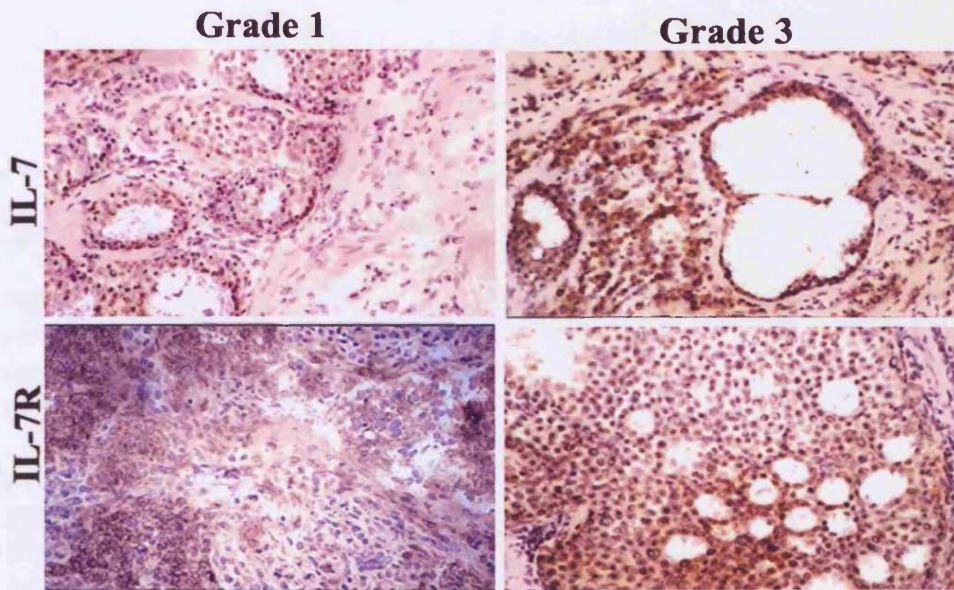


Figure 10.3 IL-7 / IL-7R immunostaining in breast cancer. Grade 3 tumours (right panel) displayed strong immunoreactivity for IL-7 (in the stroma) and IL-7R (as a cytoplasmic molecule), whereas grade 1 tumours (left panel) produced only a very weak immunostaining for these molecules (other IL-7 signalling molecules are not shown here) (magnification x200).

Table 10.3 Immunohistochemistry staining scores for IL-7, IL-7R and Jak-1 (A) and Jak-3, PI3-K and Stat-5 (B) in relation to tumour grade in background (normal) and cancer tissues.

A

<i>IHC score</i>	<i>IL-7</i>				<i>IL-7R</i>				<i>Jak-1</i>			
	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>
<i>Background</i>												
<i>G1 (n=5)</i>	1	4	0	0	5	0	0	0	0	3	2	0
<i>G2 (n=5)</i>	0	1	0	4	0	0	3	2	0	1	0	4
<i>G3 (n=6)</i>	0	1	0	5	0	1	0	5	1	2	1	1
<i>Cancer</i>												
<i>G1 (n=5)</i>	4	0	1	0	3	2	0	0	0	3	2	0
<i>G2 (n=5)</i>	0	2	0	3	0	0	1	4	0	0	0	5
<i>G3 (n=6)</i>	0	0	1	5	0	1	0	5	1	3	1	0

B

<i>IHC score</i>	<i>Jak-3</i>				<i>PI3-K</i>				<i>Stat-5</i>			
	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>
<i>Background</i>												
<i>G1 (n=5)</i>	2	2	1	0	2	3	0	0	5	0	0	0
<i>G2 (n=5)</i>	3	1	1	0	0	2	0	3	0	0	0	5
<i>G3 (n=6)</i>	0	3	2	1	0	0	2	4	0	1	0	5
<i>Cancer</i>												
<i>G1 (n=5)</i>	2	1	1	1	2	3	0	0	5	0	0	0
<i>G2 (n=5)</i>	3	0	0	2	0	2	0	3	0	0	1	4
<i>G3 (n=6)</i>	1	2	1	2	0	0	1	5	0	0	2	4

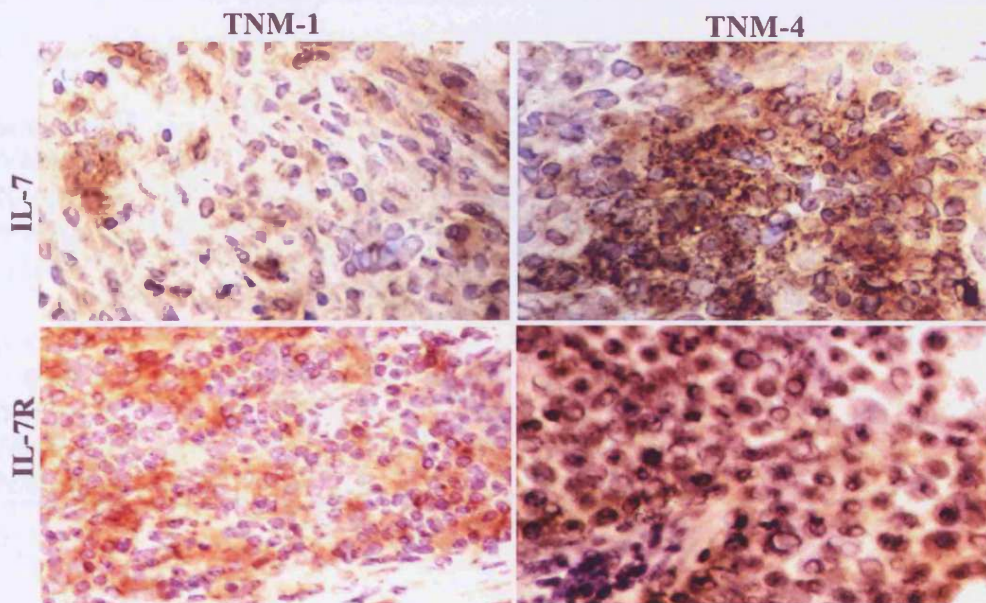


Figure 10.4 IL-7 / IL-7R immunostaining showing breast cancer tissues. TNM-4 tumours (right panel) displayed strong immunoreactivity for IL-7 and IL-7R, whereas TNM-1 tumours (left panel) produced only a very weak immunostaining for these molecules (other IL-7 signalling molecules are not shown here) (magnification x200).

Table 10.4 Immunohistochemistry staining scores for IL-7, IL-7R and Jak-1 (A) and Jak-3, PI3-K and Stat-5 (B) in relation to TNM stage in background (normal) and cancer tissues.

A

<i>IHC score</i>	<i>IL-7</i>				<i>IL-7R</i>				<i>Jak-1</i>			
	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>
Background												
<i>TNM1(n=4)</i>	2	1	0	1	1	3	0	0	2	2	0	0
<i>TNM2(n=3)</i>	1	1	0	0	0	2	0	0	0	2	0	0
<i>TNM3(n=5)</i>	0	0	0	2	0	0	1	1	0	1	3	1
<i>TNM4(n=4)</i>	0	0	1	3	0	0	0	4	1	0	2	2
Cancer												
<i>TNM1(n=4)</i>	0	3	0	1	2	2	0	0	1	1	2	0
<i>TNM2(n=3)</i>	1	1	0	0	0	2	0	0	0	0	2	0
<i>TNM3(n=5)</i>	0	0	0	2	0	0	0	2	0	2	0	0
<i>TNM4(n=4)</i>	0	0	0	4	0	1	0	3	0	1	1	0

B

<i>IHC score</i>	<i>Jak-3</i>				<i>PI3-K</i>				<i>Stat-5</i>			
	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>
Background												
<i>TNM1(n=4)</i>	2	2	0	0	3	1	0	0	0	2	2	0
<i>TNM2(n=3)</i>	0	1	0	1	0	1	0	1	0	2	0	0
<i>TNM3(n=5)</i>	0	0	1	4	0	0	1	4	0	1	0	1
<i>TNM4(n=4)</i>	0	0	0	4	0	0	0	4	0	0	1	3
Cancer												
<i>TNM1(n=4)</i>	1	1	2	0	2	2	0	0	3	1	0	0
<i>TNM2(n=3)</i>	0	0	2	1	0	1	1	0	0	1	1	0
<i>TNM3(n=5)</i>	0	1	1	3	0	0	0	5	0	0	0	2
<i>TNM4(n=4)</i>	0	0	1	3	0	0	1	3	0	0	0	4

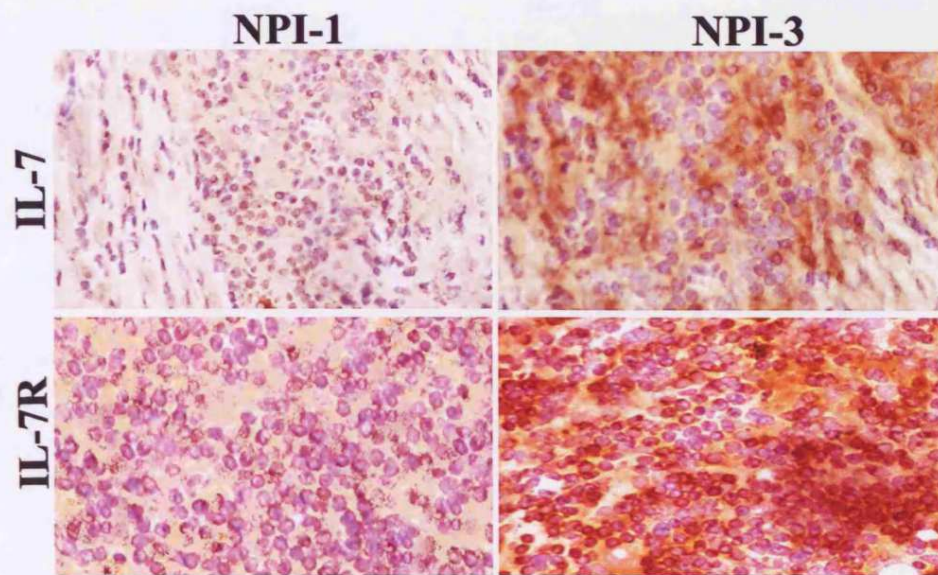


Figure 10.5 IL-7 / IL-7R immunostaining in breast cancer. Tumour tissues belong to patients who had poor prognosis as assessed by the Nottingham Prognostic Index (NPI-3, right panel) Shows strong immunoreactivity for both IL-7 and IL-7R. This is in contrast to tumours belong to NPI-1 patients that display a negative or weak immunostaining for these molecules (other IL-7 signalling molecules are not shown here) (magnification x200).

Table 10.5 Immunohistochemistry staining scores for IL-7, IL-7R and Jak-1 (A) and Jak-3, PI3-K and Stat-5 (B) in relation to tumour patients prognosis (NPI=Nottingham Prognostic Index).

A

<i>IHC score</i>	<i>IL-7</i>				<i>IL-7R</i>				<i>Jak-1</i>			
	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>
Background												
<i>NPI (n=4)</i>	2	2	0	0	3	1	0	0	1	3	0	0
<i>NPI2(n=4)</i>	0	1	1	2	0	0	2	2	1	1	0	2
<i>NPI3(n=8)</i>	0	0	1	7	0	1	0	7	4	0	1	3
Cancer												
<i>NPI (n=4)</i>	4	0	0	0	4	0	0	0	1	2	1	0
<i>NPI2(n=4)</i>	0	1	0	3	0	0	1	3	0	1	2	1
<i>NPI3(n=8)</i>	0	0	0	8	0	1	1	6	2	2	1	3

B

<i>IHC score</i>	<i>Jak-3</i>				<i>PI3-K</i>				<i>Stat-5</i>			
	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>	<i>0</i>	<i>+1</i>	<i>+2</i>	<i>+3</i>
Background												
<i>NPI (n=4)</i>	3	0	1	0	2	1	1	0	1	2	1	0
<i>NPI2(n=4)</i>	0	1	1	2	1	0	0	3	1	1	0	2
<i>NPI3(n=8)</i>	0	0	3	5	0	1	0	6	1	3	2	2
Cancer												
<i>NPI (n=4)</i>	2	1	1	0	3	1	0	0	1	2	1	0
<i>NPI2(n=4)</i>	0	1	0	3	0	0	2	2	0	3	0	0
<i>NPI3(n=8)</i>	0	1	1	6	0	2	1	5	2	4	1	1

10.4 DISCUSSION

IL-7 has been localised in both normal and breast cancer tissues immunohistochemically here. The data presented in this chapter shows that IL-7 is localised to stromal cells rather than to malignant cells and this is consistent with the RT-PCR presented in Chapter 5. Although the size of the sample used for the immunohistochemical study was small, some general patterns seem to emerge. Breast cancer tissues displayed strong positive (score 3) immunoreactivity to IL-7, PI3-K and Stat-5, whereas IL-7R, Jak-1 and Jak-3 were mainly detected in the matched background tissues. However, the least differentiated tumours (G3) stained strongly positive to IL-7 and all its downstream signalling molecules except Jak-3. This might indicate that these molecules play a role in breast cancer cells differentiation. Furthermore, it seems that the detection of IL-7 in both normal and breast cancer tissues is also correlated to the TNM stage. Tumours obtained from TNM3 and TNM4 patients stained strongly positive for IL-7, IL-7R and PI3-K compared to cancer tissues of TNM1 and TNM2 tumours (Figure 10.4). However, there was no difference in staining of Jak-1 and Jak-3. Normal background tissues (belong to the same patients with TNM3 and TNM4 stages) have also displayed strong immunoreactivity to IL-7, IL-7R, PI3-K and Stat-5 compared to TNM1 and TNM2 tumours (Table 10.5). IL-7, IL-7R, Jak-3 and PI3-K stained strongly positive in tumour tissues taken from patients with the poorer prognosis using the Nottingham Prognostic Index (NPI) (Figure 10.5). Jak-1 and Stat-5 stained strongly positive in tissues taken from NPI2 patients (moderate prognosis).

However, all molecules except Jak-1 stained strongly positive in normal background tissues that belong to patients with NPI3.

The detection of IL-7 signalling intermediates in breast cancer tissues has diagnostic and prognostic significance in assessing tumour aggressiveness, nodal status or predicting patients' outcome. The identification of these molecules in both normal and cancer tissues and the distribution pattern of these molecules seem to be un-related to the local immunity against the cancer. Furthermore, cancer tissues taken from patients with the poorer prognosis displayed a strong immunoreactivity for most of these molecules suggesting that even if levels of these molecules were related to the immunity they could still constitute a marker of assessing tumour aggressiveness and patients' prognosis. Further studies can be directed to engineer cancer cells that excessively express IL-7 and IL-7R and test their aggressiveness in animal models as well as investigating local tumour reactivity.

The development of more objective criteria to analyse immunohistochemical results will help to measure these molecules in more reliable and quantitative fashion. However, the specimens in this study was analysed by two independent observers and any disagreement was resolved by discussions and re-evaluating the slides until agreement reached. Furthermore, the recent invention of intra-operative histochemical imprint technology will further add to the value of detecting these molecules as they might give an intra-operative hint of tumour

differentiation, nodal involvement and predict prognosis (Motomura *et al*, 2000; Ratanawichitrasin *et al*, 1999; Rubio *et al*, 1998).

CHAPTER ELEVEN

GENERAL DISCUSSION

11.1 IL-7 is a lymphangiogenic factor

Metastatic spread is a common denominator of almost all malignant neoplasms (Hanahan and Weinberg, 2000). It is well established that the initial metastatic route for carcinomas is the lymphatic system to the regional lymph nodes (Pepper, 2001; Weaver, 2001). Lymph node metastasis is considered to be a major determinant of prognosis in almost all solid cancers. Therefore studying lymphangiogenesis is crucial and might help to establish new strategies in the war against cancer. Despite the huge data regarding angiogenesis (formation of new blood vessels) and its regulation in cancer, research in lymphangiogenesis has been so far very limited. The recent detection of specific biological markers for the lymphatic endothelium has made studying lymphangiogenesis feasible. This thesis investigated this important processes particularly its regulation by cytokines.

Regulation of lymphangiogenesis by cytokines is an attractive research area as they are known to be involved in many intercellular autocrine and / or paracrine mechanisms (Adams *et al*, 1991; Duncan *et al*, 1994; Freiss *et al*, 1993; Hasday *et al*, 1990; Ryan *et al*, 1993; Speirs *et al*, 1995; Vanroozendaal *et al*, 1992). It has been found recently for example that IL-8 could mediate angiogenesis *in vivo* (Koch *et al*, 1992) in breast cancer. Here, the effects of a range of 13 different cytokines were tested on the expression on the so far known specific lymphatic markers. Amongst all the cytokines, IL-7 was found to specifically increase the expression of these markers in human endothelial cells (HECV). IL-7 did not have an effect on the expression of the angiogenic factors in these cells suggesting that IL-7 might have lymphangiogenic properties. IL-7 exerted its maximal effects within 1 – 4 hours incubation as shown by both qualitative and

quantitative RT-PCR. Therefore, it was concluded from this initial study that IL-7 might have an impact on the process of lymphangiogenesis and further investigations were carried out using this cytokine.

11.2 The IL-7 induced lymphangiogenesis is VEGF-D dependent

In the last 2-3 years, there have been several studies that clearly demonstrated the importance of tumour lymphangiogenesis. However, most of these studies used experimental xenotransplanted tumour models and they are largely dependent on the activation of VEGFR-3, a tyrosine kinase receptor, by one of its only known ligands, VEGF-C and VEGF-D (Karpanen *et al*, 2001; Mandriota *et al*, 2001; Skobe *et al*, 2001a; Skobe *et al*, 2001b; Stacker *et al*, 2001). However, there have been no studies available in connection to the regulation of lymphangiogenesis. Here, the effects of IL-7 on the expression of VEGFs (VEGF-A, VEGF-B, VEGF-C and VEGF-D) and their receptors (VEGFR-1, VEGFR-2 and VEGFR-3) in endothelial cells were investigated. While, IL-7 did not affect the expression of the angiogenic factors; VEGF-A, VEGF-B and their receptors, VEGFR-1 and VEGFR-2 respectively in endothelial cells, it was found to significantly increase the expression of VEGF-D. Moreover, this effect of IL-7 was seen within 1-4 hours that is consistent with its effects on the expression of lymphatic markers (LYVE-1, prox-1 and podoplanin) in endothelial cells. Furthermore, the expression of VEGFR-3 was also increased by IL-7 with the best expression seen between 1-4 hours. VEGF-D is 48% identical to VEGF-C (Achen *et al*, 1998b; Yamada *et al*, 1997b). Although at

initial experiments, IL-7 was found to increase the expression of VEGF-C in endothelial cells as was shown by the RT-PCR, this was not consistent with repeating the experiment and therefore it was concluded that IL-7 does not have a significant effect on VEGF-C. Additionally, IL-7 did not have any effect on the expression of VEGF-A, VEGF-B, VEGFR-1 or VEGFR-2 in endothelial cells, indicating the lymphangiogenic potential of this cytokine.

The intercellular interactions between many cytokines are considered to be one of the deciding factors determining the fate of tumour and or endothelial cells function. Proliferative responses induced by the MAPK and PI3-K pathways can be activated by both IL-7 and VEGFs signalling (Chapter 1). Cross talks between pathways of different cytokines do exist, for example IL-7 induces tyrosine phosphorylation and activation of PI3-K, which is implicated in the VEGFs-induced endothelial cell survival via activation of its downstream target serine kinase Akt/PKB (Gerber *et al*, 1998; Xia *et al*, 1996). Furthermore, cross talks do exist between the MAPK and the PI3-K pathways as phosphorylation of Raf by Akt resulted in inhibition of the Raf-MEK (MAP kinase) – ERK pathway (Zimmermann and Moelling, 1999). VEGFR-3 can also strongly activate Stat-5 (Korpelainen *et al*, 1999); also activated and phosphorylated by IL-7 (Foxwell *et al*, 1995) suggesting that Stat-5 activation is involved in the regulation of lymphatic endothelium.

The specificity of IL-7 towards the lymphatic markers was determined by the use of neutralising antibodies. The inclusion of neutralising anti-VEGF-D antibody to the IL-7 treated HECV cells has reversed the increased lymphatic expression

in these cells suggesting that the effects of IL-7 on the expression of LYVE-1, podoplanin and prox-1 are VEGF-D dependent. Neutralising antibodies against IL-7 and VEGF-D had no effect on the expression of the angiogenic marker, PECAM as well as on VEGF-A, VEGF-B, VEGFR-1 and VEGFR-2.

IL-7 activated its receptor, IL-7R and its downstream signalling intermediates in endothelial cells as revealed by the immunoprecipitation study. As IL-7 itself was not expressed on endothelial cells, it was therefore proposed that in the body IL-7 exerts its effects probably via a paracrine mechanism. Within the tumour microenvironment, among other types of cells, haematopoietic, inflammatory, immune, endothelial and malignant cells interact with each other via a complex network of extracellular signals, such as cytokines. Therefore, IL-7 produced by other cells of stromal origin might stimulate endothelial and / or malignant cells via a paracrine mechanism.

The lymphangiogenic process starts by budding and sprouting from endothelial cells that involve endothelial cell growth, proliferation and migration. The detection of a fully functional IL-7R in endothelial cells has led to further exploration of the effects of IL-7 on endothelial cells growth. IL-7 has significantly accelerated the growth of HECV cells as shown by four different assays including the MTT, Hoescht 33258, crystal violet and PicoGreen[®] assays. IL-7 functions primarily as a growth and anti-apoptotic factor for haematopoietic cells (Rich *et al*, 1993; Silva *et al*, 1994). Cell maturation and differentiation induced by IL-7 are probably due to its trophic action by inhibiting apoptosis. IL-7 maintains the viability of cells by repressing a 'death-inducing' factor and /

or activating a 'life-promoting' factor (Hofmeister *et al*, 1999). Furthermore, IL-7 could prevent cell death by inhibiting some pro-apoptotic proteins like Bid, Bad or Bax (Hofmeister *et al*, 1999).

11.3 The effects of IL-7 are mediated via a Wortmannin sensitive pathway

To dissect downstream IL-7 signalling, inhibitors to its main signalling pathways (the Jak-Stat and the PI3-K pathways) were used. AG490 has been known to inhibit the autokinase activity of both Jaks and Stats, although originally it is a potent inhibitor of epidermal growth factor receptor kinase autophosphorylation. Jak-3 Inhibitor 1 is a known potent inhibitor of Jak-3 and has no effects on Jak-1, Jak-2, Zap/Syk or Src kinases. Wortmannin, a fungal metabolite that acts as a potent, selective, cell-permeable and irreversible inhibitor of PI3-K in purified preparations and cytosolic fractions. In this study, Wortmannin was found to significantly abolish the IL-7 induced growth of endothelial cells. Furthermore, Wortmannin has also significantly decreased the IL-7 induced expression of both LYVE-1 and VEGF-D in these cells. Therefore, it was concluded here that IL-7 exerts its growth stimulatory and lymphangiogenic effects mainly via the PI3-K pathway in endothelial cells.

11.4 IL-7 and endothelial cells functions

The other element of lymphangiogenesis involves endothelial cell migration. The *in vitro* migration assay was proved to be a very useful method in assessing cell migration. Treatment of HECV cells with IL-7 (20 ng/ml) has significantly

increased their migration distance. The inclusion of neutralising anti-IL-7 antibodies has reversed the IL-7 induced endothelial cell migration almost to the control level, indicating the specificity of the test. Furthermore when these cells were treated with VEGF-D, a similar effect to that of IL-7 was seen. Interestingly, using anti-VEGF-D antibodies had completely abolished the effects of IL-7 on endothelial cells migration, suggesting that this effect of IL-7 is probably mediated by a VEGF-D dependent mechanism.

IL-7 has also significantly enhanced the ability of HECV cells to generate microtubules both *in vitro* and *in vivo*. In the *in vitro* assay IL-7 significantly increased both number and mean tubular length produced by these cells. This effect matched exactly that produced by VEGF-D. As in the migration assay, using neutralising anti-IL-7 and anti-VEGF-D antibodies has reversed this effect of IL-7, confirming that this effect of IL-7 is first specific and second VEGF-D dependent. Furthermore, specificity was further proved by the inclusion of a non-specific irrelevant antibody. Using ribozyme transgenes technology, IL-7R was successfully knocked out from HECV cells. Two ribozymes were initially used, one only proved to be able to knock out the receptor (IL-7R) from these cells. This was confirmed at both gene and protein levels. IL-7R null HECV had blunt response to IL-7, but not towards VEGF-D, in relation to tubule formation *in vitro*, indicating the specificity of IL-7. In this regards, the use of ribozymes proved to be a successful means of confirming specificity.

As research in lymphangiogenesis has been very limited, *in vivo* models have been exclusively used in studying angiogenesis rather than lymphangiogenesis

(Hu *et al*, 1993; Strieter *et al*, 1992). The lack of a reliable lymphatic marker that is suitable for paraffin sections has been the main obstacle to initiate proper *in vivo* Lymphangiogenesis models. Recently, antibodies against VEGFR-3 (Clarijs *et al*, 2001; Kitadai *et al*, 2001a) and LYVE-1 (Carreira *et al*, 2001 and Chapter 5) that work on paraffin embedded tissue sections were used. However, so far, most studies involved VEGFR-3 as a lymphatic marker (Clarijs *et al*, 2001; Kitadai *et al*, 2001a; Partanen and Paavonen, 2001; Sleeman *et al*, 2001). Although VEGFR-3 is highly specific marker for normal adult lymphatic vessels, its upregulation in angiogenesis in some tumours and other pathological processes like granulation tissue has made the specificity of this marker unclear. Here, using anti-LYVE-1 monoclonal antibodies proved to be very successful in differentiating between lymphatic and angiogenic characteristics of endothelial cells. In the nude mice model, IL-7 significantly increased the formation of microvessels in Matrigel pockets injected into these mice. Immunostaining of the IL-7 induced tubules with anti-mouse anti-LYVE-1 antibody displayed a strong reactivity compared to staining with anti-von Willebrand (Factor VIII) antibody. Therefore, the tubules developed in Matrigel sections are of lymphatic rather than angiogenic origin. Furthermore, There was significantly higher number of these vessels if breast cancer cells (MDA MB-231) treated with IL-7 were used. The use of MDA MB-231 cells attracted a significantly higher number of tubules and this effect was increased with IL-7. In addition to endothelial cells, the vascular endothelial growth factors are also known to be expressed in human cancer cells including breast cancer (Kurebayashi *et al*, 1999; Salven *et al*, 1998). It is not yet known, however, whether human VEGF family members

particularly, VEGF-C and VEGF-D are similar to those in the mice or can indeed induce their receptor, VEGFR-3 on mice endothelial cells. However, it is known that the human recombinant IL-7 that was used in the experimental model is structurally and functionally similar to mouse IL-7 and therefore IL-7 might also have stimulated the autocrine secretion of VEGF-D from mouse endothelial cells. However, the increased lymphangiogenesis was noted in the mice injected with MDA MB-231 over those injected with HECV alone might suggest that the production of VEGF-D from breast cancer cells exceeded that from HECV cells.

It was concluded from the growth and migration assays as well as from the *in vivo* model that IL-7 induces the generation of lymphatic tubules both *in vitro* and *in vivo* probably via a VEGF-D dependent pathway.

11.5 The effects of IL-7 on breast cancer cells

The other aspect of this thesis explored the effects of IL-7 on breast cancer cells. As stated in the introduction chapter, IL-7 is involved in several solid cancers including colorectal (Maeurer *et al*, 1997; Watanabe *et al*, 1995), oesophageal (Oka *et al*, 1995), renal (Trinder *et al*, 1999), head and neck squamous cell carcinoma (Paleri *et al*, 2001) and others. Although IL-7 and IL-7R were expressed in normal breast and breast cancer tissues IL-7 gene was not detected in breast cancer cells (MDA MB-231 and MCF-7). However, as with endothelial cells, a fully functional receptor, IL-7R was detected and found to be able to activate its downstream signalling molecules, Jak-1, Jak-3, PI3-K and Stat-5 as

shown by the immunoprecipitation study. There it was possible to study the role of IL-7 in breast cancer cells.

The expression of the VEGF family members in breast cancer cells, MDA MB-231, MCF-7 and BT-483 was at variable levels. While VEGFs -A, -B and -C were consistent, the expression of VEGF-D was either weak or absent. IL-7 increased the expression of VEGF-D in breast cancer cells at both mRNA and protein levels. This effect was similar to that produced by IL-7 on HECV cells suggesting that the signalling pathways of IL-7 in both cells are similar. Additionally, IL-7 has been found to significantly accelerate the growth of breast cancer cells (MDA MB-231 and MCF-7) using *in vitro* growth assays. Four different growth assays were used and despite the different sensitivities of these tests, this effect of IL-7 was persistent and conclusive. It was observed from those growth assays that IL-7 induced the growth of these cells mainly by activating the PI3-K pathway as Wortmannin has completely abolished the effects of IL-7 when included in the culture medium of these cells. It has been reported that PI3-K is frequently activated in breast cancer and ER α could bind its p85 α subunit and activate the Akt2 pathway (which is important for malignant transformation) in an oestrogen-independent manner (Sun *et al*, 2001). There are several studies indicating the use of Wortmannin as an anti-inflammatory and anti-neoplastic agent (Boehle *et al*, 2002). These data suggest that inhibition of the PI3-K pathway could be a potential target for cancer therapies. The IL-7 induced breast cancer cell growth was also inhibited by Jak-3 inhibitor-1, which is a known selective inhibitor to Jak-3. Jak-3 is a non-receptor tyrosine kinase

that plays an important role in coordinating signals received through IL-7R in both endothelial and breast cancer cells as shown by the phosphorylation studies. However, the use of its selective inhibitor did not affect the IL-7 induced endothelial cell growth. Therefore, It is concluded that IL-7 could induce the growth of breast cancer cells *in vitro* via Jak-3 and a Wortmannin sensitive pathways. This may have an important impact on breast cancer development, progression and aggressiveness and thus might have diagnostic and prognostic implications. The development of IL-7R blocking molecules or the use of selective inhibitors to the downstream signalling pathway of IL-7 should be further explored in solid tumours including breast cancer.

11.6 Expression of IL-7 and its receptor signalling complex bears important clinical implications

Collectively, the lymphangiogenic and growth promoting effects of IL-7 on both endothelial and breast cancer cells have lead to investigate its clinical significance in human breast cancer tissues. Levels of IL-7, its receptor and their downstream signalling molecules were found to be aberrantly expressed in breast cancer and correlated with various clinicopathological characteristics of patients. Using real time quantitative RT-PCR to analyse 163 normal and cancer tissues showed that IL-7 does play a role in breast cancer. IL-7 and most of its signalling pathway molecules expression is significantly higher in breast cancer tissues. Levels of expression were higher in the most undifferentiated cancers and in those with higher lymph nodes metastatic rate. The association of high levels of expression with higher lymphatic spread was particularly interesting as it suggests the lymphangiogenic role of IL-7 in breast cancer. As IL-7 increased

the growth of breast cancer cells *in vitro*, It seems that over-expression of IL-7 and its signalling intermediates in the highly undifferentiated and higher TNM tumours may not be a desired phenomenon and cannot merely be explained by an accelerated immune host response alone. While the activation of IL-7 signalling complex may indicate an accelerated immune system in and around tumour tissues, it seems that the cellular immunity is not sufficiently potent in these regions to be significant enough against tumour cells. In fact It has been known for some time that patients with solid malignancies have profound depression in their cellular immunity, especially in the local tumour microenvironment (Oka *et al*, 1993; Wang *et al*, 1991). In fact, this over-expression may be detrimental to the patients, as patients with poor prognosis (i.e. the group with NPI3) have significantly raised levels of IL-7, IL-7R, Jak-3 and PI3-K. The case is further strengthened by the data that patients who died from breast cancer also exhibited significantly higher levels of these molecules than those who remained disease free.

Expression of IL-7 and its signalling molecules was also detected immunohistochemically in breast cancer tissues. Interleukins, including IL-7 have a short half-life and therefore rapid freezing of tissue samples preserves cytokine levels in the tissues. IL-7 was localised as a cytoplasmic molecule in inflammatory and other stromal cells, whereas IL-7R was detected as a transmembrane protein on breast cancer cells. All other molecules involved in the IL-7 signalling were mainly identified as cytoplasmic or nuclear proteins in breast cancer cells. Despite the relatively small sample size, an association was

made between levels of IL-7 and its molecules expression and tumour grade, TNM stage and patients prognosis. However, in addition to sample size, the scoring system used was subjective. Nevertheless, the detection of expression of these molecules in breast cancer tissues exhibited a specific trend that indicates a role for IL-7 in breast cancer.

In summary, IL-7 has a lymphangiogenic role in endothelial systems. IL-7 exerts this effect via inducing endothelial cells growth and migration through a VEGF-D dependent mechanism. Furthermore, IL-7 promotes breast cancer growth mainly via Jak-3 and Wortmannin sensitive pathways. Levels of expression of IL-7 are elevated in breast cancer tissues and found to be associated with poor tumour differentiation, higher lymph node metastatic rate, poorer prognosis and a reduced survival (Figure 11.1). This of course has an impact on breast cancer growth and metastasis.

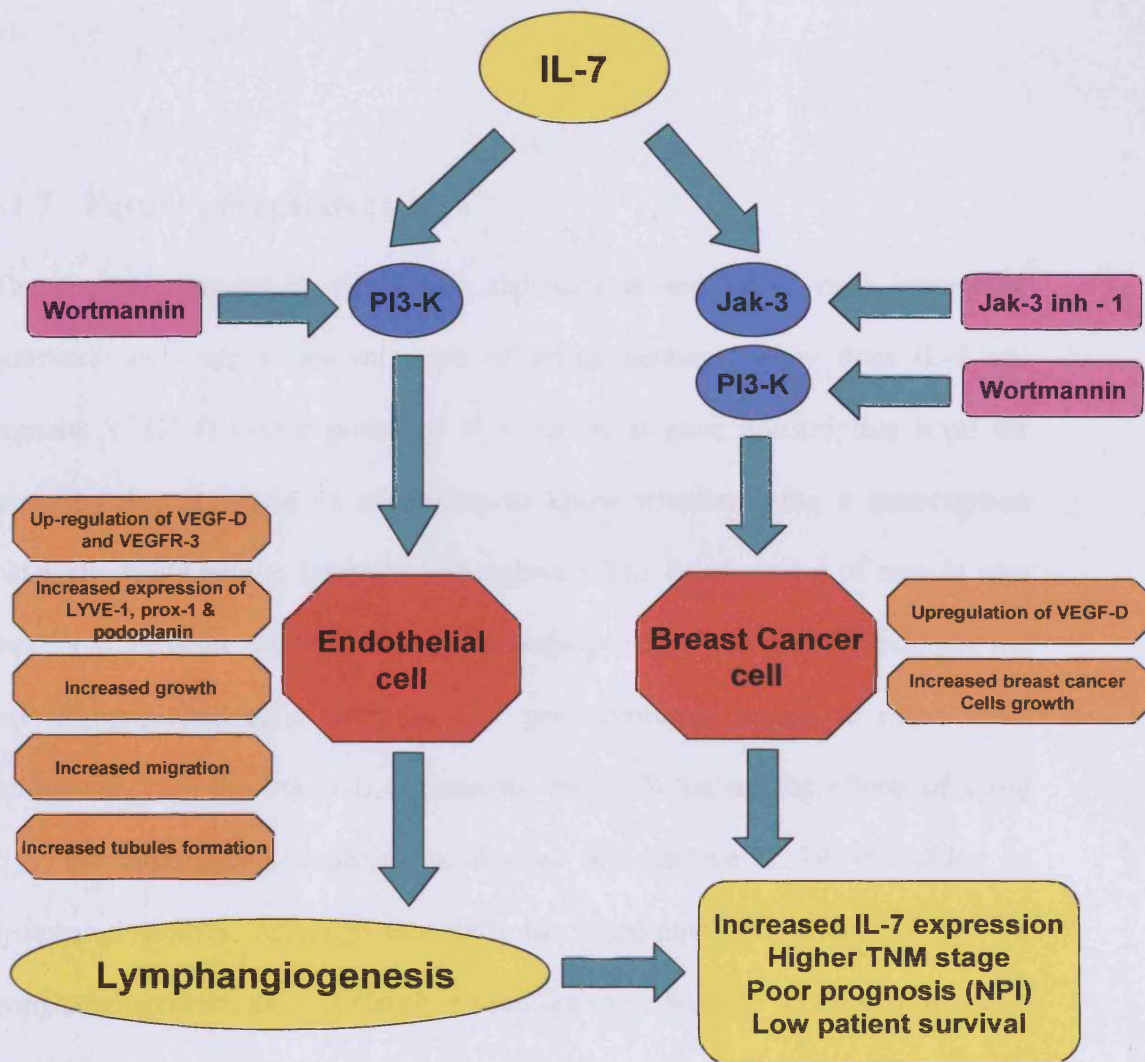


Figure 11.1 IL-7 is a lymphangiogenic growth factor in human endothelial cells. IL-7 increases lymphatic specific markers (LYVE-1, prox-1 and podoplanin) expression and up-regulates VEGF-D and VEGFR-3 in these cells. Furthermore, IL-7 induces the growth, migration and tubules formation in endothelial cells. These effects of IL-7 on endothelial cells are mediated via a Wortmannin (PI3-K inhibitor) sensitive pathway. In breast cancer cells, IL-7 up-regulates VEGF-D expression at both mRNA and protein levels. Additionally, it induces breast cancer cells growth via both PI3-K and Jak-3 pathways. The pattern of IL-7 (and its downstream signalling molecules) expression in breast cancer tissues suggests a significant association between IL-7 with higher lymphatic metastatic rate, higher TNM stage, poor prognosis and lower patient survival in human breast cancer.

11.7 Future perspectives

The results presented in this thesis and summarised above raise interesting questions and suggest several areas of future research. How does IL-7 up-regulate VEGF-D over-expression? If it occurs at gene transcription level for example, then it would be interesting to know whether using a transcription blockade would help to interrupt this pathway. The development of new *in vivo* models to study the role of IL-7 in lymphangiogenesis is needed, for example the use of transfected cells with the IL-7 gene promoter region to make them produce IL-7, or the use of IL-7 transgenic mice. What are the effects of using IL-7 inhibitors like neutralising antibodies or selective IL-7R blockades on lymphangiogenesis. Although this study has highlighted the effects of IL-7 on lymphangiogenesis, there is clearly a need for more work to be done in this area in order to fully understand the role of this cytokine as a regulator of this process. Prevention of lymphatic metastasis of cancers, including breast cancer, remains a long-term goal.

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