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Lymph Node Involvement in Breast Carcinoma Metastasis

Christina LeBedis

Department of Surgery, Division of Surgical Research
McGill University, Montreal, Quebec, Canada

August 2000

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of:

Master of Science

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor, Dr. Pnina Brodt for the guidance and encouragement she gave me throughout my studies as a Master's student. I am indebted to Dr. Brodt for the opportunity to work in scientific research and learned much from her expertise. It was a privilege to work under her supervision.

I thank Lucia Fallavollita, Abdel Khatib, Roya Navab, Tarek Boutros, Joseph Idigo, Amir Samani, Maria Kontogiannea, Amy Wong, Keguan Chen and Betty Tam for their invaluable technical advice, helpful discussion, support and friendship. I am grateful for having them as colleagues and could not have managed without them!

I would also like to acknowledge Keewhan Choi, Chung-Hae Lee, Eun-Hee Park, Roksana Nasseri, Nancy Morin, Betty Giannias, Daniel White and Irene Siderenko for their generous assistance.

A special thanks to Alex Jackson for his tireless help and continuous encouragement.

Finally, I would like to thank my family and friends for their love and support.

CONTRIBUTIONS TO THESIS

Keguan Chen is responsible for the isolation of the two lymph node stromal cells lines, STA4 and STB12, and for the writing of the lymph node stromal cells isolation protocol. Betty Tam performed the TGF-β receptor ligand binding assays (autoradiography and gamma radioactivity counts) and is credited for the writing of the ligand binding protocol. The remainder of the experiments and writing are the work of Christina LeBedis.

ABSTRACT

Regional lymph node metastases in breast cancer patients have important implications for staging, prognosis and treatment. Although recent studies established that tumor cells migrate sequentially through lymph node chains, the movement of lymph and cells within the lymph node itself is not yet well understood and remains a subject of active investigation. Since lymph node stromal cells remain largely uncharacterized with respect to cell surface markers and function, their role in regulating the growth and invasion of disseminated cancer cells, including breast carcinoma has, to date, been virtually unexplored. In the present study, we asked whether peripheral lymph node cells could modulate the growth of breast carcinoma cells and, thereby, contribute to the progression of the metastatic process. Primary cultures of rat peripheral lymph node stromal cells were obtained by limiting dilution and two sublines, STA4 and STB12, with breast carcinoma growth-promoting activities were isolated. Immunocytochemistry performed on these cells revealed that they express vimentin, S-100 and fibronectin, but neither cytokeratin nor von Willebrand factor indicating that they are stromal and dendritic in origin. Several functional studies were performed using media conditioned by STA4 and STB12 cells. The [^{3}H]-thymidine incorporation assays revealed that STA4- and STB12-conditioned media increased DNA synthesis in rat, TMT-081, and human, MCF-7 and Hs578t, breast carcinoma cell lines by 3 - 4.2 fold as compared to the control cells cultured in serum depleted medium. RT-PCR analysis revealed the presence of transcripts for multiple growth factors including IGF-1, IGF-2, bFGF, EGF, HGF, PDGF-α and TGF-β in both lymph node stromal cell lines. In addition, PDGF-β was detected only in STA4 cells. Antibody-mediated depletion of different growth factors from these conditioned media identified IGF-1 and EGF as the major mitogenic factors for all of the breast carcinoma cell lines while PDGF-α and HGF depletion had variable effects on the tumor cells. Finally, the soft agar assay was used to test the effect of STA4- and STB12-derived growth factors on the clonogenicity of breast carcinoma cells. We found that the clonogenicity of these cells increased by up to approximately 50 fold depending on the tumor type and lymph node stromal cell-conditioned media used. When taken together, these findings suggest that the lymph node stroma may not be a passive bystander in the

process of lymph node metastasis, but could instead contribute to this process through the elaboration of growth and tumorigenicity-enhancing factors.

RESUMÉ

Les métastases des nodules lymphatiques sont très importantes pour la stadification, le traitment et le pronostic du cancer du sein. Bien que des études récentes aient établies que la migration des cellules tumorales à travers la chaîne des nodules lymphatiques se fait par étapes successives, le déplacement de la lymphe et des cellules dans le nodule lymphatique lui-même n'est pas encore bien compris et demeurent un sujet de recherches très actives. Les cellules stromales des nodules lymphatiques sont mal caractérisées relativement aux marqueurs de surface cellulaire ainsi que'à leur fonction. En conséquence, leur rôle dans la régulation de la croissance et l'invasion des cellules cancéreuses disséminées, y compris dans le cancer du sein, est demeuré inexploré jusqu'à ce jour. Dans cette étude, nous avons questionné le rôle des cellules des nodules lymphatiques dans la modulation de la croissance des cellules cancéreuses et leur contribution à la progression du processus métastatique. Des cultures primaires des cellules stromales de nodules lymphatiques d'un rat ont été obtenues par la technique de dilution limitée. Deux sous-lignées cellulaires, STA4 et STB12, dotées d'activités promotrices de croissance du cancer du sein furent isolées. L'immunocytochimie réalisesé sur ces lignées a révélé qu'elles expriment la vimentine, la S-100 et la fibronectine, mais pas la cytokératine ni le facteur von Willebrand, ce qui confirme leur origine stromale et indique qu'elles pourraient être d'origine dendritique. Le milieu conditionné des cellules STA4 et STB12 a été utilisé dans des tests fonctionnels. La technique d'agar mou a été utilisée pour vérifier l'effet des milieux conditionnés sur la clonogénicité de cellules cancereuses de sein, TMT-081 (rat), Hs578t (humain) et MCF-7 (humain). Nous avons trouvé que la clonogénicité de ces cellules augmente d'un facteur supérieur à 50 fois, selon le type cellulaire ou le milieu conditionné utilisé. L'effet mitogène de ces milieux a été mesuré par la méthode d'incorporation de [^{3}H]-thymidine dans des cultures à une couche cellulaire. Nos résultats ont révélé que les milieux conditionnés des lignées STA4 et STB12 augmentent la synthèse d'ADN dans les trois lignées cellulaires par un facteur de 3 - 4.2 par rapport aux lignées cellulaires témoins cultivées dans un milieu dépourvu de sérum. Par RT-PCR nous avons montré que les deux lignées cellulaires, STA4 et STB12, expriment l'ARN messager de

plusieurs facteurs de croissance tels que IGF-1, IGF-2, bFGF, EGF, HGF, PDGF-α, TGF-α et TGF-β. L'ARN messager pour le PDGF-β a été détecté seulement dans les cellules STA4. Enfin, la déplétion de différents facteurs de croissance contenus dans les milieux conditionnés par la méthode des anticorps spécifiques, montre que IGF-1 et EGF sont les facteurs de croissance prédominants pour la prolifération des trois types cellulaires, tandis que la privation du milieu conditionnés de PDGF-α et HGF ont des effets variables sur la prolifération de ces cellules. En tenant compte des études précédentes, nos résultats suggèrent que le stroma des nodules lymphatiques ne constetuerait pas une voie de passage passive dans le processus métastatique, mais au contraire, contribuerait activement à ce processus par l'élaboration de facteurs de croissance et de facteurs tumorigènes.

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ABBREVIATIONS

APC: Antigen-presenting cell

CM: Conditioned media

ECM: Extracellular matrix

EGF: Epidermal growth factor

FGF: Fibroblast growth factor

FRC: Fibroblastic reticular cell

HEV: High endothelial venule

HGF: Hepatocyte growth factor

IGF: Insulin-like growth factor

IGFR: Insulin-like growth factor receptor

mAb: Monoclonal antibody

PDGF: Platelet-derived growth factor

RT-PCR: Reverse transcription-polymerase chain reaction

SFM: Serum-free medium

TGF: Transforming growth factor

TNM: Tumor-node-metastasis

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Chapter 1

LITERATURE REVIEW

1.A. INTRODUCTION

Metastasis of neoplastic cells from the primary tumor to other tissues represents the major cause of morbidity and mortality in cancer patients and the most formidable barrier to the successful treatment of cancer (Wells, 2000; Nakamura *et al*, 1997). Despite progress in diagnosis and treatment of malignant tumors, the mechanisms of cancer progression remain elusive (de Jong *et al*, 1998a). Metastatic growth is the end result of a complex cascade of interdependent steps including the detachment of cells from the primary tumor, migration through the extracellular matrix (ECM), penetration of basement membranes of blood vessels and lymphatics, circulation through vascular flow, invasion of remote organs and formation of independent colonies with their own growth factors and vascular supply (Favoni and de Cupis, 2000; Lee *et al*. 1998; Anan *et al*., 1996). Not all malignant tumors are metastatic suggesting that the metastatic phenotype may be distinct from the tumorigenic phenotype (Sobel, 1990).

1.B. BREAST CANCER INCIDENCE AND RISK FACTORS

Globally, breast cancer is the most common type of cancer affecting women (Parkin *et al*., 1999). While breast cancer is the second leading cause of cancer deaths in American women, second only to lung cancer since 1987, it is the leading cause of death in women aged 40-59. In the year 2000, approximately 1,220,100 new cases of invasive cancer will be diagnosed in the United States. Breast cancer alone is expected to account for 184,200 of the new cases (182,800 in females and 1,400 in males). The number of deaths from breast cancer among females began to decline an average of 1.8% per year between 1990 and 1996. For example, breast cancer deaths were highest in 1995 at 43,844 and have declined to 41,943 in 1997. If this trend continues, it is anticipated that in the year 2000, there will be approximately 41,200 deaths from breast carcinoma in the United States; 40,800 among women and 400 among men. Increased screening, leading to the detection of cancers at an earlier stage as well as the development of more effective treatment strategies are most likely responsible for the decline in breast cancer-related mortality rates (Greenlee *et al*., 2000).

The risk factors for breast cancer include gender, age, race, genetic risk factors (i.e., mutated BRCA1, BRCA2 and p53 genes), a family history of breast cancer,

personal history of breast cancer, ionizing radiation, early onset of menarche, late age of menopause and lifestyle-related risk factors such as use of oral contraceptives, not having children, use of estrogen replacement therapy, use of alcohol and obesity (DeVita, 1997). The lactating phenotype represents the ultimate level of differentiation in the human breast and it is thought that repeated periods of tissue-specific differentiation overrule the accumulation of carcinogenic and cancer-promoting events during a lifetime (Snedeker and Diagustine, 1996). Despite the recognition of these risk factors, approximately 50% of the women who develop breast carcinomas do not exhibit any of these identifiable risk factors (DeVita, 1997).

The new understanding of breast cancer biology and the groundbreaking identification of oncogenes and tumor suppressor genes, in general, have not yet translated into effective therapy (Sledge, 1996). The microenvironment of the breast is thought to play an active role not only in the normal differentiation of the breast, but also in the induction and progression of cancer. It is hoped that a better understanding of the role of host microenvironments in the development and progression of neoplasia will lead to identification of novel targets and development of new avenues for therapeutic intervention for solid tumors (Ronnov-Jessen *et al.*, 1996; Petersen *et al.*, 1998).

Adenocarcinoma is a general term that is used to classify any carcinoma that has its origin in glandular tissue. There are several types of adenocarcinoma which account for nearly all varieties of breast cancers. Breast cancer generally arises from the epithelial lining of the large or intermediate sized ducts (ductal carcinoma) or from the terminal ducts of the lobules (lobular carcinoma). These carcinomas may be either *in situ* or invasive. Invasive cancers which arise from the intermediate ducts are known as "invasive ductal" or "infiltrating ductal" carcinoma. Ductal carcinoma *in situ* (DCIS) is a pre-malignant lesion which often presents unilaterally and can progress to invasive lobular cancer if left untreated (Sakorafas and Tsiotou, 2000). Lobular carcinoma *in situ* identifies patients with increased risk for developing breast cancer in either breast. Bilateral breast cancer (~1% of all breast cancers) is found more often in familial breast cancers, women younger than age 50 and in tumors with lobular histopathology. Paget's disease (~1%) is frequently misdiagnosed as dermatitis or a bacterial infection because the first symptom is often itching or burning of the nipple with superficial erosion or

ulceration. Inflammatory carcinoma (~3%) is the most malignant form of breast cancer with metastases normally having occurred by the time of presentation. Patients usually present with a rapidly growing mass that is edematous and erythematous. These tumors often infiltrate the breast diffusely. The incidence of male breast cancer is approximately 1% of that in women yet, the prognosis of breast carcinoma is worse for men than for women because systemic masses usually exist by the time the patient comes to medical attention with a hard, ill-defined, non-tender mass. Curative treatment of breast cancer is possible for patients with stage I and II disease while patients with stage III and IV disease can often expect only palliation (Rakel, 2000).

1.C. BREAST CANCER PROGRESSION AND STAGING

Lymph nodes may play a crucial role in cancer progression. The process which occurs between initiation of a primary tumor and the development of overt lymph node metastases is still relatively obscure (Wittekind, 2000).

The anatomic location of a lymph node containing metastatic cancer cells provides an important indication of the primary site of the malignant lesion. The axillary lymph nodes constitute the lymphatic filter of the breast and are generally the first site of metastasis for breast cancer (Borgstein *et al.*, 2000; Cox *et al.*, 1998). Regional lymph nodes may entrap and/or delay circulating tumor cells that have infiltrated lymphatic sinuses by developing complex immune reactions against tumor cell antigens. These immune processes include tumor cell destruction by macrophages or by T lymphocytes via a priming mechanism involving antigen presentation by cells such as dendritic cells (Gong *et al.*, 2000; Ichii *et al.*, 2000; Mulders *et al.*, 1999). T lymphocyte priming may include one or more of the following: 1. increase in the growth and expansion of tumor infiltrating lymphocytes (TILs); 2. upregulation of CD4+ and CD8+ cell proliferation 3. augmentation of T cell-mediated tumor lysis; 4. enhancement of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6) mRNA expression by TILs (Mulders *et al.*, 1999).

The diagnosis, prognosis and treatment of cancer depend largely on the presence and degree of tumor metastasis. A statistical relation exists between tumor size and incidence of metastases in which the probability of finding metastases in the regional

lymph nodes increases in proportion to the volume of the primary tumor. Other morphological features of primary tumors that correlate with the probability of lymph node metastases include tumor histology (carcinoma vs. sarcoma), poor grade of differentiation and invasion of lymph vessels (Wittekind, 2000; Ioachim, 1994).

The lymph node remains the most reliable parameter in the staging and prognosis of carcinomas through biopsy and histologic evaluation (Camp *et al.*, 2000, Canavese *et al.*, 2000; Sandrucci *et al.*, 1999; Shetty, 1997). Tumor staging is performed according to the internationally accepted tumor-node-metastasis (TNM) system. The classification stages are as follows: Stage 0, pre-invasive neoplasia; Stage I, tumor confined to the organ of origin; Stage II, direct tumor spread outside of the organ of origin; Stage III, metastasis to regional lymph nodes; and Stage IV, metastasis to distant sites (Fielding, 1992). Each stage in the TNM indicates diminishing patient prognosis since the tumor at the time of assessment has spread more extensively. Lymph node metastasis is often associated with poor prognosis in potentially resectable solid epithelial tumors (Passlick and Pantel, 2000). Breast carcinoma prognosis is directly related to the number of axillary lymph nodes involved and the quantity of tumor cells within the individual lymph nodes (Ioachim, 1994). For instance, with no axillary lymph node involvement, the five year survival rate is approximately 80%. However, if four or more lymph nodes contain metastases, the disease-free survival rate drops to about 21% (Kumar *et al.*, 1992). Sadly, a significant number of apparently lymph node-negative patients die early of metastatic disease implying that current staging procedures underestimate tumor cell dissemination (Leong, 2000; Passlick and Pantel, 2000).

1.D. THE SENTINEL LYMPH NODE

The current standard surgical management for breast cancer consists of resection of the primary tumor and axillary lymph node dissection for axilla staging (Sandrucci *et al.*, 1999; McIntosh and Purushotham, 1998). However, the need for axillary dissection has been questioned since there is, at present, no conclusive evidence that axillary lymphadenectomy improves patient survival and only about 30-40% of all invasive breast cancers are lymph node-positive (Lagares-Garcia *et al.*, 2000; Jatoi, 1999; Sun *et al.*, 1998). In addition, the value of axillary lymphadenectomy has been brought under

scrutiny because it can produce a wide range of complications such as parasthesia of the axilla, shoulder and upper arm due to costobrachial nerve injury, wound infection, hematoma, seroma, drain complications and acute and chronic lymphedema which all result in treatment delays (Cox *et al.*, 1998; McIntosh and Purushotham, 1998; Albertini *et al.*, 1996). Given such morbidity, there is an incentive to avoid axillary surgery in women who are lymph node-negative. The alternative procedure, axillary sampling, was historically associated with a higher rate of false negatives as a result of skip metastases. Skip metastases are defined as metastases to level II and III axillary nodes without involvement of level I nodes and increased risk that inadequate surgery would understage the axilla (Cox *et al.*, 1998). It should be noted, however, that previous studies arbitrarily divided the axillary lymph nodes into levels I, II and III without any formal lymphatic mapping. When accurate lymphatic mapping is employed, skip metastases are rare (Albertini *et al.*, 1996; Van Lancker *et al.*, 1995).

Sentinel lymph node mapping and resection have recently emerged as a promising alternative to axillary lymphadenectomy (Krag *et al.*, 1998). In 1977, Cabanas proposed that removal and examination of sentinel nodes, the lymph nodes that are the first to receive drainage from a tumor, could be minimally invasive and be used to determine whether more extensive lymphadenectomy should be performed. Metastasis of breast carcinoma cells to the axillary lymph nodes has been shown to occur sequentially (Crossin *et al.*, 1998; Kapteijn *et al.*, 1998). Recently, techniques have been developed that allow the identification of positive regional lymph nodes without clearing the entire lymphatic basin (McIntosh and Purushotham, 1998).

Morton and colleagues (1992) used a vital blue dye to identify the lymphatics which drained into the sentinel nodes in melanoma patients. In addition, radiocolloids have been successfully employed in the identification of axillary sentinel lymph nodes in breast cancer (Borgstein *et al.*, 1998; Veronesi *et al.*, 1997; Albertini *et al.*, 1996). Combining the vital blue dye staining method with radiocolloid imaging has been shown to offer the most accurate identification of the sentinel lymph node (Canavese *et al.*, 2000; Cox *et al.*, 1998; McIntosh and Purushotham, 1998). Sentinel lymphadenectomy with multiple sectioning and histopathologic examination can predict the presence or absence of axillary node metastases in patients with breast cancer, but further studies are

needed to investigate its value with respect to long-term regional control and effect on patient survival before it can replace axillary lymphadenectomy as the preferred staging technique (Noguchi *et al.*, 1999). Although sentinel lymph node mapping is still under investigation and questions concerning the optimal technique and clinical applications still remain, it has the potential to be a powerful device for the assessment of axillary nodal status with minimal morbidity while obviating the need for axillary lymphadenectomy in lymph node-negative patients.

1.E. THE NORMAL BREAST

The highly regulated balance of proliferation, differentiation and degeneration of cells in the mammary gland is subject to fine control by hormones, growth factors and cross-talk between epithelial cells and stromal fibroblasts (Rasmussen and Cullen, 1998; Normanno and Ciardiello, 1997; Koli and Keski-Oja, 1996). Autocrine and paracrine mechanisms affect the morphological and functional changes comprising organogenesis in the embryo, pubertal growth in the adolescent and lactation in the adult (Rasmussen and Cullen, 1998). The main components of the female breast are lobules (milk-producing glands), ducts (milk passages that connect the lobules to the nipple) and stroma (fatty tissue and ligaments surrounding the ducts and lobules, blood vessels and lymphatic vessels).

Each breast consists of 15 to 25 independent glandular units called breast lobes, each consisting of compound tubulo-acinar glands whose function is secretion of milk to nourish newborns. The lobes are embedded in a mass of adipose tissue which is subdivided by a dense collagenous septa arranged radially at different depths around the nipple. Each lobe is drained by a lactiferous duct which emerges onto the surface of the nipple. Breast lobes are subdivided into breast lobules consisting of alveolar ducts. The ducts and alveoli are composed of the same fundamental structure: an inner continuous layer of luminal cells and an outer layer of myoepithelial cells (Ronnov-Jessen *et al.*, 1996; Junqueira *et al.*, 1992). The organization of epithelial cells into branching ducts and alveoli depends on the action of various soluble factors, cell-cell and cell-ECM interactions, and the establishment of epithelial cell polarity. The epithelial cell

membrane is segregated into apical and basolateral domains by tight junctions (Eaton and Simons, 1995).

Surrounding the breast epithelium is a stromal compartment made up of fibroblasts, adipocytes, ECM proteins and vascular elements (Rasmussen and Cullen, 1998). The composition of this mesenchymal tissue is critical for normal breast development (Clarke *et al.*, 1992). Stromal appearance and function can vary significantly depending on its location in the normal breast. The stroma of the nipple/areola area contains substantial numbers of smooth muscle cells and dense collagen while the intralobular stroma, which is in closest association with the secretory epithelium, is loose, cellular and highly vascular, containing fine collagen fibers and reticulin (Junquiera *et al.*, 1992). The interlobular stroma, in contrast, is dense, fibrous and less cellular than the intralobular stroma because its main function is structural. The fibroblastic cell populations within these morphologically distinct stromal tissues are poorly characterized and it is now clear that there is considerable phenotypic heterogeneity among fibroblastic populations (Spanakis and Brouty-Boye, 1997; Schor *et al.*, 1994; Sappino *et al.*, 1990).

1.F. THE LYMPH NODE

Lymph nodes are peripheral lymphoid organs arranged at strategic locations in small chains where they drain the lymphatic vessels of specific regions. They function in lymphopoiesis, lymph filtration, antigen processing and the generation of immune responses (Ichii *et al.*, 2000; Fu and Chaplin, 1999; Ioachim, 1994). Relatively inactive lymph nodes are only a few millimeters long, but they greatly increase in size during immune reactivity (Burkitt *et al.*, 1993). The lymph node is comprised of three distinct regions: 1. the cortex or B lymphocyte (B-cell) region which includes the lymphoid follicles and is associated with humoral immunity; 2. the paracortex or T lymphocyte (T-cell) dependent region which is involved in cellular immunity; and 3. the medulla, the site of plasma cell proliferation and antibody production (Burkitt *et al.*, 1993; Junqueira *et al.*, 1992). Lymphatic sinuses are lined by reticulo-endothelial cells and carry lymph through the lymph node from the afferent to the efferent lymphatics by a mechanism that has not been fully elucidated.

In the lymph node, soluble signals and cells carried by the lymph encounter lymphocytes emigrating from the blood. The interaction and subsequent transfer of information between immune cells and material carried by the lymph is facilitated by the molecular and cellular architecture of the lymph node (Gretz *et al.*, 1996). The lymphatic stroma includes the capsule, trabeculae and a network of reticular cells and reticulin fibers associated with ECM which comprise the supporting framework of the lymph node and an ever-changing population of lymphocytes (Burkitt *et al.*, 1993). Reticulin fibers play an important role in the filtration of lymph, retention of foreign substances and anchorage of activated macrophages, lymphocytes, plasma cells and polymorphonuclear leukocytes. The reticular network interweaves throughout the lymph node and many of the fibers that cross the lymphatic sinuses continue into the paracortex (Ushiki *et al.*, 1995). This network of reticulin fibers and fibroblastic reticular cells (FRCs) provides a three-dimensional scaffold with a large surface area that facilitates cell migration and fosters interactions between soluble factors including antigens and antigen-presenting cells and lymphocytes migrating from tissues via afferent lymph. In addition, this network provides a delivery system that directs soluble mediators to critical sites such as the high endothelial venules (HEVs) to recruit lymphocytes from the blood (Lee, 1999; Gretz *et al.*, 1996; Anderson and Shaw, 1993).

Antigen presenting cells (APCs) arriving in the lymph node via lymph fluid are thought to transmigrate through the interfollicular regions and "cling" to the scaffold provided by the reticular network. Lymphocytes enter the lymph node primarily through specialized blood vessels called HEVs. These lymphocytes are then thought to move into the paracortex from HEVs and migrate along the reticular network interacting with APCs as they go. Transmigration of T-cells into the lymph node via the HEV is regulated by T-cell adhesion receptors such as L-selectin and integrins, ligands on HEV cells, extracellular matrix proteins and cytokines released by FRCs (e.g., IL-1, IL-8, TNF-α, IFN-γ and TGF-β) (Gretz *et al.*, 1996; Anderson and Shaw, 1993; Szekanecz *et al.*, 1992). Fibronectin, an ECM protein, is concentrated along reticulin fibers and provides a particularly suitable substrate for T-cell adhesion and migration (Gretz *et al.*, 1996; Shimizu *et al.*, 1991). Thus, the reticular network seems to play more than a structural

role in the lymph node by facilitating lymphocyte contact with APCs in the paracortex, where initial lymphocyte activation is thought to occur (Gretz *et al.*, 1997, 1996).

Immune accessory cells such as dendritic cells and histiocytes originate in the bone marrow, migrate to peripheral tissues and enter the lymph nodes through both the lymph and blood. They are part of the mononuclear-phagocyte system and have important roles in the processing and presentation of antigens to lymphocytes.

Dendritic reticular cells are derived from precursors in primary lymphoid tissues that migrate into secondary lymphoid tissues and reside primarily in germinal centers where they play an important role in both normal and pathological immune responses (Kapasi *et al.*, 1998). Characterization of their function has been difficult because of their scarcity and multiple maturation/differentiation states. Accumulating evidence suggests that these migratory cells can be recruited to areas of peripheral inflammation where they process antigens and present them to T lymphocytes in the context of MHC molecules (Hajek and Butch, 2000; Mentzer, 2000). Dendritic cells are also capable of transporting antigens to regional lymph nodes via afferent lymphatics. In the paracortex of the lymph node, dendritic cells interact with B and T lymphocytes initiating humoral and cellular immune responses by providing membrane-bound and soluble activation signals (Chapoval *et al.*, 2000; Mentzer, 2000).

Defective dendritic cell function has been implicated in the escape of tumors from immune system control. Data from Almand *et al.* (2000) indicate that dendritic cell dysfunction in cancer patients is the result of decreased numbers of competent dendritic cells and the accumulation of immature hematopoietic cells. A study by Gabrilovich *et al.* (1999) found that dendritic cells isolated from patients with breast cancer had a decreased ability to stimulate T-cells. While the mechanisms and clinical significance of these phenomena need to be investigated further, immunotherapies utilizing dendritic cells for antigen presentation to T-cells are currently being developed which show promise in treating cancer (Chapoval *et al.*, 2000; Gong *et al.*, 2000; Hajek and Butch, 2000; Mulders *et al.*, 1999).

Histiocytes, progeny of circulating monocytes, accumulate in the lymph node sinuses or infiltrate the lymph node paracortex under stimulatory conditions. Depending on their reactive stage, histiocytes may engulf foreign material, nuclear debris or

antigenic proteins which are subsequently presented to lymphoid cells leading to initiation of an antibody response (Ioachim, 1994).

1.G1. GROWTH FACTORS

Multicellular organisms rely on intercellular communication via growth factors, cytokines and hormones to mediate processes such as embryonic development, tissue differentiation and systemic responses to wounds and infection. The complex pathways mediating cell growth, differentiation, migration and apoptosis are regulated, in part, by growth factors that act as stimulatory or inhibitory modulators (Favoni and de Cupis, 2000).

Growth factors are polypeptides which exert their effects by binding to specific high affinity cell membrane receptors which generally possess intrinsic protein kinase activity and can initiate a cascade of intracellular signaling events culminating in activation of gene transcription and the resulting biological responses (Favoni and de Cupis, 2000; Fedi *et al.*, 1997). Some peptide growth factors are stored in the ECM and can be released by targeted proteolysis. They generally diffuse over short distances and act locally via autocrine or paracrine mechanisms. A response is considered autocrine when the growth factor stimulates the same cell in which it is produced. Those that act in a paracrine fashion regulate the growth of neighboring cells (Cullen *et al.*, 1991). Growth factors are present in a variety of tissues and their receptors are ubiquitous with cells generally expressing multiple receptors. They often act synergistically in the sense that exposure of a cell to one growth factor potentiates the mitogenic effect of another growth factor. Several factors will often act in concert to regulate growth (Anderson *et al.*, 1990). For instance, TGF-β can induce the expression of the PDGF receptor and the genes that encode the PDGF chains (Taylor and Khachigian, 2000; Kaetzel *et al.*, 1993). Moreover, growth factors operate at different points in the cell cycle thereby complementing each other's functions (Holland *et al.*, 1997). The coordinated cellular growth during development, tissue maintenance and homeostasis depends on precise regulation of cellular proliferation by the growth factors.

Several growth factors involved in cellular proliferation act via membrane receptors with intrinsic tyrosine kinase activity. The tyrosine kinase receptors share a

common structure: a transmembrane glycoprotein with an extracellular binding domain, a single hydrophobic transmembrane region and a cytoplasmic domain that contains a tyrosine kinase catalytic domain. After ligand binding, the receptors dimerize (with the exception of IGF-1R and insulin receptors which are expressed as dimers) and this leads to activation of their kinase activity and autophosphorylation. This begins a phosphorylation cascade which reaches the nucleus where phosphorylation of nuclear transcription factors results in their activation or inactivation. Nuclear transcription factors then interact with the regulatory regions of growth control genes. In this way, growth factors, acting at the cell surface, can modulate cell growth (Loef, 2000; Pizzo and Poplack, 1997). For a summary of the growth factors to be discussed please consult Table 1.

1.G2. GROWTH FACTORS IN CANCER

Genetic aberrations in growth factor signaling pathways are inextricably linked to cancer. Malignant cells arise through a stepwise progression of genetic events characterized by the abnormal expression of growth factors or components of their signaling networks (Favoni and de Cupis, 2000; Wells, 2000; Thiery and Chopin, 1999). Autocrine and paracrine stimulation of cell proliferation by tumor-derived growth factors is thought to be an important mechanism for tumor growth and progression (Ariad *et al.*, 1991). An imbalance between growth-stimulatory and growth-inhibitory factors can result in inappropriate growth stimulation, deficient growth inhibition of tumor cells or a combination of both (Ji *et al.*, 1994). Many genes that act early in growth factor signal transduction have been identified as oncogenes (Fedi *et al.*, 1997; Ariad *et al.*, 1991). Constituitive activation of growth factor signaling pathways through alterations in these gene products is thought to contribute to the development of most, if not all, human malignancies (Fedi *et al.*, 1997). The loss of requirement for specific growth factors is common in many types of cancer cells and could be mediated by: 1. increased growth factor production (an autocrine mechanism); 2. increased or altered growth factor receptor expression; 3. constitutive growth factor receptor activation or 4. activation of a pathway that bypasses the requirement for growth factor receptor expression or function (Goustin *et al.*,1986). Novel pharmacological approaches for cancer are being developed

which are designed to interfere at various steps of the growth factor signaling pathway (Favoni and de Cupis, 2000; Thiery and Chopin, 1999).

Several growth factors and growth factor receptors have been implicated in breast cancer progression. Growth factors and their receptors may stimulate or block proliferation directly when produced by the tumor cells themselves (autocrine mechanism) or by stromal cells (paracrine mechanism) and indirectly by their effect on neovascularization (El-Ashry *et al.*, 1994). Breast tumors are comprised of epithelial, stromal and vascular elements and interactions between these various cell types is essential for tumor growth. Although the stromal and vascular elements are not transformed themselves, they grow abnormally along with the malignant epithelial cells (Cullen *et al.*, 1991). Several growth factors have been shown to be expressed in invasive breast cancer including bFGF, PDGF-αα, PDGF-ββ, TGF-α and TGF-β and receptors EGFR, PDGF-αR and PDGF-βR (Brandt *et al.*, 2000; de Jong *et al.*, 1998b; Anan *et al.*, 1996; Panico *et al.*, 1996; Brown *et al.*, 1995; Coltrera *et al.*, 1995; Seymour *et al.*, 1994; Toi *et al.*, 1994). The correlation between growth factor expression, proliferation and angiogenesis was found to be particularly strong when both the growth factor and its receptor were expressed, suggesting the importance of autocrine or paracrine mechanisms in the stimulation of growth and angiogenesis in breast cancer (de Jong *et al.*, 1998b).

1.G3. INSULIN-LIKE GROWTH FACTORS

Members of the insulin-like growth factor (IGF) family play a pivotal role in embryogenesis, normal growth and development and have recently been implicated in the growth and metastatic spread of several human tumors including breast carcinoma (Shim and Cohen, 1999; Yu and Berkel, 1999; Rasmussen and Cullen, 1998; Long *et al.*, 1994). IGFs were first identified as serum factors, named somatomedins, that interacted with growth hormone to stimulate skeletal tissues. IGFs serve as endocrine, autocrine and paracrine stimulators of mitogenesis, survival and cellular transformation (Grimberg and Cohen, 2000; Grothey *et al.*, 1999; Ellis *et al.*, 1996). Insulin-like growth factor 1 (IGF-1) and IGF-2 are synthesized in many tissues (predominantly the liver) and act via receptors that are present on many cell types (Wilson, 1998).

IGF-1 and IGF-2 are 7.5 kDa single polypeptide chains sharing 48 and 50% amino acid homology with insulin, respectively, and 70% amino acid homology with each other. They act as both circulating peptide hormones and locally-acting growth factors (Wilson, 1998; Pizzo and Poplack, 1997; Clarke *et al.*, 1992). However, IGF-1 and IGF-2 are not equivalent in terms of their biological activity probably due to their different affinities for the two IGF receptors (Baker *et al.*, 1993). In addition to their transmembrane receptors, IGF-1 and IGF-2 activity is modulated by several IGF binding proteins (IGFBPs) (Perks and Holly, 2000; Yu and Berkel, 1999; Hwa *et al.*, 1999; Rasmussen and Cullen, 1998). IGFBPs are involved in the vascular transport of IGFs, prolong their plasma half-life, modulate their interactions with receptors and influence their growth promoting activity (Pizzo and Poplack, 1997). During normal post-natal growth, IGF-1 synthesis is regulated by growth hormone and it is the principle mediator of growth hormone functions until puberty (Shim and Cohen, 1999; Yu and Berkel, 1999; Humbel, 1990). IGF-2 is widely expressed by immature organogenic cells during fetal development, but its physiological role is not well understood because it is not tightly regulated by growth hormone (Humbel, 1990).

Three membrane-bound receptors bind IGFs: the insulin receptor (IR), the IGF-1 receptor (IGF-1R) and the IGF-2 receptor (IGF-2R). IR and IGF-1R are structurally related membrane-bound tyrosine kinase receptors (Pizzo and Poplack, 1997; Ellis *et al.*, 1994). They have a unique heterotetrameric structure (130 kDa + 85 kDa)$_2$ consisting of two disulfide bonded α chains containing the ligand-binding domain and two transmembrane β chains that contain the intracellular kinase domain. IGF-1R binds IGF-1 with the highest affinity, followed by IGF-2 and insulin (Wilson, 1998; Pizzo and Poplack, 1997; Ellis *et al.*, 1994; Cullen *et al.*, 1991b). Ligand-binding to IGF-1R is thought to cause conformational change in the dimeric receptor that initiates signal transduction. A crucial component to IGF-1R signal transduction is the phosphorylation of the insulin receptor substrate (IRS) family which mediates interactions with SH2 domain-containing proteins. Phosphorylated IRS binds and activates PI-3 kinase as well as binds Grb2, linking IGF-1R to ras signaling pathways (Petley *et al.*, 1999; Pizzo and Poplack, 1997; Baserga *et al.*, 1994).

IGF-2R is a transmembrane protein with a short cytoplasmic region and is homologous to the mannose-6-phosphate receptor. IGF-2R binds both IGF-1 and IGF-2, but not insulin. Unlike IR and IGF-1R, IGF-2 has no tyrosine kinase activity. Some evidence suggests that IGF-2R is associated with a G protein-coupled receptor signaling system. Two high affinity mannose-6-phosphate binding sites on IGF-2R serve to transport mannose-6-phosphate containing proteins, principally proteases, from the Golgi apparatus to the lysosomes. In addition, IGF-2R is involved extracellularly with secreted lysosomal proteins (i.e., cathepsin D) and other proteins containing mannose-6-phosphate such as the TGF-β precursor, EGF receptor and proliferin (Lee *et al.*, 1998; Wilson, 1998; Pizzo and Poplack, 1997; Vignon and Rochefort, 1992).

IGF-1 and IGF-2 are potent mitogens for a wide variety of normal and malignant cells and promote anchorage-independent growth of breast carcinoma cells (Yu and Berkel, 1999; Surmacz *et al.*, 1998). Their mitogenic activity is mediated through binding to the IGF-1R. Perturbations of the IGF family have been identified in cancer (Shim and Cohen, 1999).

Many human tumors have increased IGF ligand or receptor expression suggesting an autocrine or paracrine role in their growth (Yu and Berkel, 1999; Li *et al.*, 1998; Guvakova and Surmacz, 1997; Pizzo and Poplack, 1997). As mitogens and anti-apoptotic agents, IGFs may be important in carcinogenesis, possibly increasing the risk of cellular transformation by enhancing cell turnover. *In vivo*, tissue IGF bioactivity is determined by circulating IGF-1 and IGFBP levels and by local production of IGFs, IGFBPs and IGFBP proteases such as kallikreins, cathepsins and matrix metalloproteinases that enhance IGF-1 availability by cleaving IGFBPs (Giovannucci, 1999; Rajah *et al.*, 1995). High plasma IGF-1 and low IGFBP-3 levels have been independently associated with greater risk of breast cancer among pre-menopausal women (Grimberg and Cohen, 2000; Giovannucci, 1999; Shim and Cohen, 1999; Lee *et al.*, 1998).

Recent data suggests that the anti-apoptotic effect of IGF-1 may contribute to the reduced drug-sensitivity of many cancer cells *in vitro* and *in vivo* (Grothey *et al.*, 1999). IGF-2 has been linked to the development of several tumors including breast carcinoma (Li *et al.*, 1998; Pizzo and Poplack, 1997). IGF-2 can be expressed as an autocrine

growth factor in some breast cancer cells and its expression may result in the hormone independence that is characteristic of malignant progression (Li *et al.*, 1998; Rasmussen and Cullen, 1998; Cullen *et al.*, 1992; Daly *et al.*, 1991). IGF-1 mRNA transcripts, on the other hand, are rarely localized to malignant breast carcinoma cells, but are predominantly expressed in stromal fibroblasts surrounding the breast epithelium, suggesting that IGF-1 exerts predominantly paracrine effects on breast epithelial cells (Rasmussen and Cullen, 1998; Ellis *et al.*, 1994; Cullen *et al.*, 1992). IGF-1 is commonly expressed in fibroblasts derived from non-malignant biopsy specimens, while IGF-2 is often detected in fibroblasts adjacent to malignant tissue (Giani *et al.*, 1996; Singer *et al.*, 1995; Cullen *et al.*, 1992). It has been postulated that paracrine influences, mediated by soluble factors released by tumor epithelium, are able to specifically increase expression of IGF-2 in breast stroma by a process of clonal expansion (Singer *et al.*, 1995; Ellis *et al.*, 1994). IGF-2 can therefore enhance tumor growth via autocrine and paracrine mechanisms (Sciacca *et al.*, 1999).

IGF receptors are widely expressed in benign breast disease and in human breast cancer cell lines (Surmacz *et al.*, 1998). IGF-1R levels are higher in cancerous than in normal breast tissue or benign mammary tumors (Lee *et al.*, 1998; Guvakova and Surmacz, 1997; Happerfield *et al.*, 1997). Inappropriate IGF-1R signaling is thought to be involved in malignancy owing to its role in cell cycle progression and cell survival (Yu and Berkel, 1999; Resnicoff *et al.*, 1995; Baserga *et al.*, 1993). Interference with IGF-1R activation, expression or signaling inhibits growth and induces apoptosis in breast cancer cells. Recent studies have established the involvement of IGF-1R in the regulation of breast cancer cell growth, motility and adhesion (Guvakova and Surmacz, 1997). The expression or function of IGF-1R in breast cancer is modulated by other growth factors (especially PDGF and EGF), oncogenes (e.g., SV40 T antigen and c-myb), tumor supressor genes (e.g., WT1 and RB) and by different humoral factors (e.g., estrogen, progesterone, IGF-2 and interleukin-1) (Surmacz *et al.*, 1998; Baserga *et al.*, 1994). Thus, it is thought that several transforming agents may exert their growth-promoting effects through direct or indirect activation of the IGF autocrine loop. The IGF-1R and estrogen receptor are usually co-expressed and the two signaling systems are engaged in a complex functional cross-talk controlling cell proliferation (Happerfield *et*

al., 1997; Kenney and Dickson, 1996). The role of IGF-1R in breast cancer etiology, especially in metastatic progression, is a subject of active investigation (Surmacz *et al.*, 1998). The emerging view is that abnormally high levels of IGF-1Rs may contribute by increasing tumor mass and/or they may contribute to tumor recurrence by promoting proliferation, cell survival and cell-cell interactions (Surmacz *et al.*, 1998; Guvakova and Surmacz, 1997).

1.G4. EPIDERMAL GROWTH FACTOR

Epidermal growth factor (EGF), a 6 kDa single polypeptide chain of 53 amino acids, was first described as a peptide that stimulates the premature eyelid opening and tooth eruption in newborn mice and was later recognized for its ability to stimulate the growth of cultured cells (Cohen and Carpenter, 1975; Hollenberg and Cuatrecasas, 1973). The functions of EGF include mitogenesis, angiogenesis, growth inhibition, tissue repair, regeneration and the acquisition of differentiated phenotypes (Sabiston, 1997). EGF and its related peptides (e.g., TGF-α) play an important role in regulating the proliferation and differentiation of a variety of normal and malignant mesenchymal and epithelial cells including mammary epithelial cells (Wilson, 1998; de Cupis and Favoni, 1997; Normanno and Ciardiello, 1997).

The EGF receptor (EGFR) is a 170 kDa transmembrane protein that is present on the surface of a large variety of cells including epithelial and mesenchymal cells such as keratinocytes, vascular smooth muscle cells, chondrocytes, fibroblasts and mammary gland cells (Wilson, 1998). EGFR dimerizes upon binding EGF or TGF-α thereby activating its endogenous tyrosine kinase activity and mediate its effects via MAP kinase, ras, Shc, Gap, Jak-1, STAT-1, PI-3 kinase or PLCgamma (Humphreys and Hennighausen, 2000; Boonstra *et al.*, 1995; Earp *et al.*, 1995). EGFR regulates proliferation of multiple tissues during fetal development, adulthood and pregnancy (Dickson *et al.*, 1992). In normal epidermis, EGFR is important for autocrine growth, suppression of terminal differentiation, promotion of survival and regulation of cell migration during epidermal morphogenesis and wound healing (Hudson and McCawley, 1998).

EGFR overexpression results in impaired mammary gland development *in vitro* and *in vivo* reducing the differentiation potential of the mammary epithelium and inducing epithelial cell transformation (Brandt *et al.*, 2000). Modulating agents of the EGFR signaling pathway such as anti-EGFR antibodies, anti-her2/neu antibodies and specific kinase inhibitors are under clinical evaluation as imaging agents and have been used to block the action of this pathway thereby preventing the development, growth and survival of many types of human cancer cells (Humphreys and Hennighausen, 2000; Waksal, 1999; Pizzo and Poplack, 1997). Additionally, overexpression of EGFR in malignancies such as breast cancer has proven to be efficacious in stratifying patients with respect to poor prognosis (Ebert *et al.*, 2000; Fox and Harris, 1997; Gasparini *et al.*, 1992; Klijn *et al.*, 1992).

1.G5. HEPATOCYTE GROWTH FACTOR

Hepatocyte growth factor (HGF) was initially characterized as a potent stimulator of hepatocyte proliferation (Nakamura *et al.*, 1986). Independently, Stoker *et al.* (1987) discovered a protein called scatter factor, so named for its unique ability to "scatter" colonies of epithelial cells. It was subsequently found that these two factors are structurally identical (Weidner *et al.*, 1991).

HGF is synthesized by fibroblast cells as an inactive 87,000 MW single polypeptide chain precursor. After processing by serine proteases, the processed protein forms a dimer composed of α (69 kDa) and β (34 kDa) subunits linked by a disulfide bond (Stella and Comoglio, 1999; Soriano *et al.*, 1998). HGF is thought to act in a primarily paracrine fashion and promotes the proliferation and motility of epithelial cells (Bone,1998).

All of the biological effects of HGF are elicited by binding to its receptor, a 190 kDa transmembrane protein encoded by the *c-met* proto-oncogene with intrinsic tyrosine kinase activity (Jin *et al.*, 1997). The *c-met* receptor is expressed predominantly by cells and tumors of epithelial origin (Tsao *et al.*, 1993; Yang and Park, 1993; DiRenzo *et al.*, 1991).

The HGF/*c-met* pathway is thought to be involved in tumor growth, invasion and metastasis (Stuart *et al.*, 2000; van der Voort *et al.*, 2000; Stella and Comoglio, 1999). In

various carcinomas including breast cancer, the *c-met* receptor is overexpressed suggesting that it may contribute to cancer progression (Liu *et al.*, 1992; Giordano *et al.*, 1989). HGF is also capable of inducing angiogenesis *in vitro* and *in vivo* as it is a potent mitogen for endothelial cells, can upregulate endothelial cell matrix metalloproteinase activity and promotes extracellular matrix remodeling (Aoki *et al.*, 2000; Wang *et al.*, 2000; Jin *et al.*, 1997; Rosen *et al.*, 1997).

1.G6. FIBROBLAST GROWTH FACTORS

Fibroblast growth factors (FGFs) were initially characterized as growth factors that are mitogenic for cultured fibroblasts and vascular endothelial cells (Gospodarowicz *et al.*, 1976). The FGF family currently has nine recognized members which have a high degree of homology and varying affinity for the FGF receptors (Ornitz et al, 1996; Hughes and Hall, 1993; Miyamoto *et al.*, 1993). The FGF family is comprised of single chain polypeptide growth factors that range in size from 14-18 kDa and have an affinity for heparin and glycosaminoglycans.

FGFs are essential molecules for mammalian development (Ornitz *et al.*, 1996). They are produced by a variety of cell types including epithelial, stromal, endothelial and neural cells (DeVita, 1997). FGFs have growth-stimulatory effects on endothelial cells, epithelial cells and fibroblasts and, in addition, have transforming effects on fibroblasts (Basilico and Moscatelli, 1992; Wellstein *et al.*, 1990). Two members, acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF), have been described as having angiogenic and chemotactic functions and may also have a direct proliferative effect on tumor cells (Nugent and Iozzo, 2000; Pichon *et al.*, 2000; Sabiston, 1997; McLeskey *et al.*, 1996).

The cellular activities of aFGF and bFGF are mediated through cell surface receptors with an intracellular tyrosine kinase domain (Gerwins *et al.*, 2000; Muenke and Schell, 1995; Johnson and Williams, 1993). FGF receptors (130 kDa) dimerize upon ligand binding, autophosphorylate and bind intracellular substrates leading to downstream effects such as cell proliferation (Wennstrom *et al.*, 1991). Variant FGF receptors have different expression patterns and affinity for FGF ligands conferring the

specificity of their actions (Johnson and Williams, 1993; Givol and Yayou, 1992; Partanen *et al.*, 1992).

Members of the FGF family have been implicated in several cancers including breast cancer (Nurcombe *et al.*, 2000; Marsh *et al.*, 1999; Zhang *et al.*, 1999). A study by Song *et al.* (2000) demonstrated elevated levels of aFGF and bFGF in the conditioned media of solid and metastatic tumors. These conditioned media induced broad spectrum resistance of cancer cells to chemotherapeutic drugs with diverse structures and actions (e.g., paclitaxel, doxorubicin, 5-fluorouracil). Other groups have also reported increased FGF expression and aggressiveness of breast cancer cells (Marsh *et al.*, 1999; Smith *et al.*, 1999; Visscher *et al.*, 1995), but this appears to be controversial (Colomer *et al.*, 1997). FGF receptor expression in breast carcinoma tissues was found to be ubiquitous, although the abundance of particular transcripts varied (McLeskey *et al.*, 1994). *In vivo* data demonstrate that FGF receptors are expressed on tumor cells and can be used to target tumors for growth inhibition (Lappi, 1995).

1.G7. PLATELET-DERIVED GROWTH FACTORS

Platelet-derived growth factors are a family of dimeric protein molecules synthesized by differentiated, non-dividing blood cells as well as on platelets, placenta, preimplantation embryos and endothelial cells (Pantazis *et al.*, 1990). Platelet-derived growth factor (PDGF) was originally purified from blood platelets where it is stored as a component of the α granules (Ross and Vogel, 1978). The main functions of PDGF on connective tissue cells (e.g., fibroblasts and smooth muscle cells) and certain other cell types include mitogenic signaling, chemotaxis, vasoconstriction and angiogenesis. PDGF has also been implicated in wound repair, embryogenesis and neoplasia (Heldin and Westermark, 1999; Betsholtz and Raines, 1997; Fedi *et al.*, 1997; Sabiston, 1997; Anan *et al.*, 1996).

PDGF is a ubiquitous growth factor that has three isoforms (αα, αβ, ββ), each with specific dimeric receptors on target cells (Fedi *et al.*, 1997; Claesson-Welsh, 1994). PDGF consists of structurally similar α (14-18 kDa) and β (16 kDa) chains combined via disulfide bonds to form homo- and heterodimers (Heldin and Westermark, 1999).

PDGF exerts its stimulatory effects on cell growth, motility and differentiation by binding two structurally-related protein tyrosine kinase receptors, α and β (Heldin *et al.*, 1998). There is evidence that the receptor subunits interact differentially with the three dimeric PDGF ligands providing fine regulation of ligand/receptor interactions. Receptors for PDGF are found on a variety of mesenchymal cells (Goustin *et al.*, 1986, 1985). Overactivity of PDGF has been implicated in nonmalignant conditions characterized by an increased cell proliferation such as atherosclerosis, desmoplasia and fibrotic conditions while overproduction of PDGF is thought to be involved in growth stimulation of human tumors (Heldin and Westermark, 1999).

Little is known about the role of PDGF in breast cancer. PDGF is secreted by most breast carcinoma cells and PDGF-β receptors have been demonstrated in malignant breast tissue (Bhardwaj *et al.*, 1996). In this study by Bhardwaj *et al.*, PDGF-β receptors were present only on stromal cells surrounding the breast carcinoma cells suggesting paracrine stimulation of adjacent stromal tissue by malignant epithelial cells in human breast tumors. PDGF appears to be involved in both tumor angiogenesis and tumor metastasis (Westphal *et al.*, 2000; Ariad *et al.*, 1991; Liotta *et al.*, 1991). A study by Anan *et al.* (1996) showed that PGDF-α mRNA expression of tumors with lymph node metastases was as great as 92.3% which was 2 fold higher than that of tumors without lymph node spread. In addition, *in vitro* evidence suggests that PDGF is a potential angiogenic factor (Lindahl *et al.*, 1999). Increased PDGF plasma levels is correlated with poor prognosis and treatment failure in patients with advanced breast cancer (Seymour and Bezwoda, 1994; Ariad *et al.*, 1991). These findings implicate PDGF as a potential factor in the development of breast cancer metastasis.

1.G8. TRANSFORMING GROWTH FACTORS

Transforming growth factors (TGFs) play important roles in normal growth and development. TGF-α and -β are unrelated molecules whose actions are distinct: TGF-α is a potent mitogen while TGF-β is inhibitory for most cells (Pizzo and Poplack, 1997). TGF-α was originally characterized by its ability, in combination with TGF-β, to induce the anchorage-independent growth of normal rodent fibroblast cells. Its presence in a

variety of human tumors, and in cell lines transformed by both chemical carcinogens and viral oncogenes, underscored its potential involvement in malignant transformation (Coffey *et al.*, 1986; Luetteke and Michalopoulos, 1985).

TGF-α (~6 kDa) has significant sequence and structural homology to epidermal growth factor (Wilson, 1998). EGF-related peptides and their receptors play an important, but not fully elucidated role in epithelial cell physiology and pathophysiology. TGF-α is a principal molecule in the normal and neoplastic development of the mammary gland. TGF-α is often found in epithelial cells, embryos, placenta and many transformed cells. As mentioned previously, TGF-α's effects are mediated by binding EGFR.

Overexpression of EGF-related growth factors and their receptors during progression from normal epithelium to carcinomas has been demonstrated for several tissues including breast, endometrium, cervix and ovary (Ebert *et al.*, 2000; Johnson *et al.*, 2000). TGF-α mRNA and protein have been identified in breast cancer cells *in vivo* and *in vitro* (Humphreys and Hennighausen, 2000; Schroeder and Lee, 1997). Transgenic mouse models have established that TGF-α overexpression can induce hyperproliferation, hyperplasia and carcinoma. These studies demonstrate that TGF-α, in cooperation with several oncogenes, can facilitate proliferation and transformation of the mammary epithelium (Humphreys and Hennighausen, 2000; Schroeder and Lee, 1997). Taken together, these findings suggest that TGF-α plays an important role in the genesis and/or maintenance of breast cancer.

Transforming growth factor β (TGF-β) is a multifunctional cytokine whose properties include the potent regulation of cell proliferation (either stimulatory or inhibitory effects depending on the cell type), differentiation, morphogenesis, extracellular matrix formation, inflammation, wound healing, angiogenesis and extracellular proteolysis (Sabiston, 1997; Pizzo and Poplack, 1997; Arrick, 1996; Kalkhoven *et al.*, 1996; Koli and Keski-Oja, 1996). The human TGF-β family consists of 25 kDa proteins composed of two identical 112-amino acid polypeptides that are closely related both structurally and functionally (Sabiston, 1997; van Roozendaal *et al.*, 1995).

TGF-β usually forms homodimers, but heterodimeric forms also exist in certain cell types (Ogawa *et al.*, 1992).

TGF-β is regulated at the levels of expression, secretion and activity as well as at the level of receptor expression and cellular responsiveness. TGF-β is typically synthesized and secreted in a latent form that is activated by proteolytic cleavage or acidic pH (Pizzo and Poplack, 1997). Activation of TGF-β is crucial for its regulation, since many cells are able to produce latent TGF-β and possess TGF-β receptors (Khalil, 1999; Massague, 1990; Sporn and Roberts, 1990). TGF-β is also regulated by members of the steroid hormone superfamily and it has been proposed that TGF-β acts as a local mediator of the various actions of steroid hormones (Koli and Keski-Oja, 1996; Herman and Katzenellenbogen, 1994; Jeng *et al.*, 1993).

There are three TGF-β receptor subtypes: type I, type II and type III (Boyd *et al.*, 1990). The TGF-β receptors are membrane-bound receptors that are distinct from other growth factor receptors in that they have serine-threonine kinase catalytic activity. Type II receptor can bind TGF-β independently of type I receptor, but can not transmit the growth inhibitory signal alone. Type I and II TGF-β receptor must form a heterodimeric complex to be functional (Pizzo and Poplack,1997; Geiser *et al.*, 1992). Type III receptor, also known as betaglycan, lacks a serine-threonine kinase domain and does not directly participate in signaling, but appears to regulate the presentation of TGF-β to type II receptor or may be involved in sequestering or clearing bioactive TGF-β. It was found that diminished expression of type II receptor is a frequent cause of TGF-β insensitivity in breast tumor cells (Kalkhoven *et al.*, 1995; Ji *et al.*, 1994; Sun *et al.*, 1994).

Both TGF-β and its receptor are expressed ubiquitously in normal and transformed cells (Koli and Keski-Oja, 1996). TGF-β is normally expressed in breast tissue and is an important regulator of the normal mammary epithelial cell proliferation and mammary gland development (Daniel *et al.*, 1996; Smith, 1996; McCune *et al.*, 1992). It has been observed that many human tumors fail to respond to the normal growth-inhibiting signal of TGF-β resulting in a loss of growth control. It is thought that this loss of TGF-β-mediated growth inhibition is a critical event in breast tumorigenesis. A positive association between the expression of TGF-β by tumor cells and the

progression or survival of breast carcinoma cells has been observed in many *in vivo* studies. Possible mechanisms for these growth-enhancing effects of TGF-β include immunosuppression, angiogenesis, altering stroma formation and altering integrin and matrix metalloproteinase expression in stromal cells (Dong-Le Bourhis *et al.*, 1998; Li *et al.*, 1998; Jones *et al.*, 1997; Reiss and Barcellos-Hoff, 1997; Koli and Arteaga, 1996; Wahl, 1992; Bascom *et al.*, 1989). Several studies suggest that TGF-β can indirectly promote breast cancer progression (Arteaga *et al.*, 1993; Gorsch *et al.*, 1992; Welch *et al.*, 1990). In breast tumors, all TGF-β isoforms were identified and there is enhanced expression of TGF-β at advancing tumor edges. In lymph node metastases, TGF-β appears to be associated with disease progression (Dalal *et al.*, 1993). Due to the diverse functions of TGF-β *in vivo*, its precise biological role in breast cancer remains to be elucidated (van Roozendaal *et al.*, 1995).

1.H1. STROMAL-EPITHELIAL INTERACTIONS

Stromal-epithelial interactions mediate crucial aspects of organogenesis, developmental processes and tissue maintenance in vertebrates (Anderson *et al.*, 1996; Humphrey *et al.*, 1995; Byers *et al.*, 1994). Stromal tissue provides structural and metabolic support for tissues and organs and is comprised of various cells (e.g., fibroblasts, adipocytes, mast cells, tissue macrophages and leukocytes) and the extracellular matrix (Junquiera *et al.*, 1992). Studies have shown that a reciprocal paracrine interaction exists between stromal and epithelial cells (Charbord, 1992; Wegner and Carson, 1992). Stromal cells are known to regulate the proliferation and differentiation of epithelial cells through cytokines, cell surface adhesion molecules and growth factors (Donjacour and Cuhna, 1991).

Development of the mammary glands is initiated in the embryo, but the major part of their development occurs in the adult. Mutual and reciprocal epithelial-mesenchymal interactions are critical for both phases of development. Specific steps such as bud formation, appearance of hormone receptors and ductal elongation have been shown to be governed by epithelial-mesenchymal signaling (Robinson *et al.*, 1999).

1.H2. STROMAL-EPITHELIAL INTERACTIONS IN NEOPLASIA

Alterations in stromal-epithelial interactions occur during neoplasia and have been shown to play a role in malignant tumor cell proliferation, migration and invasion (Lazar-Molnar *et al.*, 2000; Chung, 1995; Byers *et al.*, 1994; Ellis *et al.*, 1994; Long *et al.*, 1994; Weigang *et al.*, 1994). Many similarities exist between the stroma at sites of wound repair and reactive stroma in cancer including increased stromal cell proliferation, altered expression of ECM components, neovascularization and alterations in stromal cell phenotype such as the expression myodifferentiation markers (Rowley, 1999; Gregoire and Lieubeau, 1995; Sieweke and Bissell, 1994). The stromal reaction (also known as desmoplasia) is thought to be induced by epithelial cancer cells and may reflect the attempt of normal stromal cells to re-establish the critical mesenchymal/parenchymal organization that is compromised during neoplasia (Clarke *et al.*, 1992).

Many invasive breast carcinomas are characterized by a prominent desmoplastic or stromal reaction and this led to early speculation that stromal cells play a role in breast cancer pathogenesis (Ellis *et al.*, 1994; van den Hooff, 1991). In breast cancer, the desmoplastic response is typified by an accumulation of stromal tissue composed of ECM and myofibroblasts adjacent to the carcinoma cells (Ronnov-Jessen *et al.*, 1996; Hewitt *et al.*, 1993; Brouty-Boye *et al.*, 1991). Myofibroblasts originate from fibroblasts in the mammary gland and express smooth muscle markers including smooth muscle α-actin, smooth muscle myosin heavy chain, calponin and α_1-integrin (Spanakis and Brouty-Boye, 1997; Ronnov-Jessen *et al.*, 1995; Lazard *et al.*, 1993). Evidence suggests that the stromal reaction in breast cancer is tumor-promoting (Thomasset *et al.*, 1998). The malignant breast epithelium loses its ability to respond to the external signals that regulate the differentiation of the normal breast epithelium and either develops autocrine modes of regulation or responds to alternative paracrine signals (Rasmussen and Cullen, 1998).

Stromal cells can regulate tumor cell proliferation, migration and invasion through cell-cell contact probably involving the ECM (Petersen *et al.*, 1998; Xie and Haslam, 1997; Donjacour and Cunha, 1991) and through paracrine mechanisms involving the release of multiple factors including cytokines such as IL-6, IL-11 and Leukemia Inhibitory Factor (LIF) (Bhat-Nakshatri *et al.*, 1998; Crichton *et al.*, 1996) and growth

factors such as IGF-1, IGF-2, EGF, PDGF, HGF, bFGF, TGF-α and -β (de Jong *et al.*, 1998a; Giani *et al.*, 1996; van Roozendaal *et al.*, 1996; Spanakis and Brouty-Boye, 1995; Cullen *et al.*, 1991a). Tumor growth beyond a certain size requires specific stromal-epithelial interactions in order to form a favorable microenvironment (Cullen *et al.*, 1992). The stromal reaction is thought to promote tumor invasion due, in part, to enhanced stromal expression of metalloproteinases (Benaud *et al.*, 1998; Sieuwerts *et al.*, 1998; Lochter *et al.*, 1997; Ronnov-Jessen *et al.*, 1996).

Epithelial cell-derived factors may activate stromal cells to secrete diffusable factors which can exert paracrine control over epithelial cells (de Cupis and Favoni, 1997). This is demonstrated by the following examples:

1. Breast carcinoma cells secrete PDGFs, but do not usually express PDGF receptors preventing an autocrine response (de Jong *et al.*, 1998a; Bhardwaj *et al.*, 1996). On the other hand, PDGF is a major breast fibroblast mitogen and it may stimulate IGF production in these cells. In breast tumor biopsies, IGF-1 and -2 were identified in the stroma, but not in the malignant cells suggesting that it is another potential paracrine regulator of breast carcinoma cell growth (Singer *et al.*, 1995; Cullen *et al.*, 1991; Rosen *et al.*, 1991). Stromal IGF, in turn, could stimulate epithelial cell proliferation, motility and invasion (Ellis *et al.*, 1994; Cullen *et al.*, 1991; Bronzert *et al.*, 1987).

2. HGF is a stromal-derived factor that induces mitogenic, motogenic and morphogenic responses in epithelial cells, all of which may impact on tumor progression (Matsumoto *et al.*, 1996; Weidner *et al.*, 1990). Carcinoma cells do not produce biologically significant levels of HGF, however, tumor cells do secrete soluble factors which are potent activators of HGF production in stromal fibroblasts (e.g., IL-1, bFGF and PDGF) (Nakamura *et al.*, 1997). Conditioned media from human breast carcinoma cell lines has been shown to regulate HGF production in a paracrine manner by stimulating HGF expression in a human fibroblast cell line (Byers *et al.*, 1994; Seslar *et al.*, 1993).

Tumor cells can also induce the formation of vascular elements via FGFs and PDGFs to supply oxygen and nutrients to the emerging tumors and can promote matrix protein synthesis by the tumor-associated stroma to provide a matrix for tumor anchorage and growth (Folkman *et al.*, 1989; Peres *et al.*, 1987). Moreover, tumor-derived growth

factors may act on stromal cells in a paracrine fashion to stimulate estrogen production which, in turn, can promote tumor growth (Purohit *et al.*, 1995; Simpson *et al.*, 1994).

Although the interactions between stromal cell subpopulations and adjacent cancer cells in the progression of the malignant disease remains a subject of active investigation, this evidence suggests that the bi-directional cross-talk between stromal and epithelial cells in breast cancer is mediated, in part, by the synthesis and secretion of growth factors.

1.H3. STROMAL-EPITHELIAL INTERACTIONS IN THE LYMPH NODE

Two major cell populations exist in secondary lymphoid organs; cells of hematopoietic origin and stromal cells. The interaction between these two cell populations is essential for the development and control of the immune response (Skibinski *et al.*, 1997).

Lymph node colonization in breast cancer is thought to occur sequentially (Kapteijn *et al.*, 1998; McIntosh and Purushotham, 1998), however, the movement of lymph and cells within the node is not well understood (Gretz *et al.*, 1997). As tumor cells metastasize through blood vessels and lymphatic vessels, they migrate and lodge in lymph nodes as a result of interactions between the malignant cells and resident lymph node cells (Holland *et al.*, 1997; Hanaoka *et al.*, 1995). The resulting metastasis is most likely a consequence of the adhesion molecules expressed by the tumor cells (Gendreau and Whalen, 1999; Nip *et al.*, 1995; Whalen and Sharif, 1992; Gunthert *et al.*, 1991; Brodt, 1991, 1990). Lymph node stromal cells remain largely uncharacterized and their role in the growth regulation of disseminated cancer cells including breast carcinomas has been virtually unexplored.

Examples of stromal-epithelial interactions within the lymph node come primarily from studies involving lymph node stromal cells and lymphomas. Lymph node stromal cells (CA-12) have been shown to play an important role in the growth of malignant T-lymphoma cells (CS-21) by preventing apoptotic cell death by direct cell-cell contact and enhancing lymphoma cell proliferation by secreted soluble factors (Kataoka *et al.*, 1993). This study suggested that cell-cell interaction between lymphoma and lymph node

stromal cells may play an important role in the regulation of malignant cell growth. Subsequently, it was found that a monoclonal antibody raised against CS-21 cell surface molecules suppressed apoptotic death of CS-21 cells even after they were separated from the CA-12 stromal cells and the cell surface protein responsible was identified as CD45RO. This protein appears, therefore, to mediate adhesion between the two cell types and suppress apoptosis (Hanaoka *et al.*, 1995).

A study examining the lymph node microenvironment by Ohkawa *et al.* (1989), demonstrated that lymphoid stromal (SG) cells isolated from the lymph nodes of a patient with malignant lymphoma regulated the growth of B-acute lymphoblastic leukemia (B-ALL) and T-acute lymphoblastic leukemia (T-ALL). Direct contact between SG and the lymphoid cell lines (B-ALL and T-ALL) inhibited the growth of the latter. Media conditioned by SG cells significantly increased the growth of only T-ALL cells. These findings indicated that SG cells undergo tissue-specific cellular interactions with B- and T-lymphoid cells and that they can modulate their growth by different mechanisms. Similarly, lymph node stromal cells derived from a patient with non-Hodgkin's lymphoma inhibited the proliferation of lymphoid leukemic cells and promoted leukemic cell growth by soluble factors (Tsuda *et al.*, 1990).

1.I. THE AIM OF THIS STUDY

Regional lymph node metastases in breast cancer patients have important implications for staging, prognosis and treatment. Although recent studies have elucidated that tumor cell migration through lymphatic chains is sequential, the movement of lymph and cells within the lymph node is not yet well understood and remains a subject of active investigation. Lymph node stromal cells remain largely uncharacterized with respect to cell surface marker expression and function. Their role in the growth regulation of disseminated cancer cells including those from breast carcinoma has, to date, been virtually unexplored. In the present study, we asked whether peripheral lymph node cells are able modulate the growth of breast carcinoma cells and thereby contribute to the progression of metastatic disease.

Table 1: Summary of Growth Factors

GROWTH FACTOR	DESCRIPTION	SOURCES	TARGETS	RECEPTORS
IGF-1 and IGF-2 (1, 2)	7.5 kDa. High homology among IGF-1, IGF-2 and insulin.	IGF-1 is primarily produced in adult liver and smooth muscle cells. IGF-2 is produced by the fetal liver, placenta and some tumor cells. Both factors are found in blood plasma bound to specific binding proteins.	Many cell types including epithelial and mesenchymal cells.	IGF-1 receptor (130 kDa + 85 kDa)$_2$ is a tyrosine kinase that binds both IGF-1 and IGF-2. IGF-2 receptor (260 kDa) binds IGF-2 and is homologous to the mannose-6-phosphate receptor.
HGF (3, 4, 5, 6)	Dimeric protein composed of α (69 kDa) and β (34 kDa) subunits.	HGF is produced by fibroblasts in many tissues.	Predominantly epithelial cells.	The *c-met* receptor (190 kDa) is encoded by the *c-met* proto-oncogene and has tyrosine kinase activity.
EGF and TGF-α (1, 2, 7)	6 kDa. Structural homology between EGF and TGF-α. Both are released by proteolysis of membrane-bound precursors.	EGF is found in the submaxillary glands, Brunner' s glands and possibly parietal cells. TGF-α is produced by transformed cells, placenta and embryos.	Wide variety of epithelial, mesenchymal and glial cells.	The EGF receptor (170 kDa) is a protein tyrosine kinase which is a product of the c-erbB proto-oncogene. It binds both EGF and TGF-α.
FGF (1, 7, 8, 9)	Single chain polypeptide family (14-18 kDa) with high homology among the nine members.	FGFs are produced by a variety of cell types including macrophages, epithelial, stromal, neural and endothelial cells.	Endothelial and epithelial cells as well as fibroblasts.	The FGF receptor (130 kDa) mediates its effect via intrinsic tyrosine kinase activity.
PDGF (αα, αβ and ββ) (1, 7)	Dimers of α (14-18 kDa) and β (16 kDa) for a total size of ~32 kDa. β chain is encoded by the c-sis proto-oncogene.	PDGF is produced by blood platelets, endothelial cells, preimplantation embryos and placenta.	Mesenchymal, glial and smooth muscle cells as well as placental trophoblasts.	Both α (170 kDa) and β (180 kDa) receptor types are tyrosine kinases. Type α binds all PDGF dimers, whereas type β binds αβ and ββ dimers weakly.
TGF-β (1, 7)	Dimers of 25 kDa that are secreted in latent form.	TGF-β is present in blood platelets, kidney, placenta and embryos. It is also widespread throughout adult tissues and cultured cells.	Wide variety of cell types.	Receptors type 1 (50-80 kDa), type 2 (115-140 kDa) and type 3 (280-330 kDa) all bind TGF-β_1, -β_2 and -β_3. TGF-β type 1 receptor which contains serine-threonine activity and is thought to be the main mediator of TGF-β s effects.

References: 1) Pizzo and Poplack, 1997 2) Wilson, 1998 3) Stella and Comoglio, 1999 4) Soriano *et al.*, 1998 5) Jin *et al.*, 1997 6) Matsumoto and Nakamura, 1992 7) Sabiston, 1997 8) De Vita, 1997 9) Wennstrom *et al.*, 1991

Chapter 2

MATERIALS AND METHODS

2.A. Cell Lines

The rat breast carcinoma cell line, TMT-081 (Brodt *et al.*, 1990), was a kind gift from Dr. U. Kim (Rosewell Park Memorial Institute, Buffalo, NY, USA) and the MCF-7 line (Rosenauer *et al.*, 1998) was a gift from Dr. S. Mader (University of Montreal, Quebec, Canada). Both cell lines were maintained in RPMI supplemented with 10% FCS. Human breast carcinoma cell line Hs578t (Hackett *et al.*, 1977) was obtained from the American Tissue Type Collection (Rockville, MD) and maintained in DMEM supplemented with 10% FCS.

2.B. Isolation of lymph node stromal cells

Peripheral lymph nodes were obtained from female Wistar/Furth rats (Charles River Canada, St. Constant, Quebec). The lymph nodes (inguinal, axillary and brachial) were carefully dissected out of the surrounding fatty tissues, placed in Hank's balanced salt solution (HBSS, Gibco BRL, Burlington, Ontario) containing gentamycin and finely minced with curved dissection scissors. The minced tissue was digested for 30 minutes at 37°C with 0.05% collagenase A (Boehringer Mannheim Biochemica, Montreal, Quebec) in HBSS using a shaking water bath. The digest was filtered through an 85uM Nitex nylon mesh (B & SH Thompson, Town of Mount Royal, Quebec), the filtrate washed twice by centrifugation for 10 minutes at 1200 rpm, re-suspended in RPMI medium containing 20% FCS and the cells plated in 35mm culture dishes or 24 well plates which were pre-coated with 10 μg/ml rat fibronectin (Gibco BRL, Burlington, Ontario). Twenty-four hours after plating, the cultures were gently washed to remove all lymphoid cells, new culture medium was added, and the cells fed twice weekly with RPMI containing 10% FCS. The stromal cell cultures were subsequently cloned by limiting dilutions (Yan *et al.*, 1994), the clones expanded and serum free-conditioned media collected from confluent monolayers and screened for a mitogenic effect on TMT-081 and Hs578t cells. Two subclones with growth-promoting effects, STA4 and STB12, were selected for further study.

2.C. Harvesting lymph node stromal cell-conditioned media

To harvest conditioned media, confluent monolayers of STA4 and STB12 were washed repeatedly over a 6 hour period with serum free RPMI 1640 and then incubated in serum free RPMI for 48 hours at 37°C in a 5% CO_2 incubator. The conditioned media were centrifuged at 2,000 rpm for 5 minutes to remove cells and debris, filtered through a 0.22 μm pore size filter (Millipore Corp., Bedford, MA) and stored at -20 °C until used.

2.D. Antibodies

The following primary antibodies were used: anti-vimentin mAb LN-6, anti-cytokeratin mAb C-11, anti-type I collagen mAb Col-1 and affinity purified rabbit anti-laminin antiserum (Sigma, St. Louis, MO). mAb 661 (Nip *et al.* 1995) to vitronectin was a gift from Dr. D.J. Loskutoff (La Jolla, CA), mAb 3E3 to fibronectin (Boehringer Mannheim, Laval, Quebec), rabbit antiserum to von Willebrand factor and rabbit anti-bovine S-100 (Dakopatts, Glostrup, Denmark), affinity purified goat anti-IGF-1, anti-HGF-α, anti-EGF and affinity purified rabbit polyclonal anti-PDGF-α antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies used were FITC-conjugated goat anti-mouse or anti-rabbit IgG and rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Protein A-Sepharose CL-4B beads were from Pharmacia Biotech, Inc. (Baie D'Urfe, Quebec).

2.E. Immunocytochemistry

STA4 and STB12 cells were seeded onto 4 chamber slides (LabTek; Nunc, Inc., Naperville, IL) at a cell density of $5x10^3$cells/chamber and incubated in 5 and 10% RPMI, respectively, for 24 hours at 37°C. The cells were washed with PBS and fixed with a 1:1/acetone:ethanol for 2 minutes at room temperature for vimentin and pan-cytokeratin staining or for 10 minutes in 3.7% formaldehyde for staining of ECM proteins and von Willebrand factor. The chambers were rinsed with PBS and incubated for 30 minutes at room temperature with a PBS solution containing 5% goat serum and 2% BSA to block non-specific protein binding sites. Incubations with the primary and FITC-conjugated secondary antibodies both diluted in PBS containing 2% BSA were for 1 hour each at room temperature. Chambers were rinsed in PBS and dH_2O, air dried and

coverslips mounted in a 9:1/glycerol:PBS solution containing 1.5% 1,4-Diazabicyclo-[2.2.2.]octane (Sigma, St. Louis, MO). Slides were visualized using a Zeiss Axiophot epifluorescence microscope equipped with a 35-mm film cassette Axio Mot.

2.F. [^{3}H]-thymidine incorporation assay

Tumor cells were cultured in serum free RPMI for 24 hours, dispersed with PBS containing EDTA, plated in 96 well plates (Corning Inc., Corning, NY) at a density of $3x10^3$cells/well and incubated for 48 hours in STA4- or STB12-conditioned media. Serum-free RPMI or RPMI containing 10% FCS were used to establish minimal and maximal incorporation levels. The cells were pulsed with 1 μCi/well of [^{3}H]-thymidine (DuPont Canada, Mississauga, Ontario) for 18 hours, the cells were lysed by freezing and thawing. The cell lysates were then harvested onto FilterMATs using a 12 Cell Harvester (Skatron, Sunnyvale, CA) and [^{3}H]-thymidine incorporation monitored using a Beckman LS 8000 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

2.G. RT-PCR

Total RNA was extracted from confluent monolayers of STA4 and STB12 with TRIzol™ reagent (Gibco BRL, Burlington, Ontario) according to manufacturer's instructions. Two micrograms of total RNA were reverse transcribed in a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 30 mM KCl, 8 mM $MgCl_2$, 10 mM dithiothreitol, 100 ng antisense primer, 1 mM deoxy-NTPs and 8 U of avian myeloblastosis reverse transcriptase (Pharmacia Biotech, Baie D'Urfe, Quebec). Reverse transcription was carried out using conditions described previously (Khatib *et al.*, 1999). The sense and antisense primers as well as $MgCl_2$ concentrations were as described elsewhere (Veness-Meehan *et al.*, 1997; Napoli *et al.*, 1997). The reaction mixture for cDNA amplification contained 3 U of Taq polymerase, 50 mM KCl, 10 mM Tris-HCl (Pharmacia Biotech, Baie D'Urfe, Quebec), 300 ng sense primer and 200 ng antisense primer. PCR conditions for all but IGF-2 cDNA amplifications were: 10 minute incubation at 94°C, followed by 35 cycles each consisting of 20 seconds at 94°C, 20 seconds at 60°C, 30 seconds at 72°C and each followed by a 10 minute incubation at 72°C. PCR conditions for IGF-2 were as described previously (Veness-Meehan *et al.*,

1997). Amplified cDNA fragments were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide and photographed with an Insta Doc I Gel Documentation System (BioRad, Mississauga, Ontario).

2.H. [^{3}H]-thymidine incorporation assay in the presence of TGF-β

Breast carcinoma cells were plated and treated exactly as described in the [^{3}H]-thymidine incorporation assay with the exception that the tumor cells were incubated in 3, 6, 12, 25, 50 or 100 pM of TGF-β (a kind gift from Dr. A. Philip, Montreal General Hospital, Montreal, Quebec) suspended in serum-free RPMI rather than stromal cell-conditioned media.

2.I. TGF-β_1 affinity labeling of TMT-081, MCF-7 and Hs578t cells

Iodination of TGF-β was done as detailed previously (Philip and O'Connor-McCourt, 1991). Affinity labeling techniques were performed as described previously (Tam and Philip, 1998; Dumont *et al.*, 1995). Briefly, cell monolayers were washed with ice-cold binding buffer (Dulbecco's phosphate-buffered saline with Ca^{+2} and Mg^{+2}, pH 7.4; D-PBS) containing bovine serum albumin (BSA) for 10 minutes and were labeled with 100 pM ^{125}I-TGF-β_1 in the presence or absence of 4nM non-radioactive TGF-β_1 for 3 hours at 4°C (obtained from Dr. O'Connor-McCourt, Montreal, Quebec). The receptor-ligand complexes were crossed linked with Bis(dulfocsuccinimidyl)suberate (BS3) (Pierce, Rockford, IL). The reaction was stopped by the addition of glycine and the cells were solubilized. The solubilized protein samples were run on a 3-11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel under reducing conditions. Results were analyzed using autoradiography. For analysis of TGF-β_1 affinity using the gamma counter, monolayers of cells were labeled with ^{125}I-TGF-β_1 as described above with the exception that no crosslinking was performed. Cells were then solubilized and radioactivity counted using a Cobra II gamma counter (Packard Instruments, Meridan, CT).

2.J. Growth factor depletion

Aliquots of conditioned media were incubated with an affinity purified goat polyclonal antibody to IGF-1, HGF-α, EGF or affinity-purified rabbit polyclonal anti-PDGF-α (20ug/ml) for 20 hours at 4°C with agitation. To each aliquot, rabbit anti-goat IgG-saturated Protein A-Sepharose CL-4B beads (100µl of 20% Protein A-Sepharose beads in Hepes-buffered solution, pH 7.5) were added for an additional 18-20 hours incubation at 4°C with agitation. In the case of anti-PDGF-α, no secondary antibody was used. Protein A-Sepharose beads were removed by centrifugation and stromal cell-conditioned media were filtered before use. Control conditioned media samples were treated in an identical manner without the addition of the anti-growth factor antibodies.

2.K. Soft agar cloning

To measure anchorage-independent growth, a modification of the standard soft agar cloning assay was used (Saiga *et al.*, 1987). The tumor cells were mixed with 0.8% agar (Difco Laboratories Inc., Detroit, MI), added to an equal volume of a 2x concentrated complete RPMI-FCS medium and plated onto a solidified layer of 1% agar in 35 mm petri dishes (Falcon, Montreal, Quebec) at a density of $1x10^4$cells/petri dish. The overlay was allowed to solidify and supplemented with RPMI containing 1% FCS or unconcentrated STA4- or STB12-conditioned media containing 1% FCS. The media were replenished on alternate days for 30 days. Colonies were enumerated and measured using an Diaphot-TMD inverted microscope (Nikon, Canada) equipped with an ocular square millimeter grid.

Chapter 3

RESULTS

3.A. Characterization of lymph node stromal cell lines

Cultured rat peripheral lymph node stromal cells were cloned by the limiting dilution method. Morphologically distinct cell lines were obtained and their conditioned media tested for carcinoma growth-modulating effects. Two sublines, STA4 and STB12, with growth-promoting activities were selected for further studies (Figure 1). Immunocytochemistry results shown in Figure 2 identified these cells as vimentin$^+$, fibronectin$^+$ and S100$^+$ while laminin was also detected in STB12 suggesting that they were stromal and dendritic in origin. STA4 and STB12 cells tested negative for cytokeratin, von Willebrand factor, vitronectin and collagen type I indicating that they were not derived from epithelial and endothelial cells.

3.B. Increased proliferation of breast carcinoma cells in the presence of lymph node stromal cell-conditioned media

The effects of lymph node stromal cell-conditioned media on tumor cell proliferation were measured by [^{3}H]-thymidine incorporation assays using monolayer cultures. Dividing cells incorporate [^{3}H]-thymidine into their DNA, therefore, in conditions that promote cell division, a higher [^{3}H]-thymidine incorporation will be seen. The maximal increases in [^{3}H]-thymidine incorporation as compared to the control (serum-free medium) ranged from 3 - 4.2 fold. Results shown in Figure 3 demonstrate that media conditioned by STA4 and STB12 cells were mitogenic for all three cell lines, TMT-081, MCF-7 and Hs578t.

3.C. Growth factor mRNA expression in lymph node stromal cells

Several growth factors have been implicated in stromal-epithelial interactions involving breast carcinoma cells. To investigate their potential role in the growth-promoting effect of lymph node stromal cells, RT-PCR analysis was first used to identify growth factors expressed by these cells due to its high sensitivity and ease of use. Results shown in Figure 4 revealed the presence of transcripts for IGF-1, IGF-2, EGF, TGF-α, bFGF, HGF, TGF-β and PDGF-α mRNA in both cell lines while PDGF-β mRNA was only noted in STA4 cells. These findings demonstrate that STA4 and STB12 cells

express mRNA for several growth factors that may contribute to the growth-promoting effects of lymph node stromal cell-conditioned media. These RT-PCR experiments were intended to provide a profile of growth factors potentially involved in breast carcinoma promotion for further investigation and does not serve as a quantitative analysis of growth factor RNA expression. The results also do not indicate whether the growth factor mRNAs are translated into their active, secreted protein forms. A functional assay, mAb-mediated growth factor depletion, was carried out to address this issue. Of particular interest is the finding that both cell lines express mRNA transcripts for TGF-β, a growth factor that is often thought to inhibit cell growth. Further studies were performed to examine the potential role of TGF-β on breast cancer cell lines, TMT-081, MCF-7 and Hs578t.

3.D. Inhibition of breast cancer cells by TGF-β

[^{3}H]-thymidine incorporation assays were used to assay the growth inhibition of breast cancer cell lines, TMT-081, MCF-7 and Hs578t, in the presence of varying concentrations of TGF-β. Figure 5-1 demonstrates that TMT-081 and MCF-7 cell growth was inhibited by TGF-β over a range of concentrations (3 – 100 pM) while Hs578t cells were somewhat resistant to TGF-β growth inhibition. These findings suggest that the breast carcinoma cell lines are able to bind and respond to TGF-β by exhibiting varying degrees of growth inhibition.

3.E. Expression of TGF-β receptor by breast cancer cells

TGF-β_1 affinity labeling of breast carcinoma cell lines, TMT-081, MCF-7 and Hs578t, was done to determine if they express TGF-β receptors. TMT-081, MCF-7 and HS578t cells all appear to have TGF-β receptors that bind TGF-β_1 specifically as demonstrated by the competition of binding in the presence of excess non-radioactive TGF-β_1. In addition, TGF-β receptors on TMT-081 and Hs578t cells display a higher affinity for TGF-β than MCF-7 cells (Figure 5-2). TMT-081 and Hs578t cells have types I, II and III TGF-β receptors while MCF-7 cells lower levels of type I, II and III TGF-β receptors (Figure 6). These findings are consistent with the expression of TGF-β receptor

types I, II and III on early passage human skin fibroblasts and the CRL1904 cell line from a previous study published by Tam *et al.* (1998).

3.F. Identification of IGF-1 and EGF as tumor cell mitogens in lymph node stromal cell-conditioned media

To identify the stromal cell factors that were mitogenic for the breast carcinoma cells, a mAb-based depletion assay was used. This functional assay was chosen for its ability to indicate whether or not the growth factor under investigation is present and is contributing to the growth-promoting effects of the lymph node stromal cell-conditioned media. Results of these analyses are shown in Figure 7. Antibodies to IGF-1 and EGF had the most marked effect on the mitogenic activity of STA4- and STB12-conditioned media reducing it by 51 - 92 % (IGF-1) and 21 – 66.5% (EGF). Antibodies against PDGF-α and HGF had variable effects on breast carcinoma cell growth. These data suggest that IGF-1 and EGF are major mediators of the mitogenic effect of lymph node stromal cell-conditioned media.

3.G. Enhanced clonogenicity of breast carcinoma cells in the presence of stromal cell conditioned media

Soft agar cloning is used as an *in vitro* correlate of tumorigenicity. It was used here to determine whether STA4- and STB12-derived soluble factors alter the tumorigenic properties of breast carcinoma cells. It was found that incubation in the presence of stromal cell-conditioned media significantly increased the number of agar colonies for all tumors tested (Figure 8). MCF-7 cells had the most marked response with the number of colonies increasing 50 fold and 59.2 fold in the presence of STA4- and STB12-conditioned media, respectively. The effects on clonogenicity of TMT-081and Hs578t were increased by 3.4 and 5.2 fold and by 1.4 and 2.9 fold in the presence of STA4- and STB12-conditioned media, respectively. These findings indicate that STA4- and STB12-derived soluble factors enhance the tumorigenic properties of the breast carcinoma cell lines *in vitro*.

Figure 1: Isolation of morphologically distinct lymph node cells

Ninety-six well plates were pre-coated with 10ug/ml of rat fibronectin and stromal cells (passage > 10) were seeded into the wells at a density of 0.8 cells/well in 10% FCS RPMI. The clones were expanded in tissue culture plates, serum-free conditioned media were collected and tested for a mitogenic effect on breast carcinoma cells. The positive subclones, STA4 and STB12 were selected for further study.

STA4

STB12

Figure 2: Characterization of STA4 and STB12 lymph node cells

Immunocytochemistry was performed on STA4 and STB12 cells grown on chamber slides. The cells were fixed with either 1:1/acetone/methanol for intracellular markers or 3.7% formaldehyde for extracellular markers. Primary and secondary antibodies were incubated on STA4 and STB12 cells for 1 hour each. STA4 and STB12 cells were then visualized with an epifluorescence microscope. A) STA4: Vimentin$^+$ B) STB12: Vimentin$^+$ C) STA4: Fibronectin$^+$ D) STB12: Fibronectin$^+$ E) STA4: S-100$^+$ F) STB12: S-100$^+$ G) STB12: Laminin$^+$.

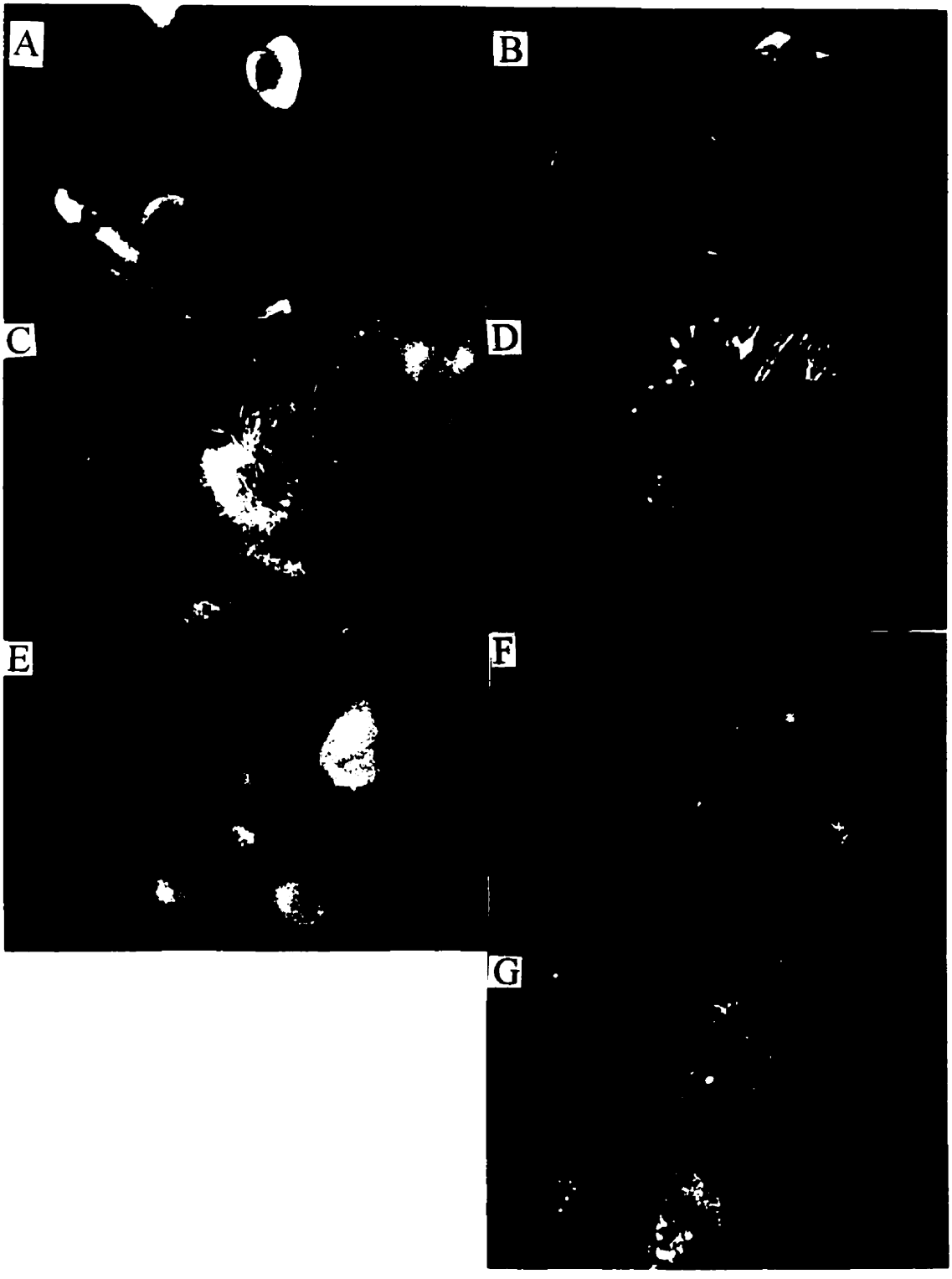

A
B
C
D
E
F
G

Figure 3: STA4- and STB12-CM causes increased proliferation of breast carcinoma cells

Breast carcinoma cells were plated in 96 well plates at a concentration of $5x10^3$cells/well with STA4 and STB12 CM. After an incubation of 48 hours, the wells were then pulsed with [^{3}H]-thymidine for 18 hours, plates were frozen then thawed to lyse the cells and [^{3}H]-thymidine incorporation was measured. Results are expressed as average fold stimulation relative to cells cultured in SFM.

Average (Standard Deviation) Fold Stimulation of Breast carcinoma cells in STA4- and STB12-CM

	STA4-CM	STB12-CM
TMT-081	4.2 (1.3)	2.98 (0.73)
MCF-7	3.31 (1.6)	3.07 (0.51)
Hs578t	4.08 (1.5)	3.84 (0.68)

Figure 4: Detection of growth factor mRNA in STA4 and STB12 cells by RT-PCR

Reverse transcription was carried out on 2 μg of total RNA extracted from STA4 and STB12 cells. The cDNA was then amplified for 35 cycles using primers for IGF-1, IGF-2, bFGF, EGF, HGF, TGF-α, TGF-β, PDGF-α and PDGF-β. PCR products were electrophoretically separated on a 1% agarose gel containing ethidium bromide. These RT-PCR assays were performed numerous times (>5 times for each type of growth factor mRNA) and representative growth factor mRNA bands were chosen for display. Negative control lanes repeatedly contained no bands (data not shown) and experimental lanes in which growth factor mRNA was amplified contained only one band at the expected molecular weight.

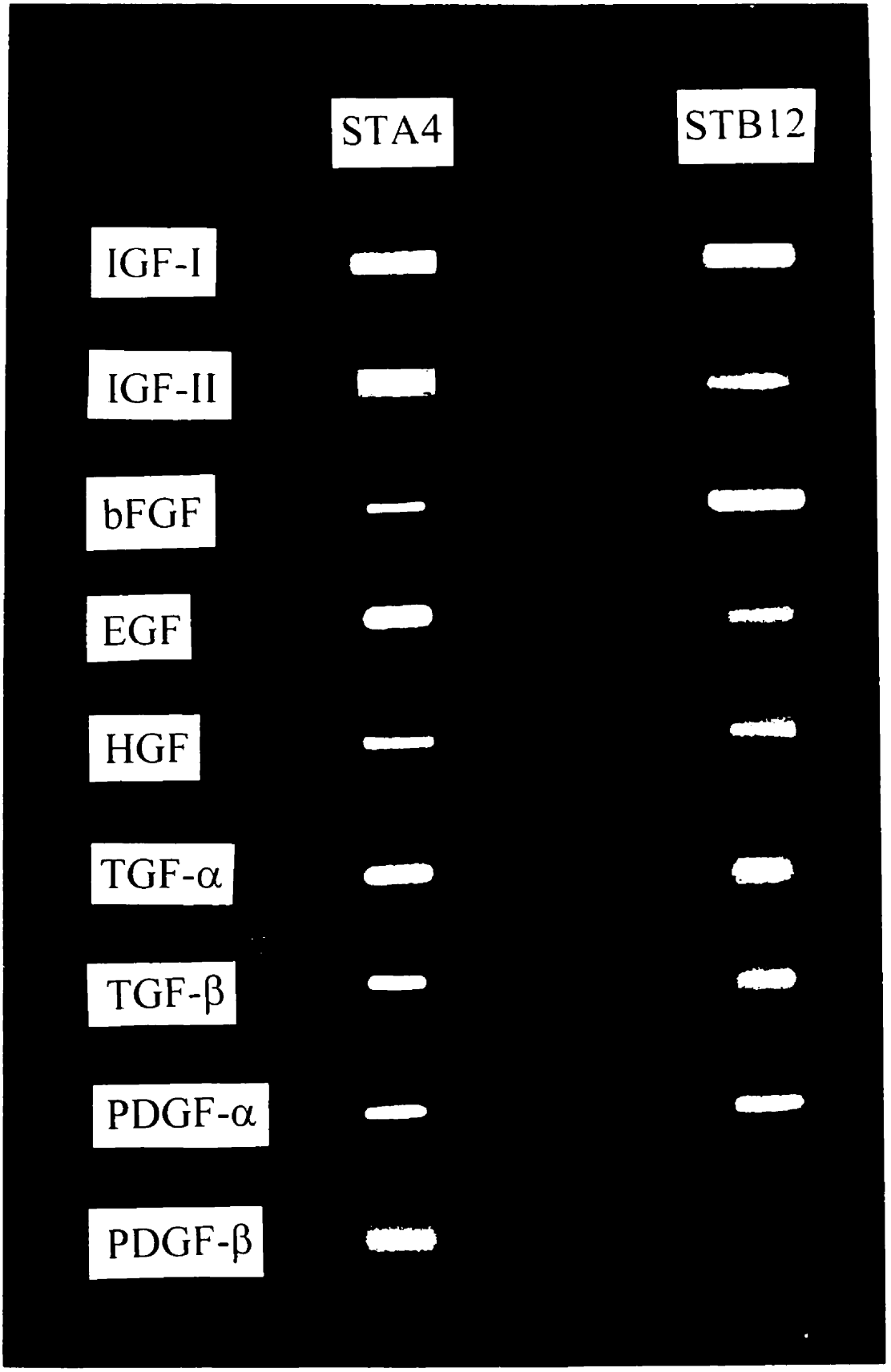
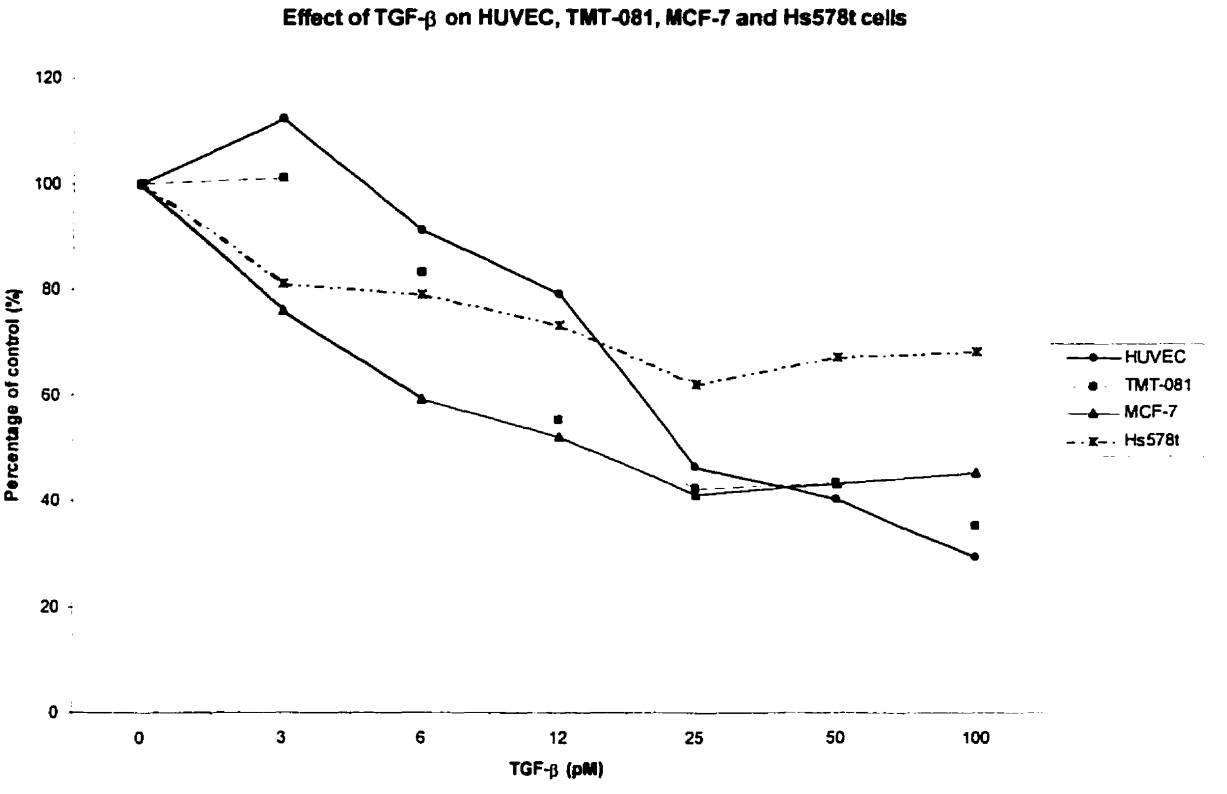

STA4
STB12
IGF-I
IGF-II
bFGF
EGF
HGF
TGF-α
TGF-β
PDGF-α
PDGF-β

Figure 5: Analysis of response of breast carcinoma cell lines to TGF-β

5-1) [^{3}H]-thymidine TGF-β assay. $5x10^3$ breast carcinoma cells were plated per well in a 96 well dish and incubated for 48 hours with several concentrations of TGF-β. The wells were then pulsed with [^{3}H]-thymidine for 18 hours, lysed by freezing and thawing and [^{3}H]-thymidine incorporation was measured. Results are expressed as a percentage of control [^{3}H]-thymidine incorporation. The results displayed are based on a representative experiment of a total of five performed. Standard deviations ranged from 2-36% (HUVEC), 5-13% (TMT-081), 1-7% (MCF-7) and 6-13% (Hs578t). 5-2) Ligand Binding Assay. Cell monolayers were washed with ice-cold binding buffer for 10 minutes and were labeled with 100 pM ^{125}I-TGF-β_1 in the presence or absence of 4 nM non-radioactive TGF-β_1 for 3 hours. Cells were then solubilized and radioactivity measured. Results indicate the reduction in radioactive TGF-β binding to TGF-β receptors due to competition from non-radioactive TGF-β.

5-1



5-2

Breast Carcinoma Cell Line	% Reduction in ^{125}I-TGF-β_1 Binding
TMT-081	85.4%
MCF-7	24.8%
Hs578t	81.7%

Figure 6: Breast carcinoma TGF-β receptor analysis

Tumor cell monolayers were labeled with 100 pM ^{125}I-TGF-β_1 in the presence or absence of varying concentrations of non-radioactive TGF-β_1 for 3 hours. Receptor-ligand complexes were crosslinked with BS3 and the reaction stopped by the action of glycine. Cells were solubilized, run on a 3-11% SDS-PAGE gel and results analyzed by autoradiography. Roman numerals I, II and III designate TGF-β receptor types I, II and III, respectively. Columns A, B (TMT-081), E, F(MCF-7) and I, J (Hs578t) were labeled with only radioactive TGF-β_1. Columns C (TMT-081), G (MCF-7) and K (Hs578t) were labeled in the presence of 0.5nM non-radioactive TGF-β_1. Columns D (TMT-081), H (MCF-7) and L (Hs578t) were labeled in the presence of 4 nM non-radioactive TGF-β_1.

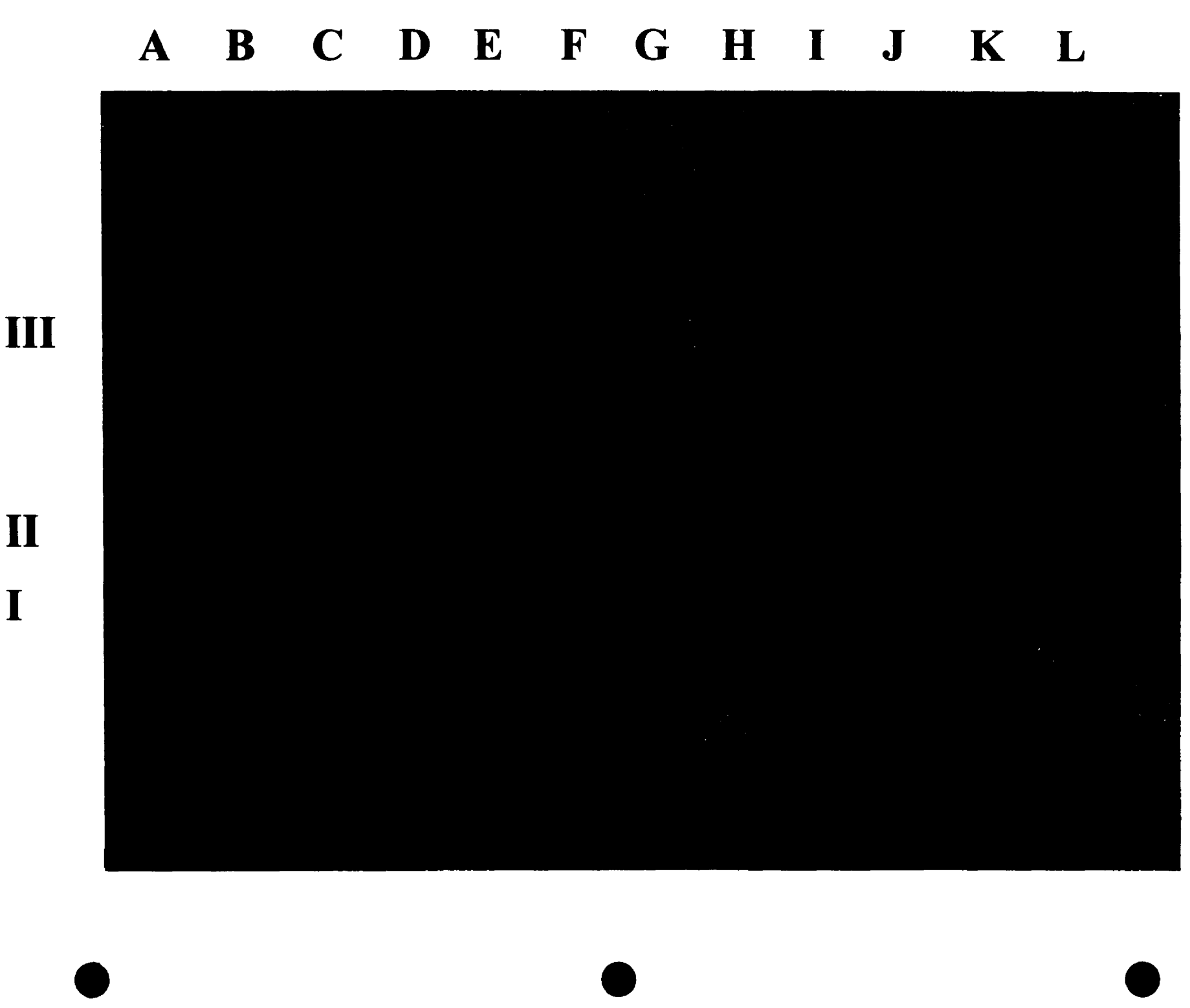
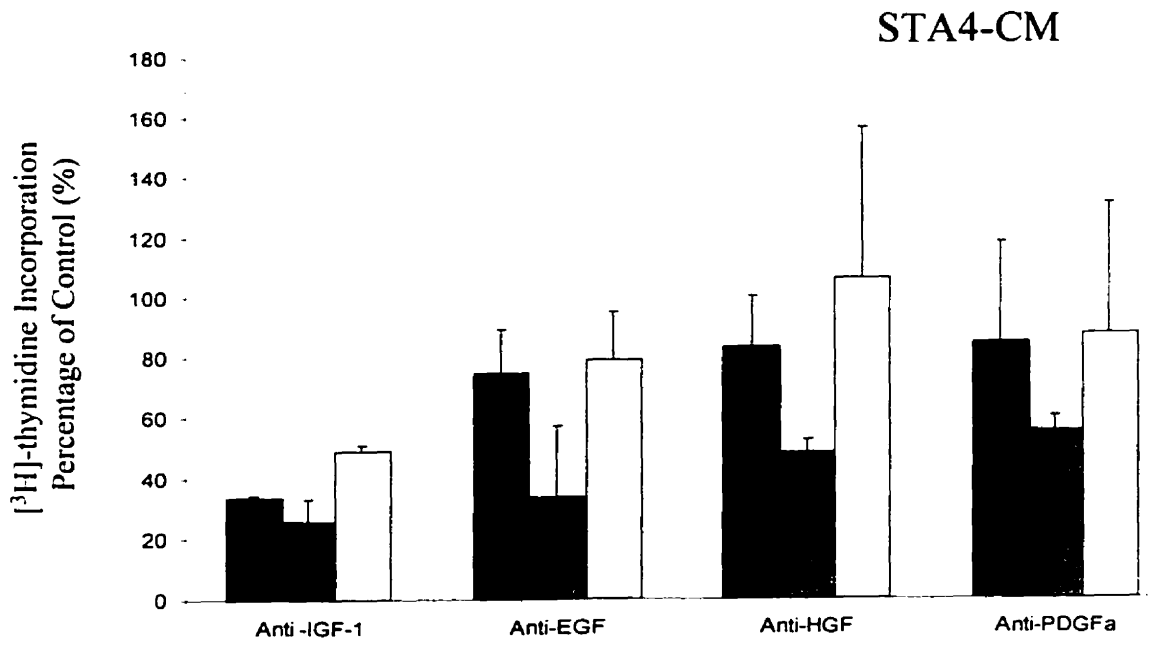

A B C D E F G H I J K L
III
II
I

Figure 7: Identification of IGF-1, EGF, PDGF-α and HGF in STA4- and STB12-CM

STA4- and STB12-CM were incubated with antibodies against IGF-1, EGF, PDGF-α and HGF for 24 hours with agitation. Protein A sepharose beads coupled to rabbit anti-goat IgG Ab were added to STA4- and STB12-CM containing the primary antibody for 18 hours. Protein A sepharose beads used with anti-PDGF-α primary antibody were not bound to any secondary antibody. Beads were removed by centrifugation and the supernatant tested immediately on breast carcinoma cells, TMT-081 (■), MCF-7 (▩) and Hs578t (□).


STA4-CM
[3H]-thymidine Incorporation
Percentage of Control (%)
180
160
140
120
100
80
60
40
20
0
Anti -IGF-1
Anti-EGF
Anti-HGF
Anti-PDGFa

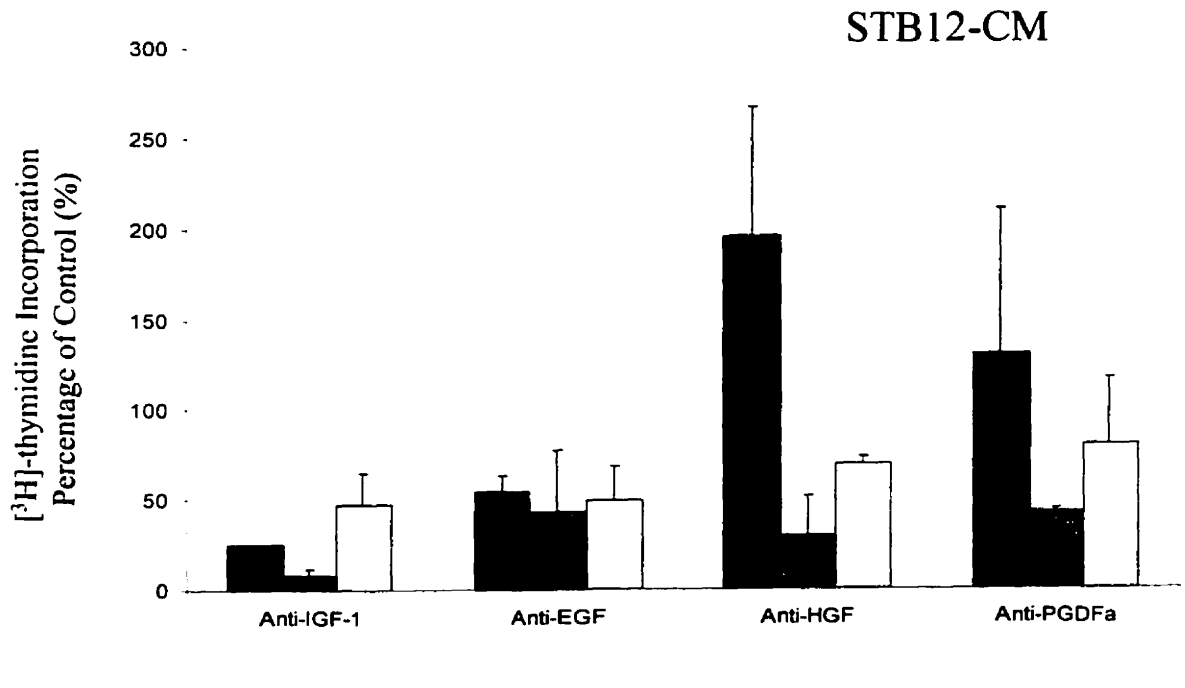
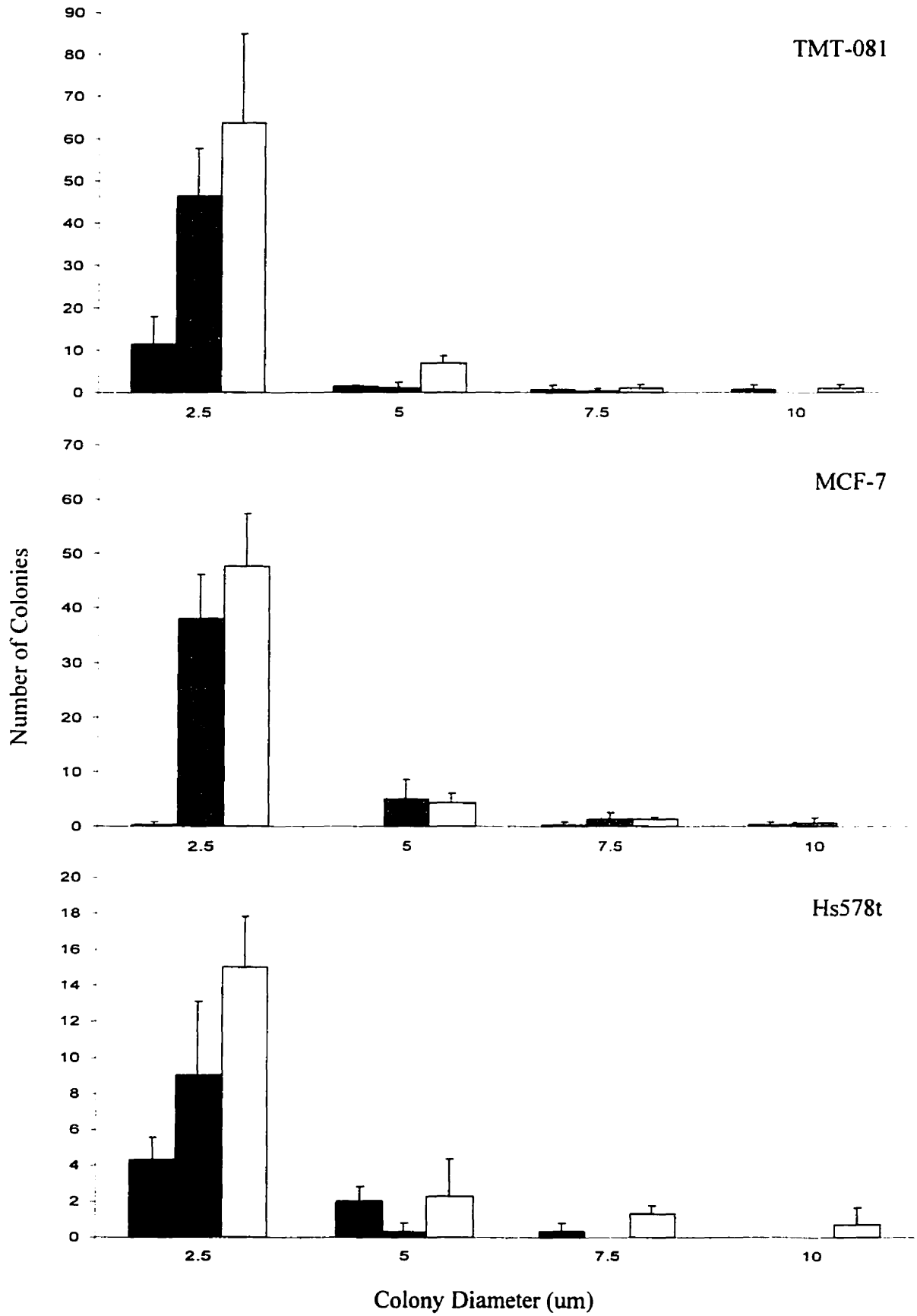

STB12-CM
[3H]-thymidine Incorporation
Percentage of Control (%)
300
250
200
150
100
50
0
Anti-IGF-1
Anti-EGF
Anti-HGF
Anti-PGDFa

Figure 8: STA4- and STB12-CM promotes anchorage-independent growth of breast carcinoma cells

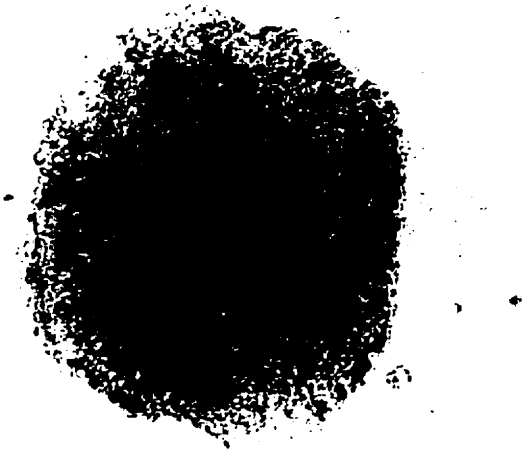
Breast carcinoma cells were seeded at a density of $1x10^4$cells/petri dish in semisolid agar and cultured for 30 days with STA4- and STB12-CM or RPMI supplemented with 1% FCS. 8-1) The results are expressed as number of colonies in 1% FCS + RPMI (■), 1% FCS + STA4 (▨) or STB12-CM (□). 8-2) Magnification 100x (10x ocular piece, 10x objective). A) MCF-7 cells cultured in 1% FCS + RPMI B) MCF-7 cells cultured in 1% FCS + STA4 CM C) MCF-7 cells cultured in 1% FCS + STB12 CM.


TMT-081
MCF-7
Hs578t
Number of Colonies
Colony Diameter (um)
90
80
70
60
50
40
30
20
10
0
2.5
5
7.5
10
18
16
14
12
8
6
4
2

A

B

C



Chapter 4

DISCUSSION

The first and most frequent site of metastatic growth for breast carcinoma are the axillary lymph nodes draining the primary tumor (Crossin *et al.*, 1998; Shetty, 1997). Lymph node metastasis is an important indicator of survival and prognosis in breast cancer (Gendreau and Whalen, 1999; Albertini *et al.*, 1996). Recent advances in lymph node staging using sentinel lymph node techniques are showing great promise while decreasing patient morbidity (Noguchi *et al.*, 1999; Krag *et al.*, 1998). Although lymph node colonization in breast cancer has been shown to occur sequentially (Kapteijn *et al.*, 1998), lymph node infiltration by tumor cells and the contribution of lymph node-metastasizing cancer cells to further tumor dissemination is still debated. Despite the lymph node's popularity as a staging tool, little is known about lymph flow through the node, stromal cell populations within the node or stromal cell function in metastatic disease. Based on previous studies (Tawil *et al.*, 1996; Brodt *et al.*, 1990), we postulated that the lymph node stroma is not a mere bystander in the process of lymph node metastasis, but may, in fact, impact on its progression (positively or negatively) through cell-cell interactions, possibly mimicking stromal-epithelial cross-talk in the primary tumor site. The present study demonstrates that peripheral lymph node-derived stromal cells can promote the growth and tumorigenic properties of breast carcinoma cells, providing the first line of evidence that the lymphatic stroma has the potential to enhance carcinoma cell growth. The results also show that different carcinoma cells vary in their response to stromal cell factors, a parameter which may play a role in the outcome of lymphatic colonization *in vivo*.

In the present study, two lymph node stromal cell lines, STA4 and STB12, were isolated and their effects on breast carcinoma cells, TMT-081, MCF-7 and Hs578t, studied. Immunocytochemical studies revealed that STA4 and STB12 cells are vimentin$^+$, fibronectin$^+$ and S-100$^+$ while STB12 cells also showed positivity for laminin. The presence of vimentin, fibronectin and laminin indicated that the two lymph node cell lines are stromal (Clark and Keating, 1995; Lerat *et al.*, 1993; Charbord *et al.*, 1990; McDonald, 1988). The S-100 protein is thought to be a marker of cells of dendritic origin (Sato and Dobashi, 1998; Uccini *et al.*, 1986; Carbone *et al.*, 1985) while the lack of cytokeratin and von Willebrand factor immunostaining suggest that STA4 and STB12

were not derived from epithelial or endothelial cells, respectively (Espinosade de los Monteros *et al.*, 2000; Penn *et al.*, 1993). Additional antibodies to stromal cell membrane antigens, fibroblast associated antigens and cytoskeletal proteins may be useful to further characterize these cells (Li *et al.*, 1995).

Tumor growth in the lymph node may depend on the same stromal factors that are important in the breast. STA4- and STB12-conditioned media stimulated breast carcinoma proliferation by up to 4 fold when compared to negative controls, suggesting that lymph node stromal cells secrete mitogenic soluble factors. Studies employing leukemia and lymphoma models have demonstrated that lymph node stromal cells can provide growth modulating and anti-apoptotic signals through cell-cell contact and via soluble factors (Peled *et al.*, 1996; Hanaoka *et al.*, 1995; Kataoka *et al.*, 1993; Tsuda, 1990; Ohkawa *et al.*, 1989). Similarly, conditioned media from breast fibroblasts have been shown to be mitogenic for several breast cancer cell lines (Ryan *et al.*, 1993). Adams *et al.* (1988) reported that fibroblasts from benign and malignant breast tumors produced conditioned media that stimulated breast carcinoma cell growth, whereas fibroblasts derived from reduction mammoplasty tissue were inhibitory. There is some discrepancy, however, as shown by experiments that found that all fibroblasts, regardless of the tissue source, stimulated breast carcinoma growth (van Roozendaal *et al.*, 1992).

To identify the growth factors mediating the mitogenic effect of lymph node stromal cell-conditioned media, RT-PCR analysis was first carried out. These studies demonstrated that STA4 and STB12 express RNA for a host of growth factors including IGF-1, IGF-2, EGF, bFGF, HGF, PDGF-α, TGF-α and TGF-β. In addition, STA4 cells express PDGF-β RNA. These growth factors are capable of eliciting many cellular responses such as mitogenesis, differentiation, transformation, chemotaxis, angiogenesis and motility (Pizzo and Poplack, 1997; Sabiston, 1997). A large body of evidence indicates that stromal-epithelial interactions in breast carcinoma are mediated by growth factors. Stromal cells can regulate tumor cell proliferation, migration and invasion through cell-cell contact probably involving the ECM (Petersen *et al.*, 1998; Donjacour and Cunha, 1991; Weaver *et al.*, 1996; Xie and Haslam, 1996) and through the

production of multiple factors including cytokines such as IL-6, IL-11 and Leukemia Inhibitory Factor (LIF) (Bhat-Nakshatri *et al.*, 1998; Crichton *et al.*, 1996) and growth factors such as insulin-like growth type 1 (IGF-1), IGF-2, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and transforming growth factors (TGF-α and -β) (de Jong *et al.*, 1998b; Giani *et al.*, 1996; van Roozendaal *et al.*, 1996; Spanakis and Brouty-Boye, 1995; Paik, 1992; Cullen *et al.*, 1991a; Rosen *et al.*, 1991). Breast carcinoma cells can, in turn, activate growth factor gene expression in stromal cells (Nakamura *et al.*, 1997; Ellis *et al.*, 1994).

Of interest is our finding that STA4 and STB12 cells express mRNA transcripts for TGF-β, a growth factor that is commonly thought to be growth inhibitory. Purified TGF-β exerted an inhibitory effect on TMT-081and MCF-7 cell growth as measured by [^{3}H]-thymidine uptake assays. However, as mentioned previously, STA4- and STB12-conditioned media were found to be mitogenic for all three breast carcinoma cell lines tested suggesting that the inhibitory effect of TGF-β was overcome. Ligand binding assays demonstrated that TMT-081 and Hs578t cells expressed type I, II and III TGF-β receptors that bind TGF-β specifically. MCF-7 cells were shown to express lower levels of all three TGF-β receptor types and bound its ligand with less affinity. This is consistent with another report showing that MCF-7 cells often gain resistance to TGF-β after long term passage in tissue culture due to inadequate TGF-β type II receptor expression (Brattain *et al.*, 1996). Also pertinent to the issue of TGF-β, are studies by van Roozendaal *et al.* (1996, 1995) which demonstrated that breast fibroblasts secrete predominantly biologically inactive TGF-β and, similarly to our results, its presence did not appear to interfere with the mitogenic effect of serum-free media conditioned by primary breast fibroblasts. It is conceivable that TGF-β concentrations in the conditioned media were too low to exert inhibitory effects and may have even been in the stimulatory range. Further studies examining TGF-β protein levels in lymph node stromal cell-conditioned media are, therefore, essential to more accurately determine its role. For example, heat-treatment of the conditioned media as described by Brown *et al.* (1990)

could yield data on the form (latent or active) of TGF-β protein secreted by STA4 and STB12.

While two of the breast carcinoma cell lines tested here were sensitive to the growth inhibiting effects of TGF-β, it is important to consider the effect of TGF-β *in vivo*. The role of TGF-β during breast cancer progression may change from an inhibitor of cell growth during the early stages of neoplasia to a potential promotor of cancer invasion and metastasis during later stages through enhancing angiogenesis, altering stroma formation, suppressing immune surveillance and altering integrin and matrix metalloproteinase expression in stromal cells (Dong-Le Bourhis *et al.*, 1998; Li *et al.*, 1998a; Jones *et al.*, 1997; Reiss and Barcellos-Hoff, 1997; Koli and Arteaga, 1996). This is supported by the observation that TGF-β production by primary breast cancers increases with advancing stages of the disease (Walker and Dearing, 1992).

Functional studies, in the form of growth factor depletion from lymph node stromal cell-conditioned media, were performed to identify the growth factors in lymph node stromal cell-conditioned media which stimulate breast carcinoma cell proliferation. Four growth factors were chosen for further investigation, namely, IGF-1, EGF, PDGF-α and HGF because of their established roles in stromal-epithelial interactions. Of these, IGF-1 and EGF were the most important mitogens for our breast carcinoma cells. This data is intriguing in view of the fact that IGF-1 has been identified as a major stromal-derived mitogen for breast carcinoma cells in stromal-epithelial interaction in the breast (Singer *et al.*, 1995; Ellis *et al.*, 1994; Cullen *et al.*, 1991b; Yee *et al.*, 1991a). The role of EGF in stromal-epithelial interactions remains unclear, however, EGFR overexpression has been implicated in epithelial cell transformation (Brandt *et al.*, 2000; Moller *et al.*, 1989). PDGF-α and HGF depletion from STA4- and STB12-conditioned media had variable effects on the breast carcinoma cell lines possibly reflecting differences in growth factor receptor expression among them. For instance, MCF-7 cells have receptors for IGFs, EGF, PDGF, FGF and TGF-β (Nurcombe *et al.*, 2000; Wosikowskia, 2000; Guvakova and Surmacz, 1999; Lee *et al.*, 1999; Yu *et al.*, 1998; Brattain *et al.*, 1996; De Leon *et al.*, 1992) while Hs578t has been documented as

expressing receptors to IGFs, EGF and TGF-β (Wickramasinghe *et al.*, 1996; Larsson and Blegen, 1994; Arteaga and Osborne, 1989). Characterization of growth factor receptors on TMT-081 cells has yet to be performed. Complicating the issue of variability of breast carcinoma cell responses to growth factor depletion is the fact that growth factors often act in concert to regulate growth. A single growth factor can also have very different effects depending on its concentration and the state of differentiation of the responding cell (Cross and Dexter, 1991). Thus, the depletion assay provides only clues as to which growth factors contribute to the proliferative effect of the conditioned media. However, the depletion assay does have the advantage of assessing whether growth factor mRNA is translated into protein and if it is functioning to regulate cell growth. Assays employing the depletion of combinations of growth factors may be necessary to address the issue of multiple growth factor actions such as synergism and potentiation.

The unique response of breast carcinoma cells to combinations of growth factors may underlie their ability to grow in different microenvironments and may ultimately govern their ability to metastasize and colonize secondary organs *in vivo*. Of particular interest to this study was the effect of STA4- and STB12-conditioned media on breast carcinoma clonogenicity. Anchorage-independent growth of the breast carcinoma cells in stromal cell-conditioned media was up to 50 fold greater than that of their negative controls. Therefore, these assays strongly suggest that lymph node stromal cell-derived soluble factors render breast carcinoma cells more tumorigenic by promoting their malignant phenotype. Many studies implicate growth factors and their receptors, such as IGF-1, EGF, HGF and TGF-β in the anchorage-independent growth of tumor cells (Chernicky *et al.*, 2000; Qiao *et al.*, 2000; Biscardi *et al.*, 1999; Dunn *et al.*, 1998; Ma *et al.*, 1998; Zhang *et al.*, 1998a; Saulnier *et al.*, 1996; Surmacz and Burgaud, 1995). For example, Osborne *et al.* (1990) demonstrated that blockade of the IGF-1R inhibits anchorage-independent growth of a panel of breast carcinoma cell lines. Similarly, a monoclonal antibody which blocks ligand interaction with EGFR was shown to significantly reduce anchorage-independent growth of tumorigenic mammary epithelial cells (Ma *et al.*, 1998). It is conceivable that the IGF-1 and EGF present in STA4- and

STB12-conditioned media which were shown to be important for the growth of breast carcinoma cells in monolayers may also be acting to promote breast carcinoma cell colony formation in soft agar. Further studies are necessary to identify the growth factors that mediate anchorage-independent growth.

The site of interaction between metastasizing tumor cells and lymph node stromal cells *in vivo* has yet to be defined. Potential mechanisms of cross-talk between cancer and stromal cells include direct cell-cell contact and/or the elaboration of soluble signals such as growth factors. Lymph node metastasizing tumor cells are thought to arrest in the subcapsular sinus. In support of this, Brodt *et al.* (1990) demonstrated that metastatic cells preferentially adhere to lymph node frozen sections and Whalen and Sharif (1992) demonstrated that the sites of preferential adhesion are the hilar and subcapsular sinuses of lymph node tissue sections. Lymph node sinuses are traversed by reticular fibers and are lined by reticulo-endothelial cells and fibroblastic reticular cells, all of which provide potential sites of tumor cell adhesion (Crivellato and Mallardi, 1997; Brodt, 1991). Observations by Kamperdijk *et al.* (1987) shed light on a possible route for soluble factor delivery between metastasizing tumor cells and lymph node stromal cells such as those which gave rise to STA4 and STB12 cell lines. While investigating the transport of lymph-borne immune complexes in lymph nodes, this group noted that the immune complexes first appeared in the subcapsular sinus either free or attached to the plasma membranes of several cell types including stromal cells. Subsequently, the complexes were seen at the periphery of lymphoid follicles and after one day, were trapped by follicular dendritic cells. It is possible that lymph borne growth factors derived from tumor cells trapped in the subcapsular sinus follow the same route of delivery into the lymph node stroma.

The course of lymph flow through the lymph node remains largely unknown, but, recently, Gretz *et al.* (1997) presented a model for the microanatomy of the lymph node and proposed a mechanism for the flow of lymph and cells. In light of evidence based on intralymphatic administration of molecular tracers showing that a functional barrier exists between the sinuses and the parenchyma, Gretz *et al.* reject the long standing

"percolation" model of lymph flow through the lymph node cortex. In its place, they propose a highly specialized conduit system, the paracortical cords, which delivers lymph-borne soluble factors from the sinuses to the abluminal surface of the HEV. According to this functional model of lymph flow, it is easy to envision delivery of soluble factors produced by tumor epithelial cells trapped in the subcapsular sinus to stromal cells of the paracortical cords. An extension to their model must be made to account for delivery of stromal cell-derived growth factors to the tumor cells. It is likely that the barrier between the sinuses and paracortical cords is compromised by tumor cell factors which either degrade the tissue themselves via proteases or upregulate protease production in lymph node stromal cells. Once this barrier is broken down, free exchange of fluids between the sinus and paracortical cord compartments would permit direct transmission of soluble factors between tumor cells and lymph node stromal cells. Immunohistological studies elucidating the spatial orientation of lymph node stromal cells and metastasizing tumor cells *in vivo* may help answer the questions of where and how these two cell types interact.

The findings presented in the current study bring into question the accepted view of the role of the lymph node in cancer progression. While only the effect of lymph node stromal cells on breast carcinoma cells was studied, it stands to reason that any type of metastasizing cancer cells may be subject to regulation by the lymph node stroma. It is also important to consider that long term culture of lymph node stromal cells *in vitro* may artificially alter their growth factor production, however, this perturbation of growth factor production may actually mimic *in vivo* conditions where tumor cells which infiltrate the node can generate an environment which activates stromal cells and induces them to secrete growth factors. The present study provides novel evidence in support of the concept that the lymph node is not just a passive filter where metastasizing breast carcinoma cells get trapped, but rather a dynamic microenvironment capable of selecting and promoting the growth of more aggressive tumor phenotypes. Further investigation of the interaction between metastasizing cancer cells and the lymph node stroma using molecular probes for growth factors and their receptors may lead to improvements in determining a patient's diagnosis and prognosis. For instance, a stromal reaction in the

form of growth factor production may help to identify micrometastases within lymph nodes such as the sentinel lymph node and reduce the incidence of false negative lymph node test results. Ultimately, stromal-epithelial interactions may serve as a predictive factor for tumor growth and patient survival. Once the mechanisms governing stromal-epithelial cross-talk are identified, they will provide potential molecular targets for the detection and elimination of lymph node metastasizing tumor cell.

Chapter 5

FUTURE STUDIES PROPOSED

1. Confirm the presence of growth factor proteins in lymph node stromal cell-conditioned media and quantitate their expression with immunoprecipitation and Western blot analysis.

2. Assess the effect of lymph node stromal cell-conditioned media on apoptosis in breast carcinoma cells.

3. Investigate whether breast carcinoma cells can induce the expression of growth factors in lymph node stromal cells and vice versa.

4. Examine whether lymph node stromal cells promote breast carcinoma growth *in vivo.*

5. Localize stromal cells within the lymph node using monoclonal antibodies developed against STA4 and STB12 stromal cells.

6. Determine the location of metastasizing breast carcinoma cells within the lymph node and their spatial relationship to the stroma.

Chapter 6

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