Studies of αvβ5 Integrin Functions in Human B Cell Precursors

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Abstract

The αVβ5 integrin is a member of integrin family that binds to different ligands such as vitronectin, fibronectin and soluble CD23 in order to mediate different biological responses such as cell growth, adhesion and metastasis. It is expressed by B cell precursors and by different acute lymphocytic leukaemia cell lines such as SMS-SB cells. This thesis is an attempt to explain how the sCD23- αVβ5 integrin interaction stimulates SMS-SB cell growth and to study the role of the αVβ5 integrin and other receptors such as PDGF receptor and CXCR4 in B cell development in the bone marrow. The maturation and differentiation of B-cells occur due to several factors that impact on gene expression in its development program. This program is divided into two main phases, the antigen-independent B-cell development phase and antigen-dependent B-cell development phase, respectively. The antigen - independent phase of B cell development starts from the pluripotent haemopoietic stem cell (PHSC) and progresses through several successive stages which are identified by somatic recombination and rearrangement of both heavy and light chain genes.

Soluble CD23 and LP (a synthetic peptide derived from soluble CD23) significantly stimulate SMS-SB growth while a smaller growth stimulation is caused by either SDF1-α or $PDGF_{AB}$. There are different signalling targets involved in the αVβ5 integrin-mediated proliferation due to its binding to either sCD23 or LP. These ligands enhance the association between the αVβ5 integrin and the PDGF receptor which promote the phosphorylation of both Jak2 and STAT5. Moreover, cell growth was reduced and the phosphorylation of Jak2 and STAT5 was also knocked down with using either PDGF receptor inhibitor (AG1295) or Jak2 inhibitor (AG490).

Both soluble CD23 and LP activate the STAT5-DNA binding and strongly increase its transcriptional activity. In addition, both ligands induce the phosphorylation of other different substrates such as STAT2, c-Src, c-yes and AMPKα2 which might be related to cell growth stimulation.

The αVβ5 integrin ligands also promote the phosphorylation of ERK1/2, p90RSK and activate a SRF transfected reporter gene. However, ERK1/2 and p90RSK phosphorylation was completely blocked by the specific MEK inhibitor (U0126). In similar context, SDF1-α stimulates the transcriptional activity of SRF but not STAT5 while $PDGF_{AB}$ does the opposite. Finally, soluble CD23 induces the proliferation of 697 and BAF03 which are other pre-B cell line models.

These data suggest that the αVβ5 integrin-ligated ligands stimulate SMS-SB cell growth by promoting different signalling pathways, mainly Jak2/STAT5 and MEK/ERK1/2 pathway.

Further work is required to determine the role of STAT5, p90RSK, c-Src and SRF in stimulating either the proliferation or apoptosis that promoted by the αVβ5 integrin-sCD23 interaction and to investigate the relationship between the activation of these targets.

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For my parents; thank you for everything

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Declaration

I hereby declare that the work presented in this thesis is my own, except where otherwise cited or acknowledged. No part of this thesis has been presented for any other degree.

Adel Alkhedaide

Abbreviations

AG 490	Tryphostin 490
AG 1295	Tryphostin 1295
AID	Activation-Induced Cytidine Deaminase
Akt	Auto- ja Kuljetusalan Työntekijäliitto
ALL	Acute lymphocytic leukaemia
AML	Acute myelogenous leukaemia
APAF-1	Apoptotic protease activating factor 1
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A gene
CLL	Chronic Lymphocytic leukaemia
CML	Chronic myelogenous leukaemia
c-Myc	Avian myelocytomatosis virus oncogene cellular homolog
CSR	Class switch recombinant
CVID	Common Variable Immunodeficiency
CXCR4	Stromal cell-derived factor 1
CXCR12	Stromal cell-derived factor 1 receptor
DCR	Dicer
EBV	Epstein Barr Virus

Elk-1	Ets-like transcription factor
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular signal-regulated kinase
Et-1	Endothelin-1
FAB	French American British system
FAK	Focal adhesion kinase
FITC	Fluorescein isothiocyanate
HCL	Hairy cell leukaemia
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIM	Hyper-IgM syndrome
HRP	Horseradish peroxidase
IFN-γ	Interferon- γ
JAK	Janus kinase
JH	JAK homology domains
JNK	c-Jun N-terminal kinase
LP	Long peptide
LSC	Lymphoid stem cell
MAPK	Mitogen – activated protein kinase
MEK	MAPK/Erk kinase
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major histocompatibility complex

MOPS	3-(N-morpholino)propanesulfonic acid
MSK	Mitogen- and stress- activated kinase
NFκB	Nuclear factor-κB
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PE	Phycoerythrin
PFHM	Protein-free hybridoma medium
PHSC	Pluripotent haematopoietic stem cell
PI3K	Phosphatidylinositol 3 – kinase
qRT-PCR	Quantitative real time polymerase chain reaction
Raf	(v-raf) murine leukemia viral oncogene homolog
Ras	Rat Sarcoma (Guanine-nucleotide-binding protein)
RGD	Arg-Gly-Asp
RIPA	Radioimmunoprecipitation assay
RKC	Arg-Lys-Cys
RSK	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SAP-1	Stomach cancer-associated protein-tyrosine phosphatase-1
SCF	Stem cell factor
sCD23	Soluble CD23

SDF1	Stromal cell-derived factor 1
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFKs	Src-family kinases
SH2	Src-homology 2 domain
shRNA	small hairpin RNA
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SRE	Serum response element
SRF	Serum response factor
STAT	Signal transducers and activators of transcription
TAD	Trans-activation domain
TBE	Tris-borate-EDTA
TBS	Tris-Buffered Saline
TBS/T	Tris-Buffered Saline with Tween 20
TCF	Ternary complex factor
TNF	Tumour necrosis factor
T-PLL	T-cell prolymphocytic leukaemia
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very Late Antigen-4
Vn	Vitronectin

WHO	World Health Organization
XIAP	X-linked inhibitor of apoptosis protein
XLA	X-linked agammaglobulinaemia

1 Introduction

1.1 B Lymphocytes

1.1.1 Introduction

The immune system is divided into two major systems, the humoral and cell- mediated immune system. Both of these classes are linked together such that each one works optimally with the other [1]. Cellular compartments of the immune system can be classified into two main groups based on their lineages; the myeloid lineage which includes monocytes, macrophages, erythrocytes, megakaryocytes, and the lymphocyte lineage which produces B or T lymphocytes [2]. The B lymphocyte is one of the major members of the immune system which plays a key role in the humoral immune system due to its ability to produce and release a vast number of immunoglobulins after its activation and differentiation into a plasma cell. B refers to Bone marrow where the generation and maturation of B cells takes place in humans. B cells, like other cellular compartments of the immune system, are generated from hematopoietic stem cells. The lineage of B-lymphocytes begins in foetal liver and B-cell lymphopoiesis takes place in bone marrow in all mature mammals [3]. It is derived from the pluripotent haematopoietic stem cell (PHSC) similar to any other blood cellular compartments [3]. The maturation and differentiation of B-cells occur due to several factors that impact on gene expression in its development program [3]. This program is divided into two main phases, the antigen-independent B-cell development phase and antigen-dependent B-cell development phase, respectively [3].

1.1.2 Antigen-independent B-cell development phase

The antigen - independent phase of B cell development occurs in the bone marrow, starting from the pluripotent haemopoietic stem cell (PHSC) and progresses through several successive stages [4]. B-cell developmental stages in this phase are characterized by somatic recombination and rearrangement of both heavy and light chain genes [5]. The earliest step of B-cell development in this phase starts with differentiation of the PHSC into lymphoid stem cells (LSC), which are also called $CD34^+$ bipotential lymphoid stem cells [5]. Under the effect of certain transcriptional factors such as PU.1, Ikaros and E2A, LSCs become either a T-cell precursor which is identified by expressing CD2 and CD7, or a B-cell precursor which is identified by expression of CD19 [6,7,8,9]. The first B lineage – committed progenitors are called pro-B cells and are characterized by the presence of both CD45R and CD43 on their plasma membrane outer surface (figure 1.1) [6]. In addition, late pro – B cells are characterized by the presence of cytoplasmic heavy chain protein of the μ class as well as CD19 (figure 1.1) [6,7]. Almost all of these markers are Ig heavy chain rearrangement related [6,7]. The process of Ig heavy chain rearrangement is completed by joining V_H to DJ_H at the end of pro-B cell maturation stage which leads to expression of μ chain on the cell surface as a part of pre-B cell receptor (pre-BcR) which appears as a marker of the transition from pro-B cell to pre-B cell (figure 1.1) [10,11]. During the early stages of pre-B cell maturation, cells are large and identified by CD43 down-regulation as well as $V_{pre\text{-}B}$ /λ5 surrogate light chain expression (figure 1.1) [6,10].

At this stage, cells undergo a rapid proliferation process which leads to increased μ chain expression on the outer plasma membrane surface [5]. Consequently, the dramatic increase

of proliferation increases the opportunity of μ chain attachment to IgL chains, which is essential for further maturation, signalling and processing [5].

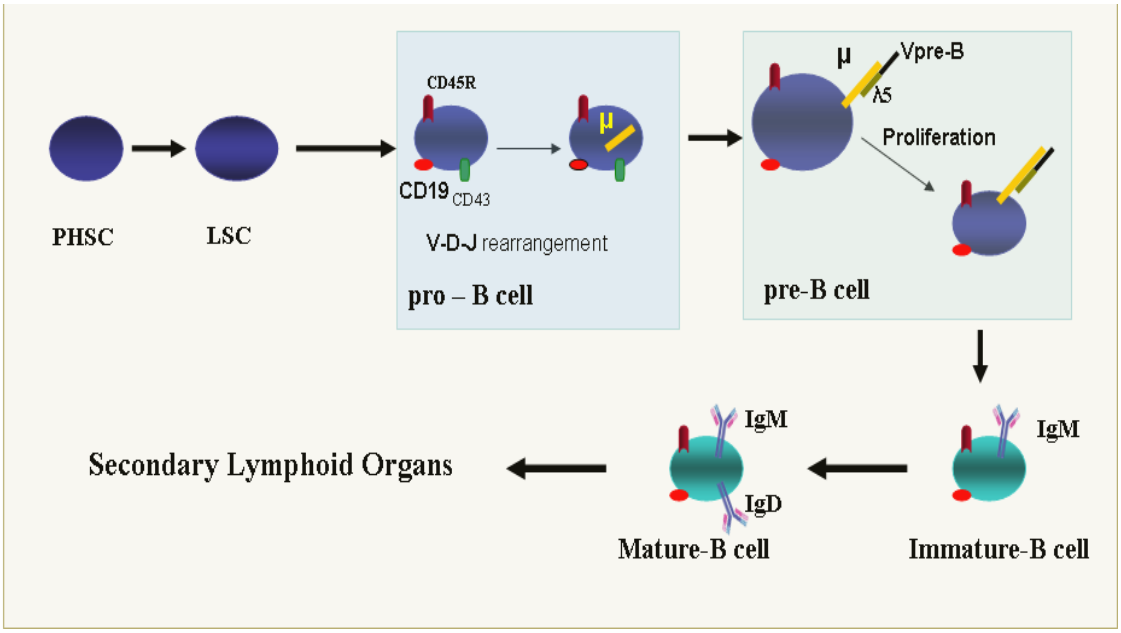


Figure 1.1 Antigen dependent B-cell development. B-cells are derived from a Pluripotent haematopoietic stem cell which is converted to a lymphoid stem cell (LSC). Due to the D_H,J_H locus rearrangement, the LSC is converted to early pro B-cell stage which is characterized by CD19,CD45R and CD43 expression. At the late pro B-cell stage the cytosolic μ heavy chain starts to appear as a phenotypic reflection of V_H-D_H-J_H rearrangement process. Consequently, these alterations lead to conversion of pro B-cell into Pre B-cell which seems morophogically large and characterized by CD19, CD45R and μ heavy chain – light chains (as the V_{pre}-B /λ5 surrogate). These cells proliferate and convert to small pre B-cells which in turn convert to Immature B- cells which express IgM on their surface. The last stage of this process is called the mature B-cell and expresses IgM and IgD and migrates to the secondary lymphoid organs such as spleen and germinal centres.

In general, the Pre-B cell stage is characterized by the presence of several markers such as $CD45R^+$, $CD43^-$ and IgL locus re-arrangement during the small late pre-B cell stage (figure 1.1) [6]. Light chain gene rearrangement and complete IgM expression on the cell surface are important markers that define the immature B cell stage [6,11]. After that, immature B cells undergo further differentiation and other gene rearrangement processes which lead to expression of both IgM, IgD and CD23 as well as CD45R on the cell surface [6,11]. The B cell is now ready to leave the bone marrow and migrate into peripheral lymphoid tissue such as spleen and lymph nodes [6,11].

1.1.3 Antigen-dependent B-cell development

The lifespan of mature B-cells in the secondary lymphoid tissues and circulation system is between several weeks to several months and to maintain the quantity of B-cells in both systems the newly-developed B-cells are eventually replaced [12].

In this phase, B-cell activation occurs by two different processes, T - cell - dependent and T-cell - independent development. In fact, the immune responses require both processes [12]. B-cell activation starts by the binding of B-cell receptor (BcR) to the specific antigen either via cognate recognition and T-cell help or through cross - linking of the BcR by antigens [1,12]. T-cell independent development occurs without MHC class II- restricted T-cell help and leads to a response to a small number of antigens [1].These thymus-independent antigens are resistant to degradation processes and can be classified into two categories depending on their mode of activation. The first category is bacterial cell wall components which belong to Gram-negative bacteria and all of them are lipopolysaccharides, while the second category is large polysaccharide molecules. The activation of B cells by the first group requires high antigen concentrations. However, the second group can activate B cells by using non-cognate T-cell help such as cytokines [1,12].

In general, T-independent antigen stimulation occurs through several signal transduction molecules such as TNFα, CD19, HS1 protein, Lyn, IL-5Rα as well as lymphotoxin α. On the other hand, in the T- dependent antigen development process, B cells undergo proliferation, differentiation and activation by the interactions between T-cells and B-cells. These interactions require the help of T helper cells (T_H) and sets of complementary cytokines [12].

1.1.4 The importance of non-lymphoid stromal cells of the bone marrow for B cell development

Isolated stem cells cannot differentiate into B cells in culture unless grown in the presence of bone marrow stromal cells which provide several important factors [11]. One of these factors is the ability of stromal cells to provide specific adhesive contacts with the B cell during all developmental stages, using particular cell-adhesion molecules and ligands [11]. Moreover, several essential soluble growth and differentiation factors are provided by stromal cells. Stem cell factor (SCF) is one of these factors which is mandatory for B cell development [11]. SCF is a member of c – kit ligand family which interacts with a tyrosine kinase receptor on the outer membrane surface of B cell precursors [11]. Another important factor is the chemokine CXCL12, also called Stromal cell-derived factor 1 (SDF1), which stimulates the proliferation of both pro-B cells and pre-B cells, and is required for the maturation of B-cell precursors [11]. In mice, both pro-B cells and pre-B cells require interleukin 7 (IL-7) produced by bone marrow stromal cells [12]. In vitro, interleukin 7 shows an effective role in cellular proliferation, survival and development. However, in humans there is no clear evidence about the roles of IL-7 in any of these processes [12].

1.1.5 Disorders caused by defects during antigen – independent B-cell development

1.1.5.1 Primary immunodeficiency

1.1.5.1.1 X- linked agammaglobulinaemia (XLA) :

XLA is one of the primary immunodeficiency disorders and is caused by a Bruton's tyrosine kinase (BTK) gene mutation [4]. This mutation occurs in X-linked agammaglobulin

(XLA) and all XLA patients have a deficiency in the percentage of normal peripheral blood B cells as a result of blocking the transition of pro- B cells to the large pre- B cells due to the mutation. BTK is a major player in signalling downstream of pre-BcR, as well as having a role in BcR activation, primarily by stimulating calcium flux [4]. In fact, BTK gene mutations lead to changes either in BTK protein folding or stability [4].

1.1.5.1.2 Common Variable Immunodeficiency (CVID) :

Individuals with this disease exhibit a low level of plasma Ig and they are susceptible to infections as well as having a variable reduction in memory B cells, class switch recombination (CSR) and B cell activation [4]. This disease is characterized by several mutations such as in the activated T cell stimulatory molecule ICOS, CD19 and TNFα receptor [4].

1.1.5.1.3 Hyper- IgM syndrome :

Hyper-IgM syndromes are humoral immunodeficiencies. Patients who are suffering from this disease have elevated serum IgM and decreased IgD and IgA serum levels together with recurrent infections [13]. This disease is characterized by several mutations and there are five types of it defined so far. HIM-1 is the most common, being an X-linked mutation in CD154 [4]. Furthermore, the defect of CD40 signalling is considered as the major reason for the disease development, because due to this defect B-cells do not recognise T-cells which blocks Ig class switch recombination (CSR) in B-cells [14]. The second type is HIM-2, a very rare type, which is an autosomal recessive inherited syndrome [15]. This type is characterized by deficiency of Activation-Induced Cytidine Deaminase (AID) which in turn leads to a

CSR defect [16]. HIM-3 is caused by mutation of the CD40 gene as well as one of the Activation-Induced Cytidine Deaminase genes (AICDA) [16,17]. The last two HIM-4 and HIM-5, are both CSR defects but have different mutations to the others [17].

1.1.5.2 Autoimmunity

Within the B-cell lineage there are several checkpoints which control B- cell selection, both in the bone marrow and the peripheral lymphoid tissues [4]. The activation or the inhibition of B-cells, which depends upon both T-cell stimulation and the B-cell itself, requires balance between the activating signals to protect our bodies from the invasive pathogens [18]. On the other hand, the imbalance between those signals could lead to either massive infections or autoantibody production and autoimmunity as a result [18].

Autoantibody production is believed to be a natural process of the adaptive immune response that arises as a consequence of the imbalance between the activating and the inhibiting signals, for example, in rheumatoid arthritis, an autoimmune disease [19]. Recently, the contribution of B-cells to several autoimmune diseases in the human being has become clear [4,19]. In this regards, the B-cell contributes to autoimmune diseases in different ways, for example by producing autoantibodies such as in systemic lupus erythematosus (SLE), by presenting autoantigen to T-cells or by producing proinflammatory cytokines [19,20].

1.1.5.3 Leukaemia and lymphomas

Several haematological and immunological studies have shown both cytogenic and molecular genetic abnormalities in malignant B-lineage cells [4]. In follicular lymphoma, the anti-apoptotic Bcl-2 gene is translocated to chromosome 14 and falls under the influence of the enhancer element (Eμ) of the Igh locus [21]. In addition, clonal Ig gene rearrangements are implicated in Hodgkin's lymphoma [22]. The second part of this review will give more details about the acute lymphocytic leukaemias.

1.2 Leukaemia

1.2.1 Introduction

Leukaemia is a Greek term; Leukos means white or clear and heima means blood. In general, Leukaemia is a definition of blood cancer which means an abnormal and uncontrolled growth of blood cells in bone marrow [23]. It occurs in either mature or immature cells [23]. Generally, there are three major classes of blood cancer; leukaemia, lymphoma and multiple myeloma. Both leukaemia and lymphoma affect lymphoid cells but there are several essential pathological differences between them. Leukaemia usually occurs in bone marrow during the lymphoid cell maturation process, while lymphoma originates in lymphatic system causing enlargement the affected tissues and is generally a solid tumour [24].

There are four major types of leukaemia which are classified according to the cell type and mode of progression :

1. **Acute lymphocytic leukaemia (ALL)** occurs in either T or B lymphocytes. In this type of leukaemia, the cells, mainly immature lymphocytes, grow rapidly [25]. Most recorded cases are children but it is also affects adults [25]. The cell line model used in this study is called SMS-SB cells which was established from the bone marrow of nine year-old girl suffering from acute lymphocytic leukaemia. These cells are B-cell precursors roughly between Pro-B cells and large pre-B cells.
2. **Chronic Lymphocytic leukaemia (CLL)** occurs in either T or B lymphocytes [26]. It is called chronic because cells grow slowly; the progression is different from patient to patient but generally takes months to years and the majority of affected

cells are mature B lymphocytes [26]. Chronic leukaemia is common in older people but it is found in different age groups [25].

3. **Acute myelogenous leukaemia (AML)** occurs in myeloid cells and it is common in adult males but can also occur in children and females [27]. In this type of leukaemia, cells grow rapidly and the bone marrow becomes very congested. There are different subtypes of AML such as acute megakaryoblastic leukaemia, acute promyelocytic leukaemia and acute myeloblastic leukaemia [27].
4. **Chronic myelogenous leukaemia (CML)** occurs in myeloid cells, mainly in adults and can occur in children though that is very rare. It is characterized by slow progression and there is one subtype of this leukaemia called chronic monocytic leukaemia [28]. Clinically, 95% of diagnosed cases were associated with BCR/ABL chromosomal translocation (Philadelphia chromosome) [29].
5. **Other types of leukaemia;** there are some other types of leukaemia such as **Hairy cell leukaemia (HCL)** which occurs mainly in adult males and is easy to treat, **T-cell prolymphocytic leukaemia (T-PLL)** which affects mature T-cells and mainly in adults. This type of leukaemia is very rare, occurs in men more than woman and is very aggressive [25]. **Large granular lymphocytic leukaemia** which affects either Natural killer cells or T-cells, is very rare and not aggressive [25]. **Adult T-cell leukaemia** is a viral infectious disease caused by HTLV. This virus infects $CD4^{+}$T-cells and makes them proliferate abnormally [25].

1.2.2 Acute lymphocytic leukaemia (ALL)

Acute lymphocytic leukaemia is a malignant disease occurring in lymphoid cell precursors and can affect either adults or children [30]. Clinical statistics show that the most common cases arise between the ages of 2 to 5 years. In addition, there is a promising development of treatment where more than 80% of cases have been cured using different kinds of regimens containing vincristine, cyclophosphamide, doxorubicin or methotrexate which also depends on genotype, phenotype and risk assessment [31, 30].

1.2.2.1 Acute lymphocytic leukaemia classification

There are different systems to classify ALL according to different biological and clinical features. Clinically, the most common classification used is called French American British (FAB) system which classifies ALL according to a number of biological features [32 ,33]. The main purpose of this system is to simplify the selection of treatment procedures including the regimen components, dose and the treatment period. In this system, ALL is classified into three primary sub-types according to size and shape of the leukaemic cells [32 ,33].

L1 is the most common in children. Morphologically, lymphoblasts are small with normal nuclear shape and are either T-cells or B-cells [34].

L2 occurs in adults more than children. In this sub-type, the affected lymphoblasts are T-cells or B-cells and they are immature and pleomorphic. Cells look large with cytogenetic and nuclear shape alterations [34].

L3 also called Burkitt's leukaemia/lymphoma which represents the least percentage of recorded cases. The affected lymphoblasts in this subtype are large with regular nuclear shape [34].

Recently, the World Health Organization (WHO) has classified the ALL subtypes into three different subgroups according to cytogenetic and immunophenotypic characterization as the following [35]:

1. Acute lymphoblastic leukaemia/lymphoma [mixture of FAB L1/L2]. This type includes:

a). Precursor B acute lymphoblastic leukaemia/lymphoma which are also in turn classified into four cytogenetic subtypes:

I. t(12;21)(p12,q22) TEL/AML-1

II. t(1;19)(q23;p13) PBX/E2A

III. t(9;22)(q34;q11) ABL/BCR

IV. T(V,11)(V;q23) V/MLL

b). Precursor T acute lymphoblastic leukaemia/lymphoma.

2. Burkitt's leukaemia/lymphoma [FAB L3].

3. Biphenotypic acute leukaemia [35].

The FAB classification system is broadly used for clinical diagnostic and treatment, whereas the WHO system could be used for either scientific research or clinical purposes.

1.2.2.2 Chromosomal abnormalities

In many developing leukaemic cells, there are detectable chromosomal alterations such as translocation, inversion, deletion or addition [30]. Nowadays, chromosomal translocations are useful for identifying and diagnosing the subtype of acute lymphocytic leukaemia [30]. Biologically, these translocations lead to activation of certain transcription factors which in turn control genes that control, either directly or indirectly, cellular differentiation, proliferation or survival [30]. About one fourth of recorded cases of acute lymphocytic leukaemia which occur during childhood are related to the TEL/AML-1 fusion protein, a consequence of translocation between chromosome 12 and 21, t(12;21)(p12,q22) [36,37]. In mice, both Tel and AML-1 genes have a central role in haemopoietic-cell maturation [38]. However, the TEL/AML-1 fusion protein is believed to be a pre-leukaemic cell inducer as it is responsible for the alterations of self-renewal processes as well as changes in survival and proliferation modes [39]. In adults, more than 30% of ALL recorded cases are related to chromosomal translocation between chromosome 9 and 22 which is called the Philadelphia chromosome (figure1.2) [30,40]. This translocation leads to fusion of the BCR protein to ABL, a non-receptor kinase, which in turn leads to downstream signaling events; the best known target pathway is the Ras-Mek-Erk signaling pathway which is considered to be involved in the regulation of genes that control cell survival, differentiation and proliferation [40].

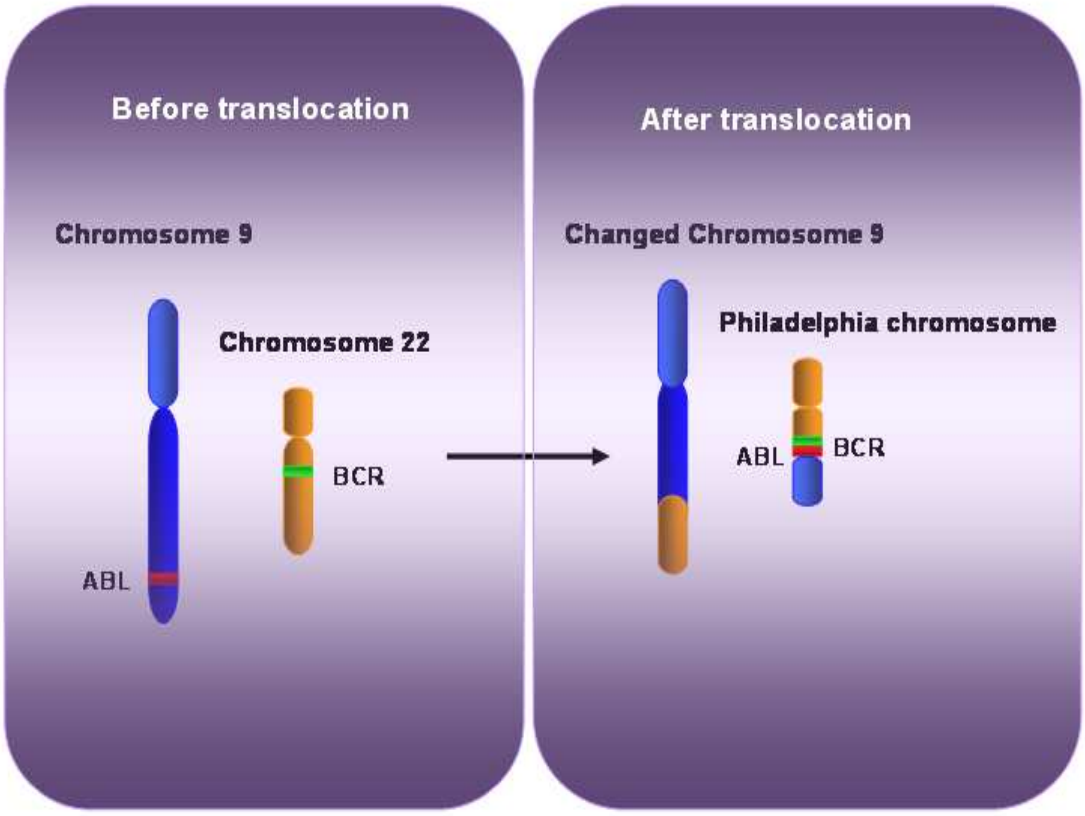


Figure 1.2 Philadelphia chromosome. One of the common chromosomal translocations found in 95% of CML cases, 30% in adult and 5% in pediatric ALL patients. The translocation occurs between chromosome 9 and chromosome 22, t(9;22) (q34;q11) ABL/BCR. This translocation produces ABL/BCR fusion protein which underlies the cell survival [29].

One of genetic lesions which induce many cases of B-cell progenitor acute lymphoblastic leukaemia is epigenetic silencing of cyclin-dependent kinase inhibitor 2A gene (CDKN2A) which encodes either the tumour suppressor p16 or p14 [37]. In the same context, several published analyses reveal a link between the alteration of the PAX5 gene and the onset of B-cell precursor lymphoblastic leukaemia [41]. Furthermore, some genetic studies identified some kinds of chromosomal deletions in different cases of B-cell precursor acute lymphoblastic leukaemia such as IKZF3, IKZF1, LEF1,TCF3 (E2A) AND EBF1 (EBF) [42].

1.3 Integrins

1.3.1 General Overview

Integrins are membrane glycoproteins which play several vital roles in communication processes between intracellular and extracellular environments by mediating various intracellular signals. In addition, integrins play a major role in the regulation of cellular shape, mobility and the cell cycle [43]. Moreover, integrins attach the cell to the tissue and are capable of revealing the status of the cell to the surrounding environment [44]. This may reflect on the main function of whole organ or tissue, such as the role of GPIIb/IIIa in platelets and the coagulation process [44].

In fact, integrins have been found in all mammals. There are many kinds of integrins and many cells have multiple types of integrin on their plasma membranes [44]. So far, 18 α subunit genes have been identified and 8 β subunit genes [45]. These subunits can form 24 different kinds of integrin receptors that mediate a large spectrum of cellular functions and behaviours [45].

1.3.2 General structure of integrins

In general, integrins are heterodimers composed of two distinct types of polypeptide chains, the α and β subunits [43]. These subunits have numerous forms which cause the variety of integrins; for example, the αV subunit forms a heterodimer with β1, β3, β5, β6 and β8 [43]. The α and β subunits have a short intracellular domain of about 40 – 70 amino acid residues, with the exception of the β4 subunit which has an intracellular domain of 1088 amino acid residues [45]. On the surface of cell, the extracellular domain of both the

α and β subunits lie close together and the N – terminal regions of each chain form a binding region for the extracellular matrix [46]. The extracellular portion of α subunits contains about 1104 amino acid residues while the extracellular portion of β subunit is composed of about 778 amino acid residues [47]. So far, we could say that the molecular weights of integrin subunits are different from one to the other but generally they are between 90 kDa – 160 kDa [48]. The N-terminal regions of αVβ3 come together to form a globular head domain that generates the ligand binding site [47]. This binding site head is linked to the membrane by a stalk which as a length of about 170Å [47]. Some mammalian α subunits have an extra 190 amino acid domain which is called the I domain (also known as vWFA domain) [49]. Moreover, most β subunits can associate with more than one α subunit; for instance, β1 is found in association with 11 different α subunits [50]. Therefore, there are several systems for classification and characterization of integrins, but the most of common one is αnβn where n is the subunit number [51]. The integrin subunits dimerization underlies the integrin binding characteristics which in turn control the function of the integrin [50]. For instance, α1β1 and α1β2 both bind to collagens or act as cell – cell adhesion molecules [50, 51]; indeed, almost all of the β2 family are involved in cellular adhesion processes [50].

Both α and β subunits contain several cations and the number of these cations depends on the presence of acidic amino acids in their structures [48]. The role of the cations in the α subunits still unknown, but they may play a role in the stabilization of the folds of protein [46]. However, the cations in the β subunits are involved in integrin – extracellular matrix interactions [47]. In addition, β subunits have four cysteine – rich repeated sequences that may play a major role in that interaction [45].

1.3.3 General functions of integrins

There are two major functions of integrins; attachment of the cell to the extracellular matrix and transduction of the signals between extracellular matrix and cytosol [52]. Moreover, integrins are involved in many biological activities, including binding of viruses, cell migration, immune activation, etc [51,52].

1.3.3.1 The role of the integrin family in cellular attachment

The extracellular head domain of the heterodimer or in some cases the extracellular portion of the individual subunit, and particularly the α subunit, contains the ligand binding sites [53]. The most well characterized target motif is an Arg-Gly-Asp (RGD) sequence which is present in many different adhesive ligands such as vitronectin and fibronectin [53]. In general, integrins bind their ligand in the presence of divalent cations such as Ca^{2+}, Mn^{2+}or Mg^{2+} [54]. However, this cationic binding dependence varies between the integrin family members. For example, magnesium enhances the binding of αVβ1 and α2β1 to their ligands but calcium does not, while the binding of αVβ3 to vitronectin can use either cation [55]. Several functional proteins which regulate cellular adhesion, migration or interaction bind to their target integrin either through the RGD motif or other motif such as the RKC motif in sCD23 [55]. This binding stimulates the intracellular domain of the integrin by causing conformational changes which may lead to cytoskeletal protein clustering or initiation of a certain signaling pathway in order to control cellular activity such as proliferation, survival or apoptosis [56]. Many published studies reveal that several integrin family members contribute to many tumour malignancy behaviours such as proliferation, survival, metastasis, and invasion [57].

1.3.3.2 Integrin - mediated signalling pathways

The ability of integrins to perform their role as transmembrane bridges between the actin filaments of the cytoskeleton and the extracellular matrix is controlled by the Ras–related GTPase family [58]. These communicating bridges are important not only for cell adhesion, but also for cell motility, migration and integrin–related signalling functions [58].

Integrin – mediated signalling events can be divided into two groups; the first one is driven by ligands that bind directly to integrins causing them to cluster and directly activate signalling pathways [59]. This pathway involves or activates cytoplasmic tyrosine kinases such as focal adhesion kinase (FAK) and serine/threonine kinases (e.g., mitogen – activated protein kinase MAPK) [59].

The second group of integrin – mediated pathways is called the "collaborative signalling pathway" which is initiated by other groups of receptors such as receptor tyrosine kinases (RTKs) which are activated by certain growth factors and which then enhance integrin activation which leads to efficient transduction of signals [59]. The affinity of integrin toward its ligands is controlled by intracellular inducers such as the active form of R-Ras which increases the binding affinity of αvβ3, α4β1 and α5β1 integrins for ligands [59].

Integrins can also modulate MAPK phosphorylation through the classical MAPK signalling pathway of Ras/Raf/MEK/MAPK (ERK). This action depends upon either FAK, Src-family kinases (SFKs) or both together [60,61]. Moreover, integrins activate several transcription factors as a downstream consequence of different signalling pathways. One of these transcription factors, NFκB, controls expression of a spectrum of

genes such as pro-inflammatory genes and some cytokines [62]. Figure 1.4 summarizes some signalling pathways modulated by different integrin family members.

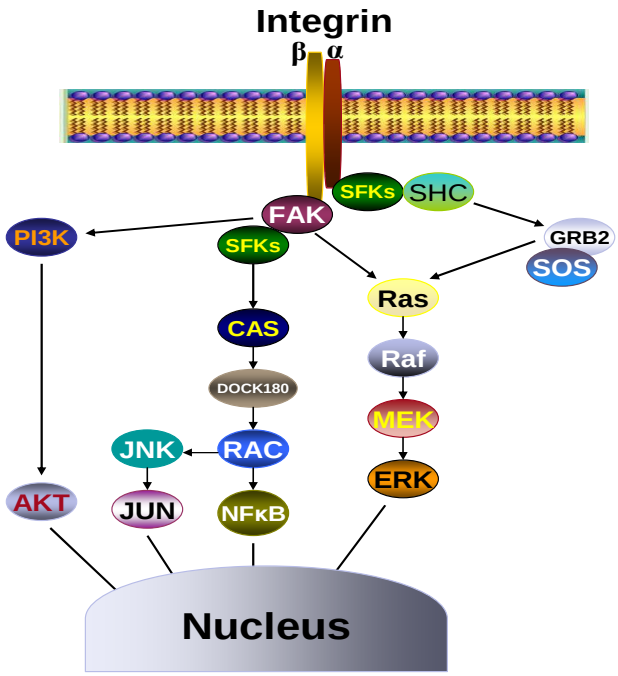


Figure 1.3 Signalling pathways modulated by different integrin family members. Integrins activate several transcription factors, in order to control different cellular activities or events such as proliferation, migration or differentiation.

1.3.3.3 Growth factor signalling mediated by integrins

Integrins are able to modulate signalling pathways which are activated by soluble growth factors and other differentiation stimuli [58]. This phenomenon has been observed in several cell lines and seems to be general. One of these pathways is the activation of RTKs which leads to the activation of mitogen signalling [58]. According to Sundberg and Rubin, the integrin – mediated activation of platelet derived growth factor - β receptor (PDGFR-β) is independent of ligand (i.e. PDGF) [63]. In contrast, a highly tyrosine –

phosphorylated portion of PDGFR associates with αvβ3 integrin in the presence of vitronectin [64]. So far, it is not clear whether the RTKs and integrins associate together directly or whether other cellular components are involved in their association [58]. Some studies suggested that the efficiency of the MAPK cascade, which is enhanced by integrin – mediated adhesion, may involve Raf recruitment and activation [65]. Other studies suggested that the activation of MAPK is enhanced by MEK rather than Raf [66]. Recently it has been clear that integrin – dependent adhesion molecules play a major role in modulating growth signalling pathways via both RTKs and the MAPK cascade, meaning that integrin – dependent adhesion molecules have an importance in the regulation of the cell cycle and apoptosis [58]. In addition, there are some reports showing that soluble growth factors, motility factors and differentiation factors require the activation of certain types of integrin [58].

1.3.3.4 Integrin – mediated apoptosis

Apoptosis is an important biological process which regulates the growth of cells, whether normal or tumour cells, and the role of integrins in the apoptotic process has been recognised since 1996 [67]. Although the caspase family proteases have a major role in modulating apoptosis in both down and up stream events and in cooperation with the Bcl-2 family, the activation of the Jun-kinase (JNK) cascade also has been shown to be an apoptotic mediator in some cell types [68]. On the other hand, the activation of the PI3K – Akt cascade has been shown to be an apoptotic antagonist [69]. Furthermore, some published work shows that FAK can be cleaved by caspase family proteases to initiate the apoptotic mechanism which, in turn, blocks the FAK – PI3K – Akt pathway [70]. The role of integrin – mediated apoptosis is to maintain the expression of Bcl-2 which blocks

caspase activation [71]. In addition, integrin – mediated signals can activate the MEKK-1 – JNK cascade which also leads to suppression of apoptosis [71]. In conclusion, integrin – mediated signalling pathways play a regulatory role in a network of cellular events such as adhesion, apoptosis, growth, proliferation, differentiation and mobility.

1.3.4 αv integrin structure and its importance

According to Nemeth (2007), the structure of αv integrin is different from the other types of integrins, because the α subunit can bind one of five different β subunits, β1, β3, β5, β6 and β8 subunit [72]. αv integrins play a major role in wound healing due to their ability to bind to vitronectin and fibronectin as well as their role as collagen receptors [73]. αvβ3 is a member of αv integrin subfamily and has an important role in tumour growth, tumour – linked angiogenesis and metastatic processes [74]. There is some evidence that has shown high levels of αvβ3 integrin on many sold tumours, such as cancers of kidney, breast, lung, prostate, ovary and skin [75,76]. Moreover, αvβ3 integrin plays a key role as a mediator in spreading and migratory properties of human melanocytes [77]. Both αvβ3 and αvβ5 are highly expressed on osteosarcoma cells and both of them can mediate adenovirus infection [78] .

Unfortunately, any functional or structural alteration in integrins may promote many health problems such as cancer, tumour growth, metastasis, coagulation, bleeding, etc [74, 79]. Hence, the recent research interest in anti-cancer therapies directed toward αv integrin [72]. In a different context, αv integrins regulate the blood vessels formation during the embryonic progression [80]. Bader and his group have investigated the role of αv integrins in vascularisation by using αv integrin knockout mice. They found about 80% of these mice die *in utero* while 20% die a few hours after birth and this was a accompanied by

extensive abnormalities in brain vessels, intestinal vessels and bleeding [80]. These findings suggested that αv integrins are important in either blood vessel formation or maturation. A supportive study was conducted by Yang and his group where they found hemorrhagic disorders in β3 knockout mice [81].

1.3.5 Integrins and leukaemia

Integrins play an essential role in cellular adhesion which in turn plays an important anti-apoptotic role in both normal and leukaemic B-cells [82]. Apoptosis was induced using monoclonal antibodies against either VCAM-1 or α4β1 integrin [83]. These findings explained the role of integrin in the cellular adhesion between leukaemic and stromal cells which is believed to be essential for precursor B leukaemic cell survival [83]. In pre-B acute lymphoblastic leukaemia, integrins modulate different signaling pathways which may underlie cell survival [84]. In the same context, integrins upregulate both Bcl-2 and Bcl-x_L genes [84]. Bcl-2 and Bcl-x_L are proteins that contribute to the anti apoptotic process. In addition, Matsunaga and his group have found that the interaction between fibronectin and VLA-4 leads to Bcl-2 upregulation as an outcome of the activation of the PI3K/Akt cascade in acute myelogenous leukaemia (AML) cell lines [85]. Furthermore, the stimulation of β1 integrin in pre-B acute lymphocytic leukaemia (ALL) inhibits both caspase-3 and caspase-7 as well as inducing the expression of two apoptosis inhibitor proteins which are called Survivin and X-linked inhibitor of apoptosis protein (XIAP) [86].

1.4 CD23

1.4.1 CD23 structure

CD23, also known as (FcεRII), is a glycoprotein with a molecular weight of 45 KDa and is usually present on many immune cell membranes, especially B – lymphocytes and monocytes [87]. In fact, it is expressed in many different cell types such as Langerhans cells, endothelial cells, platelets, follicular dendritic cells and some epithelial cells [88] In addition, this protein is sometimes present free in blood and called soluble CD23 (sCD23) [87].

Although CD23 is considered an Fc receptor, its structure is different from other Fc receptors [89]. Membrane bound, trimeric CD23 is composed of three portions; head, stalk and tail. The head portion is the C - terminal extracellular portion and is composed of three C – type lectin domains [90, 91]. The stalk also is an extracellular portion which is composed of a trimeric α helical coiled – coil containing N – linked glycosylation sites; the cytoplasmic tail is the N- terminal intracellular portion [89, 91]. The extracellular portions are composed of 277 residues while the transmembraneous domain and the cytoplasmic terminal are composed of 20 and 23 residues, respectively [92].

CD23 can bind to both CD21 and IgE simultaneously through specific binding sites in the extracellular lectin domain [93]. Two lectin domains can bind to Cε3 domains in IgE. However, the third lectin domain of CD23 expresses one to three binding sites for CD21 [94].The interaction between CD23 and IgE is Ca^{2+} dependent. However, the interaction between CD23 and CD21 is carbohydrate-dependent [88]. Figure 1.4 shows the binding sites on CD23 lectin domains, the binding site shown in yellow colour is specific for binding the αVβ5 integrin which was identified by our laboratory [95].

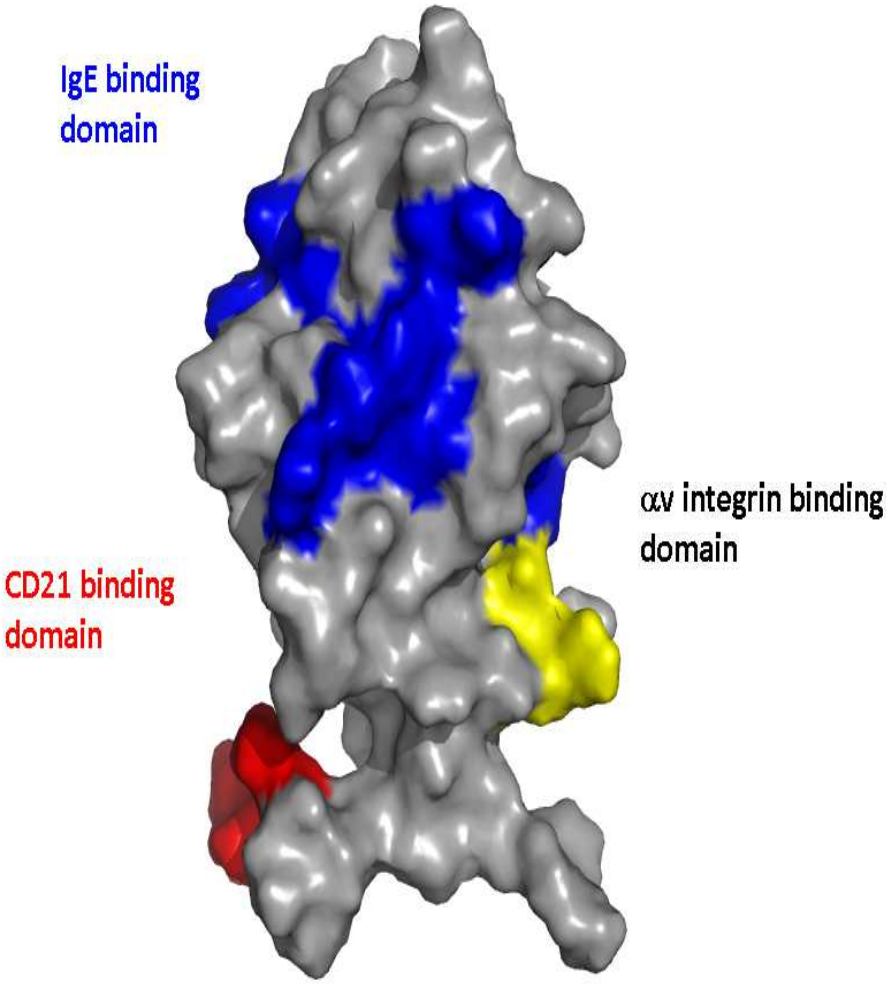


Figure 1.4 Binding surfaces on human CD23. The lectin domains of human CD23 have three distinct binding sites IgE binding sites (blue), CD21 binding site (red) and αV integrin binding site (yellow). This figure is adapted from Borland et al [95]

There are two different forms of CD23. These forms are different in their structure and location. The first form, called CD23a, is different in the N – terminal six amino acids from the other form, CD23b [88]. CD23a is usually present on B- cells while CD23b is present on a variety of cell types [88]. Furthermore interleukin – 4 is required for CD23b expression on B cells [88].

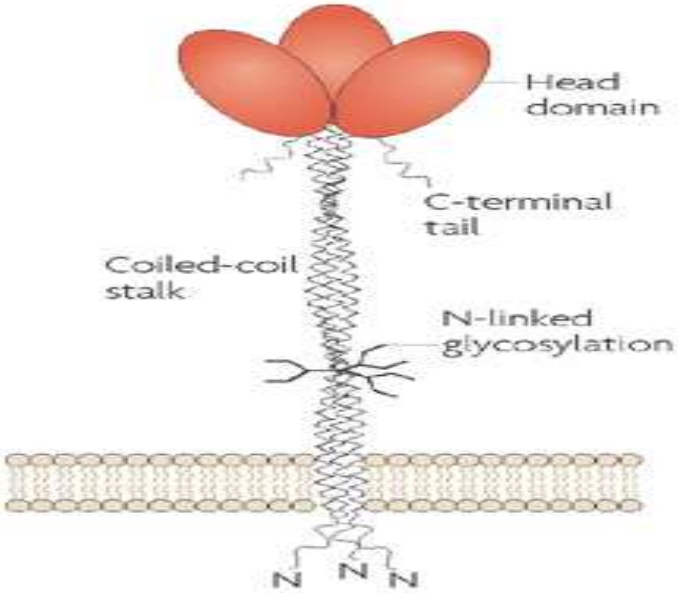


Figure 1.5 CD23 structure. It is composed of three parts, C-terminal portion which contains the binding sites, the coiled-coil stalk containing the glycosylation site and the N- terminal portion which is the cytosolic portion. This figure is adapted from (Sutton and Gould, 1993) [91].

CD23 normally undergoes proteolytic processing via metalloprotease enzymes that release soluble CD23 into serum [96]. There are many products of proteolytic cleavage of CD23 and these products differ in sizes and functions [96]. This cleavage process occurs in the coiled-coil stalk as it is susceptible to proteolysis [97]. CD23 cleavage produces 37, 33, 29, 25 and 16 KDa fragments all of which contain the C-terminal domain [96]. Some papers reported that the fragments which have molecular weight more than 25000 MW promote IgE synthesis [96,98]. On the other hand, the fragments of cleaved CD23 which have molecular weight equal or less than 25000 MW suppresses IgE synthesis [98].

1.4.2 CD23 function

CD23 binds different ligands such as immunoglobulins (IgE), glycoproteins (CD21) and viruses such as Epstein Barr Virus (EBV) [99]. CD23 plays many important roles in regulation of IgE production, control of histamine production and T – cell / B – cell interactions [100]. CD23 is capable of linking IgE with another glycoprotein CD21 and this binding is a trigger for IgE production and, according to that model, CD23 plays a major role in IgE production as a positive regulator [101]. The CD23/CD21 – IgE complex also requires other stimulation effectors such as interleukin – 4 and interleukin – 13 [99, 102]. The inducing role of these effectors is sometimes blocked by interferon γ, interferon α and prostaglandin E2. [102,103].

Moreover, the production of IgE requires T – cell / B – cell interaction, a process enhanced by CD23; this has been proved *in vitro* by using anti-CD23 monoclonal antibodies, where the results clarified that there was no interaction between T and B – cells in the presence of the CD23 antibody [104]. The other possible role of CD23 is regulating histamine production due to its binding to CD21 on the surface membrane of basophils [105]. Furthermore, a high concentration of free CD23 fragments in plasma is usually associated with inflammation and some diseases such as asthma, chronic lymphoblastic leukaemia and rheumatoid arthritis [106]. Clinically, an increased serum CD23 level was found in systemic lupus erythematosus, primary Sjogren's syndrome, glomerulonpharitis and rheumatoid synovitis [107,108,109].

In addition, soluble CD23 stimulates monocytes to produce both tumour necrosis factor – α (TNF-α) and interleukin – 1α [110]. Consequently, sCD23 interacts with interleukin – 1α to stimulate the differentiation of monocytes [110]. In this context, soluble CD23

interacts with CD11b (αMβ2) and CD11c (αXβ2) on monocytes, which drives monocytes to produce proinflammatory mediators such as interleukin-1α, interleukin-1β, interferon- γ and interleukin-6 [94]. The CD23-CD11b/CD11c interaction also increases levels of nitrate, hydrogen peroxide and other oxidative products [94].

In human B lymphocytes, soluble CD23 interacts with IgE which leads to increased Ca^{2+} and cyclic-AMP levels [111]. Moreover, membranous CD23 acts as signal transducer when cross-linked with several B cell membrane proteins such as MHC class II , CD9,CD81 and CD82 [112,113]. Recently, some researchers reported that CD23 plays an important role in supporting the survival of pre B – cells via its binding to αvβ5 integrin [95].

1.4.3 CD23 – αvβ5 integrin interaction

The αvβ5 integrin, like other integrins, is a heterodimeric transmembraneous receptor composed of two protein subunits, αV and β5 [43]. The αvβ5 integrin is one of the vitronectin receptors which means that the αvβ5 integrin plays several roles in cell attachment, survival and growth [114,115,116]. In addition, in tumours, the αvβ5 integrin is implicated to be one of the important mediators of tumour growth, metastasis and angiogenesis [95,117].

The αvβ5 integrin interacts with its ligands such as vitronectin and fibronectin via a distinct binding motif, known as the RGD motif, which is arg-gly-asp [53,118,119]. In a different context, soluble CD23 also interacts with different integrin family members and this interaction underlies the production of different cytokines such as IL-1α, IL-1β, IL-6 and IFN-γ as well as proinflammatory mediators such as interleukin-1β, interleukin-6 and tumour necrosis factor-α [106,120,121,122].

Recently, our laboratory has found that the αvβ5 integrin recognizes an arg-lys-cys (RKC) motif on sCD23 in an adhesion – independent interaction [95]. In addition, this interaction plays an important role in the growth of pre – B cells, and also activates ERK [123]. Furthermore, ERK activation was increased in the presence of SDF1 [123]. These findings suggest that the activation of ERK might underlie B-cell survival and growth, and that any enhancement or synergistic effect from the bone marrow microenvironment such as stromal cell – derived factor 1 (SDF1) and/or platelet derived growth factor (PDGF) might be delivered via ERK.

1.4.4 Other ligands related to haematopoiesis and αvβ5 integrin

1.4.4.1 Stromal cell – derived factor 1 (SDF1)

Stromal cell – derived factor 1 (SDF1 or CXCL12) is a chemokine which plays several roles during B – cell development in bone marrow [124]. SDF1 is produced by immature osteoblasts in bone marrow and by epithelial cells in different organs [125,126,127]. SDF1 is composed of 67 residues, is about 8KDa, and presents in all vertebrates in different isoforms SDF1α, SDF1β, SDF1γ, SDF1ε and SDF1φ [128,129,130]. Both SDF1α and SDF1β encoding genes are located on chromosome 10 [131]. Recently, several studies have demonstrated the ability of SDF1 to activate some types of integrins such as LFA-1, VLA-4 and VLA5 and to play a synergistic role with cytokines which might be required for cell survival and proliferation [132,133,134]. SDF1 certainly plays an essential role in haematopoietic cell maturation, survival and proliferation [135]. In addition, in various cell types including B cell precursors, SDF1 increases the intracellular calcium levels via ligation of CXCR4 [136,137]. This ligation also leads to the activation of

phosphatidylinositol 3 – kinases (PI3K) and phosphorylation of MEK and ERK as a consequence of phosphorylation of focal adhesion complexes [138]. Moreover, SDF1 stimulates the Jak/STAT pathway [139]. On the other hand, SDF1 is thought to play different pathological roles such as HIV infection enhancement, tumour growth, inflammation and angiogenesis [140,141,142]. In terms of leukaemias, CXCR4 is highly expressed in B- chronic lymphocytic leukaemia cells which enhances their response to SDF1 [143]. Meanwhile, the migration, cellular adhesion and survival of different acute lymphocytic leukaemia cell lines are enhanced in the presence of SDF1[144,145].

1.4.4.2 Platelet – derived growth factor (PDGF)

Platelet – derived growth factor (PDGF) was recognized and detected in whole blood cells but was absent in the plasma [146]. The major source of Platelet derived growth factor (PDGF) is the α – granules of platelets and it is synthesized by other cell types such as macrophages, epithelial and endothelial cells [147,148,149]. Several studies show that PDGF plays a significant role in several physiological systems such as cellular development and growth. On the other hand, PDGF is implicated in several pathological mechanisms including atherosclerosis, fibrosis and neoplasia [150,151,152].

PDGF is composed of two polypeptide chains linked together by a disulphide bond [153]. There are four distinct polypeptide chains A, B, C and D, which are assembled to form the dimer of PDGF, whether homodimers such as PDGF – AA, PDGF – BB, PDGF – CC and PDGF – DD or heterodimers such as PDGF – AB [154,155,156].

PDGF binds to its target cells via two types of receptors PDGFR – α and PDGFR – β, and both are similar to each other in their structures [157]. The PDGFR – α has a high

affinity toward A, B and C chains, while the PDGFR – β recognizes and binds to the B and D chains [158]. However, PDGF requires proteolytic cleavage before being able to bind to the PDGFR [159]. The PDGF exerts its biological role by inducing the dimerization of receptors which leads to autophosphorylation of the PDGF receptor tyrosine kinase (RTK) [160]. In turn, the RTK phosphorylates many downstream signalling proteins [160].

According to Soliven and his colleagues, PDGF stimulates both Src family kinases and sphingosine kinase in oligodendroglial progenitors [161]. Recently, some studies have shown that the PDGF induces cellular proliferation, migration and differentiation via the RAS/MAPK pathway and promotes cell survival through PI3K/Akt pathway [162,163,164]. Furthermore, PDGFR directly mediates STAT1 activation and phosphorylates STAT3 via the phosphorylation of JAK protein in fibroblasts [165,166]. In addition, Paukku and his colleagues have published that PDGF induces the activation of both STAT5 and c-Src in mammalian cells [167]. PDGF induces cell growth via STAT5 phosphorylation in different types of leukaemia such as AML and CML [168,169,170]. Figure 1.6 summarizes some of signalling pathways mediated by the PDGF; Ras/Raf/MEK/ERK pathway, JAK/STAT pathway and PI3K/Akt pathway.

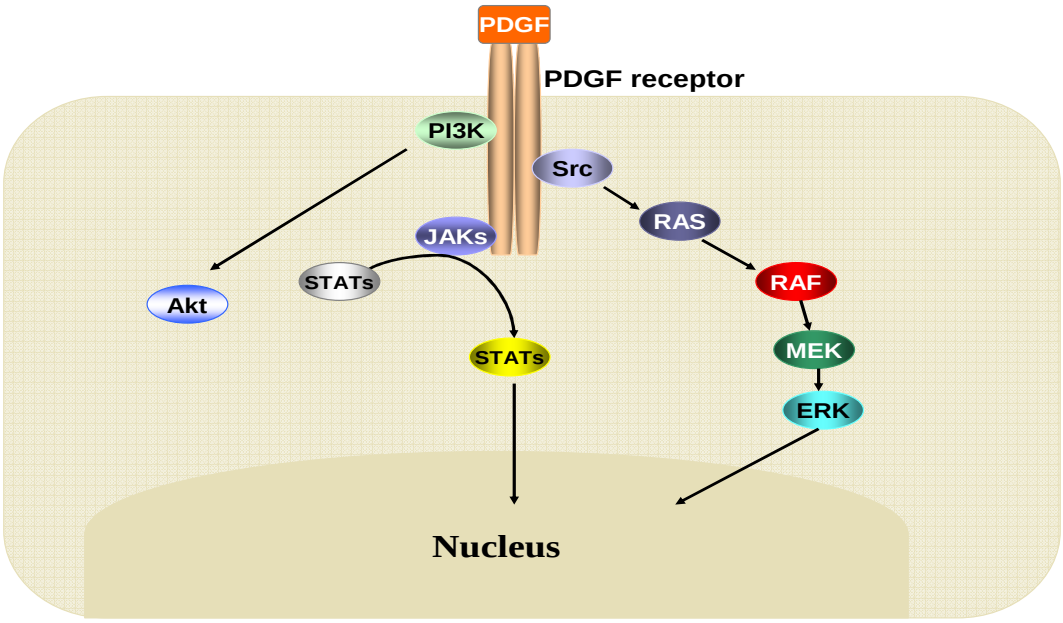


Figure 1.6 PDGF signalling. PDGF induces several signalling pathways depending on the cell type and condition.

1.4.4.3 Vitronectin

Vitronectin is a 75 kDa glycoprotein present in the extracellular matrix and plasma [171, 172]. Vitronectin has different binding sites so that it is a multifunctional protein [171]. One of these binding sites is the Arg-Gly-Asp (RGD) motif which is located in the N-terminal domain and is involved in the cellular attachment via the vitronectin-integrin crosslink [173]. In humans, the vitronectin gene is located in the long arm of chromosome 17 [174].

Vitronectin plays several biological roles such as cellular adhesion, spreading and migration [173,175]. In addition, vitronectin is involved in physiological processes such as haemostasis via its binding to heparin, the immune response by its binding to the

terminal complex in the complement protein cascade and in cell proliferation as a co-stimulant growth factor [171,173,175].

Pathologically, vitronectin is implicated in several disease processes such as arteriosclerosis, degenerative central nervous system disorders, fibrosis and membranous nephropathy [176]. Vitronectin is involved in angiogenesis and tumour growth due to its interaction with αvβ3 integrin [177]. Moreover, vitronectin plays an important function in wound healing via its binding to some of integrin family members [178].

1.5 Signalling pathways related to cell growth, survival and proliferation

1.5.1 JAK/STAT signalling pathway

Janus Kinases are intracellular proteins identified as tyrosine kinases involved in cell survival and growth stimulated by different growth factors, cytokines and interferons [179, 180]. At present, there are four members of the JAK family in mammals, JAK1, JAK2, JAK3 and Tyk2 [181]. The molecular weights of these kinases are between 120 and 140 KDa and they have similar structure [181]. Each JAK family member is composed of seven highly conserved domains called JAK homology domains (JH) [182,183]. The kinase activity domain is in the C-terminal domain, which is called the JH1 domain, and this domain is followed by JH2, a kinase-related domain [184]. The structural differences between JAKs are located in the remaining five domains in which the degree of conservation varies from one to the other [182]. JAKs are activated by transmembraneous cytokine and growth factor receptors. Normally, these receptors are associated with one member of the JAK family via the intracellular domain [185,186,187]. These receptors undergo ligand-driven conformational changes leading to either multimerization, such as with cytokine and interferon receptors, or dimerization, as noted in PDGF and growth factor receptors [188,189]. This action leads to JAK phosphorylation and phosphorylated JAKs in turn either phosphorylate the receptor itself or other downstream substrates [187].

The major JAKs substrates are called signal transducers and activators of transcription (STATs) [190]. The STAT family is composed of seven members STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [190]. In humans, STAT1 and STAT4 genes are located on chromosome 2 whereas STAT2 and STAT6 genes are located on

chromosome 12 [191,192]. The other STAT family members STAT3, STAT5a and STAT5b genes are located on chromosome 17 [191,192]. Structurally, STATs are different from other transcription factors because they have Src-homology 2 (SH2) domains [191,193]. These domains are responsible for STAT dimerization and activation [191]. Once STAT becomes phosphorylated at the SH2 domain the dimerization will start between SH2 domain of one monomer and the C-terminal of phosphotyrosine-binding domain of the other [194,195]. The activation of STATs produces either heterodimers such as STAT1-STAT2 and STAT1-STAT3 or homodimers such as STAT1 and STAT3 [193,194].

In general, the JAK/STAT pathway plays several different roles in mammals and other vertebrates corresponding to the stimuli, cell type and biological context. Many published studies have revealed that the JAK/STAT pathway mediates several cellular and biological functions such as proliferation, apoptosis and growth in response to either cytokines, interferons or growth factors.

In haematopoietic cells, the JAK/STAT pathway was identified in different cases either in normal or malignant cell lines. In 1997 Lacronique and his colleagues identified TEL-Jak2 fusions in early B-precursor acute lymphoblastic leukaemia as a product of t(9;12)(p24;p13) translocations [196]. Similar data were shown in chronic myeloid leukaemia but with additional translocations t(9;15;12)(p24;p15;p13) [197]. In both cases, the transcription factor Tel is fused to the JH1 domain of Jak2 and consequently activates STAT1 and STAT5 [198,199, 200]. In addition, both STAT3 and STAT5 were found activated in response to different cytokines and growth factors in several leukaemic cell line models [200,201,202, 203]. Moreover, the anti-apoptotic role of STAT3 has been shown in multiple myeloma in which the activated STAT3 in turn promotes Bcl-2 and Bcl-x_L expression [204]. Several published papers have reported that gamma interferon-activated

site (GAS) is the main site that activated STATs bind in order to regulate their target genes in response to different stimuli such as cytokines, hormones and growth factors [205,206, 207]. Figure 1.7 describes the general activation steps of JAK/STAT pathway that occur in different cell types in mammals.

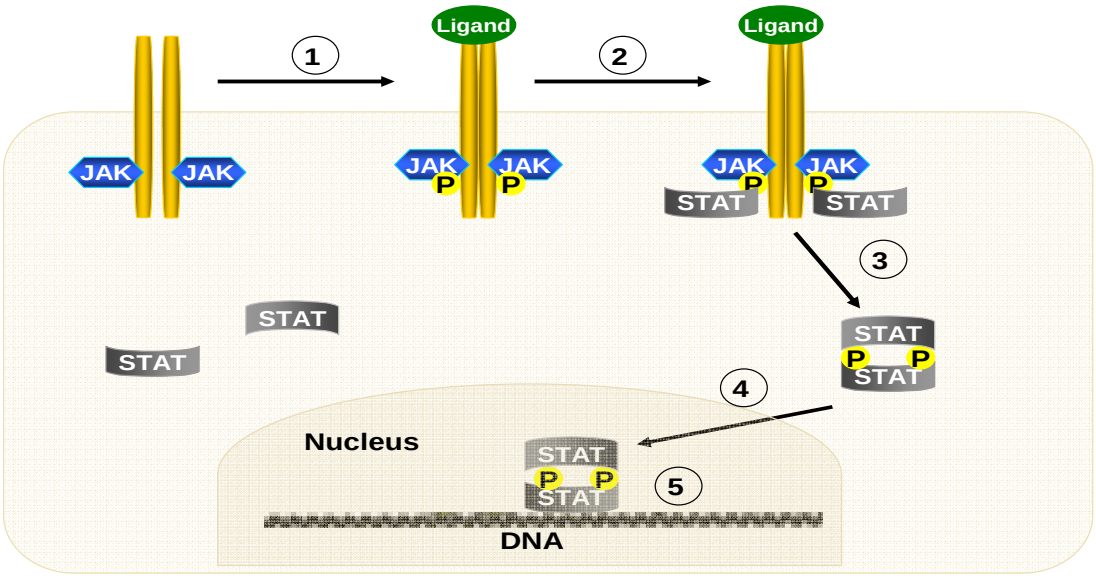


Figure 1.7 JAK/STAT pathway. Dimerization and auto-phosphorylation of Tyrosine kinase receptors can be caused by different ligands such as interleukins and growth factors. (1) Receptor tyrosine kinase and Jak phosphorylation by ligand-driven conformational changes. (2) Phosphorylated receptor kinase recruits and phosphorylates STATs. (3) Phosphorylated STAT forms active dimers. (4) Activated STAT translocates into the nucleus. (5 The activated STAT in turn binds DNA at specific binding sequence mainly (GAS) and activates the transcription of target genes.

1.5.1.1 STAT5 structure and function

STAT5 is a name of two highly conserved STAT family monomers STAT5a and STAT5b. STAT5 was identified in 1994 by Groner and his group in mammary epithelium and was called at that time Mammary Gland Factor (MGF) [208]. STAT5a is composed of 793 amino acids whereas STAT5b contains 786 amino acids. The

similarity between the proteins reaches up to 96% and the variability between them is located in the C-terminal transactivation domain [209, 210,211].

The structure of either STAT5a or STAT5b is similar to other STAT protein; composed of N-terminal domain followed in order by coiled coil, DNA binding domain, linker region, SH2 and transactivation domains which is in the C-terminus [211,212]. The linker region is responsible for dimerisation which is essential for the transcriptional activity of STAT5. The dimerisation produces either homodimer (STATa/a) or heterodimer (STAT5a/b), however, it requires the phosphorylation of either of them at a specific target residue, at Tyrosine 694 for STAT5a and Tyrosine 699 for STAT5b [208, 213]. The phosphorylation event in either isoform typically occurs by the receptor associated tyrosine kinase proteins Jaks, Jak1, Jak2 or Jak3 [214]. The phosphorylation of Jak occurs via receptor tyrosine kinase and then the activated Jak in turn cross-phosphorylates the receptor tyrosine kinase which recruits either STAT5a or STAT5b and binds to any of them via SH2 domains and phosphorylates a specific tyrosine residue which induces STAT5 dimerisation [215, 214, 216]. STAT5 dimers translocate into the nucleus in order to regulate the target genes [214].

STAT5 regulates a wide range of genes depending on the cell type, the stimuli and the cell condition. The most well known gene is Bcl-X_L the gene that encodes Bcl-X_L protein that acts as an anti-apoptotic protein [217, 218, 219,220]. Many published reports have revealed that STAT5 regulates various genes in different species and cell types. Some studies show that STAT5 regulates cytokine genes and cyclin D [221, 222,223].

In general, STAT5 plays different biological and pathological roles such as preventing cell death, cell cycle stimulation, cytokine secretion and tumour growth [223, 224, 221, 225].

In normal conditions, IL-7 plays an essential role in early B-cell development via activating STAT5, and STAT5a/b is also required for survival of $CD8^+$ T cells [226, 227]. In addition, STAT5 controls Bcl-6 expression in germinal centre B cells which leads to self-renewal and differentiation of human memory B cells [228]. Furthermore, STAT5 activation was found associated with different leukaemias due to its role in apoptotic prevention or cell cycle regulation [219, 229, 230].

1.5.2 The ERK1/2 mitogen-activated protein kinase pathway

ERK1 and ERK2 are intracellular proteins expressed in almost all cell types with molecular weights 43 and 41 KDa, respectively [231]. Both ERK1 and ERK2 proteins have 85% an identical core where the substrate binding sites are located [231, 232]. Furthermore, the activation site which contains the phosphate acceptor residues tyrosine and threonine is located in a TEY motif in the activation loop in both ERK1 and ERK2 [232, 233]. The two proteins are activated by different stimuli such as serum, cytokines, growth factors and several G protein-coupled receptor ligands [232, 233].

The activation of ERK1/2 was found linked with several biological actions such as cell growth, survival and proliferation in different cell types. Moreover, there is some evidence that the activation of ERK is required for cell cycle progression, precisely for cell transition from G1 phase into S phase which means that the activated ERK is also required for DNA synthesis [234, 235, 236].

The Ras/Raf/MEK/ERK signaling pathway is the main upstream signaling cascade for ERK and ERK seems to be the key target of this pathway. However, ERK is involved in different downstream signaling pathways illustrated in figure 1.6. ERK is identified as a kinase of either c-Fos or c-Myc in order to induce cyclin D1 gene expression which in

turn controls the cell cycle [237,238]. Phosphorylated ERK is also responsible for Elk-1 phosphorylation at multiple Ser/Thr-pro sites [239]. The activated Elk-1 interacts with the serum response factor (SRF) to form the ternary complex factor (TCF) which in turn regulates serum response element (SRE) transcriptional activity [240, 241, 242]. According to Marais and his group, the activation of the SRF-DNA interaction is ERK2 dependent [242]. In other contexts, ERK is identified as a kinase of p90RSK, MSK and c-Jun [243, 244, 245].

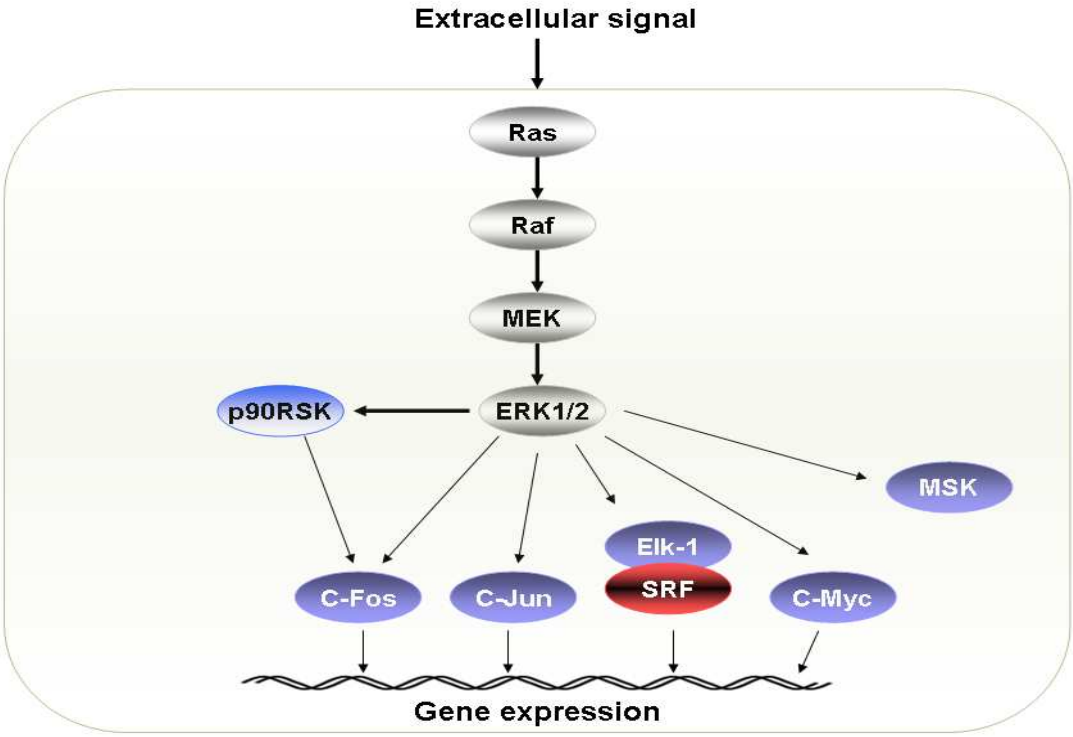


Figure 1.8 The Ras/Raf/MEK/ERK cascade. ERK1/2 plays a key role in several cellular activities. The activation of ERK1/2 via the Ras/Raf/MEK regulates expression of many genes via activating different transcription.

The MAP Kinase pathway is implicated in several physiological disorders such as tumour growth, metastasis and angiogenesis [246, 247, 248]. In addition, the superoxide activated-Ras/Raf/MEK/ERK pathway is implicated in renal dysfunction, dementia and Parkinson's disease [249, 250, 251].

In haematopoiesis, the Ras/Raf/MEK/ERK cascade plays different roles depending on the cell type and the maturation step it is involved in. The activation of MEK/ERK signaling by stem cell factor (SCF) stimulates the haematopoietic stem cell proliferation [252]. Furthermore, the activation of MEK/ERK pathway as a downstream effecter of cytokine receptors is involved in different maturation steps in either lymphoid or myeloid lineages [253, 254]. On the other hand, activated ERK was found in about 75% of AML cases in a study conducted by Ricciardi and his colleagues [255]. In addition, ERK1/2 seems to be a significant indicator for both B-ALL and T-ALL patients [256, 257]. In the same context, the MEK inhibitor U0126 reduces growth of either AML or CML cell lines [258, 259].

1.5.3 Calpain

Calpain is a biochemical system composed of three components; two calcium-dependent proteases, called μ-calpain and m-calpain, and the third component is a peptide called calpastatin which serves as an inhibitor for both μ and m Calpains [260]. These three proteins are present in almost all cell types in all vertebrates but in variable ratios depending on the cell type, tissue and species [260]. Both Calpains μ and m are cytoplasmic cysteine proteases playing several biological roles in cell mobility and embryonic development due to cleaving the cytoskeletal proteins [261] , [262]. In addition, both μ and m Calpains play different roles in cell signaling and apoptosis via cleaving caspase-7, -8, -9 and caspase-3 or via activating caspase activators such as APAF-1 and cytochrome C [263, 264].

1.6 Research aims

The major aim of this study is to investigate the role of the sCD23- αVβ5 integrin interaction in survival of acute lymphocytic leukaemia cells and to answer the question how the interaction leads to regulation of growth.

The specific aims of this project are to:

(a) Study the role of both sCD23 and vitronectin in B-cell progenitor growth.

(b) Study the synergy of PDGF(AB) and SDF1-α with sCD23 in B-cell precursors growth.

(c) Study the activation of kinases in signaling pathways due to the effects of CD23 and vitronectin.

(d) Investigate the role PDGF receptor in the signaling events caused by sCD23-αVβ5 integrin interaction.

2. Materials and methods

2.1 Chemicals and reagents

All routine chemicals and reagents unless otherwise listed in Table 2.1 and Table 2.2 were purchased from Sigma -Aldrich Company Ltd, Poole, UK and Fisher Scientific UK Ltd, Leicestershire, UK.

Table 2.1 Chemicals and reagents used in this study

Chemical / reagent	Supplier
MES SDS Running Buffer (NuPAGE®)	Invitrogen, Paisley, UK
MOPS SDS Running Buffer (NuPAGE®)	
NuPAGE 10% Bis-Tris Gel 1.0mm	
NuPAGE 4-12% Bis-Tris Gel 1.0mm	
3MM chromatography paper	Whatman, Springfield Mill, Maidstone, UK
3MM blotting paper	
PROTRAN nitrocellulose transfer membrane	
Nucleic acid transfer membrane Hybond-N+	Amersham Life Science Ltd. Buckinghamshire, UK.
Prestained Protein Marker, Broad Range (7-175 kDa)	New England BioLabs, Hitchin, Hertfordshire, UK
[Methyl-^{3}H] Thymidine	GE Healthcare. Pollards Wood Nightingales Lane, UK
X-tremeGENE 9 DNA Transfection reagent	Roche Diagnostics Limited, Charles Avenue, Burgess Hill, West Sussex UK
X-tremeGENE HP DNA Transfection reagent	
Mirus TransIT-TKO Transfection reagent	Cambridge Bioscience Ltd, Munro House, Cambridge, UK
FuGENE HD Transfection reagent	Promega UK Ltd, Southampton, United Kingdom.

Table 2.2 Kits were used in this study

KIT	SUPPLIER
Nuclear Extract KIT	ACTIVE MOTIF Rixensart, Belgium Catalog No: 40010
Proteome Profiler Antibody Arrays	R&D SYSTEMS, Abingdon, UK

KIT	SUPPLIER
STAT5b EMSA KIT	Panomics Catalog No; AY1405 via Caltag-Medsystems Limited, Whiteleaf Business Centre, 11 Little Balmer, Buckingham, MK18 1TF
Catch and Release Immunoprecipitation KIT	Millipore (U.K.) Limited, Building 6 Croxley Green Business Park, Watford
Calpain Activity Assay KIT	Abcam, Cambridge Science Park, Cambridge, UK
Luciferase Assay System	Promega UK Ltd, Southampton, United Kingdom.
ONE-Glo™ Luciferase Assay System	

2.2 Ligands, inhibitors and Antibodies

All ligands and inhibitors used in this study were purchased from different suppliers are listed in Table 2.3. Table 2.4 summarized the information about the antibodies which are used in this study and the supplier of each.

Table 2.3 Peptides and inhibitors were used in this study

Peptides/inhibitors	supplier
Recombinant Human CD23/Fc epsilon RII.	R&D SYSTEMS, Abingdon, UK
Recombinant Mouse CD23/Fc epsilon RII.	
Recombinant Mouse IL-3	
Vitronectin	Sigma -Aldrich Company Ltd, Poole, UK
PDGF-AB	
SDF-1α	Millipore (U.K.) Limited, Building 6 Croxley Green Business Park, Watford
Long peptide (derived fromCD23)	Mimitopes, Victoria, Australia
Tryphostin AG490 (Jak2 inhibitor)	ENZO Life Sciences Ltd, Matford Court, UL
Tryphostin AG 1295 (PDGFRβ inhibitor)	
MEK inhibitor (U0126)	Promega Ltd, Southampton, Hampshire, UK
PI3kinase inhibitor (LY 294002)	New England BioLabs, Hitchin, Hertfordshire, UK

Table 2.4 Antibodies were used in this study

Antigen	Source	Clone/Cat. No.	Isotype	Supplier	Conjugate
Human β3 integrin	Mouse monoclonal	MHF4	IgG1	Santa Cruz Biotechnology, Inc. Heidelberg, Germany	FITC
Human CD45	Mouse monoclonal	BRA- 55	IgG1	Sigma -Aldrich Company Ltd, Poole, UK	FITC
Human CD5	Mouse monoclonal	UCTH2	IgG1	BD Bioscience, Oxford, UK	FITC
Human CD10	Mouse monoclonal	HI10a	IgG1		PE
Human CD19	Mouse monoclonal	TB28-2	IgG1		FITC
Human IgM	Mouse monoclonal	G20-127	IgG1		FITC
Human CD34	Mouse monoclonal	563	IgG1		PE
Human αVβ5 integrin	Mouse monoclonal	P5H9	IgG1	R&D SYSTEMS, Abingdon, UK	PE
Human CXCR4	Mouse monoclonal	12G5	IgG1		FITC
Human CD23	Mouse monoclonal	138628	IgG1		PE
Human PDGFRβ	Mouse monoclonal	PR7212	IgG1		FITC
Human Phospho-ERK1/2 (T202/Y204)	Rabbit polyclonal	FTE12	IgG		–
Phospho-STAT5 (Y699)	Rabbit polyclonal	ZBS02	IgG		–
Rabbit IgG	Goat polyclonal	FIN03	IgG		HRP
Human IgG	Goat polyclonal	A0170	IgG	Sigma -Aldrich Company Ltd, Poole, UK	HRP
Rabbit IgG	Goat polyclonal	A6667	IgG		HRP
Mouse IgG	Goat polyclonal	A4416	IgG		HRP
Rabbit IgG	Goat polyclonal	7074	IgG	New England BioLabs, Hitchin, Hertfordshire, UK	HRP
Non-human antigens	Mouse monoclonal	GO1	IgG1	DAKO, Cambridgeshire, UK	FITC
Non-human antigens	Mouse monoclonal	GO1	IgG1		PE

Table 2.4 Antibodies were used in this study

Antigen	Source	Clone/Cat. No	Isotype	Supplier	Conjugate
Human STAT5	Rabbit polyclonal	9363	IgG	New England BioLabs, Hitchin, Hertfordshire, UK	–
Human PDGFRβ	Rabbit monoclonal	28E1	IgG		–
Human Phospho-p90RSK(S308)	Rabbit monoclonal	9D9	IgG		–
Human Phospho-p90RSK (T359/S363)	Rabbit polyclonal	9344	IgG		–
Human Phospho-p90RSK(T573)	Rabbit polyclonal	9346	IgG		–
Human RSK1/RSK2/RSK3	Rabbit monoclonal	32D7	IgG		–
Human Phospo-p44/42 MAP Kinase	Rabbit monoclonal	197G2	IgG		–
Human Akt	Rabbit polyclonal	9272	IgG		–
Human Phospho-Akt (S473)	Rabbit monoclonal	193H12	IgG		–
Human p44/42 ERK1/2	Rabbit monoclonal	137F512	IgG		–
Human NF-kappa B2 p100/p52	Rabbit polyclonal	4882	IgG		–
Human Phospho-JAK2 (Y1007/1008)	Rabbit monoclonal	C80C3	IgG		–
Human CREB	Rabbit monoclonal	48H2	IgG		–
Human Phospho-CREB (S133)	Rabbit monoclonal	87G3	IgG		–
Human JAK2	Rabbit polyclonal	NG1548937	IgG	Millipore (U.K.) Limited, Building 6 Croxley Green Business Park, Watford	–
Human Phspho-JAK2 (Y1007/1008)	Rabbit polyclonal	30220	IgG		–
Human β5 integrin	Rabbit polyclonal	AB1926	Whole serum		–
Human Phospho-STAT5a/b (Y694/699)	Mouse monoclonal	8-5-2	IgG		–

2.3 Buffers

The following table is listed the buffers which were routinely used:

Table 2.5 Buffers were used in this study

Buffer	Composition
PBS	130mM NaCl, 27mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4 (pH 7.2)
TBS	50mM Tris HCl (pH 7.4), 150mM NaCl
TBS/T	50mM Tris HCl (pH 7.4), 150mM NaCl + 0.1% (v/v) Tween20
Stripping buffer	200mM Glycine, 0.1% (w/v) SDS, 1% (v/v) Tween20. (pH 2.2)
Blocking buffer	TBS/T, 5% (w/v) either non-fat dried milk or BSA
Tris HEPES-SDS running buffer	100mM Tris HCl (pH 8.0), 1% (w/v) SDS, 100mM HEPES.
Transfer Buffer	25mM Tris HCl, 192mM Glycine, 20% (v/v) ethanol.
4X SDS sample buffer	200mM Tris HCl (pH 6.8), 40% (v/v) glycerol, 0.4% (w/v) Bromophenol, 8% (w/v) SDS.
RIPA buffer	50mM Tris HCl (pH 7.4), 150mM NaCl,1mM EGTA.
Lysis buffer	RIPA buffer, 1mM PMSF, 1mM Na-deoxycholic acid, 1% (v/v) NP40, 1mM Na_3VO_4, 2ug/ml Leupeptin
1X TBE buffer	100mM Tris HCl, 0.9mM Boric acid, 0.01mM EDTA.

2.4 Plasmids and Oligonucleotides

Plasmids were used as a reporter vectors in this study and oligonucleotides are listed in the following table.

Table 2.6 Plasmids and oligonucleotides were used in this study

Plasmid/Oligonucleotide	Supplier
pGL4.34[luc2P/SRF-RE/Hygro] Vector	Promega UK Ltd, Southampton, United Kingdom.
TransLucent GAL4-STAT5B TAD fusion protein expression vector, pTL-STAT5B.	Panomics Inc. And Caltag MedSystems Ltd, Cambridge, UK

TransLucent luciferase reporter vector, pTL-Luc	Panomics Inc. And Caltag MedSystems Ltd, Cambridge, UK
TransLucent GAL4-BD controll vector, pTL-BD	
SignalSilence Control siRNA (Fluorescein Conjugate)	New England BioLabs, Hitchin, Hertfordshire, UK

2.5 Cell lines and culture

The main cell line model used in this study, SMS-SB cells, was derived from a female patient at the leukemic phase of acute lymphocytic leukaemia [265]. These cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2mM fresh glutamine, (100U/ml) penicillin and (100µg/ml) streptomycin at 37º C and 5% CO_2 in a humidified incubator. For experimental purposes, these cells were adapted and grown in protein-free hybridoma medium (PFHM) supplemented with (100U/ml) penicillin and (100µg/ml) streptomycin at 37º C and 5% CO_2 in a humidified incubator. The other two cell line models used in this study are called BAF03 and 697. BAF03 is a murine pro-B cell line and was cultured in RPMI- 1640 medium with 50µM 2-ME (mercapto-ethanol) and 1ng/ml murine IL-3 as well as 10% (v/v) heat-inactivated foetal calf serum, 2mM fresh glutamine, (100U/ml) penicillin and (100µg/ml) streptomycin [266]. The 697 is a pre-B cell line extracted from a 12-year boy diagnosed with Acute Lymphocytic Leukaemia (ALL) [267]. This cell line was cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2mM fresh glutamine, (100U/ml) penicillin and (100µg/ml) streptomycin at 37º C and 5% CO_2 in a humidified incubator.

2.6 Proliferation assay

2.6.1 Using tritiated thymidine

Cells were harvested and washed twice in PFHM then plated at a density of 5000 cells/100µl in each well of a 96-well flat bottom plate in PFHM. The plate was incubated for 72 hours at 37º C and 5% CO_2 in a humidified incubator in the presence or absence of stimulants, soluble CD23, long peptide (LP); a peptide was derived from soluble CD23 and contains the RKC motif, vitronectin, SDF1-α and PDGF-AB. Then cells were pulsed with 0.3µCi/well (5.5×10^{-6} µmol) tritiated thymidine ([^{3}H]-TdR) and re-incubated at 37º C and 5% CO_2 in a humidified incubator for 6 hours before harvesting and determination of incorporation by liquid scintillation spectrometry.

2.6.2 Using Alamar blue

Cells were harvested and washed twice in PFHM then plated at a density of 5000 cells/100µl in each well in 96-well white flat bottom plates in PFHM. In the case of 697 and BAF03, cells were plated in PHFM medium supplemented with 1% (v/v) FCS. The plate was incubated for 48 hours at 37ºC and 5% CO_2 in humidified incubator in the presence or absence of stimulants as noted above. In the case of using inhibitors, cells were incubated with either Tyrphostin AG490 or Tyrphostin AG1295 for 3 hours at 37º C and 5% CO_2 before plating. The plate then was incubated at the same conditions for a further 24 hours after adding 10µl of Alamar blue dye (44 µM resazurin salt) to each well [268]. Plates were read in a fluorimeter at 544nm excitation filter and 590nm emission filter.

2.7 Flow Cytometry

SMS-SB cells were harvested and washed three times in PBS buffer supplemented with 0.5% (w/v) BSA. Cells then were resuspended in the same buffer to a final density 5 x 10^6 cells/ml and 25ul of cells (1 x 10^5) was transferred to a 5ml tube for staining. Cells were Fc-blocked by treatment with 1ug of human IgG/10^5 cells for 15 minutes at room temperature prior to staining. Cells then were incubated for 45 minutes at 4ºC with 1µg of either FITC-conjugated or R-Phycoerythrin-conjugated mouse monoclonal antibodies raised against the human receptor or marker of interest. Following the incubation cells were washed twice in 4ml PBS buffer supplemented with 0.5% (w/v) BSA. Finally, cells were resuspended in 300µl of PBS buffer for flow-cytometric analysis.

2.8 Protein Estimation assay (Bradford's method)

A protein concentration standard curve was plotted by using gradient concentrations (0, 5, 10, 15, 20,25,30,35 and 40 µg/ml) of BSA. Either standards or samples were diluted in dH_2O before adding 1ml of Bradford's reagent (0.01% (w/v) Coomassie Blue G250, 5.1% (v/v) H_3PO_4 and 5% (v/v) Ethanol). Samples and standards were incubated at room temperature for 10 minutes and the light absorption was detected by spectrophotometry at A_{595} nm. The unknown protein concentrations were each determined in accordance with the linear range of the resulting standard curve.

2.9 Antibody arrays

2.9.1 Principle of the assay

This assay is intended to analyze the phosphorylation profiles of kinases and their protein substrates. It is a simultaneous detection of the levels of phosphorylation of 46 kinase phosphorylation sites, using capture and control antibodies spotted in duplicate on nitrocellulose membranes. These antibodies catch their targets in the lysates during overnight incubation. The captured targets then will be visualized by biotinylated detection antibodies. Some signals will produced when the membrane developed by streptavidin-HRP followed by chemiluminescent detection reagents. Each capture spot corresponds to the amount of phosphorylated protein bound.

2.9.2 Procedures

SMS-SB cells were harvested and washed twice in PFHM and adjusted to a density of 10^7 cells/ml in PFHM. Four universal tubes each contained 10^7 cells/ml; one of them was a control without treatment and at 0 time, the rest of them were treated by either 5µg/ml of long peptide or 250ng/ml of sCD23 and incubated at 37º C and 5% CO_2 in a humidified incubator for 5, 10 and 20 minutes time course. The cells were washed twice in ice-cold phosphate-buffered saline to quench the reaction. After the second wash the supernatants were discarded and the pellets were resuspended in 1ml of lysis buffer supplied with the antibody array kit. Samples then were incubated on ice for 30 minutes on rocking platform at 20xg, then centrifuged at 14000 xg for 5 minutes at 4ºC. Supernatants were then transferred into a clean 1.5 ml microcentrifuge tube. The

Bradford protein estimation was carried out for the soluble fractions before using them for the array protocol. Each membrane to be used was blocked by incubating in 1ml of array buffer which is supplied with the array membranes for 1 hour on a rocking platform. The lysates were then diluted to 120µg/ml using the array buffer and applied onto the blocked membranes. Membranes then were incubated overnight at 4ºC. On the following day membranes were washed three times by 1x wash buffer for 10 minutes each. After the third wash each membrane was incubated with 1ml of array buffer containing 20µl of detection antibody cocktail for 2 hours at room temperature on a rocking platform. Membranes were washed again three times by 1x wash buffer for 10 minutes each followed by incubating with streptavidin-HRP for 30 minutes at room temperature on a rocking platform. Three washes were applied to the membranes with 1x wash buffer for 10 minutes each. Finally, membranes were developed by the chemiluminescence substrate for three minutes and exposed several times for different periods on the X ray film.

2.10 Western blotting

SMS-SB cells were harvested and washed twice with protein-free media (PFHM) and adjusted to a density of 5×10^6 cells/ml. Two ml of cells either treated or untreated were washed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EGTA) and lysed in RIPA buffer containing (1mM Na_3VO_4, 1% (v/v) NP40, 1mM Na deoxycholate, 1mM PMSF and 2µg/ml Leupeptin). Protein concentrations were estimated by Bradford's method. The same protein concentration of lysates was loaded per lane on a 4-12% NuPAGE Novex Bis-

Tris gradient gels using the MES buffer; both were supplied from Invitrogen. Proteins were separated on the gels at 180V and 70mA for 1 hour. Separated proteins were transferred onto nitrocellulose membrane in the presence of transfer buffer at 30V and 220mA for 90 minutes using (XCell SureLock) blotting system supplied from Invitrogen. The membrane then was blocked by TBS/T buffer containing either 5% non fat milk or BSA according to the antibody supplier protocol and then rinsed once with TBS/T buffer. The membrane was incubated with primary antibody for either four hours at room temperature or overnight at 4 º C according to the supplier protocol and washed by TBS/T three times for 5 minutes each before incubating for one hour with HRP-linked secondary antibody. After that the membrane was washed three times by TBS/T buffer for 5 minutes each. Ultimately, the membrane was developed by using the chemiluminescence substrate and exposed several times with different periods on the X ray film.

2.11 Immunoprecipitation

SMS-SB cells were harvested and washed twice with PFHM and adjusted to a density of 5×10^6 cells/ml. Two ml of cells either treated or untreated were washed twice in ice-cold PBS buffer and lysed in 500µl of HEPES buffer containing (1% (w/v) Octyl-β-D-glucopyranoside, 0.5% (v/v) Protein inhibitor cocktail). The protein concentrations were estimated by Bradford's method. 400µl containing 400µg of protein from each sample and the control was incubated for 30 minutes with 4µg antibody against the target protein in a spin column purchased from Millipore which contains precipitation slurry, and 1µg antibody capture affinity ligand supplied with the kit. The spin columns were washed twice with 1x wash buffer. Target proteins

were eluted by applying three cycles of adding 70µl of 2x elution buffer and centrifugation at 2400 xg for 30 seconds. The eluates were collected after each elution cycle. The eluates were run on a 4-12% NuPAGE Novex Bis-Tris gradient gels using the MES buffer supplied from Invitrogen and immunoblotted as described in western blotting section.

2.12 Electrophoretic-Mobility Shift Assay (EMSA)

SMS-SB cells were harvested and washed twice with PFHM and adjusted to a density of 5×10^6 cells/ml. Two ml of cells either treated or untreated during the time course were washed once with 5ml of ice-cold PBS buffer containing phosphatase inhibitors supplied with the nuclear extraction kit (purchased from Active Motif Company). Samples were spun down at 4ºC for 5 minutes at 2400 xg. The pellets were lysed by 500µl hypotonic buffer supplied with the kit and incubated for 15 minutes on ice on a rocking platform at 20 xg. Samples were vortexed at high speed each with 25µl detergent supplied with the kit and centrifuged at 14000xg for 30 seconds at 4ºC. Pellets were resuspended in 50µl complete lysis buffer and incubated for 30 minutes on ice on a rocking platform at 20 xg. The suspensions were centrifuged at 4ºC for 10 minutes at 14000xg. The supernatants were collected and the protein concentration was measured by Bradford's method. Three micrograms of each sample was incubated for 5 minutes at room temp with (1µl of STAT5b biotinylated probe [CAGAATTTCTTGGGAAAGAAAAT], 1µg poly d(I-C), 2µl of 5x binding buffer and 3µl of nuclease-free water). Mixtures were incubated at 15°C for 30 minutes in a thermal cycler. Samples were mixed with 1 µl of loading dye and run on 6.0% non-

denaturing polyacrylamide gel with pre-chilled 0.5X TBE. Separated protein/DNA complexes on the gel were transferred onto N^+ nylon membranes purchased from Amersham company. Membranes were baked at 80ºC for an hour for protein/DNA cross linking and blocked for 15 minutes in a blocking buffer. Membranes were incubated for 30 minutes with streptavidin- HRP followed by three washing cycles with 1x wash buffer for 8 minutes each. At the last step membranes were incubated in detection reagent supplied with the kit for 5 minutes and then for 5 minutes again with the chemiluminescence substrate before exposing several times for different periods on the X ray film.

2.13 Calpain activity assay

SMS-SB cells were harvested and washed twice with PFHM and adjusted to a density of 2×10^6 cells/ml. For the short term stimulation study, cells were divided into three 50ml tissue culture tubes each containing 6ml of cellular suspension; one was a control without treatment, one was treated with 1.8nM vitronectin and one treated with 33uM long peptide. The three tubes were incubated at 37º C and 5% CO_2 in a humidified incubator. From the control tube 1ml of cells was immediately incubated on ice as time 0 control. One ml from each tube was removed and immediately incubated on ice at each time point (3,15,25 and 40 minutes). However, for the long term stimulation study the same procedure was followed but the differences were the time points which were 30 minutes, 1, 2 and 4 hours. Samples were then centrifuged at 14000xg for 30 seconds at 4ºC. Supernatants were discarded and the pellets were resuspended in 100µl of extraction buffer supplied with the kit and incubated on ice for 20 minutes with gentle mixing by tapping from time to time. Samples then

centrifuged at 10000xg for 1 minute at 4ºC. Supernatants were collected and the total protein was estimated by using Bradford's reagent. In 96-well white flat bottom plate samples were plated in 60µg total protein/ 85µl of extraction buffer.10µl of 10x reaction buffer was added into each well followed by adding a specific Calpain substrate, which is called AC-Leu-Leu-Tyr-7-amino-4-Trifluoromethylcoumarin (AC-LLY-AFC).The plate was incubated for in the dark at 37º C. Fluorescent activity was measured by a fluorimeter at 355nm excitation filter and 505nm emission filter.

2.14 Control for siRNA Transfection efficiency

SMS-SB cells were harvested and washed twice with PFHM and adjusted to a density of 3×10^5 cells/ml. In a 24 well tissue culture plate, 250µl of cells were plated in each well. The oligo/Transfection reagent complex was formed in three different ratios to examine transfection efficiency and toxicity, 3µl of Control siRNA (Fluorescein Conjugate) were diluted in 50µl PFHM and mixed with either 1µl,2.5µl or 4µl of Transfection reagent. The mixtures then were vortexed and incubated at room temperature for 15 minutes before adding each of them to the cells in the appropriate well. The plate then was incubated at 37º C and 5% CO_2 in a humidified incubator. After 48 hours cells were harvested and washed twice with PBS and resuspended in 500ul of PBS. Finally, cells were assayed by flowcytometry.

2.15 Reporter gene experiments

The plasmid/Transfection reagent complex was formed by diluting 3µg of pGL4.34[luc2P/SRF-RE/Hygro] Vector in 180µl PFHM without antibiotics and

mixed with 8µl of X-tremeGENE HP Transfection reagent. The mixture was incubated for 25 minutes at room temperature. In terms of the STAT5 reporter vector assay, the complex was formed by diluting 1.5µg TransLucent GAL4-STAT5B (TAD) fusion protein expression vector with 1.5µg of TransLucent luciferase reporter vector in 180µl PFHM without antibiotics and then adding 8µl of X-tremeGENE HP Transfection reagent. Meanwhile, SMS-SB cells were harvested and washed twice with PFHM and adjusted to a density of 3×10^5 cells/ml. 90µl of cells/well were plated in 96-well white flat bottom plate and mixed with 10µl of plasmid/Transfection reagent complex. The plate was incubated at 37º C and 5% CO_2 in a humidified incubator for 48 hours before adding stimulants (sCD23, LP and vitronectin) and re incubating at the same conditions for 6 hours as described in Chapter 4 . Cells then were lysed and treated with luciferen substrate. The luciferase activity was estimated in three minutes after adding the substrate by illuminometer .

2.16 Data analysis

Results are shown as mean plus or minus standard error of the mean (SEM) of triplicate experiments. Data were normalized by dividing each result by the control. Statistical comparison was performed using the Student paired t test. The minimal level of significance was $P<0.05$. Each result represents an accumulated data for at least three experiments that were carried out in three different dates unless otherwise stated. In terms of western blots, EMSA blots and the antibody membranes the data were quantitated by using a software called Image J (National Institutes of Health), to estimate the optical density of each experimental sample.

RESULTS

3. SMS-SB cells growth in response to different αvβ5 integrin ligands and other proteins related to B-cell progenitors growth.

3.1 Introduction

There are several factors which stimulate growth and maturation of B-cell precursors during their development in bone marrow. As mentioned in the introduction chapter, stromal-derived factor (SDF1) is one of the important growth and survival factors for B-cell progenitors which integrates with other growth factors such as interleukin-4, interleukin-7 and Platelet-derived growth factor (PDGF) [269, 270, 271]. In this study, the cell line model used is SMS-SB, which is a line derived from a nine-year old girl diagnosed as an acute lymphocytic leukaemia patient [265]. These cells express the cytoplasmic μ heavy chains but do not secrete them [265]. In addition, they do not express IgM and light chains [265]. Phenotypically, SMS-SB cells seem to be located between two stages of B-cell progenitors development; pro-B cell and pre-B cell [265].

On the other hand, CD23 is highly expressed on chronic lymphocytic leukaemia cells but was not detected in acute lymphocytic leukaemia cells [272, 273]. However, soluble CD23 was highly increased in the serum of 42 chronic lymphocytic leukaemia patients comparing with 32 normal individuals [274]. In terms of SMS-SB cells, they do not express CD23, CD11b-CD18, CD11c-CD18 and CD21 [275]. Furthermore, CD23 sustains bcl-2 protein levels in SMS-SB cells in order to prevent apoptosis when they are cultured in low cell density [275]. This action occurs when soluble CD23 binds the αvβ5 integrin [95]. Although αvβ5 integrin binds its specific ligands such as vitronectin

and fibronectin through an RGD motif, it binds CD23 via a tripeptide motif Arg-Lys-Cys (RKC) located close to the C-type lectin domain of CD23 [95].

The main purpose of this chapter is to investigate the ability of different ligands to stimulate SMS-SB growth at the minimal density, and in addition, to find out which signalling targets are involved in the action of sustaining SMS-SB cell growth.

3.2 Results

3.2.1 Phenotypic characterisation of SMS-SB cell line

There are several markers used to identify the B-cell development stages. As mentioned previously SMS-SB cells are B-cell progenitors extracted from an acute lymphocytic leukaemia patient. The aim of this experiment is to identify the development stage that SMS-SB cell represents and to investigate the expression of growth-related receptors which this study will focus on, mainly the αVβ5 integrin as well as PDGFR and CXCR4 (the SDF-1 receptor).

Figure 3.1 clearly shows that SMS-SB cell express CD19 a major determinant of B-cell lineage and, to a lesser extent, CD45. However, SMS-SB cells do not express CD34, a marker for the very early stage of haematopoietic cell development. In addition, these cells do not express some pre-B cell markers such as μ-chain, or CD10 and they do not express CD5 which is most likely a marker of T-cell and human foetal lymphoid tissues [276] as shown in figure 3.1. In terms of the receptors that this project is to focus on, SMS-SB cells do not express CD23 and β3 integrin but, they clearly do express CXCR4, PDGF receptor and αVβ5 integrin as shown in figure 3.1. These data suggest that SMS-SB cell is a Pro-B cell and probably at the late developmental stage of pro-B cell because they also express cytoplasmic μ-chain, (data not shown) [265].

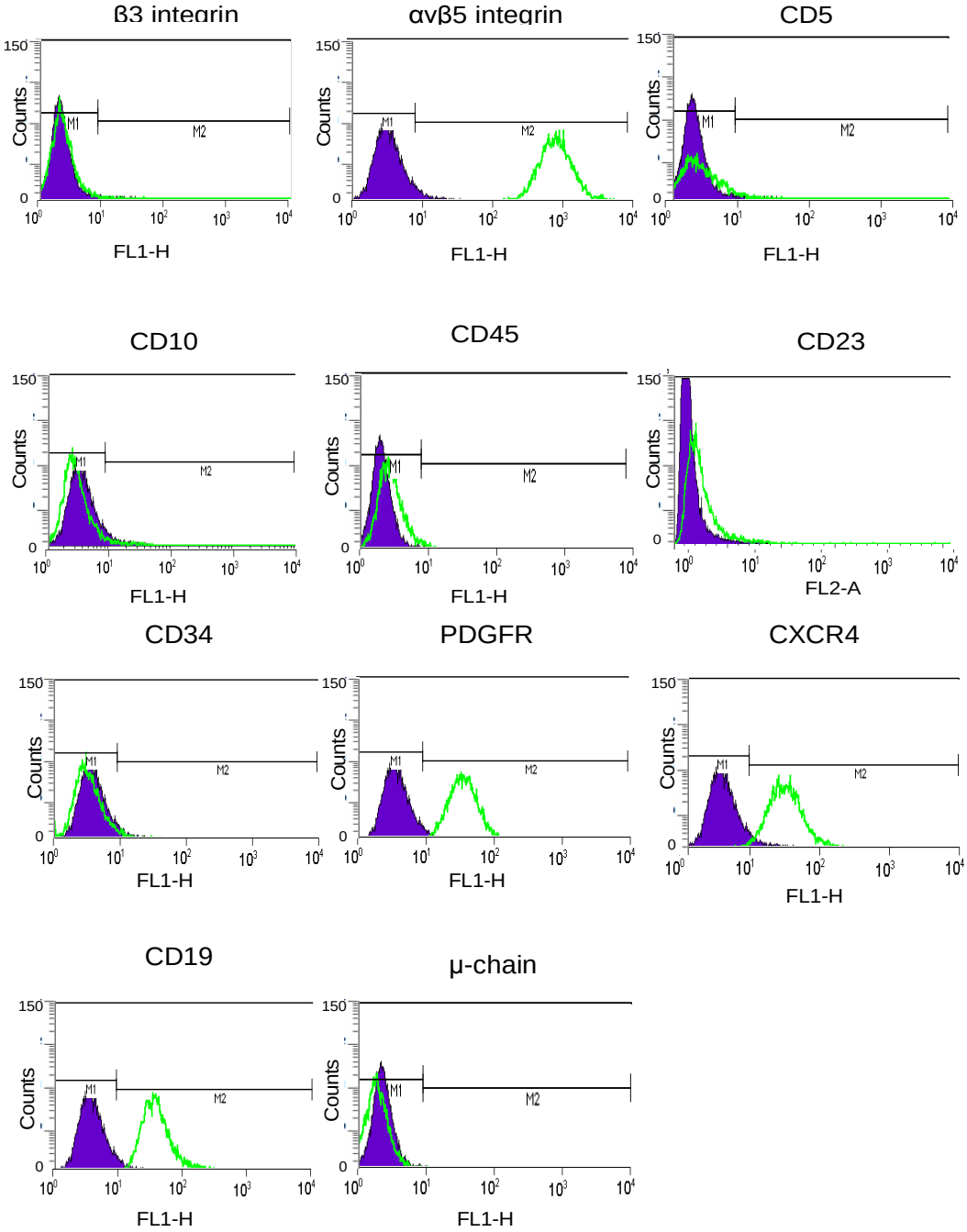


Figure 3.1 Phenotypic characterization of SMS-SB cell. SMS-SB cells were harvested and washed twice with PBS buffer supplemented with 0.5% (w/v)BSA and adjusted at $5x10^6$ cell/ml. 25µl of cells were incubated individually with 10µl of either R-Phycoerythrin or FITC conjugated antibodies to CD23, CD19, CD5, CD10, CD45, µ-Chain, β3 integrin, αVβ5 integrin, PDGF receptor, CXCR4 or CD34. The negative controls were Mouse IgG_1-PE conjugated and Mouse IgG_1-FITC conjugated. Shown expressing was assessed by using FACS system. FACS staining for the negative controls is shown as blue shaded area and for the each MAb is shown as a green line. These data are representative of at least three independent staining experiments.

3.2.2 SMS-SB cell growth is cell density dependent.

There are two purposes for this experiment. The first aim is to investigate the effect of cell density on SMS-SB cell growth which has published by our laboratory already [271]. According to Tsai et. Al (1994). SMS-SB cells express and release PDGF as an autocrine factor which is also implicated to sustain cell growth at high density. The other goal of this experiment is to compare the accuracy of Alamar blue, a fluorescent dye and tritiated thymidine incorporation in the context of growth estimation.

Briefly, Alamar blue is a solution of resazurin salt added in a non-fluorescent oxidized/blue form, which undergoes reduction caused by oxygen consumption metabolism of living cells [277]. The reduced form of the resazurin salt is a fluorescent/pink, which is called resorufin, and can be measured by a fluorimeter with 544nm excitation filter and 590nm emission filter [277]. The tritiated thymidine [^{3}H]-TdR proliferation assay depends upon measuring the amount of [^{3}H]-TdR incorporated into DNA by using a scintillation counter [278].

Panel (a) in figure 3.2 clearly shows that SMS-SB cells proliferate about eight-fold when plated at 5X10^5 cell/ml compared with those plated at 5X10^4 cell/ml, about five-fold when plated at 2.5X10^5 cell/ml, and about three-fold when plated at 1X10^5 cell/ml compared with those plated at 5X10^4 cell/ml. These data were collected by using the thymidine incorporation method. Statistically, the growth increase of cells was significant over the three biological replicates in which the *P* value was less than 0.05.

Panel (b) in figure 3.2 shows similar data using Alamar blue. Surprisingly, Alamar blue method gives a similar reading to [^{3}H]-TdR method in which P values of three biological replicates are less than 0.001. In general, these data suggest that SMS-SB

cell growth is cell density-dependent and that either Alamar blue or [^{3}H]-TdR corporation can be used to detect proliferation accurately.

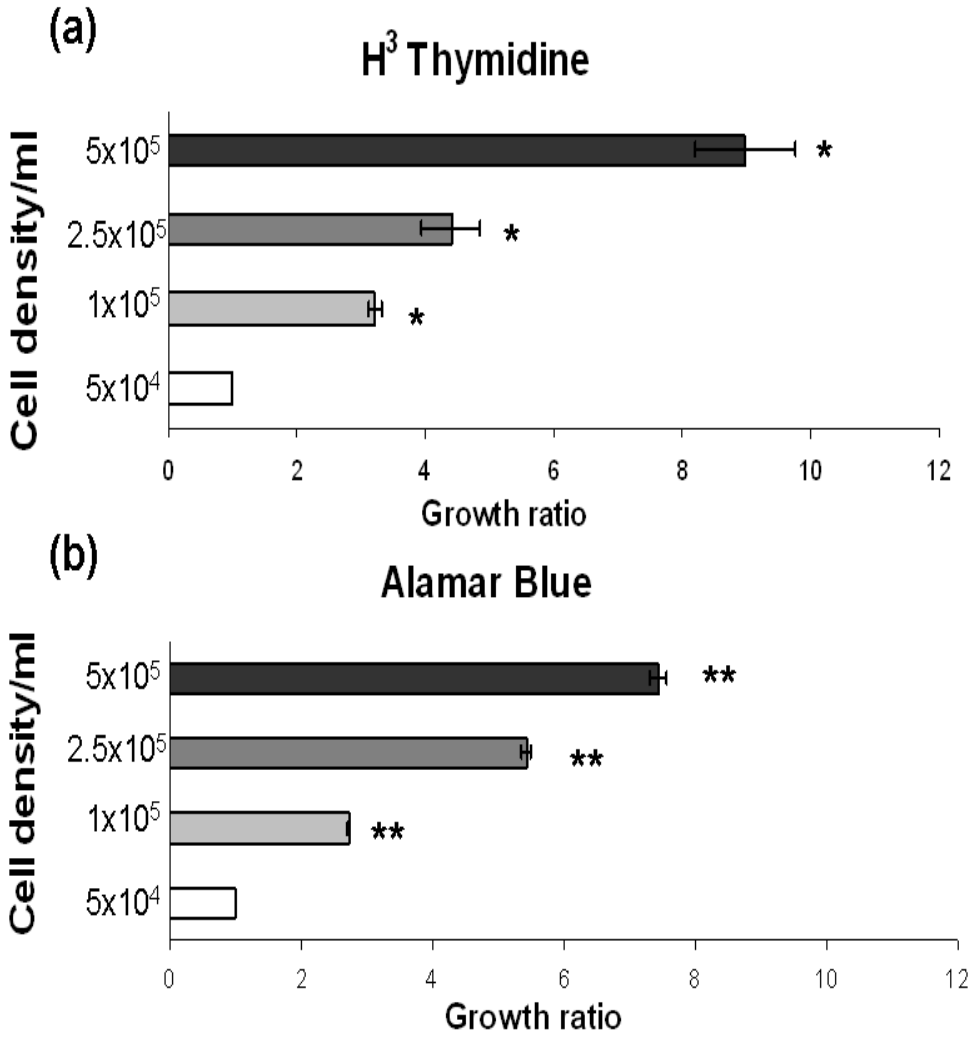


Figure 3.2 Density dependent SMS-SB cell growth. SMS-SB cells were cultured at the indicated cell densities for 48 hours at 37ºC and 5% CO_2.(a) Cells were pulsed for the final six hours with 0.3µCi/well [^{3}H]-TdR and harvested onto filter mats for liquid scintillation spectrometry.(b) Cells were incubated for 24 hours further with 4.4uM resazurin salt (Alamar blue) and the fluorescence was estimated in a fluorometer at 544nm excitation filter and 590nm emission filter. ** $P < 0.001$, * $P < 0.05$ versus corresponding $5x10^4$ cell/ml. (These data represent three independent experiments for each).

3.2.3 Soluble CD23 and LP stimulate proliferation of SMS-SB cells while vitronectin does not

In this experiment, three different stimuli were used to investigate the role of αVβ5 integrin in SMS-SB cell growth. LP is a synthetic peptide derived from soluble CD23 with a length of 15 amino acids and contains the RKC motif, a motif via which soluble CD23 binds to the αVβ5 integrin. The third ligand is Vitronectin, one of the matrix ligands which binds to the αVβ5 integrin via its RGD motif.

Figure 3.2 clearly shows that SMS-SB cell growth is dose-dependent when cells are stimulated by either sCD23 or LP (the RKC motif-containing ligands). In panel (a) SMS-SB cell growth was increased about 10-fold following stimulation with LP at a concentration of 66μM (10μg/ml) and about 9-fold with 33μM (5μg/ml). Both of these stimulations were statistically significant different from unstimulated controls (*P* values were less than 0.005 for three independent experiments). A similar trend was obtained using sCD23 stimulation (panel (b)) which gives about 8 fold stimulation when added at 20nM (500ng/ml) and about 6 fold with 10nM (250ng/ml). Data analysis for three independent experiments shows that both of these stimulations are significant (*P* values are less than 0.005 and 0.05, respectively). On the other hand, SMS-SB cells did not respond to increasing concentrations of Vitronectin 0.36-3.6nM (50-500ng/ml) as shown in figure 3.2 panels (c). These data suggested that sCD23 (the RKC containing ligand) could stimulate αVβ5 integrin to modulate B-cell precursor growth whereas matrix ligands (the RGD containing ligands) could not.

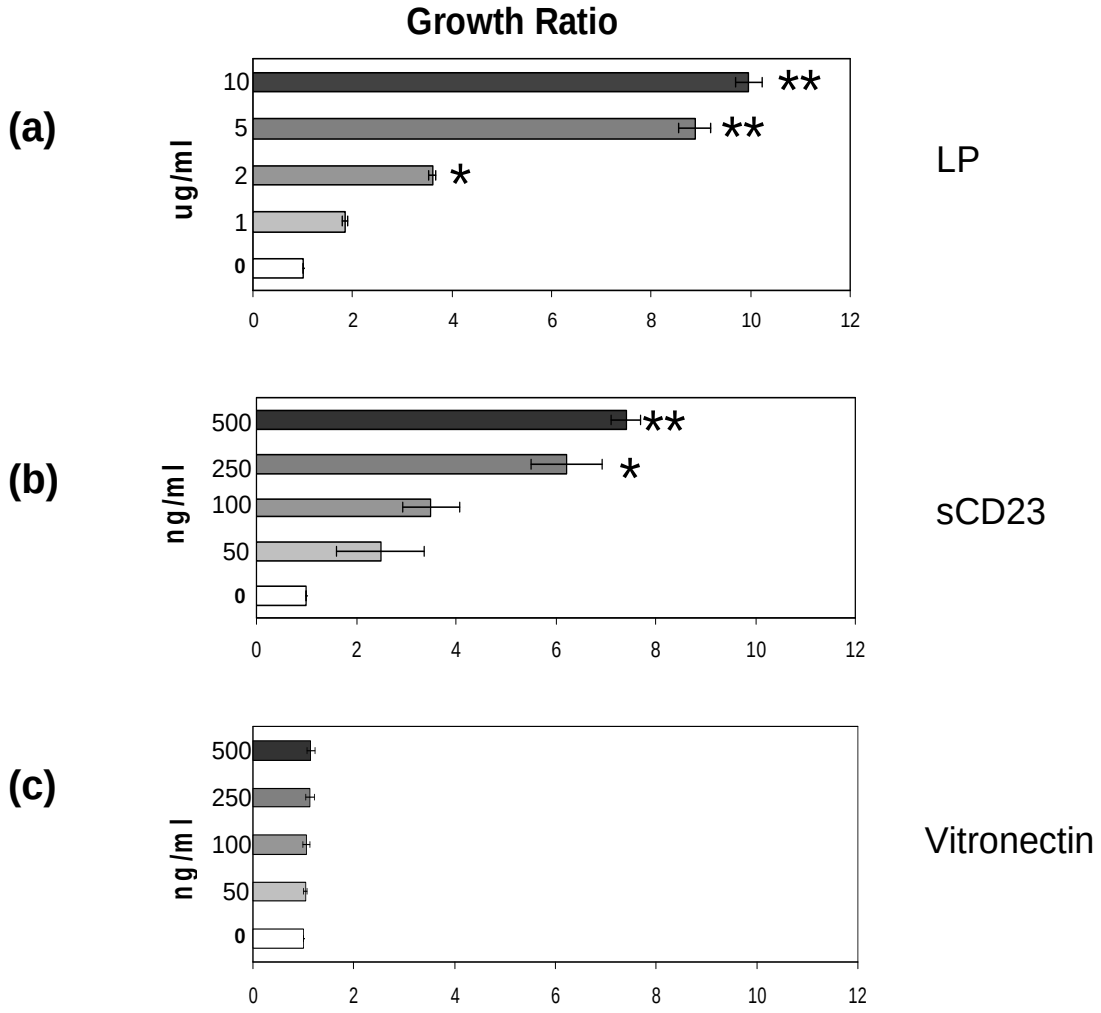


Figure 3.3 αVβ5 integrin mediates growth of SMS-SB cells when ligated by sCD23 and LP (a peptide derived from sCD23 contains RKC motif), but not vitronectin. SMS-SB cells (5×10^4 cells / ml in protein-free medium) were stimulated with the indicated concentrations of stimulants for 48hr, then processed for Alamar Blue fluorescence (sCD23 and Vitronectin treated plates) or were pulsed with 0.3μCi/ well tritiated thymidine (LP treated plates).** $P < 0.005$, * $P < 0.05$ versus corresponding untreated cells. (These data represent three independent experiments).

3.2.4 Stromal cell –derived factor 1α (SDF1α) Stimulates SMS-SB cells proliferation. And enhances SMS-SB cells growth stimulated by LP.

Stromal cell-derived factor-1 plays an important role in B-cell development [124].The following experiments were carried out to determine to what extent SDF-1 itself can stimulate SMS-SB cell growth, and also to investigate the effect of SDF-1 on cell growth stimulated by LP. Normally, the plasma levels of SDF-1 are between 8-20ng/ml [279]. The SDF-1 levels either in peripheral blood or bone marrow are dramatically increased in AML, HIV infections and Non-Hodgkin's lymphomas [279, 280, 281].

Figure 3.4 Panel (a) shows that a modest increase of SMS-SB cell growth is correlated with increasing concentrations of added SDF-1α. This growth increase peaked at 250ng/ml (31.25nM) SDF-1α, and was statistically significant (*P* value was less than 0.05 for three independent experiments) compared to the cells were grown without treatment.

In Panel (b), SDF-1α strikingly increases the cell growth stimulated by 5ug/ml (33μM) LP. Roughly, about a 20% further growth increase was observed when cells were co-stimulated by either 100ng/ml (12.5nM) or 250ng/ml (31.25nM) SDF-1α, the differences were statistically significant (*P* values were less than 0.05 for three independent experiments) compared to the cells were stimulated with LP alone.These data suggested that SDF-1α enhances cell-growth stimulated by LP.

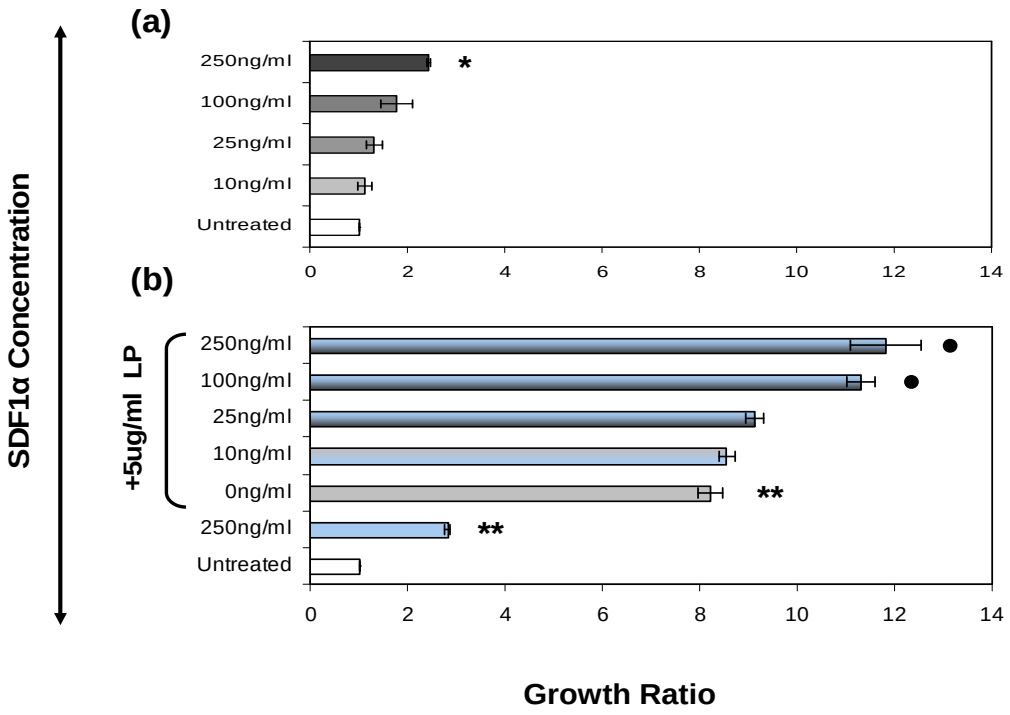


Figure 3.4 SDF1α stimulates growth of SMS-SB cells and enhances SMS-SB cells proliferation stimulated by LP. SMS-SB cells (5×10^4 cells / ml in protein-free medium). Panel (a) cells were stimulated with the indicated concentrations of SDF1α for 48hr, and then pulsed with 0.3μCi/ well tritiated thymidine. * $P < 0.05$ versus corresponding untreated cells. Panel (b) cells were cultured with either 31.25nM (250ng/ml) of SDF1α or 33μM (5μg/ml) of LP or with 5μg/ml of LP plus increasing concentrations of SDF1α for 48 hours, then processed for Alamar Blue fluorescence. ** $P < 0.005$ versus corresponding untreated cells. • $P < 0.05$ versus corresponding cells stimulated with LP alone. (These data represent three independent experiments).

3.2.5 Platelet Derived Growth Factor ($PDGF_{AB}$) Stimulates SMS-SB cells proliferation. And enhances SMS-SB cells growth stimulated by LP.

There are five different isoforms of PDGF ; $PDGF_{AA}$, $PDGF_{BB}$, $PDGF_{CC}$, $PDGF_{DD}$ and $PDGF_{AB}$ [146, 155, 156]. Many studies have been published over the last two decades demonstrates that PDGF family members are implicated in stimulating the growth of various cancers, angiogenesis, atherosclerosis and liver fibrosis [282, 283, 284, 285]. In leukaemias, $PDGF_{AB}$ is implicated in AML cell growth, and is also expressed in CML, pre-B acute lymphocytic leukaemias (particularly SMS-SB cells) and chronic eosinophilic leukaemia [286, 271, 287].

The purpose of this experiment was to investigate the role of $PDGF_{AB}$ as a growth factor for SMS-SB cells in sustaining cell growth. The other aim was also to examine any synergistic effect of $PDGF_{AB}$ with soluble CD23, which is represented by LP.

Figure 3.5 panel (a) shows that SMS-SB cell growth gradually increased with increasing $PDGF_{AB}$ dose. The statistical analysis for three independent experiments shows that there is a significant growth increase (*P* value was less than 0.005) at 250ng/ml (82.5nM), with an increase of three-fold compared to the cells were grown without treatment.

In terms of co-stimulation, panel (b) PDGF has enhanced by more than 20% cell-growth stimulated by 5μg/ml LP. This additional growth increase was significant at 100ng/ml and 250ng/ml of $PDGF_{AB}$ (*P* values were less than 0.05 and 0.005, respectively, for three independent experiments) compared to the cells stimulated with LP alone.

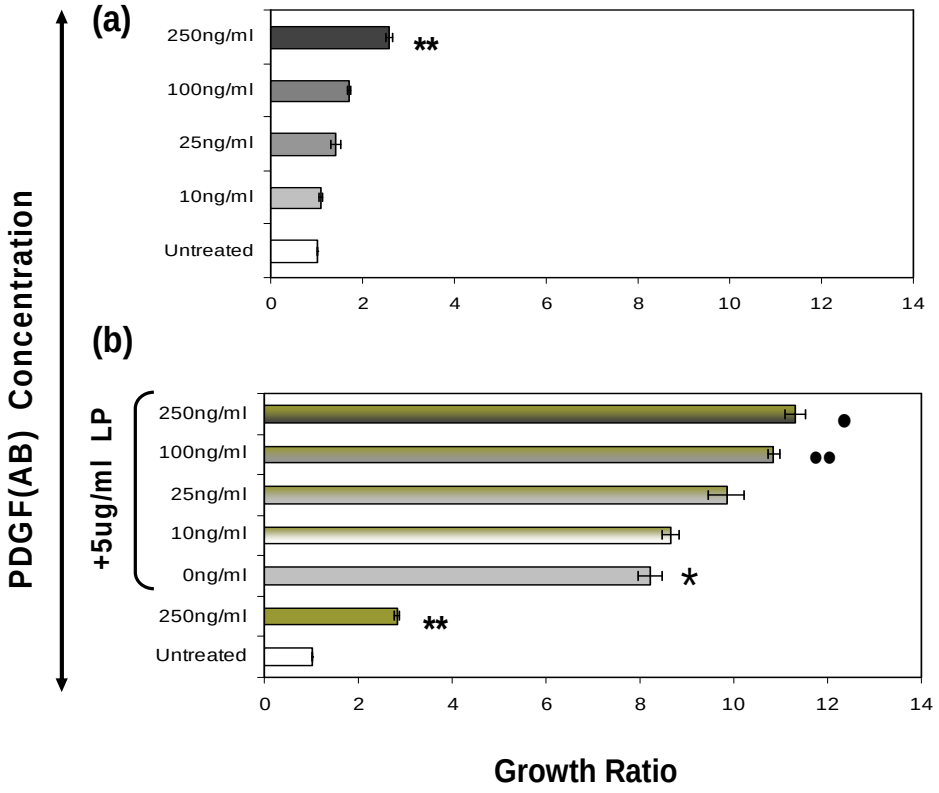


Figure 3.5 $PDGF_{AB}$ stimulates growth of SMS-SB cells and enhances cell growth stimulated by LP. SMS-SB cells (5×10^4 cells / ml in protein-free medium), panel (a) cells were stimulated with the indicated concentrations of $PDGF_{AB}$ for 48hr, then pulsed with 0.3µCi/ well tritiated thymidine. ** $P < 0.005$ versus corresponding untreated cells. Panel (b) cells were cultured with either 8.3nM (250ng/ml) of PDGF-AB or 33µM (5µg/ml) of LP or with 5µg/ml of LP plus increasing concentrations of PDGF-AB for 48 hours, then processed for Alamar Blue fluorescence. ** $P < 0.005$, * $P < 0.05$ versus corresponding untreated cells. • $P < 0.05$, •• $P < 0.005$ versus corresponding cells stimulated with LP alone. (These data represent three independent experiments).

3.2.6 Soluble CD23 and LP stimulate the phosphorylation of different kinase substrates

The previous data raised the question of how LP or soluble CD23 stimulates the cell growth and which signalling pathway is recruited by the αVβ5 integrin as a consequence of its interaction with specific ligands.

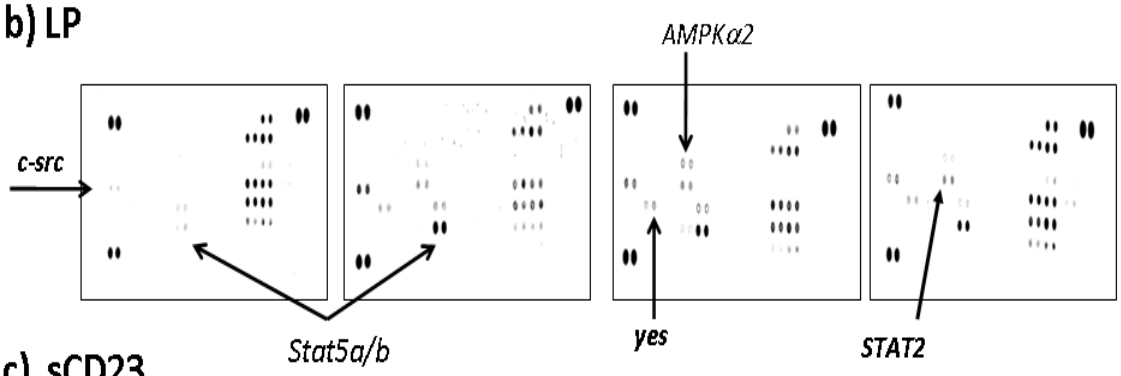
One of several recent methods for screening cell signalling pathways is an antibody array technique. This method depends on a group of antibodies being spotted individually on a nitrocellulose membrane in duplicate. These antibodies are raised against different human cell signalling proteins in phosphorylated form.

This experiment revealed that either soluble CD23 or LP activates phosphorylation of several kinases and signal transducers. Figure 3.6 shows the antibody array blots and their densitometric analysis for lysates of SMS-SB cells either untreated, as a control, or treated with either sCD23 or LP for a 20 minutes time course. Both stimuli increase phosphorylation of STAT5a/b at Tyrosine 699 which is clearly observed after five minutes of stimulation and sustained over the 20 minutes time course. The other signal transducer was STAT2 which is phosphorylated at Tyrosine 689. Furthermore, both ligands stimulated the phosphorylation of Src at Tyrosine 419 but with LP that event came five minutes earlier. In addition, a Src family member (Yes) is phosphorylated at Tyrosine 426 and AMPKα2 at Threonine 172. These data might open a broad story involving different signaling pathways for different purposes such as growth, survival or anti apoptosis.

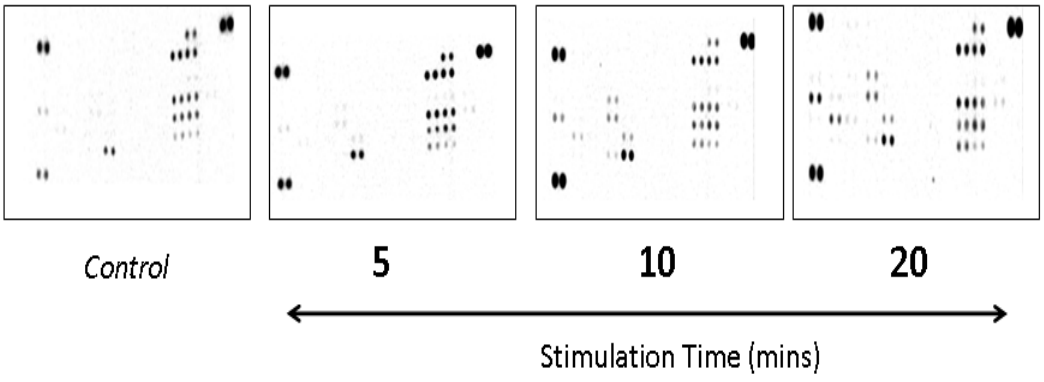
a)

	(1,2)	(3,4)	(5,6)	(7,8)	(9,10)	(11,12)	(13,14)	(15,16)	(17,18)
A	Positive control	p38α T180/Y182	ERK1/2 T202/Y204, T185/Y187	JNK pan T183/Y185, T221/Y223	GSK-3αβ S21/S9	-	p53 S392	-	Positive control
B	-	MEK1/2 S218/S222, S222/S226	MSK S376/S360	AMPKα1 T174	Akt S473	Akt T308	p53 S46	-	-
C	TOR S2448	CREB S133	HSP27 S78/S82	AMPKα2 T172	β-Catenin	p70 S6 kinase T389	p53 S15	p27 T198	Paxillin Y118
D	Src Y419	Lyn Y397	Lck Y394	STAT2 Y689	STAT5a Y699	p70 S6 kinase T421/S424	RSK1/2/3 S380	p27 T157	PLCγ-1 Y783
E	Fyn Y420	Yes Y426	Fgr Y412	STAT3 Y705	STAT5b Y699	p70 S6 kinase T229	RSK1/2 S221	c-Jun S63	Pyk2 Y402
F	Hck Y411	Chk-2 T68	FAK Y397	STAT6 Y641	STAT5 a/b Y699	STAT1 Y701	STAT4 Y693	eNOS S1177	Negative control
G	Positive control	-	Negative control	-	-	-	-	-	-

b) LP



c) sCD23



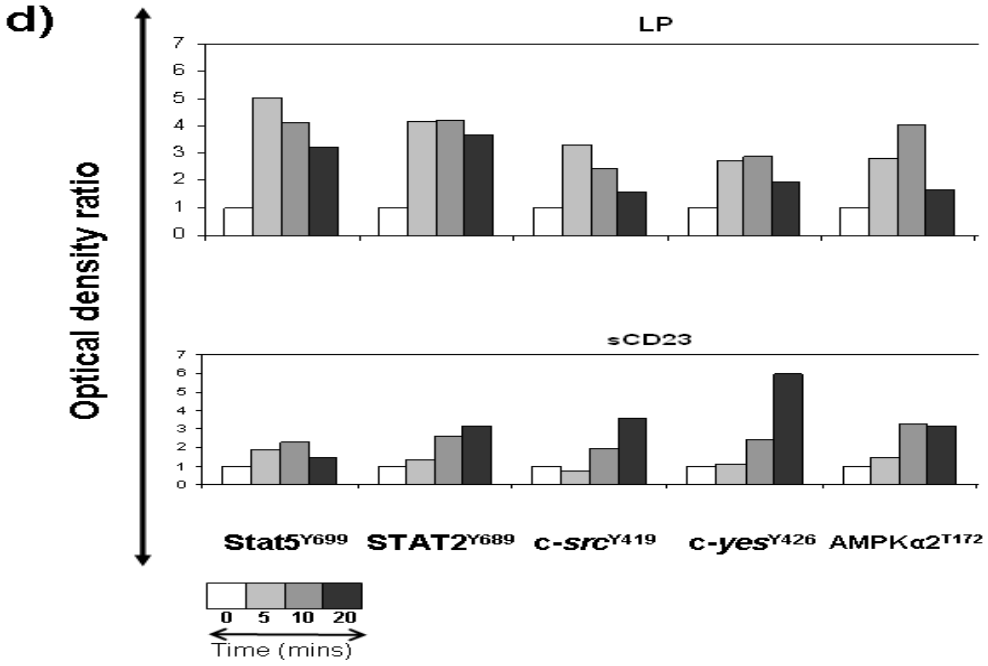


Figure 3.6 sCD23 and LP stimulate different signalling targets. SMS-SB cells were stimulated with either the CD23-derived LP, (33µM) or with sCD23 itself (10nM) for the indicated times and aliquots of lysates added to individual antibody arrays and binding determined by ECL Panels ***b***) and ***c***). Panel ***a***) shows the map of the target substrates printed on a nitrocellulose membrane and panel ***d***) shows the densitometry data.

3.3 Conclusion

The data presented in this chapter clearly indicate that SMS-SB cells express the αVβ5 integrin, PDGF receptor and CXCR4, which are related to the cell growth as demonstrated in the first chapter of this thesis. In addition, these cells express CD19, one of the common B-cell precursor markers; however, they do not express the μ-heavy chain on the cell surface which suggests that these cells might represent the pro-B cell development stage. Consequently, figure 3.3, 3.4 and 3.5 show that these receptors are related to the cell growth after stimulating them with $PDGF_{AB}$, SDF1-α, sCD23 and LP. Furthermore, figure 3.2 has also shown that SMS-SB cell growth is density dependent. These data are strongly compatible with the data published by Borland, G. et al, Tsai, L.H. et al and Acharya, M. et al. [95, 123, 271].

In order to investigate how the αVβ5 integrin is involved in cell growth stimulation, the antibody array data demonstrated in figure 3.6 show that both sCD23 and LP stimulate multiple signalling targets such as STAT5, STAT2, c-Src, c-yes and AMPKα2 which poses several questions about the link between STAT5 phosphorylation and cell proliferation, and about how the αVβ5 integrin can induce the phosphorylation of STAT5.

Therefore, the next chapter will concentrate on confirming the antibody array data and on investigating STAT5 activity as well as focusing on investigating the relationship between the αVβ5 integrin stimulation and the phosphorylation of STAT5.

4. Soluble CD23 and LP activate Jak2/ STAT5a/b pathway due to a unique PDGFRβ-αVβ5 integrin association.

4.1 Introduction

STAT5a/b is a heterodimeric protein composed of STAT5a (94KDa) and STAT5b (92KDa). The difference between these two proteins is in the 12 C-terminal amino residues [288]. STAT5a and STAT5b genes are located on chromosome 17 [192]. Several studies over the last ten years have revealed some critical roles of STAT5a/b in the immune system. STAT5a/b is required for T-cell proliferation, activation and tolerance mainly in response to IL-2 [289, 290, 291]. In addition, STAT5a/b is involved in haematopoietic cell adhesion and migration [292].

On the other hand, the Jak2/STAT5 signalling pathway is implicated to play a crucial role in cell survival, proliferation and apoptosis of several leukaemias in response to a wide range of cytokines and growth factors [293, 294, 295].

This chapter will concentrate mainly on investigating the role of the Jak2/STAT5a/b signalling pathway in SMS-SB cell growth stimulated by either sCD23 or LP to validate the data obtained from the antibody array experiments demonstrated in the previous chapter. The other important purpose for this chapter is to investigate how does the αVβ5 integrin contribute to the Jak2/STAT5ab signalling pathway.

4.2 Results

4.2.1 αVβ5 integrin mediates STAT5 phosphorylation due to its binding to different ligands

The data later are based on simple western blotting technique. There are two purposes for these experiments. The first aim is to validate the data that was obtained from the antibody array experiment. The second aim is to investigate the difference between different ligands that bind to αVβ5 integrin via different binding motifs in activating STAT5 phosphorylation. Three ligands in this experiment have been tested, soluble CD23 and LP which both bind by the RKC motif, and vitronectin which binds to the αVβ5 integrin via the RGD motif.

Figure 4.1 clearly shows that LP activates the phosphorylation of STAT5 at tyrosine 699 after five minutes of stimulation and sustains that event over the 20 minutes time course. Similar data were observed with stimulating the cells with soluble CD23 instead of LP as shown in figure 4.2. In contrast, as shown in figure 4.3 vitronectin does not show any significant stimulation. These data suggest that the αVβ5 integrin activates STAT5 phosphorylation when it binds to the soluble CD23 but not when the vitronectin binds.

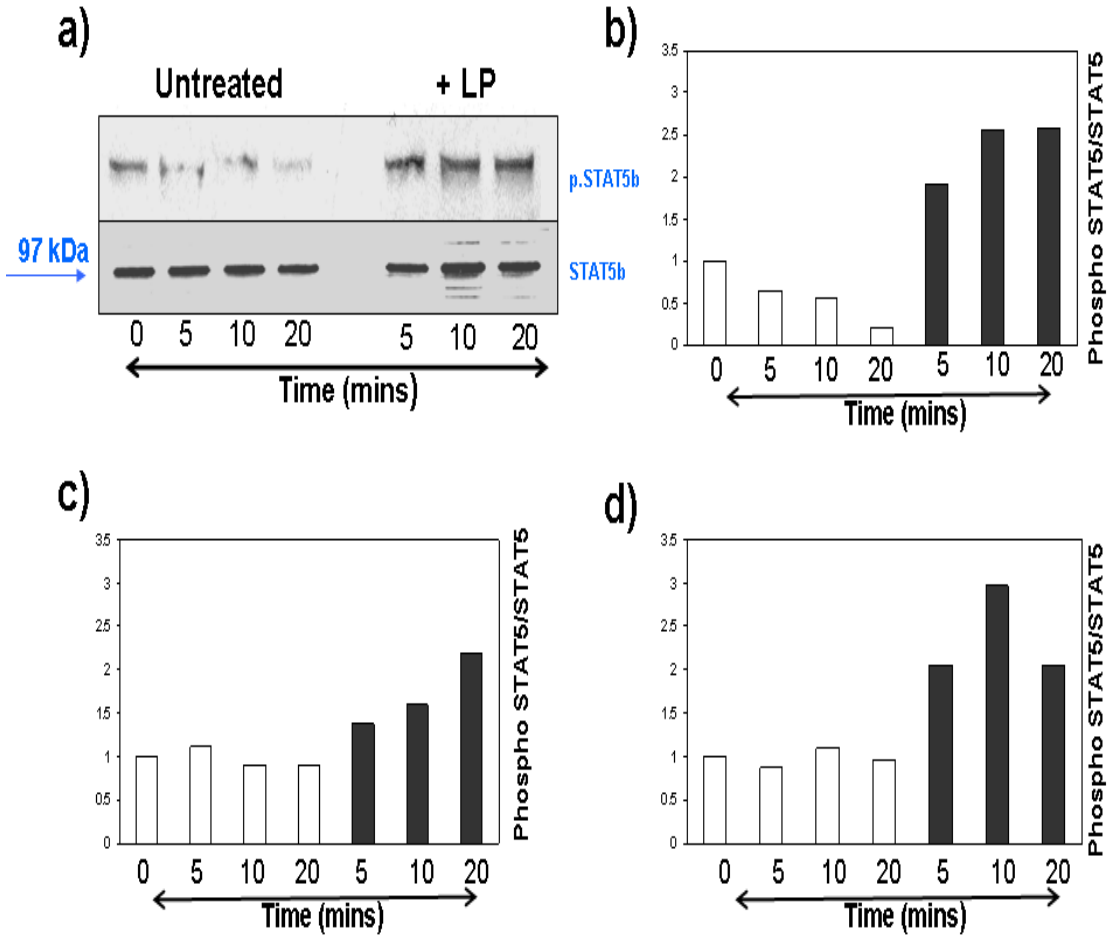


Figure 4.1 LP Stimulates the αVβ5 integrin to promote STAT5b phosphorylation. Lysates were extracted from cells either stimulated by 33μM LP or without stimulation for the indicated time course and, after western blotting, probed with antibodies to phosphorylated STAT5b then stripped and re-probed with antibodies to STAT5b protein Panel a). Panel b) is the densitometric analysis for the phospho-STAT5/STAT5 ratio demonstrated. The other Panels c)and d) are the densitometric analysis for the phospho-STAT5/STAT5 ratios for another blots of two individual experiments have been carried out in different weeks. Black bars represent phospho-STAT5/STAT5 ratios in stimulated cells in all cases, and the white bars represent the unstimulated cells.

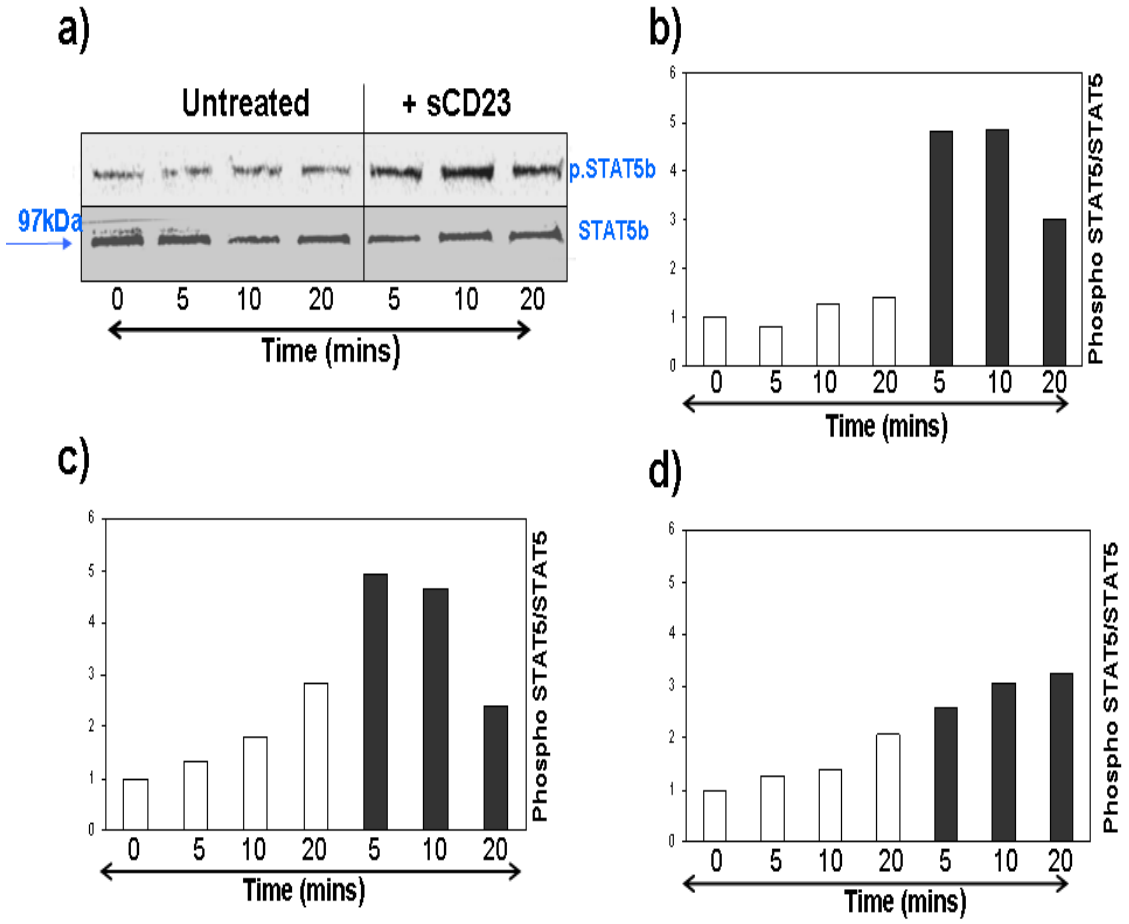


Figure 4.2 Soluble CD23 Stimulates the αVβ5 integrin to promote STAT5b phosphorylation. Lysates were extracted from cells either stimulated by 10nM sCD23 or without stimulation for the indicated time course and, after western blotting, probed with antibodies to phosphorylated STAT5b then stripped and re-probed with antibodies to STAT5b protein Panel a). Panel b) is the densitometric analysis for the phospho-STAT5/STAT5 ratios demonstrated. The other Panels c)and d) are the densitometric analysis for the phospho-STAT5/STAT5 ratios for another blots of two individual experiments have been carried out in different weeks. The white bars represent the unstimulated cells while the black bars represent the stimulated cells.

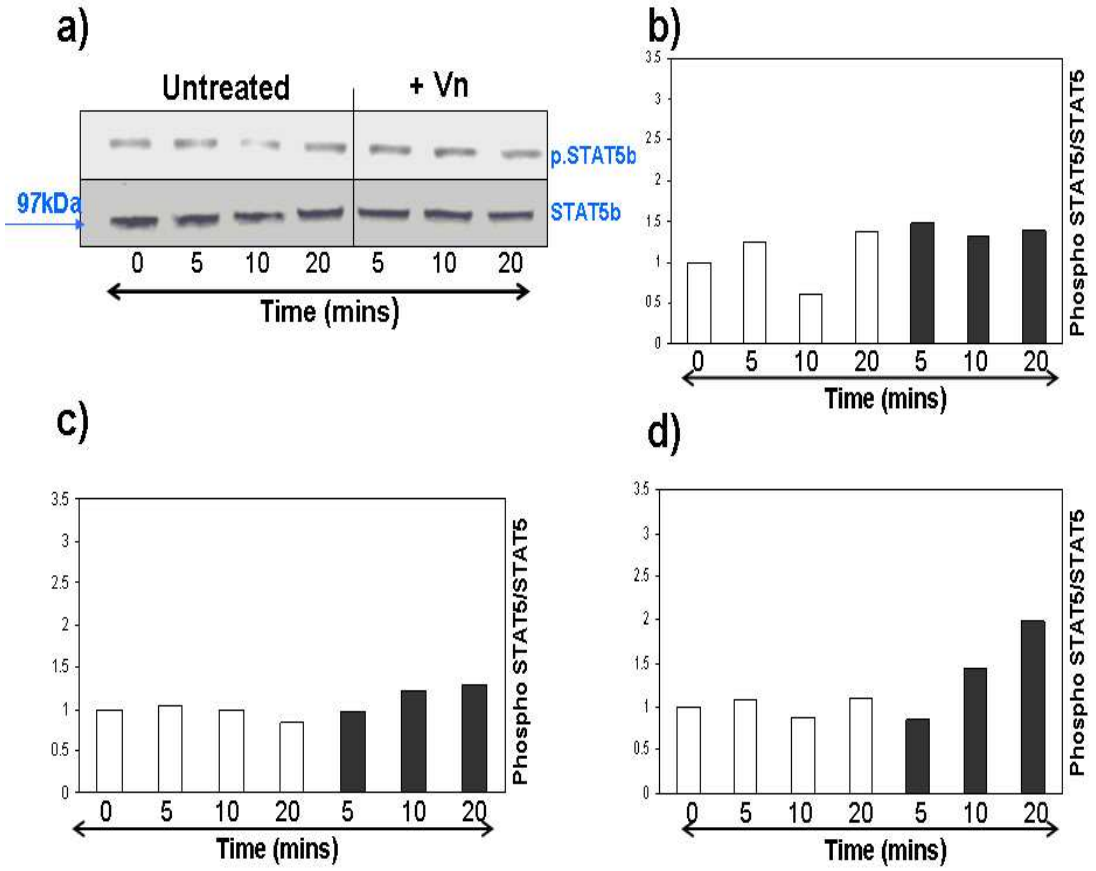


Figure 4.3 Vitronectin does not clearly stimulate the αVβ5 integrin to promote STAT5b phosphorylation. Lysates were extracted from cells stimulated by 1.8nM vitronectin or without stimulation for the indicated time course and, after western blotting, probed with antibodies to phosphorylated STAT5b then stripped and re-probed with antibodies to STAT5b protein Panel a). Panel b) is the densitometric analysis for the phospho-STAT5/STAT5 ratios demonstrated. The other Panels c)and d) are the densitometric analysis for the phospho-STAT5/STAT5 ratios for another blots of two individual experiments have been carried out in different weeks. The white bars represent the unstimulated cells while the black bars represent the stimulated cells.

4.2.2 Both soluble CD23 and LP strongly induce STAT5 / DNA binding but vitronectin causes a smaller induction

STAT5a/b is a member of the STAT proteins which are transcription factors playing different biological roles in cell survival, growth and apoptosis [227, 191]. The active form of STAT5b interacts with the DNA at specific binding sequence CAGAATTTCTTGGGAAAGAAAAT [296, 297].

Therefore to test binding activity, the Electrophoretic-Mobility Shift Assay (EMSA) was used to test the transcription factor/DNA binding activity by using the specific binding sequence for probing STAT5 binding.

Figure 4.4 clearly shows that the STAT5/DNA interaction is strongly enhanced after 15-30 minutes of stimulating cells with either 33µM LP or 10nM soluble CD23 compared to untreated cells. In contrast, a minimal STAT5/DNA interaction activation was observed using vitronectin as stimulant. These data give an additional confirmation of the antibody array data and nicely fit the results obtained by the western blotting.

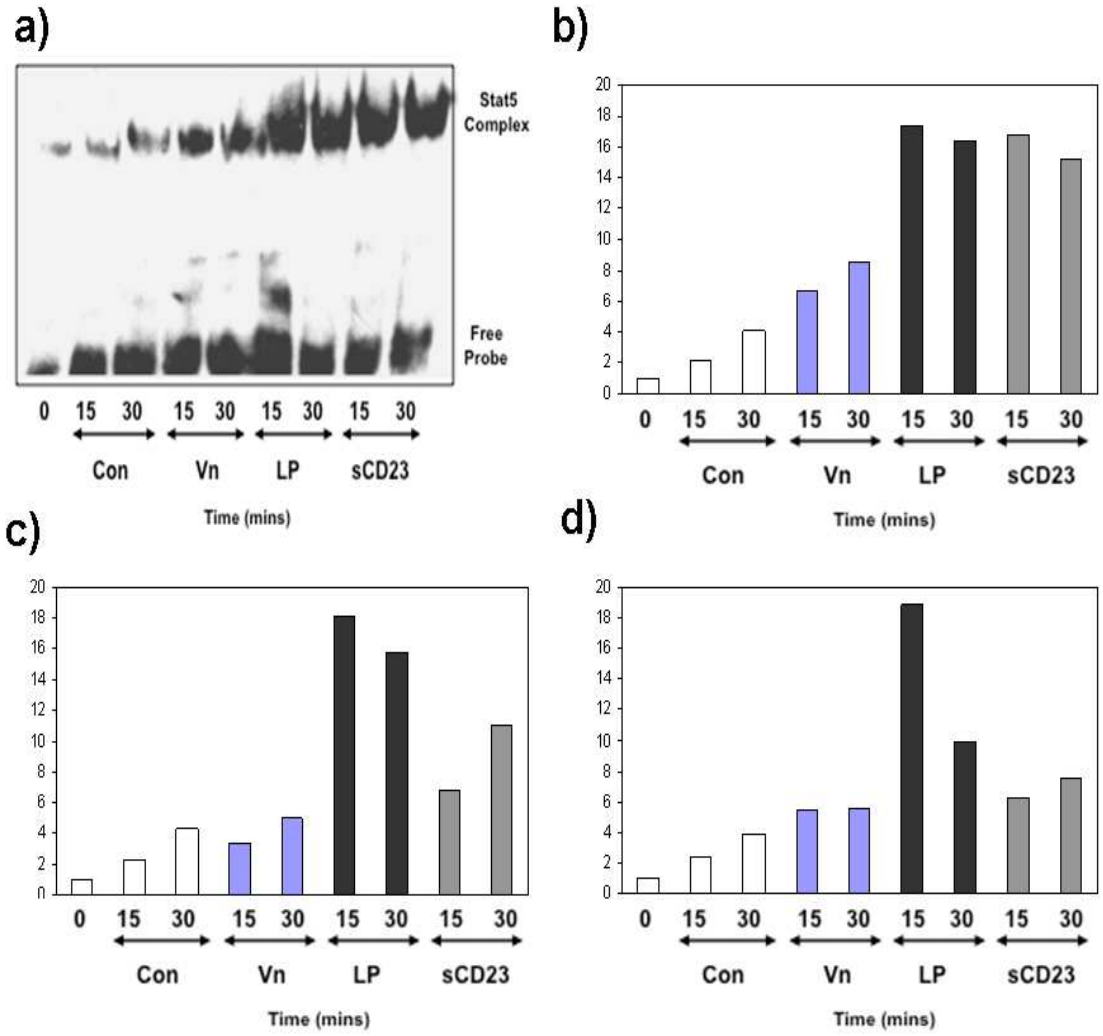


Figure 4.4 Stimulation of SMS-SB cells by sCD23, LP and vitronectin activate STAT5b/DNA binding activity. (a) Nuclear extracts (3µg) of either stimulated or un stimulated SMS-SB cells were mixed with biotin-labelled STAT5 probe and protein/DNA complexes were separated on a non-denaturing gel, transferred to a nylon membrane and detected using strepatvidin-HRP. **(b)** The band intensities were quantitated by densitometry for the demonstrated gel. **(c)** and **(d)** represent the band intensities for two further experiments.

4.2.3 Both soluble CD23 and LP induce STAT5b transcriptional activity

In this experiment, SMS-SB cells have been transiently co-transfected with two plasmids, an expression vector containing a trans-activation domain (pTL-TAD) of STAT5b fused to the Gal4-DNA binding domain (DBD). The other vector is a Gal4-responsive luciferase plasmid (pTL-Luc). Therefore, when STAT5b becomes activated it will activate luciferase expression via inducing Gal4 expression. Ultimately, the STAT5b transcriptional activity is measured by estimating luciferase activity in a luminometer. Cells were then stimulated with either 33µM LP, 10nM sCD23 or 1.8nM vitronectin 48 hours following the transfection.

The data shown in figure 4.5 clearly indicate a significant luciferase activation caused by both LP and sCD23 which abut double of luciferase activation compared with untreated co-transfected cells.

On the other hand, vitronectin does not give a significant response which is compatible with the previous data. So far, the data obtained from the previous work suggest that the αVβ5 integrin modulates the activation of STAT5b via it binding to the soluble CD23 and LP the RKC containing ligands. However, these data raise two important questions about the role of STAT5 in the cell-growth stimulated by both ligands and how the αvβ5 integrin can mediate the STAT5 phosphorylation.

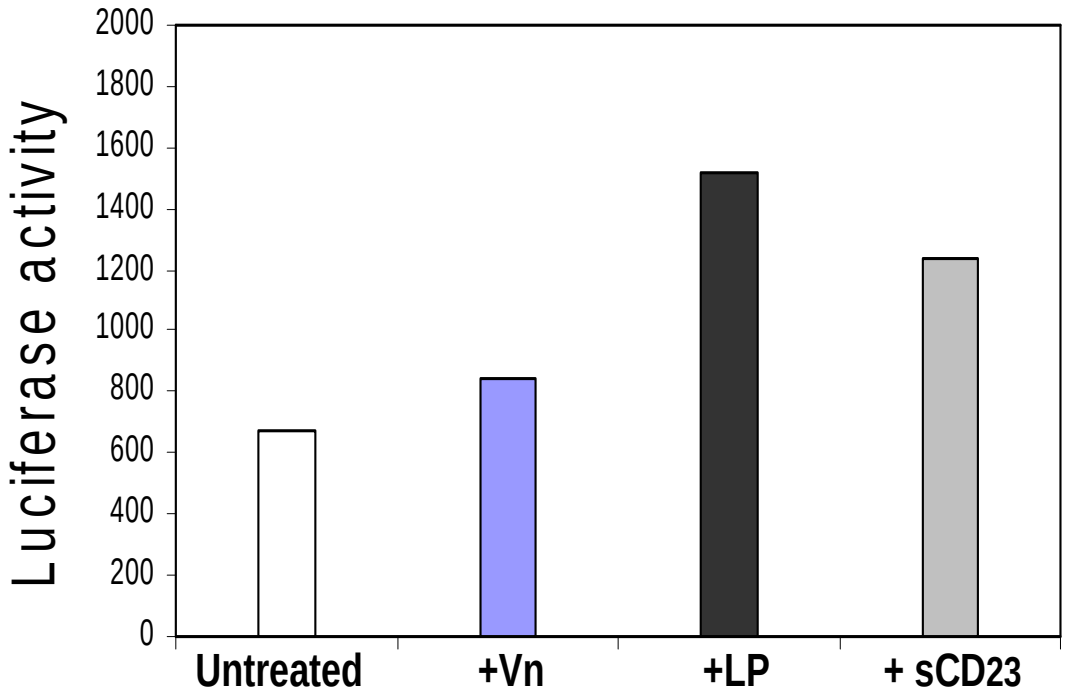


Figure 4.5 LP and sCD23 clearly stimulate STAT5b responsive reporter vector but vitronectin (Vn) causes less stimulation. SMS-SB cells were transiently co-transfected by (pTL-luc) and (pTL-TAD) STAT5b responsive vector. Cells were incubated for 48 hours at 37^{o}C and 5%CO2 followed by adding the stimuli; 1.8nM Vn (blue bar), 33µM LP (black bar) and 10nM sCD23 (grey bar). After 6 hours further incubation, the luciferase activity was assessed by using luciferase substrate in a plate illuminometer. (These data are representative of three independent experiments).

4.2.4 LP and Soluble CD23 stimulate the phosphorylation of Jak2 while vitronectin shows less stimulation

There are several Jak/STAT pathways playing different biological roles in response to various cellular stimuli. STAT5 usually lies downstream of two Jak isoforms, Jak2 and Jak3 depending on the ligand, cell type and the receptor for ligand. However, any Jak family member needs to be phosphorylated in order to phosphorylate the downstream target. The phosphorylation of Jak2 at tyrosine 1007/1008 is potentially required for Jak2 auto- or trans-phosphorylation and for Jak2 kinase reactions [298]

Therefore, the aim of this experiment is to investigate the effect of LP, sCD23 and vitronectin on Jak2 phosphorylation for answering the question of how STAT5 is eventually phosphorylated by these ligands due to their interaction with the αVβ5 integrin.

Figure 4.6 shows a western blot of cellular extracts of either treated or untreated cells during a 20 minutes time course which has been probed by anti-phospho Jak2 $^{Y1007/1008}$ which is also stripped and reprobed by anti-total Jak protein. As clearly shown in this figure, LP significantly increases Jak2 phosphorylation levels comparing with the control (untreated cells). Similarly, increased levels are observed with soluble CD23 stimulation but not with vitronectin.

The data shown in figure 4.6 suggest that the trend of Jak2 phosphorylation might fit the trend of STAT5 phosphorylation in response to the same αVβ5 integrin-ligands. However, further investigation needs to be done to disclose whether Jak2 is the STAT5 kinase or not.

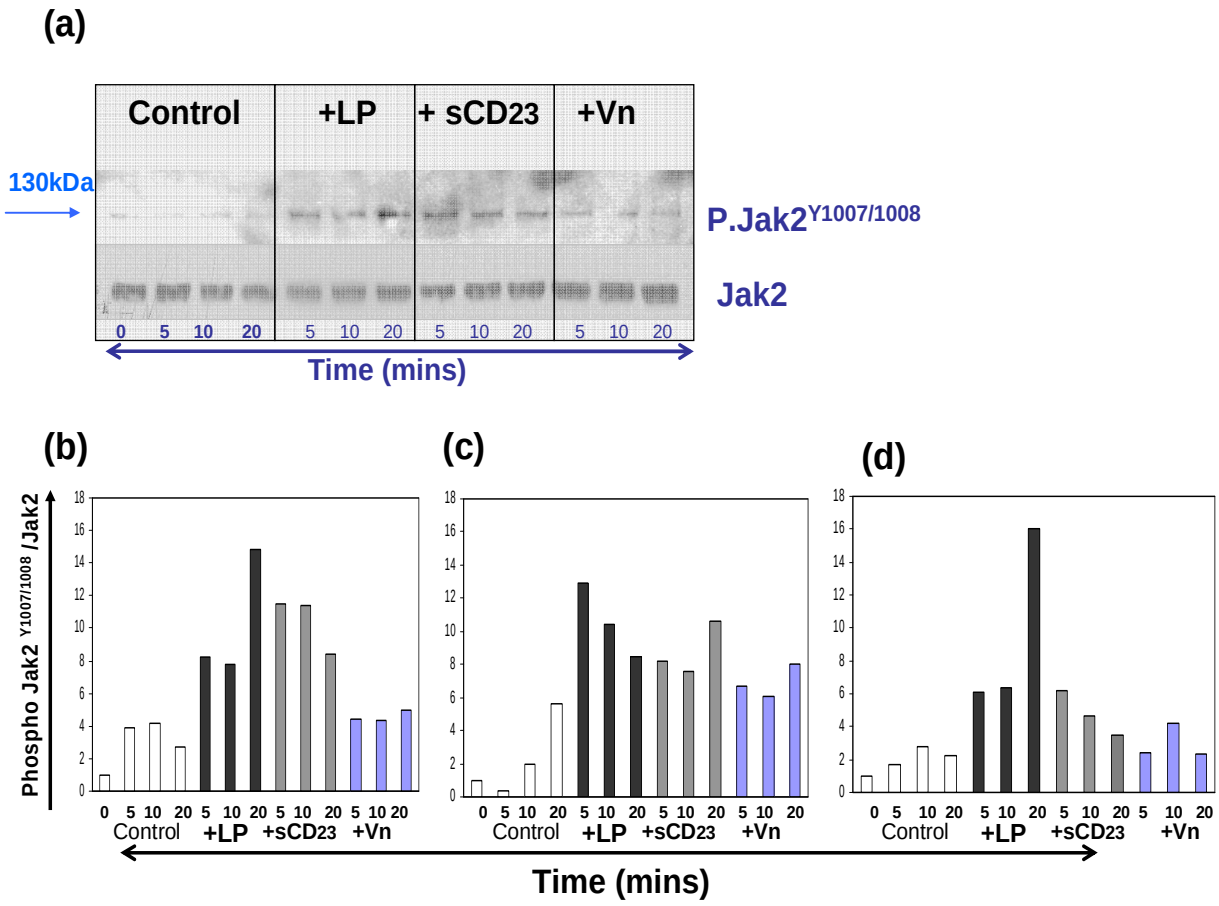


Figure 4.6 Stimulation of SMS-SB cells by sCD23, LP and vitronectin increase Jak2 phosphorylation. Lysates were extracted from cells either stimulated with 33µM LP, 10nM sCD23 or 1.8nM vitronectin or without stimulation for the indicated time course and, after western blotting, probed with antibodies to phosphorylated Jak2 then stripped and re-probed with antibodies to Jak2 protein Panel a). Panel b) is the densitometric analysis for the phospho-Jak2/Jak2 ratios demonstrated. The other Panels c)and d) are the densitometric analysis for the phospho-Jak2/Jak2 ratios for another blots of two individual experiments have been carried out in different weeks. Black bars are for LP stimulated cells, grey bars are for sCD23 stimulated cells and the blue bars are for vitronectin treated cells.

4.2.5 Either Jak2 inhibitor (AG490) or PDGF receptor inhibitor (AG1295) reduces both SMS-SB cell growth and STAT5 phosphorylation

AG490 and AG1295 are synthetic compounds belonging to a large family called tyrphostins. Biologically, tyrphostins are protein tyrosine kinase (PTK) inhibitors which are designed for blocking their selective target [299]. In some references they are called Tyrphostin-490 and Tryphostin-1295.

The chemical structure of the AG490 is N-benzyl-3,4-dihydroxybenzylidenecyanoacet-amide. Wang *et al* reported that AG490 inhibits Jak2, STAT1 and STAT3 tyrosine phosphorylation [300]. In other context, Meydan *et al* showed that AG490 induces the programmed death of human B-precursor leukaemic cells [301]. The chemical structure of AG1295 is 6,7-Dimethyl-2-phenylquinoxaline and it is a selective PDGF receptor kinase acting as a competitive inhibitor for the ATP binding site of the kinase [302].

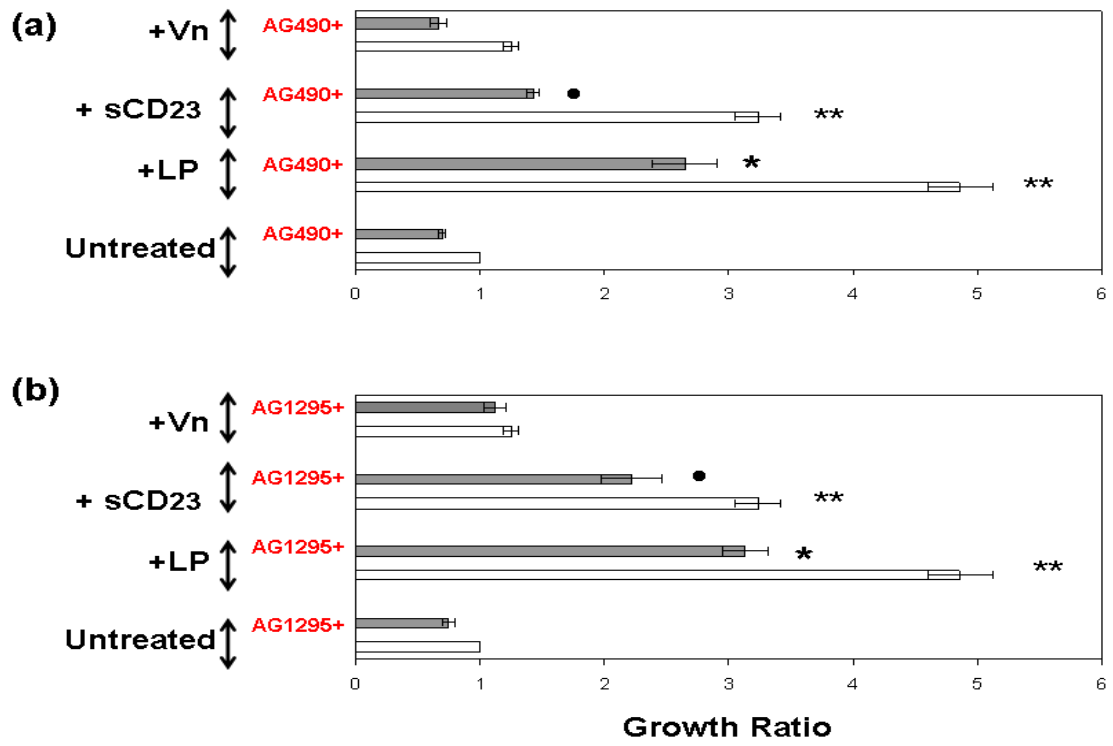
In order to verify whether Jak2 is a kinase of STAT5a/b or not 100µM AG490 was incubated with SMS-SB cells cultured in protein-free hybridoma medium for three hours before adding the stimuli. Panel(a) in figure 4.7 clearly shows that AG490 reduces the cell growth stimulated by LP by more than 30% and the *P* value for three individual experiments was less than 0.05. Similar growth reduction occurs with cell growth stimulated by soluble CD23 with a similar outcome of the statistical analysis for three independent experiments. In the case of vitronectin, there is no clear growth stimulation and the effect of AG490 is similar to untreated cell growth.

In the same context, AG490 strongly reduces the phosphorylation of both Jak2 and STAT5 in either untreated cells or cells stimulated by LP or vitronectin as shown in panel (c) figure 4.7.

The purpose for using the PDGF receptor kinase inhibitor (AG1295) is to investigate the possible role of PDGF receptor kinase in Jak2/STAT5 phosphorylation and also to examine its role in SMS-SB cell growth stimulated by different αVβ5 integrin.

In figure 4.7, panel (b) clearly shows that AG1295 also reduces the cell growth induced by either LP or soluble CD23 in which SMS-SB cells have been incubated with 25μM of AG1295 for three hours before proceeding to either the proliferation assay or cell signalling investigation by using western blotting technology. The cell growth stimulated by either ligand was significantly reduced by about 30% and the *P* values for three independent experiments was less than 0.05 for both ligands.

Panel (c) in the same figure shows that AG1295 interrupts both Jak2 and STAT5 phosphorylation. These data suggest that somehow the αVβ5 integrin might recruit both PDGF receptor kinase and Jak2 in order to activate STAT5 due to its binding to different ligands, particularly the RKC motif containing ligands.



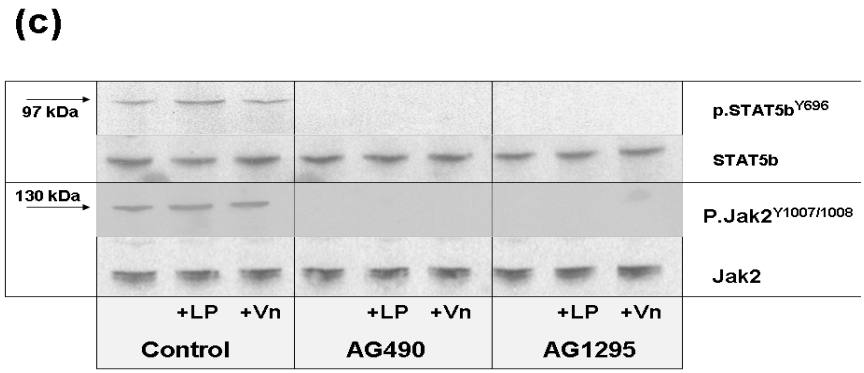


Figure 4.7 AG490 and AG1295 reduce SMS-SB cells proliferation stimulated by LP , sCD23 and both inhibitors interrupt Jak2/STAT5 phosphorylation. Panel (a) and (b) SMS-SB cells (5 $\times 10^4$ cells / ml in protein-free medium) were pre incubated with either 100µM AG490 or 25µM AG1295 for three hours and then stimulated by either 33µM of LP, 10nM sCD23 or 1.8nM vitronectin for 48hr, then processed for Alamar Blue fluorescence. ** $P < 0.005$ versus corresponding untreated control. * $P < 0.05$ versus corresponding cells stimulated with LP, • $P < 0.05$ versus corresponding cells stimulated with sCD23 and ** $P < 0.05$ versus corresponding untreated cells). Panel (c) lysates were extracted from SMS-SB cells were pre incubated with either 100µM AG490 or 25µM AG1295 for three hours at 37ºC and 5%CO_2 in a humid incubator and stimulated by either 33µM LP or 1.8nM vitronectin for 5 minutes before extraction. After western blotting, membranes were probed with antiphospho STAT5b^{Y699} then stripped and re-probed with antibodies to STAT5b protein or probed with antiphospho Jak2$^{Y1007/1008}$ then stripped and re-probed with antibodies to Jak2 protein. These data are representative of three independent experiments.

4.2.6 SMS-SB cells fail to accept control siRNA transfection

RNA interference is one of the recent useful tools to knockdown any gene of interest selectively. Briefly, siRNA is a small double stranded RNA that undergoes a cleavage process by a multidomain enzyme called Dicer (DCR) in order to form an RNA-protein assembly which in turn down regulates the gene that has a compatible sequence to the single strand of siRNA [303]. This mechanism is called RNA-silencing [304]. Normally, the RNA-silencing mechanism plays an important role in immune responses to viruses especially in plants and Drosophila [305, 306]. In mammalian cells, there is no clear relation between the expression of small siRNA and the interferon machinery [307]. However, the RNA-silencing system plays a crucial role in regulating gene expression in plants, insects and mammals [308, 309].

In molecular biology, the small interfering RNA has become one of the useful techniques to study either gene or protein functions by knocking down a certain gene selectively [310]. The strategy of using siRNA for knocking down a gene is based on transfecting the cells with a specific siRNA which needs an optimized transfection protocol, reagents and cellular conditions which are different from one cell type to the next [310].

Before going forward with using siRNA for knocking down either Jak2, PDGF receptor or STAT5 in SMS-SB cell, a control siRNA (fluorescein conjugated) was transfected into SMS-SB cells in different concentrations to assess the transfection efficiency by flowcytometry.

Figure 4.8 panel (a) clearly shows that the cell viability is affected by the transfection reagent. The cell viability is reduced to about 20% when incubated 24 hours after the control siRNA transfection with 4µl of transfection reagent

comparing to cell viability without transfection. The *P* values for three independent experiments is less than 0.005.

On the other hand, the transfection efficiency increases with increasing the transfection reagent volume figure 4.8 panel (b). However, there is a critical reduction in cell viability accompanied with the siRNA transfection efficiency. Therefore, using siRNA technology is not suitable for cell signalling study in SMS-SB cells.

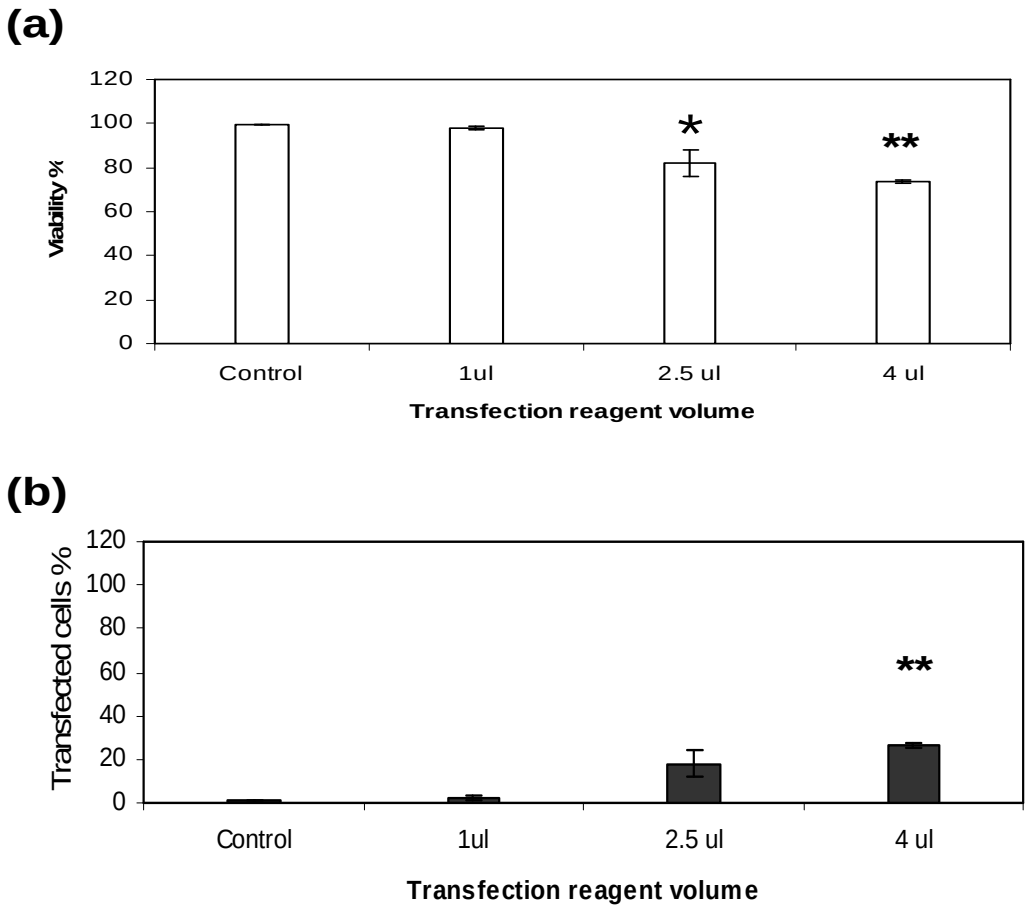


Figure 4.8 SMS-SB cell viability is reduced with increasing siRNA transfection efficiency. SMS-SB cells were transfected with control siRNA (Fluorescein conjugated) by the indicated transfection reagent volumes. Panel (a) represents the cell viability 24 hours post-transfection. Panel (b) represents the percentage of viable transfected cells measured by a flowcytometer 24 hours post-transfection. * $P < 0.05$, ** $P < 0.005$ versus corresponding untransfected cells**.** These data represent three independent experiments.

4.2.7 Platelet derived growth factor receptor (PDGFRβ) and αVβ5 Integrin interaction study

In general, integrins activate several signalling pathways in order to initiate different biological events in response to a wide range of ligands. Several studies over the last two decades have revealed that integrins are able to activate cell signalling pathways in different ways such as receptor clustering, receptor cross-linking or cytoskeleton proteins assembly [311, 312, 313, 314]. In fibroblasts, Zemkove and colleagues have reported that the β subunit of PDGF receptor forms cell surface bridges with either β1 or β3 integrins after stimulating the cells with transglutaminase [315].

The previous data suggested that the αVβ5 integrin mediates the activation of Jak2/STAT5 pathway via its binding with either sCD23, LP or slightly with vitronectin and that activation is interrupted by either Jak2 or PDGF receptor kinase inhibitors. Therefore, the questions raised are how the αVβ5 integrin contributes to the Jak2/STAT5 pathway and what is the role of PDGF receptor in that event. In order to answer these questions an immunoprecipitation strategy was carried out to examine the possible link between PDGF receptor and αVβ5 integrin.

Figure 4.9 clearly shows that the β subunit of PDGF receptor is present in the β5 integrin immunoprecipitates of either treated or untreated cells. The middle panel of figure 4.9 shows that β5 integrin is also present in the PDGF-β receptor immunoprecipitates. In addition, Jak2 protein was found in PDGF-β receptor immunoprecipitates but not with β5 integrin as shown in the bottom panel.

These data give a further conformation of Jak2/STAT5 pathway activation via the αVβ5 integrin and might suggest that there is an association between the integrin and PDGF receptor complex.

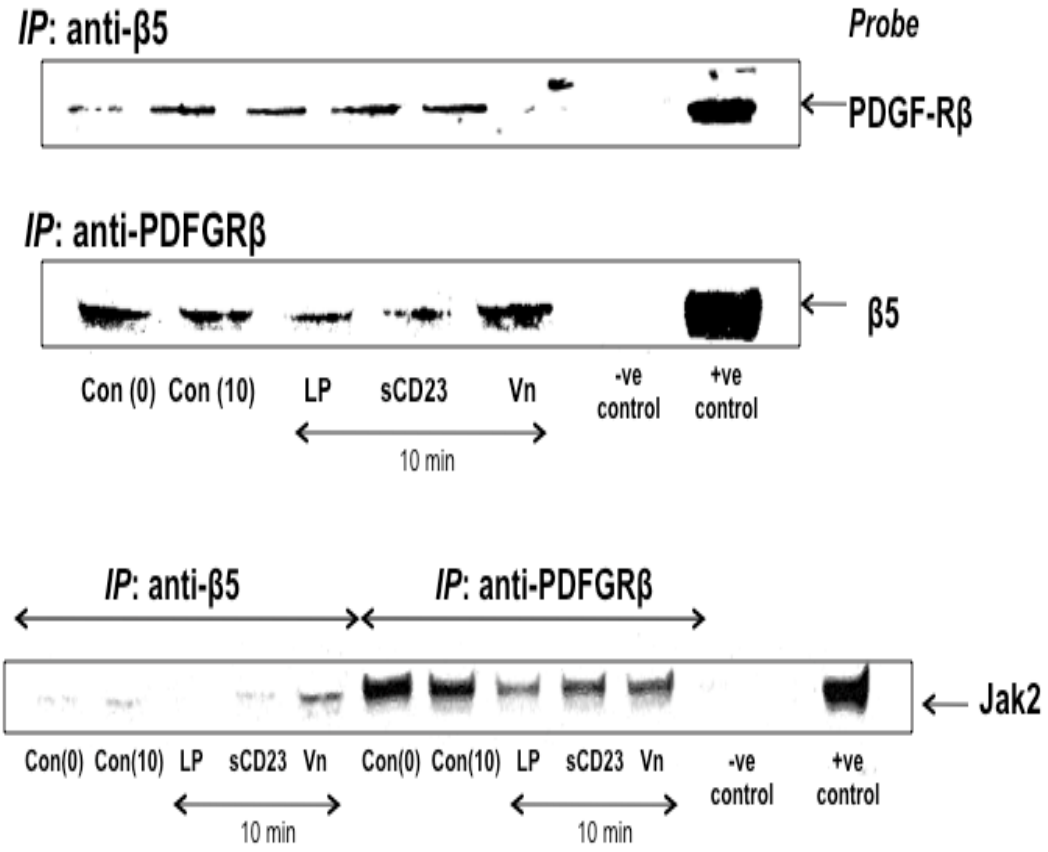


Figure 4.9 PDGF receptor-β and αVβ5 interact. Control and stimulated lysates from SMS-SB cells were immunoprecipitated with antibodies to PDGFR-β or αVβ5, and the separated precipitates probed with anti-PDGFR-β, anti-β5 or anti-Jak2 antibodies as indicated. The negative control is a cell lysate immunoprecipitated with non-immune IgG, while the positive control is a lysate blotted directly. (These data represent one of three independent experiments).

4.3 Conclusion

The data presented in this chapter establish that the αVβ5 integrin stimulates the phosphorylation of STAT5 due to its binding to either sCD23 and LP. As shown in figure 4.1 and 4.2, sCD23 and LP strongly activate the phosphorylation of STAT5. In contrast, figure 4.3 demonstrates that vitronectin does not show clear activation. Moreover the data shown in figure 4.4 and 4.5 indicate that both LP and sCD23 stimulate STAT5/DNA binding and transcriptional activity with minimal activation shown with vitronectin.

In addition, the data presented in figure 4.6 show that the αVβ5 integrin activates Jak2 phosphorylation due to its binding with either sCD23, LP or vitronectin. SMS-SB cell growth and Jak2 phosphorylation was blocked by using either Jak2 or PDGF receptor inhibitors as shown in figure 4.7.

Interestingly, the data obtained from the immunoprecipitation strategy strongly suggest that there is an association between the αVβ5 integrin and the PDGF receptor which might influence the stimulation of the Jak2/STAT5 signalling pathway via the αVβ5 integrin. However, further experiments need to be done to explain the nature of αVβ5 integrin- PDGF receptor association, for example by using confocal microscopy and fluorescence resonance energy transfer .

In a different context, according to Acharya et al (2009), both sCD23 and LP stimulate ERK1/2 phosphorylation which might be one of the key targets that might underlie the SMS-SB cell growth mediated by the αVβ5 integrin. Therefore, the next chapter will proceed to investigate the role of both sCD23 and LP in the activation of the ERK1/2 signalling pathway and to study the effect of SDF1-α and $PDGF_{AB}$ on STAT5 and Serum response factor (SRF) transcriptional activation. Furthermore, it will contain an

examination of the effect of sCD23 in activating the proliferation of 697 and BAF03 cell line models in order to study the importance of the αVβ5 integrin in promoting the survival and proliferation of B-cell progenitors and acute lymphocytic leukaemia cell line models.

5. αVβ5 integrin mediates the phosphorylation of multiple kinase substrates due to its binding to soluble CD23, LP and Vitronectin

5.1 Introduction

Many published studies over the last two decades have revealed that several signalling pathways contribute to cell proliferation and apoptosis. One of these pathways is the Ras/Raf/MEK/ERK cascade. Our laboratory has published that the sCD23-αVβ5 integrin interaction activates ERK phosphorylation [123]. Therefore, the main aim of this chapter is to follow up on the ERK phosphorylation stimulated by sCD23 to look for downstream targets of phosphorylated ERK such as p90RSK and SRF. The other aim is to investigate the effect of either PDGF-AB or SDF1-α on the activation of STAT5b and SRF reporter vectors in SMS-SB cells.

This chapter will include an attempt to investigate if there is a role of the αVβ5 integrin in controlling Calpain enzymatic activity in SMS-SB cell due to integrin interaction with different ligands. Calpain is a calcium-dependent endopeptidase that plays a crucial role in activating caspase pathways in response to either intrinsic or extrinsic factors and also cleaves some cytoskeletal proteins that involved in signal transduction pathways mediated by integrins particularly β integrins [316, 317]. Moreover, Pfaff et al have reported that Calpain cleaves β integrin cytoplasmic domain [318].

The last purpose of this chapter is to investigate the effect of sCD23-αVβ5 integrin interaction in activating STAT5 in two different cell lines represent different stages in B-cell lineage.

5.2 Results

5.2.1 Soluble CD23, LP and vitronectin activate p90RSK at four residues in different trends

The p90RSKs are 90KDa serine/threonine kinases which are also called ribosomal S6 kinases. In humans, there are four isoforms of RSKs, RSK1, RSK2, RSK3 and RSK4, as well as two structurally-related proteins, mitogen and stress- activated kinase-1 and -2 (MSK1 and MSK2) [319, 320, 321].

Several published articles show that RSKs lie downstream of the MEK/ERK pathway in response to different stimulants such as growth factors, cytokines and hormones [322, 244, 323, 320]. According to Jensen et al the phosphorylation of the linker region between N- and C- termini is required for the kinase activation of p90RSK [324]. Six different phosphorylation sites have been clearly identified in p90RSK, and four of them are located in the linker region; these are Ser221, Thr 573, Ser 363 and Ser 380 [324, 325, 326]. In addition, Threonine 573 has been identified as the binding site for ERK1/2 [325, 327]. The p90RSK plays different biological roles as it is a growth factor- and cytokine-responsive cell signalling element, for instance in cell proliferation, differentiation and motility [328, 329, 330, 331].

In fact, the purpose for studying p90RSK activation after stimulating the cells by sCD23, LP and vitronectin is to extend the earlier ERK phosphorylation studies to seek targets downstream of ERK1/ERK2.

Figure 5.1 clearly shows that p90RSK phosphorylation at Thr573 increased within five minutes of adding LP, and was sustained over the 20 minutes time course; a similar effect occurs on p90RSK at Thr359, Ser363 and Ser380. Soluble CD23 and vitronectin also give similar impact on these four residues but to different extents and

at different times during the 20 minutes experimental time course. On the other hand, there is a clear increase in p90RSK phosphorylation at Thr359, Ser363 and Thr573 in unstimulated cells but that always came after 20 minutes of incubation at the same conditions.

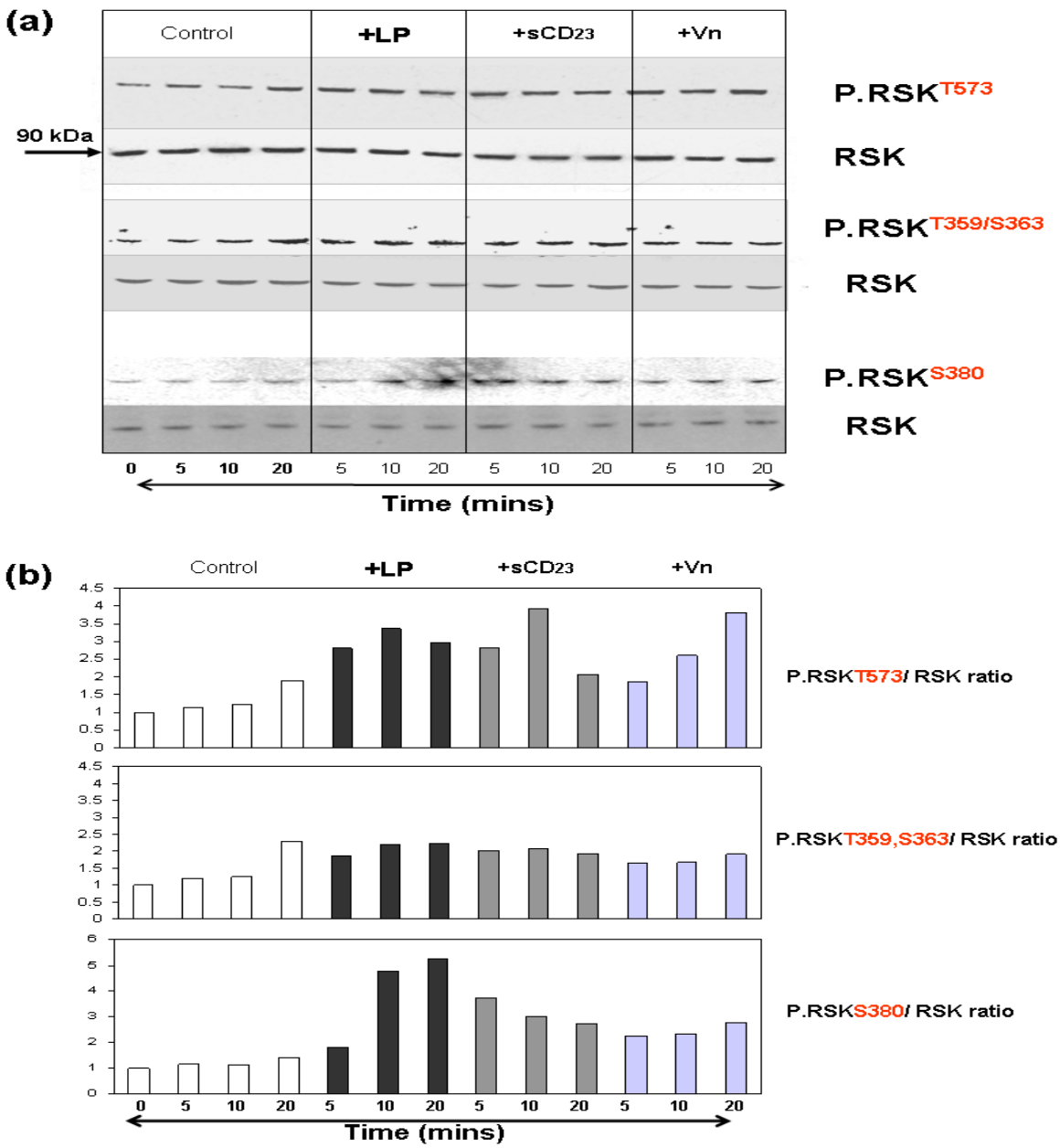


Figure 5.1 Stimulation of SMS-SB cells by sCD23, LP and vitronectin increase p90RSK phosphorylation at different residues. Lysates were extracted from cells either stimulated by 33µM LP, 10nM sCD23 or 1.8nM vitronectin, or without stimulation for the indicated time course and, after western blotting, probed with antibodies to phosphorylated p90RSK(Thr573), (Thr359,Ser363) or (Ser380) then stripped and re-probed with antibodies to p90RSK protein (Panel a). Panel b) is the densitometric analysis for the phospho-p90RSK/RSK ratios. Black bars are for LP stimulated cells, grey bars are for sCD23 stimulated cells and the blue bars are for Vitronectin treated cells. These data are representative of at least three independent experiments.

5.2.2 The MEK1/2 inhibitor U0126 inhibits the phosphorylation of both ERK1/2 and p90RSK modulated via the αVβ5 integrin

U0126 is a MEK1/2 inhibitor initially identified as an inhibitor of AP-1 transactivation [258]. The chemical structure of U0126 is1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene [332]. In the last ten years U0126 has been broadly used in the context of studying the role of MEK/ERK pathway in many cell types and under many different conditions.

According to Acharya et al [123] ERK1/2 is phosphorylated in SMS-SB cells after 30 minutes of stimulation with sCD23 [123]. In this experiment, SMS-SB cells have been incubated with 5µM of U0126 for 30 minutes and then stimulated with either sCD23, LP or vitronectin for 10 minutes. In the other part of the experiment, SMS-SB cells were either stimulated with sCD23, LP or vitronectin or without stimulation for 10 minutes which represents a control.

Figure 5.2 panel (b) clearly shows that LP, sCD23 and vitronectin stimulate the phosphorylation of ERK1/2 at T202/Y204 and T185/Y187 after 10 minutes of the stimulation to different levels. This event is strikingly absent in cells pre-incubated with U0126. The same figure shows that U0126 strongly inhibits the phosphorylation of p90RSK at all four residues (Ser380, Thr 359, Ser 363 and Thr 573) that are stimulated by the same ligands at the same time point. These data suggest that the phosphorylation of both ERK1/2 and p90RSK by these ligands is MEK-dependent. However, it is not clear yet whether the phosphorylation of p90RSK lies downstream of ERK1/2, which is a good fit the work reviewed in this

chapter, or another kinase such as ERK5. In fact, these possibilities require further study.

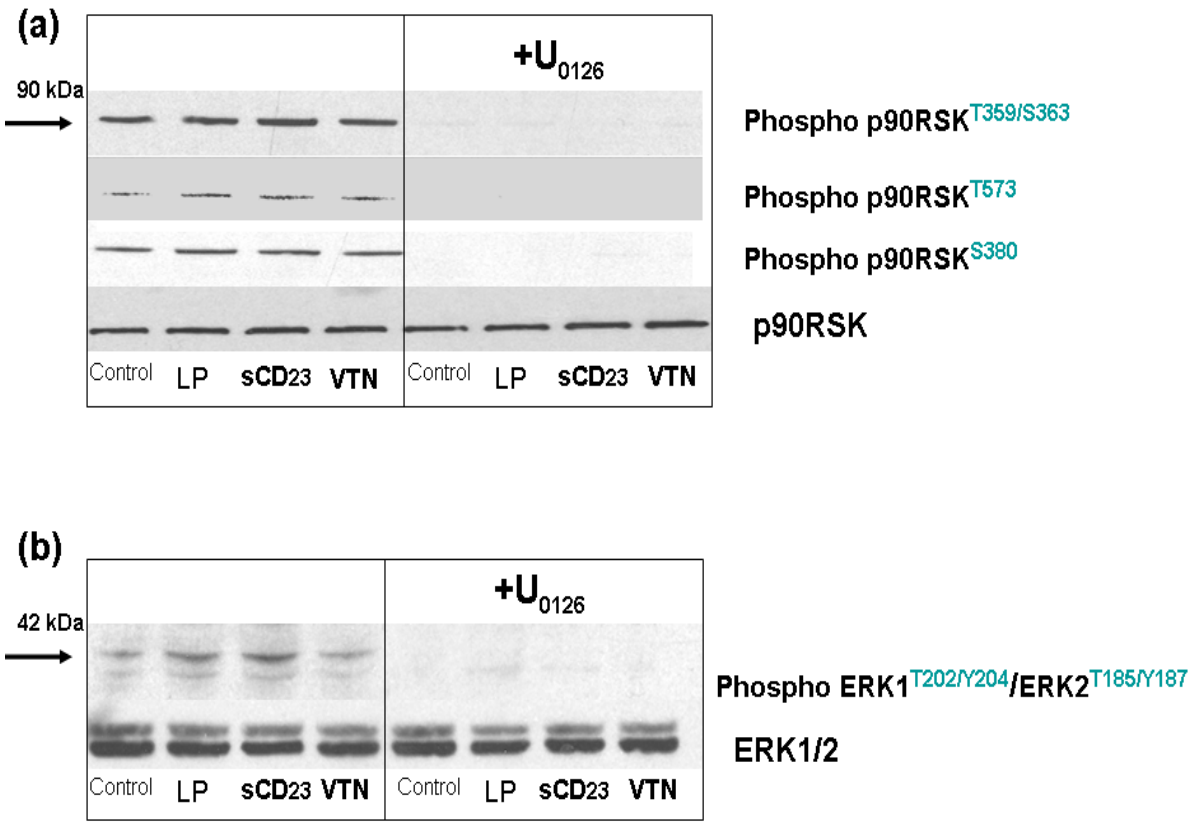


Figure 5.2 U0126 inhibits both ERK1/2 and p90RSK phosphorylation mediated by αVβ5 integrin. SMS-SB cells were pre-incubated either in presence or absence of 5μM U0126 for 30 minutes and stimulated for 10 minutes by the indicated ligands or without stimulation as a control. After western blotting, (a) the separated proteins were probed with antibodies to phosphorylated p90RSK$^{T359/S363}$, p90RSK^{T573}, p90RSK^{S380} or with antibodies to p90RSK protein. Panel (b) the separated proteins were probed with antibodies to phosphorylated ERK1$^{T202/Y204}$/ERK2$^{T185/Y187}$ or with antibodies to ERK1/2 protein. (These data represent one of three independent experiments).

5.2.3 Neither LP nor vitronectin affects calpain enzymatic activity during either chronic or acute time course windows

The calpain activity assay is based up on the detection of emitted light by a fluorimeter due to cleaving a specific calpain cleavable substrate (Ac-LLY-AFC).

There are two purposes for measuring calpain activity in the context of the αVβ5 integrin-mediated signaling pathways. The first aim is to investigate the role of calpain in cleaving cytoskeletal proteins due to stimulating SMS-SB cells by either LP or vitronectin and for that purpose the calpain activity was measured over a 0,3,15,25 and 40 minutes time course. The other aim is to investigate the extrinsic effect of either LP or vitronectin on apoptosis pathways and for that reason the calpain activity was estimated within a 0, 0.5,1,2 and 4 hours time course.

Figure 5.3 shows that there is no clear effect on calpain activity with either LP or vitronectin stimulation with the exception of 20% increase following vitronectin stimulation at the 3 minute time point. These data suggest that the calpain system is not involved in cell signaling pathways mediated by αVβ5 integrin due to its interaction with sCD23,LP or vitronectin.

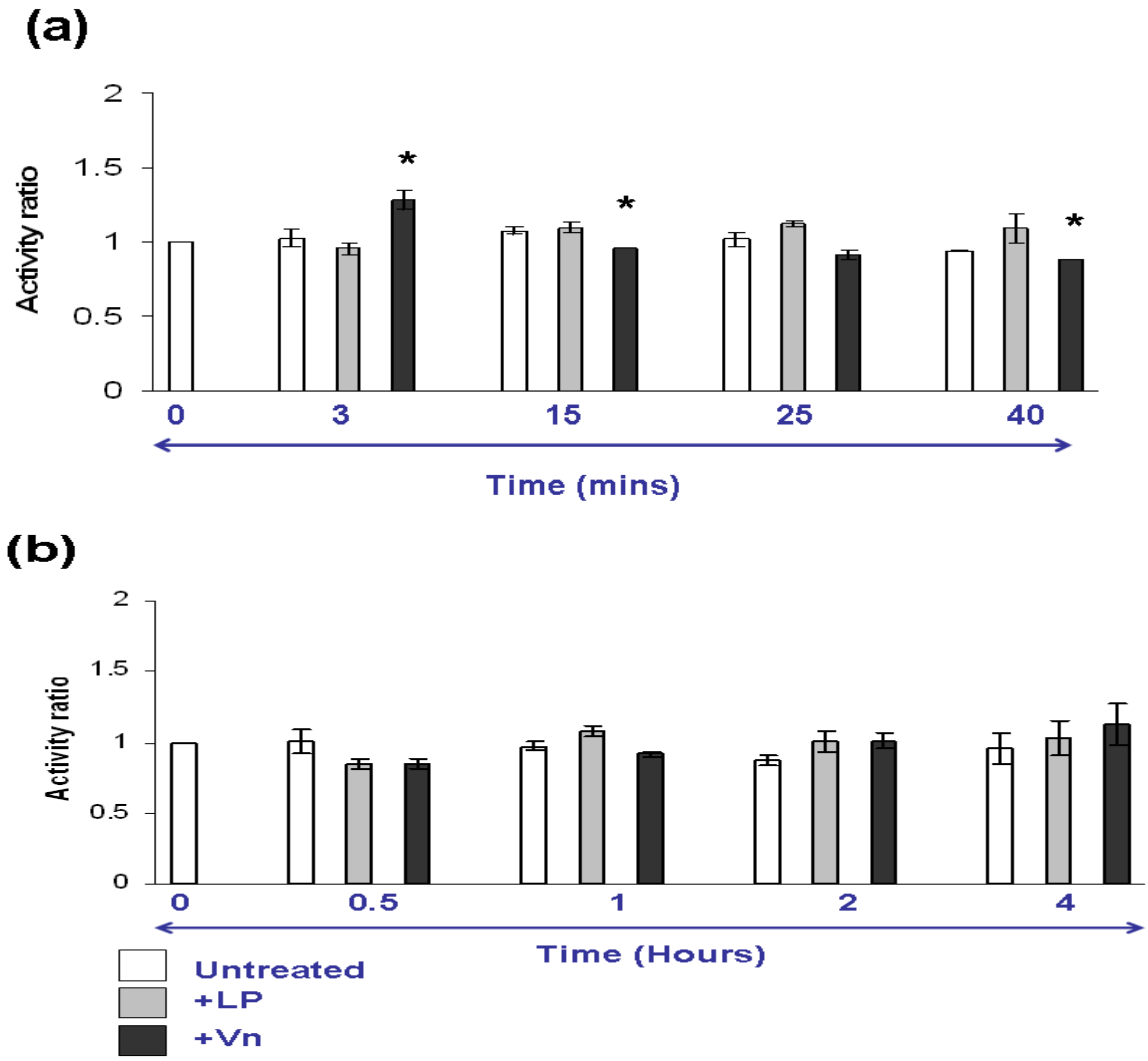


Figure 5.3 There is no change in Calpain activity in SMS-SB cells stimulated by either LP or vitronectin within either short or long time course. SMS-SB cells were either stimulated by LP or Vitronectin or without stimulation for the indicated time points. Cellular proteins were extracted in the absence of protease inhibitors. 60µg total protein/ 85µl of extraction buffer were mixed with 10µl of 10x reaction buffer and 5µl of Calpain substrate provided from abcam followed by an hour incubation at 37ºC in the dark. The reaction was measured by fluorimeter at 355nm excitation filter and 505nm emission filter. Panel (a) demonstrates the Calpain activity within short time course and panel (b) demonstrates the Calpain activity within long time course experimented. * $P < 0.05$ versus corresponding untreated cells at the same time point. (These data represent three independent experiments).

5.2.4 Vitronectin, sCD23 and LP activate SRF-responsive luciferase vectors

Serum response factor (SRF) is a nuclear protein that binds the Serum Response Element (SRE) in order to regulate the transcription of set of genes that control normal cell growth, apoptosis, and other cellular activities [333, 334]. Several published studies show that SRF forms different ternary complexes with different transcription factors such as Elk-1, SAP-1 and Ets-1 in order to regulate a wide range of genes [335, 336, 337]. Moreover, serum stimulation strongly increases SRF transcriptional activity [338]. The phosphorylation of SRF lies downstream of different kinases mainly ERK2, p90RSK and JNK [339, 340, 341, 342].

The investigation of the effect of sCD23,LP and vitronectin on SRF activation is to follow up the data that were obtained at the beginning of this chapter and in order to identify downstream signalling outputs of both ERK1/2 and p90RSK.

As shown in figure 5.4 the αVβ5 integrin ligands clearly increase the luciferase activity in SMS-SB cells which have been transiently transfected with pGL4.34 [luc2P/SRF-RE/Hygro] vector. In this experiment, a 10% (v/v) of serum was used as a positive control which shows very clearly that the serum stimulates SRF activation up to 3-fold compared to untreated cells. These data suggest that the αVβ5 integrin activates the transcriptional activity of SRF via its interaction with different ligands.

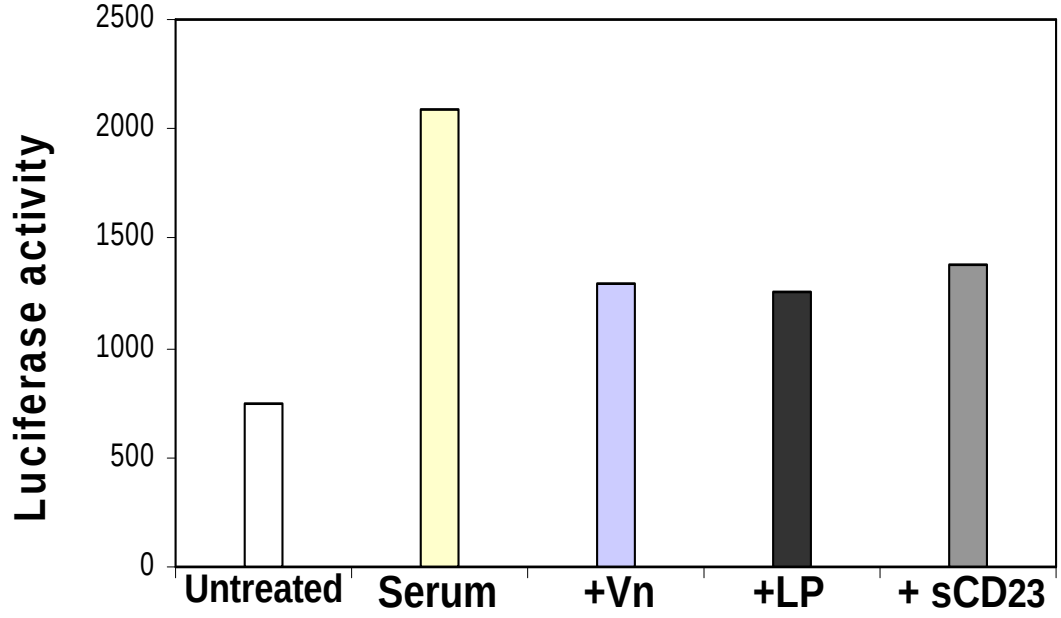


Figure 5.4 Vitronectin, LP and sCD23 stimulate SRF responsive reporter vector. SMS-SB cells were transiently transfected by pGL4.34[luc2P/SRF-RE/Hygro] vector. Cells were incubated for 48 hours at 37^{o}C and 5% CO_2 followed by adding the stimuli 1.8nM Vn (blue bar), 33µM LP (black bar), 10nM sCD23 (grey bar) and 10% (v/v) serum, as a positive control,. After 6 hours further incubation, the luciferase activity was measured by using luciferase substrate in a plate luminometer. (These data are for one representative of three independent experiments).

5.2.5 SDF1-α stimulates SRF reporter vector but does not stimulate STAT5 reporter vector

In the third chapter of this thesis, we have shown that SDF1-α stimulates SMS-SB cell growth at 31.25nM. However, the SDF1-α stimulation was much less than the growth stimulated by LP, a soluble CD23 derivative, and also SDF1-α enhances the LP stimulation by about 20%. Therefore, the aim of investigating the effect of SDF1-α in both STAT5 and SRF reporter activation is an attempt to study the link between growth stimulated by both sCD23 and SDF1-α in order to find out key points of that event.

In figure 5.5 panel (a) SDF1-α does not show a considerable effect on STAT5 responsive vector, while sCD23 has significantly stimulated that reporter. On the other hand, SDF1-α activates the SRF responsive vector but also not as much as sCD23 stimulation (figure 5.6 (b)).

In addition, SDF1-α fails to cause additional stimulation of either the STAT5 and SRF reporter activity when added together with sCD23. These data suggest that SDF1-α does not mediate STAT5 activation but it does activate SRF at a limited level.

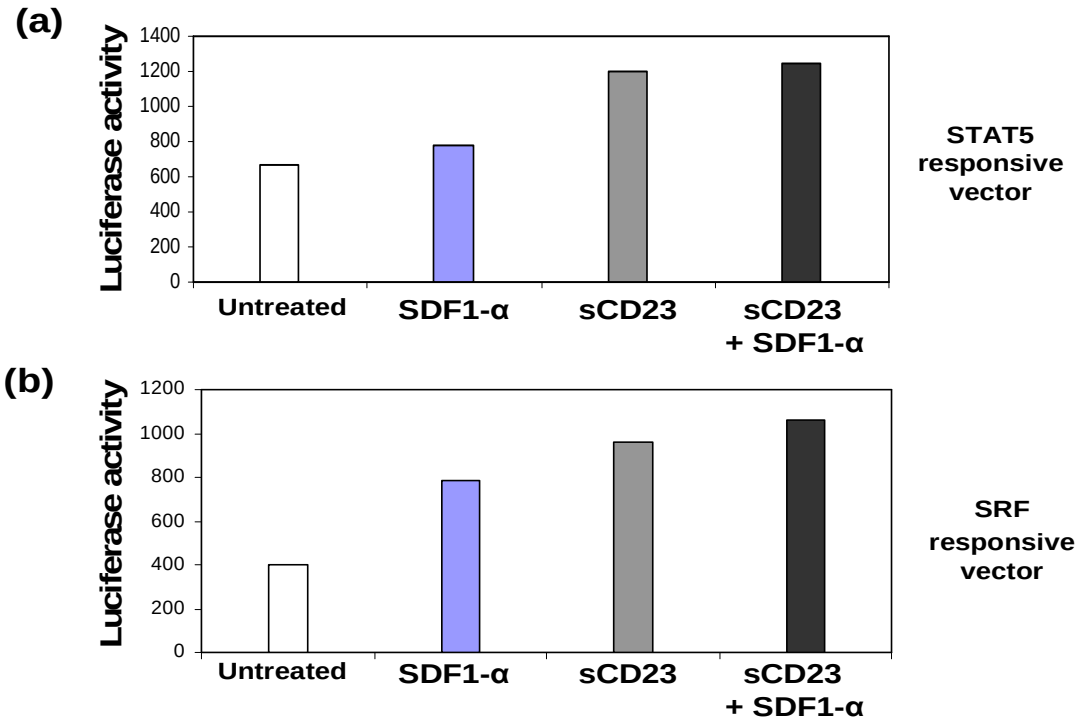


Figure 5.5 SDF1-α stimulates SRF responsive reporter vector but not STAT5 reporter vector. SMS-SB cells were transiently co-transfected with (pTL-luc) and (pTL-TAD) STAT5b responsive vectors panel (a), or transfected with pGL4.34[luc2P/SRF-RE/Hygro] vector panel (b). Cells were incubated for 48 hours at 37^{o}C and 5%CO2 followed by adding the stimuli; 31.25nM recombinant SDF1-α (blue bar), 10nM sCD23 (grey bar) or both together (black bar). After 6 hours further incubation, the luciferase activity was measured by using luciferase substrate in a plate illuminometer. (These data are for one representative of three independent experiments).

5.2.6 PDGF stimulates STAT5 transcriptional activity but does not stimulate SRF

The previous data show that PDGF stimulates SMS-SB cell growth and enhances the cell growth stimulated by the LP, a sCD23-derived peptide. In addition, there appears to be an association between PDGF receptor-β and αVβ5 integrin due to its interaction with the specific ligands. The purpose for examining the effect of PDGF in stimulating the transcriptional activity of both STAT5 and SRF is to follow up the previous data and to find out whether STAT5 and SRF can be activated by the PDGF receptor.

Figure 5.6 panel (a) shows that PDGF stimulates the transcriptional activity of STAT5 which fits well the literature review of JAK/STAT pathway and the data shown in the two previous chapters. In the same panel, sCD23 shows even further stimulation compared to the untreated control cells.

However, PDGF does not show a clear stimulation in activating the SRF responsive vector as demonstrated in panel (b), whereas sCD23 shows a highly significant stimulation. On the other hand, PDGF does not enhance either STAT5 or SRF reporter activated by sCD23. These data suggest that PDGF stimulates STAT5 transcriptional activity to a certain extent but does not drive the activation of SRF.

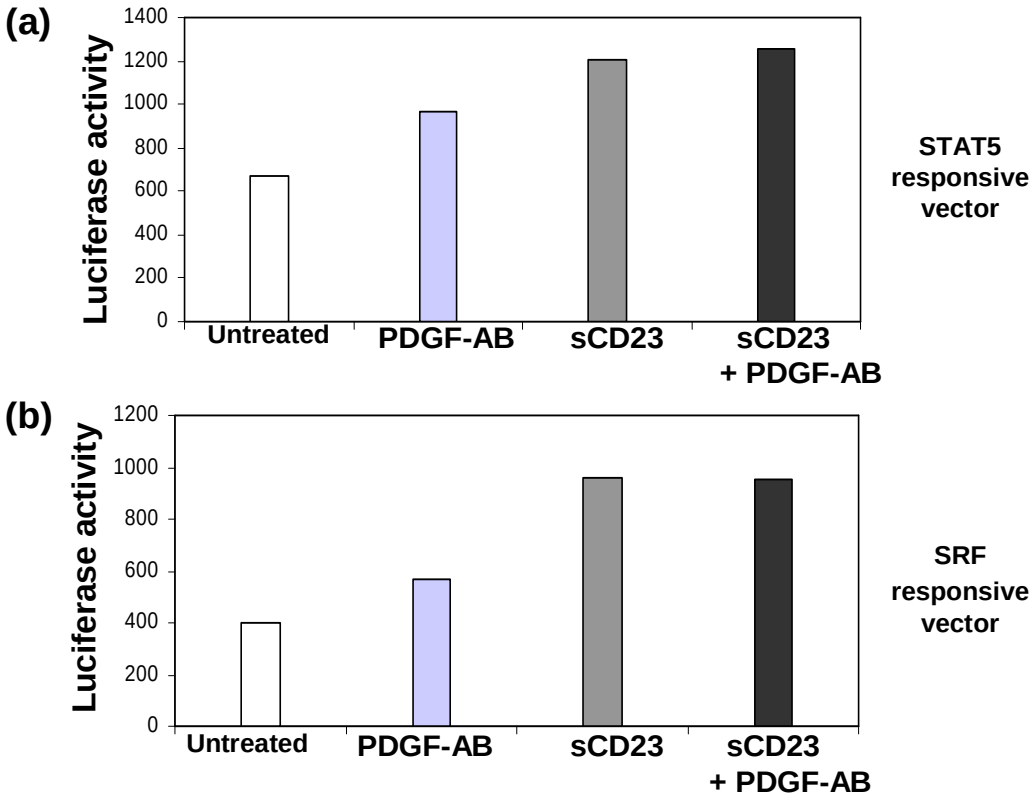


Figure 5.6 PDGF stimulates STAT5 transcriptional activity but does not stimulate SRF. SMS-SB cells were transiently co-transfected with (pTL-luc) and (pTL-TAD) STAT5b responsive vector (panel (a)) or transfected with pGL4.34 [luc2P/SRF-RE/Hygro] vector (panel (b)). Cells were incubated for 48 hours at 37^{o}C and 5% CO2 followed by adding the stimuli; 8.3nM PDGF-AB (blue bar), 10nM sCD23 (grey bar) or both together (black bar). After 6 hours further incubation, the luciferase activity was measured by using luciferase substrate in a plate illuminometer. (These data are for one representative of three independent experiments).

5.2.7 Soluble CD23 induces the growth of 697 and BAF03 cell line models

697 is a cell line derived from the bone marrow of a 12 year-old boy diagnosed with acute lymphocytic leukaemia (ALL) [267]. The 697 cell represents the pre-B stage of development of B-cell in which expresses the cytoplasmic and surface μ-chains [267]. Therefore, the 697 cells seem to represent the next developmental stage of B cell development compared to SMS-SB cells.

The other cell line to be examined is called either BAF03 or BA/F3. These cells are lymphoblasts derived from murine bone marrow and cannot grow without exogenous IL-3 supplementation [343, 266]. These cells do not express IgM which is a marker for both immature and mature B lymphocytes [343].

In fact, the aim of this experiment is to ask whether the sCD23 stimulates the growth of these cell lines as it does with the SMS-SB cells. Figure 5.7 (panel a) clearly shows that 1μg/ml (40nM) of sCD23 significantly stimulates 697 cell growth up to about 2.5-fold compared to untreated cells (*P* value is less than 0.005).

Similarly, 2μg/ml sCD23 stimulates the growth of the BAF03 cells up to 2-fold , which is also significant (*P* value is less than 0.005) as shown in figure 5.7 (panel b). These results suggest that the sCD23-αvβ5 interaction stimulates the growth of different stages of B cell precursors.

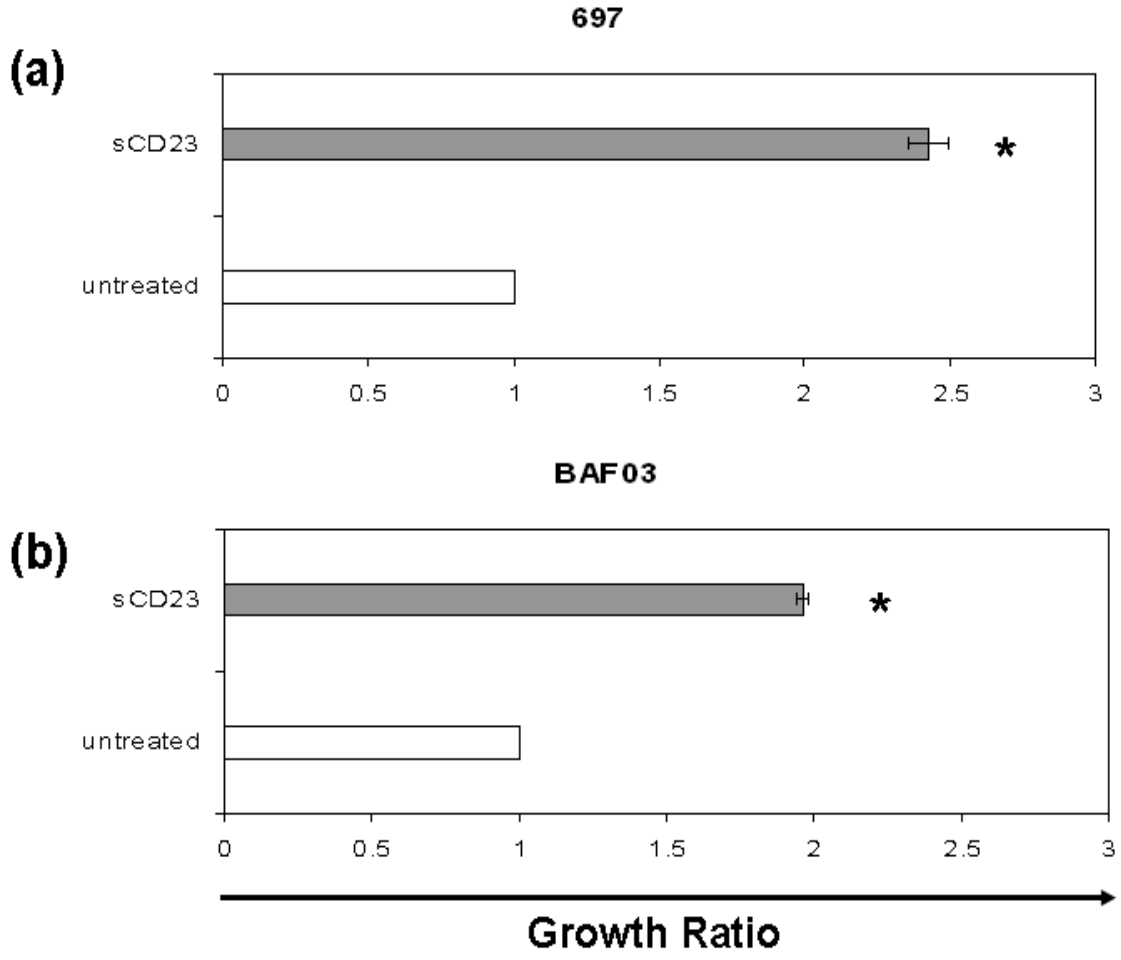


Figure 5.7 sCD23 stimulates growth of 697 and BAF03 cells. Panel (a) 697 cells were cultured at (5×10^4 cells / ml in protein-free medium supplemented by 1% (v/v) FCS) and stimulated with 40nM of sCD23 for 48hr (gray bar). Panel (b) BAF03 cells were cultured at (5×10^4 cells / ml in protein-free medium supplemented by 1% (v/v) FCS) and stimulated with 80nM of sCD23 for 48hr (gray bar). Cells then were processed for Alamar Blue fluorescence followed by 24hr further incubation at the same conditions. * $P < 0.005$ versus corresponding untreated cells. (These data represent three independent experiments).

5.3 Conclusion

The data presented in this Chapter show that αVβ5 integrin activates p90RSK phosphorylation at four different residues Ser380, Thr 359, Ser 363 and Thr 573 due to its binding to the extracellular matrix ligands represented by vitronectin and the soluble ligands such as CD23 and LP as shown in figure 5.1. The phosphorylation of these residues is required for the p90RSK kinase activation [324, 325, 326]. Moreover, the data shown in figure 5.2 indicate that the phosphorylation of p90RSK by these ligands was completely blocked by the specific MEK1/2 inhibitor (U0126) which suggests that the activation of MEK is essential for the phosphorylation of both ERK1/2 and p90RSK. Although these data are consistent with several published reports it is not clear yet whether ERK1/2 is the kinase of the p90RSK or not which needs further investigations.

The data presented in figure 5.5 show that SDF1-α activates the SRF reporter construct but not the STAT5 construct. In contrast, $PDGF_{AB}$ stimulates the STAT5 transcriptional activity but not the SRF. These findings may suggest that $PDGF_{AB}$ involves the Jak2/STAT5 pathway while SDF1-α involves the MEK/ERK/p90RSK/SRF signalling pathway, in order to stimulate SMS-SB proliferation.

Finally, soluble CD23 activates the growth of both 697 and BAF03 cells at different concentrations 40nM and 80nM respectively which indicates that the sCD23-αvβ5 interaction might be important in inducing the growth and survival in ALL B cells.

6 Discussion

The data presented in this thesis show that the αVβ5 integrin, PDGF receptor and CXCR4 are expressed on SMS-SB cells. These receptors stimulate cell growth to characteristic levels in response to different ligands. As shown in the first results Chapter, the αVβ5 integrin mediates SMS-SB cell growth when it binds either LP or sCD23 which both contain the RKC motif. In addition, both SDF1-α and PDGF-AB stimulate SMS-SB proliferation to a limited extent.

Moreover, sCD23, LP and vitronectin induce the JAK2/STAT5b signalling pathway to different levels and at different times over a 20 minutes time course. In addition, the third Chapter of this thesis showed that sCD23, LP and vitronectin stimulate the phosphorylation of ERK1/2 and p90RSK, and induce SRF transcriptional activity in SMS-SB cells.

6.1 SMS-SB cell phenotypic characterization

SMS-SB is a cell line derived from a female patient in the leukemic phase of lymphocytic leukaemia [265]. Smith and colleagues found that these cells express cytoplasmic μ-chain [265]. The data shown in the first results Chapter of this thesis show that these cells express CD19 which is the major marker of B-cell progenitors. However, these cells do not express CD34 which is a determinant of the early stage of antigen- independent phase of B-cell development. In addition, the SMS-SB cell does not express CD5, a marker of B-cells in both human fetal and postnatal lymphoid tissues [276]. Furthermore, these cells do not express some pre-B cell determinants such as CD10 and μ-chain at the cell surface. The data shown in this thesis and the data

presented by Smith et al suggest that the SMS-SB cell probably represents the stage between pro-B and pre-B cells. Figure 6.1 demonstrates the possible stage of SMS-SB cells within the B-cell lineage scheme. Figure 3.1 shows that SMS-SB cells express αVβ5 integrin, but not αVβ3 integrin, and also express the PDGF receptor and CXCR4: expression of these markers is consistent with data from other reports [265, 123]

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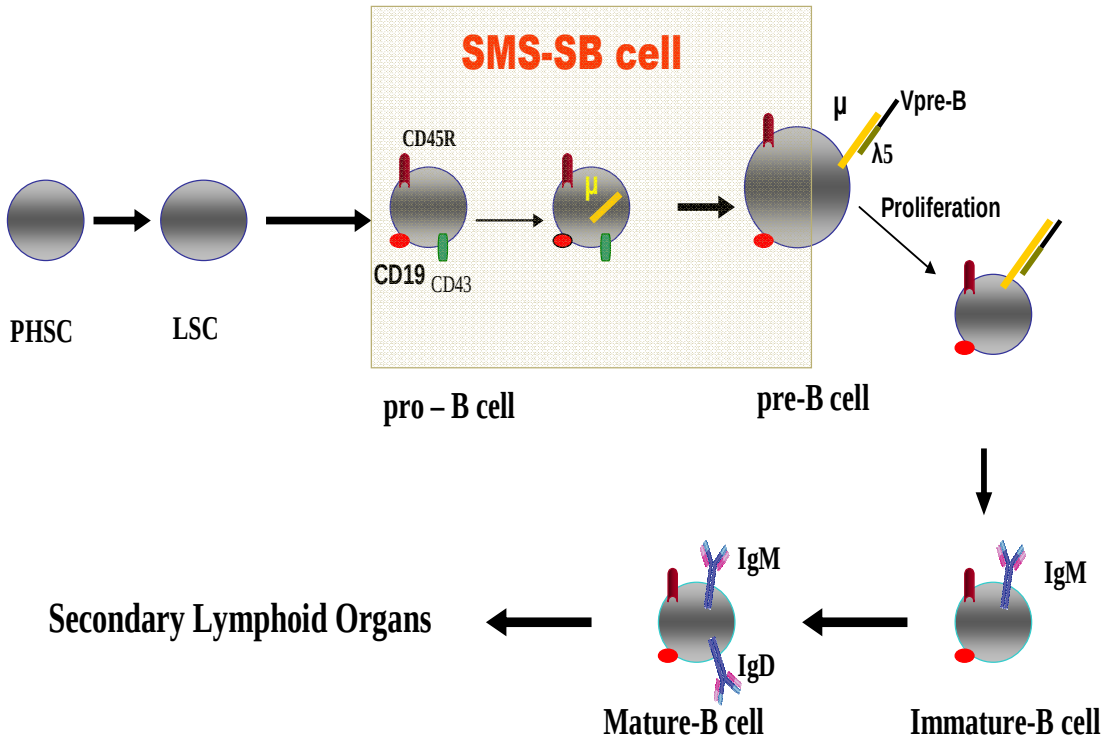

Figure 6.1 B-cell development stages in Bone Marrow. SMS-SB cell represents the area between pro-B and pre-B cell according to the data shown in the third Chapter and Smith RG et al [265].

6.2 SMS-SB cell growth mediated via different soluble ligands

The proliferation data presented in Chapter 3 show that SMS-SB cell growth is increased in response to different stimuli. This growth increase is different from one ligand to the other and it is dose-dependent.

In this regard, figure 3.2 showed that SMS-SB cell growth is density-dependent which is compatible with Tsai et al who showed that SMS-SB cells express PDGF as an autocrine growth factor [271]. The fact that these cells fail to sustain their own growth at low density which might indicates that the expression of the PDGF in these cells requires an extra communication between the cells such as cell-cell adhesion and the αVβ5 integrin might play a role in that kind of adhesion. This hypothesis needs further work such as knocking the αVβ5 integrin down using Lentiviral small hairpin RNA (shRNA) followed by a cell density-dependent growth study. In addition, the cell density growth experiments in this study were assessed by two different methods; Alamar blue and tritiated thymidine incorporation, and there is a robust signal-to-noise ratio in both of them which means either of these methods could be used for mammalian cell proliferation studies.

Stimulation of SMS-SB cells using either sCD23 or LP, (the RKC motif-containing ligands) which act via the αVβ5 integrin induced significant growth increase. LP stimulates SMS-SB cell growth at the concentrations of 33 and 66µM and sCD23 shows a similar effect. Both LP- and sCD23-driven growth stimulation seems to be dose-dependent as shown in figure 3.3. On the other hand, a matrix ligand containing the RGD motif, vitronectin, does not stimulate the cell growth, which suggests that the αVβ5 integrin stimulates SMS-SB when it binds RKC motif-containing ligands

but not RGD motif-containing ligands. These data are consistent with results published by both Acharya et al [123] and Borland et al [95]. In the same context, figure 3.4 and figure 3.5 clearly demonstrated that either $PDFG_{AB}$ or SDF1-α induces SMS-SB cell growth to modest levels, and both ligands significantly enhance the cell growth stimulated by LP. These data are also compatible with the data shown by Acharya et al [123] and Tsai et al [271]. However, more investigations should be done to determine whether that significant proliferative response to the different ligands used in this study, is specific for SMS-SB cells and other types of leukaemia as shown in figure 5.7 or important at a specific stage of B-cell development. One useful approach is studying the effect of these ligands and the role of the αVβ5 integrin in normal B-cell precursors.

6.3 Different kinase substrates stimulated by either sCD23 or LP

The antibody array data clearly showed that multiple signalling pathways might control how the αVβ5 integrin mediates the growth increase stimulated by sCD23. A surprising observation presented in figure 3.6 is that both LP and sCD23 strongly induce the phosphorylation of STAT5 at tyrosine 699, and moderately on STAT2 at tyrosine 689. This seems to be unusual in that the αVβ5 integrin rarely mediates the phosphorylation of any STAT family member according to the work reviewed in the first Chapter of this thesis. In addition, this event was consistent over the 20 minute time course experimented. The other observation on the array is that both LP and sCD23 stimulate the phosphorylation of c-Src at tyrosine 419. This event appeared after 5 minutes of LP stimulation and was increased gradually over the 20 minutes of

sCD23 stimulation. Similar trends are observed with c-yes which was phosphorylated at tyrosine 426. c-yes is a member of Src family kinases and both c-yes and c-Src can regulate different signalling pathways involved in cell growth, motility, survival and differentiation [344, 345]. Therefore, the phosphorylation of c-yes with the same trends of phosphorylation of c-Src by both ligands might represent a good technical control. Both LP and sCD23 activate the phosphorylation of the AMP-activated protein kinase α2 (AMPKα2) at threonine 172, a protein that plays a crucial role in ATP generating mechanisms and protein synthesis [346, 347]. In fact, the phosphorylation of AMPKα2 in response to both ligands might be involved in energy supply for the cell growth stimulated by these ligands or might be involved in a protein synthesis regulation; this merits further investigations.

6.4 STAT5b activation

In normal conditions, IL-7 plays an essential role in early B-cell development via activating STAT5, and STAT5a/b is also required for survival of $CD8^+$ T cells [226, 227]. In addition, STAT5 controls Bcl-6 expression in germinal centre B cells which leads to self-renewal and differentiation of human memory B cells [228]. However, STAT1, STAT3 and STAT5 were found to be activated in response to different cytokines and growth factors in several leukaemic cell line models, particularly in early B-precursor acute lymphoblastic leukaemia and chronic myeloid leukaemia and this activation mainly lies downstream of JAK2 [196, 197, 200]. Moreover, Catlett-Falcone et al showed that constitutive STAT3 signalling activation underlies the apoptosis resistance of multiple myeloma cells in which the activated STAT3 in turn activates Bcl-2 and Bcl-x_L expression [204].

The data illustrated in this thesis show that the αVβ5 integrin mediates the phosphorylation and activation of STAT5b due to binding specific ligands. As shown in figures 4.1 and 4.2, both sCD23 and LP stimulate STAT5 phosphorylation at tyrosine 699 over the 20 minutes time course while vitronectin does not. The phosphorylation of STAT5 at this specific residue is required for STAT5 activation [208, 213]. These data validate the results obtained by the antibody array and also highlight another difference between the RGD- and RKC- containing stimuli. In addition, the phosphorylated STAT5 has DNA binding and transcriptional activity as shown in figures 4.4 and 4.5. These figures demonstrated that LP and sCD23 activate cell growth and STAT5 to similar extents which suggest that STAT5 plays a key role in these stimulations. In the case of vitronectin, this ligand shows a minimal activation of STAT5 which also fits well with the proliferation data presented in Chapter 3.

In fact, several studies have shown that STAT5 regulates a range of genes in different cell types. In terms of SMS-SB cell growth stimulation, there are some candidate genes that might be regulated by STAT5 such as Bcl-x_L in the context of anti-apoptotic mechanisms, cyclin D $_{or}$ STAT5 might regulate the expression of PDGF, the autocrine factor $_{that}$ might underlie the cell density growth dependence. Therefore, it would be very interesting to identify the STAT5 target genes in response to soluble CD23- αvβ5 integrin interaction. In this regard, a chromatin immunoprecipitation strategy (Chip-Sequencing) is one useful technique which uses a genome sequencer to provide the sequences of DNA fragments that have been immunoprecipitated with an antibody against a target transcription factor such as STAT5 [348]. In fact, the Chip-Sequencing technique would identify a primary gene network which will subsequently need a further investigation to study the effect of the CD23- αvβ5

integrin interaction on the regulation of the candidate genes in the network. Nowadays, DNA microarray technology is one of very common method that is used to study the gene expression levels. This technique might be useful as a follow-up to Chip-Sequencing data together with a further independent gene expression estimation such as quantitative real time polymerase chain reaction (qRT-PCR) [349, 350].

6.5 The role of JAK2

The JAK protein family members are the main kinases for STATs [189, 190]. The JAK2/STAT5 signalling pathway was identified in different haematopoietic malignant cell line models, particularly in early B-precursor acute lymphoblastic leukaemia in which the cells express TEL-Jak2 fusions as a product of t(9;12)(p24;p13) translocations, and in chronic myeloid leukaemia with additional translocations t(9;15;12)(p24;p15;p13) [196, 197]. In both cases the transcription factor Tel is fused to the JH1 domain of Jak2 and consequently activates STAT1 and STAT5 [198, 199, 200].

In this regard, the data shown in the fourth Chapter of this thesis clearly demonstrate that both LP and sCD23 stimulate the phosphorylation of JAK2 at tyrosine 1007/1008 with similar trend of STAT5 activation as shown in figure 4.6. The phosphorylation of Jak2 at these sites is potentially required for Jak2 auto- or trans-phosphorylation and for Jak2 kinase reactions [298]. Moreover, both STAT5 and JAK2 inductions were knocked down by using either PDGF receptor inhibitor (AG1295) or JAK2 inhibitor (AG490) and both inhibitors reduce the cell growth in the absence or presence of stimulation as shown in figure 4.7. Although there is a concern about the selectivity of

these inhibitors, these accumulated data suggest that the STAT5 activation mediated via the αVβ5 integrin might be JAK2 dependent and that also need more investigation on the role of either Jak1, Jak3 or Tyk2 in that event [351,352,353]. Unfortunately, one of the obstacles that been faced in this project is the difficulty of transfecting SMS-SB cell with siRNA which is usual in all lymphocytes, however, it would be good to try using the Lentiviral shRNA to study STAT5 phosphorylation in either Jak2 or PDGF receptor knocked out SMS-SB cells.

6.6 PDGF receptor-αVβ5 integrin cross linking

Several studies over the last two decades have revealed that integrins are able to mediate cell signalling pathways in different ways such as receptor clustering, receptor cross-linking or cytoskeleton protein dynamics [311, 312, 313, 314].

Zemkove and colleagues have reported that the β subunit of PDGF receptor forms cell surface bridges with either β1 or β3 integrins after stimulating fibroblasts with transglutaminase [315]. Sundberg and Rubin have published that the stimulation of β1 integrin activates the phosphorylation of the PDGF receptor [63].

The data presented in figure 4.9 show that the β subunit of PDGF receptor is co-immunoprecipitated with the β5 integrin after stimulating SMS-SB cells with LP, sCD23 or vitronectin; the β5 integrin is present in the PDGF receptor immunoprecipitates. Therefore, these data are consistent with results published about the association of PDGF receptor with other integrins. Furthermore, JAK2 is present in the PDGF-receptor immunoprecipitates but not in the β5 integrin precipitates which is also consistent with results of Ihle et al [185, 186] . Although these

data do not explain the nature of this interaction, whether it is a physical cross-link or due to receptor clustering, there are several useful techniques that can elucidate the nature of this association such as confocal microscopy and fluorescence resonance energy transfer. Moreover, investigating the interaction between the αvβ5 integrin and the PDGF receptor in different B-cell malignant cell lines would be valuable to understand and disrupt the proliferation mechanism and anti-apoptosis.

In conclusion, there are different scenarios to explain how the αVβ5 integrin mediates the activation of the JAK2/STAT5 signalling pathway and figure 6.2 summarizes one of these possible scenarios.

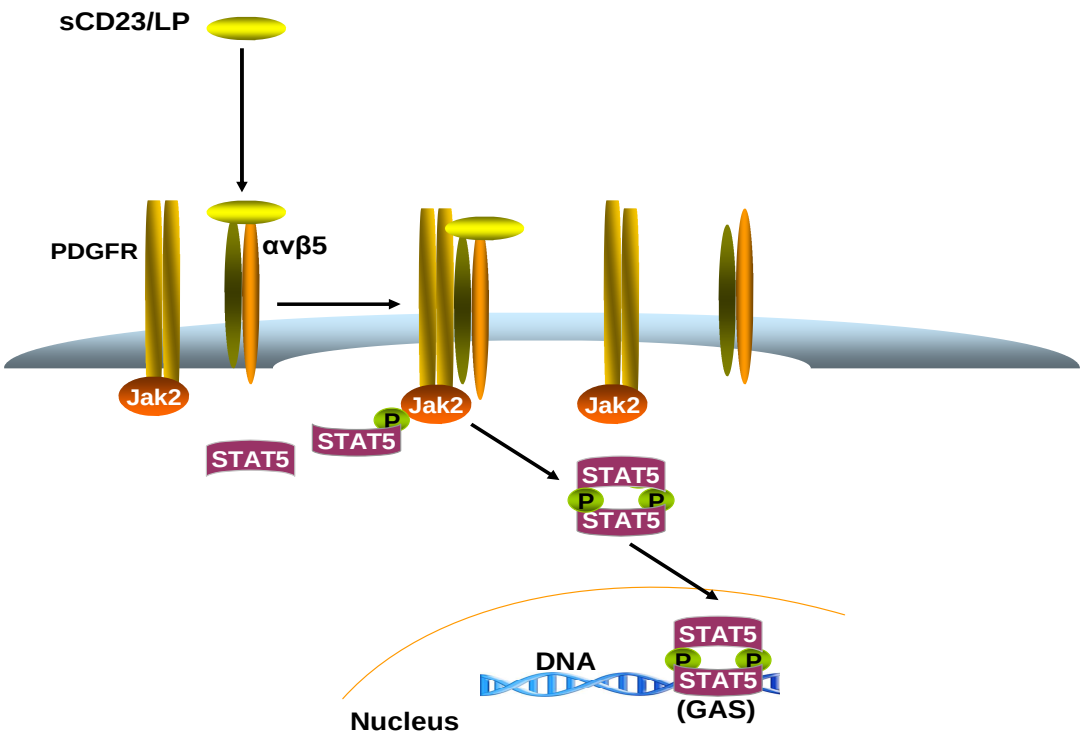

Figure 6.2 Scenario for αVβ5 -mediated STAT5 activation. The binding of the αVβ5 integrin to the stimuli (LP or sCD23) induces PDGF receptor- αVβ5 integrin association and that propagates JAK2/STAT5 pathway. The activated STAT5 in turn regulates the transcription of certain genes that might be related to either cell growth or apoptosis.

6.7 The phosphorylation of ERK and p90RSK

As mentioned in the introduction, activation of ERK1/2 was found to be linked with several biological actions such as cell growth, survival and proliferation in different cell types. Moreover, there is some evidence that the activation of ERK is required for cell cycle progression, and more precisely for cell transition from G1 phase into S phase, which means that the activated ERK is also required for DNA synthesis [234, 235, 236].

In haematopoiesis, the Ras/Raf/MEK/ERK cascade plays different roles depending on the cell type and the maturation step it is involved in. The activation of MEK/ERK signaling by stem cell factor (SCF) stimulates haematopoietic stem cell proliferation [252]. Furthermore, the activation of the MEK/ERK pathway as a downstream effecter of cytokine receptors is involved in different maturation steps in both lymphoid and myeloid lineages [253, 254]. On the other hand, activated ERK was found in about 75% of AML cases in a study conducted by Ricciardi and his colleagues [255]. In addition, increased ERK1/2 seems to be a significant indicator for both B-ALL and T-ALL patients [256, 257]. In the same context, the MEK inhibitor U0126 reduces growth of either AML or CML cell lines [258, 259].

In terms of SMS-SB cells, Acharya and her colleagues have shown that sCD23 activates the phosphorylation of ERK1/2 and that this event was enhanced when cells were co-stimulated with SDF1-α. The data illustrated in figure 5.2 confirmed that sCD23 and LP stimulate the phosphorylation of ERK1/2 and that is inhibited by the MEK inhibitor U0126; this might mean that the phosphorylation of ERK1/2 is MEK dependent and that also is compatible with the work reviewed in this study.

Several studies have shown that RSKs lie downstream of the MEK/ERK pathway as a response to different stimulants such as growth factors, cytokines and hormones [322, 244, 323, 320]. There are six different phosphorylation sites that have been clearly identified in p90RSK, and four of them are located in the linker region between the N- and C-terminal domains (Ser 221, Thr 359, Ser 363 and Ser 380); the phosphorylation of this region has been shown to be required for the activity of the p90 RSK [324, 325, 326]. In addition, Threonine 573 has been identified as the binding site for ERK1/2 [325, 327]. The p90RSK plays different biological roles as it is a growth factor- and cytokine-responsive cell signalling element in cell proliferation, differentiation and motility [328, 329, 330, 331]. In the SMS-SB model, either sCD23, LP or vitronectin activates the phosphorylation of p90RSK at four different residues (Ser380, Thr 359, Ser 363 and Thr 573), with different trends and levels as clearly shown in figure 5.1. In addition, figure 5.2 shows that the MEK inhibitor U0126 completely inhibits p90RSK phosphorylation with either ligand. However, it is not clear yet whether the phosphorylation of p90RSK is via ERK1/2, via MEK directly or other MEK dependent kinase such as ERK5, as published by Pearson et al [354].These data raised several questions about the role of p90RSK in the context of SMS-SB cell growth stimulated by the αvβ5 integrin. It would be very interesting to investigate the role of p90RSK in Bad phosphorylation and anti-apoptotic action, and to examine the link between p90RSK and other possible downstream targets such as SRF, c-Fos and c-Jun using either Lentiviral shRNA or p90RSK inhibitors.

6.8 The activation of SRF

Serum response factor (SRF) regulates the transcription of sets of genes that regulate normal cell growth, apoptosis, and other cellular activities [333, 334]. SRF forms distinct ternary complexes with different transcription factors such as Elk-1, SAP-1 and Ets-1 in order to regulate a wide range of genes [335, 336, 337]. Moreover, serum stimulation strongly increases SRF transcriptional activity [338]. The phosphorylation of SRF lies down stream of different kinases, mainly ERK2, p90RSK and JNK [339, 340, 341, 342].

The SRF transcriptional activity study presented in Chapter 5 shows that the αVβ5 integrin activates SRF to drive a reporter gene construct in SMS-SB cells transiently transfected with that vector. Figure 5.4 shows a significant activation increase after stimulating the cells with vitronectin, LP or sCD23, as well as with serum stimulation, compared to unstimulated cells. The data for ERK1/2 and p90RSK activation, in the presence or absence of U0126, and the SRF reporter results raised several questions drawing the complexity of signalling downstream of αVβ5 integrin, and these questions are summarized in figure 6.3.

Therefore, further work needs to be done to determine whether SRF lies downstream of MEK/ERK1/2 and possibly p90RSK. In addition, it would be promising to investigate which possible proteins form a ternary complex with SRF by using a co-immunoprecipitation technique. Furthermore, it is not clear yet what kind of link exists between the activation of c-Src, ERK1/2, p90RSK, SRF, STAT5 and JAK2.

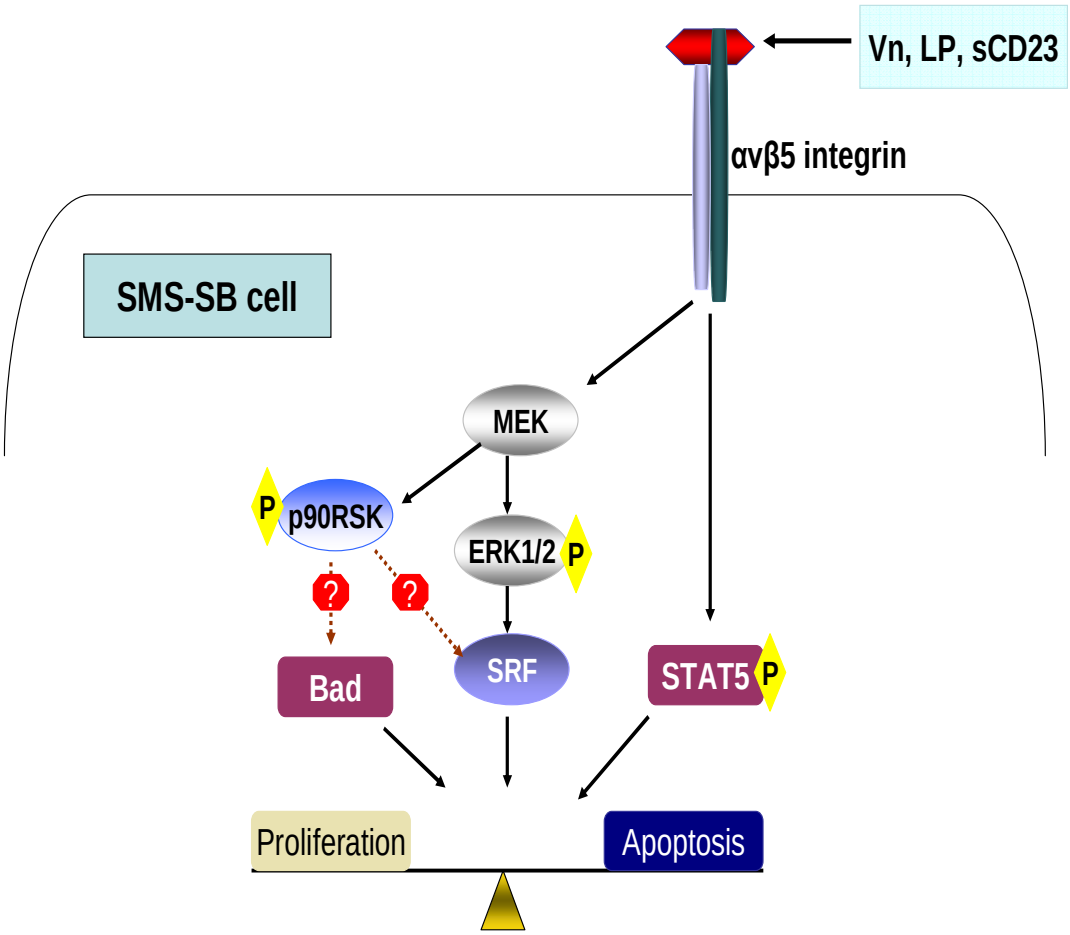

Figure 6.3 The activation of ERK1/2, p90RSK, SRF and STAT5 leaves many question marks in order to understand how the sCD23-αVβ5 integrin interaction sustains the SMS-SB cell growth.

6.9 Calpain activity study

Both Calpains, μ and m are cytoplasmic cysteine proteases that play several biological roles in cell mobility and embryonic development due to cleaving cytoskeletal proteins [261, 262]. In addition, both μ and m Calpains play different roles in cell signaling and apoptosis via cleaving caspase-7, -8, -9 and caspase-3 or via activating caspase activators such as APAF-1 and cytochrome C [263, 264].

The data presented in figure 5.3 clearly show that there is no clear effect on calpain activity following stimulation of the αVβ5 integrin with either LP or vitronectin for a short time course (0-40 minutes) or also for along time course (0.5-4 hours). However, these data are not enough to say that there is no caspase involvement after stimulating the αVβ5 integrin with its specific ligands. Moreover, changing the substrate used in this experiment (AC-LLY-AFC) with other substrate such as Suc-LLVY-aminoluciferin or (EDANS)-Glu-Pro-Leu-Phe-Ala-Glu-Arg-Lys-(DABCYL) might give different outcomes and lead to different conclusion.

6.10 The activation of STAT5 and SRF by either SDF1-α or PDGF-AB

SDF1 plays an essential role in haematopoietic cell maturation, survival and proliferation [135]. In addition, in various cell types including B cell precursors, SDF1 increases the intracellular calcium levels via ligation of CXCR4 [136, 137]. This ligation also leads to the activation of phosphatidylinositol 3 – kinases (PI3K) and

phosphorylation of MEK and ERK as a consequence of phosphorylation of focal adhesion complexes [138]. Moreover, SDF1 stimulates the Jak/STAT pathway [139]. On the other hand, SDF1 is thought to play different pathological roles such as HIV infection enhancement, tumour growth, inflammation and angiogenesis [140, 141, 142]. In terms of leukaemias, CXCR4 is highly expressed in B- chronic lymphocytic leukaemia cells which enhances their response to SDF1 [143]. Meanwhile, the migration, cellular adhesion and survival of different acute lymphocytic leukaemia cell lines are enhanced in the presence of SDF1 [144, 145]. However, the data presented in figure 5.5 clearly show that SDF1-α on its own significantly simulates the transcriptional activity of SRF but does not stimulate STAT5. These data are consistent with the work reviewed above and the proliferation data shown in the third Chapter of this thesis.

In a wider context, several studies have shown that the $PDGF_{AB}$ induces cellular proliferation, migration and differentiation via the RAS/MAPK pathway and promotes cell survival through the PI3K/Akt pathway [164, 163]. In addition, PDGF induces the activation of both STAT1 and STAT3 via the phosphorylation of JAK proteins in fibroblasts [165, 166]. Moreover, $PDGF_{AB}$ activates STAT5 in different types of leukaemia such as AML and CML [168, 169, 170].

The data illustrated in figure 5.6 show that STAT5 transcriptional activity is significantly induced by $PDGF_{AB}$ whereas there is no effect on SRF activity. These data strongly support the proliferation data presented in Chapter three and are consistent with the work reviewed in this thesis. In contrast, both stimuli do not provide any further stimulation to the vectors beyond that induced by sCD23.

To conclude, SDF1-α activates SRF but does not activate STAT5 while $PDGF_{AB}$ activates STAT5 but does not activate SRF and that probably means each stimuli plays its role in a different way. Similarly, it would be interesting to identify which genes might be regulated by STAT5 in response to $PDGF_{AB}$ and to compare these genes with the set of genes regulated by STAT5 in response to either sCD23 or vitronectin. In addition, using qRT-PCR might give another comparison study on candidate gene expression levels in response to these ligands. In terms of SDF1, further experiments need to be done to examine the effect of SDF1 in promoting the phosphorylation of p90RSK and other ERK1/2 downstream targets such as c-Fos.

6.11 The growth of 697 and BAF03 cells with soluble CD23 stimulations

The data illustrated in Chapter 5 (figure 5.7) demonstrates that soluble CD23 significantly increases the 697 cell growth at the concentration of 40nM, and also stimulates the proliferation of murine transformed cells (BAF03) at 80nM. Acharya and colleagues [123] have shown that soluble CD23 stimulated the proliferation of Blin-1 cells, a pre-B cell line model extracted from the bone marrow of a 11-year old boy with ALL. Moreover, they have shown that soluble CD23-derived peptide (LP) stimulates the RS4;11 cell growth which is another ALL cell line model. These data, together with the results presented previously, might suggest that soluble CD23 underlies the proliferation of several acute lymphocytic leukaemia cells from different origins. However, the data shown previously leave some challenging questions about the cell signalling pathways that are involved in each cell line model due to the αVβ5

integrin-sCD23 interaction, especially JAK2/STAT5, MEK/ERK1/2, p90RSK and SRF, and also about the role of sCD23 in normal pre-B cell growth and maturation.

6.12 Conclusion

This study has attempted to understand how the αVβ5 integrin mediates SMS-SB cell growth due to its binding to the model soluble ligands represented by the soluble CD23 and LP (a synthetic peptide derived from soluble CD23). The data presented in this thesis can be summarized as the following:

- The αVβ5 integrin promotes SMS-SB proliferation after binding to sCD23 and LP, which both contain an RKC motif, while there is no proliferation after vitronectin binds via the RGD motif.
- Both SDF1 and PDGF stimulate SMS-SB growth weakly, and enhance growth promoted by LP.
- Both sCD23 and LP stimulate the phosphorylation of different kinase substrates including STAT5a/b and STAT2.
- Stat5B phosphorylation is induced by sCD23 or LP, and slightly by vitronectin.
- sCD23 and LP strongly stimulate Stat5 DNA binding and transcriptional activity, but vitronectin causes minimal activation.
- αVβ5 integrin and PDGF receptor are co-immunoprecipitated from SMS-SB cells, and Jak2 is co-immunoprecipitated with PDGF receptor but not αVβ5.

- SMS-SB cell proliferation and Phosphorylation of Stat5b are clearly reduced by both Jak2 kinase inhibitor (AG490) and PDGF receptor kinase inhibitor (AG1295).
- Furthermore CD23, LP and vitronectin promote the phosphorylation of MAPK, p90RSK and induce SRF transcriptional activity.
- Soluble CD23 promotes the growth of 697 and BAF03 cell lines.

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