Chapter I

Introduction
1.1 Placenta

The placenta is an ephemeral organ present in female vertebrates during gestation. The organ functions as a foetomaternal organ with two components, the foetal part (Chorion frondosum), and the maternal part (Decidua basalis). The placenta receives nutrients, oxygen, antibodies, and hormones from the mother's blood, and passes out waste from the fetus (Vause et al 2005). It forms a barrier (called the "placental barrier"), which filters out some substances that could harm the fetus. The placental barrier does not allow the two bloods from the mother and embryo to mix in order to avoid the possible transfusion of different blood types.

Deoxygenated fetal blood passes through umbilical arteries to placenta. At the junction of umbilical cord and placenta, the umbilical arteries branch radially to form chorionic arteries. Chorionic arteries also branch before they enter into the villi. In the villi, they form an extensive arteriocapillary venous system, bringing the fetal blood extremely close to the oxygenated blood containing maternal blood, to facilitate the gaseous exchange between fetus and mother.

In addition to the transfer of gases and nutrients, the placenta also has endocrine activity. It produces several hormones as progesterone, placental lactogen, estrogen, Somatostatin, relaxin, and human chorionic gonadotrophin (hCG). These hormones are required for maintaining normal pregnancy (Ferretti et al 2007).

Bearing the hormones, placenta also expresses various growth factors as- Epidermal growth factor (EGF), Platelet derived growth factor (PDGF), Insulin like growth factor (IGF-1&2), Colony-stimulating factor-1 (CSF1), Vascular endothelial growth factor (VEGF), Corticotrophin-releasing factor (CRF), Growth hormone (GH), Transforming growth factor (TGF)-β, Placental growth factor (PIGF) (Ferretti et al 2007).
Placenta is also known to produce some matrix degrading enzymes as matrix metalloproteinase-9 (MMP-9), Heparanase, Urokinase, plasminogen activator (uPA) (Ferretti et al 2007).

Fig 1.1: Diagram showing the anatomical position of Placenta.

The organ Placenta is known to possess various medicinal properties for time immemorium. Infact many asian countries had been using various indigenously made placental medicinal preparations for long time, some as early as 5000 years.

China

The Placenta has been used for more than 5000 years, in Chinese traditional medicine, as a tonic remedy under the name of 'Shikasha', and is, as the ultimate source of life, the subject of much modern research in the development of new medicines for presently incurable diseases.

According to the record from an ancient Chinese masterpiece, the placenta was dried and made into powder or buried under the soil in order to obtain its
decomposed substance for nourishment or as anti-ageing formula. In his quest for immortality, Shi Huang Ti had discovered the placenta as his ultimate secret formula.

In the search for immortality, many preparations used the umbilical cord as their main ingredients.

Japan

In ancient Japan, one of the most treasured medicines was found to contain placenta and its therapeutic effects were very potent for strengthening the body, relieving fatigue, slimming and other chronic diseases. In the Japanese tradition, the placenta and umbilical cord from childbirth were used as offering on the altar.

Korea

The placenta is also mentioned in details in their famous medical encyclopedia. As a whole, the placenta is found to be very effective for relieving fatigue and prevent ageing. It is excellent for eliminating anxiety and fear. It is also being valued for its ability in strengthening and invigorating the body.

Based on the historical data, regarding the usage of placental extract, a new therapy “Human Placental Therapy” came into light in early twentieth century. The development of this therapy, can be traced back to the 1930’s when Soviet surgeon Vladimir Filatov noticed, that pregnant women were remarkable healthy during the third trimester of pregnancy and for a six-month period following delivery. He pioneered the early research and experimentation that led to the development of term placenta therapy (TPT). This therapy involves the injections of Term Placenta Extract (TPE) and the implantation of a certain amount of full-term placenta (after a normal baby is born), under the skin and that is the Term Placenta Implant (TPI). Placenta cells increase the capillary collateral network by stimulating the development of collateral buds. They dilate the blood vessels and thus improve circulation of the blood throughout the organism. Placenta therapy has a
hypotensive and a strong diuretic effect. The placenta of the very early fetus is rich in cells provided with pituitary qualities while the placenta of the mature fetus acts more after the manner of cells of the sex glands. In cellular therapy, the placenta assures the nutrition of fetal cells.

In general TPT (term placenta therapy) optimizes the immune, hormonal and circulatory systems and can be used for many conditions. Based, on the above principle, in 1950, two types of injections containing extract of human placenta, "Melsmon" and "Laennec", were developed in Japan. According to the experience of many physicians in Europe and other countries this therapy is found to be very effective for the following conditions:

**General indications:**
- Chronic fatigue syndrome
- Stress & anxiety
- Insomnia; memory problems.
- Prior or after surgery, chemotherapy or radiation therapy.
- People that want to keep their health in top condition.
- To improve the quality of life of patients with cancer.
- Prevention and treatment of Geriatric conditions like senile dementia, Parkinson's and Alzheimer's disease.

**Immune system conditions:**
- Auto-immune diseases, like Rheumatoid arthritis, Generalized or Discoid Lupus, Multiple Sclerosis, and autoimmune Hepatitis; Degenerative diseases like Osteoarthritis.
- Chronic infections like AIDS, Hepatitis C, Lime disease, Tuberculosis, etc.
- When the immune system is down because of steroid therapy, cancer therapy or depression.
Circulatory disorders:
• Arteriosclerosis, High Blood Pressure, Poor venous circulation.
• Tinnitus, Chronic dizziness.
• Complication of patients with Diabetes: Kidney insufficiency, peripheral neuropathy.
• Migraine/tension headaches.

Hormonal system:
• In men: Sexual dysfunction, prostate problems, diminished libido, poor energy.
• In women: Dry skin (premature aging). PMS syndrome; Menopause syndrome. Pelvic congestion, Nocturia, Poor energy.

Taken together, the clinically proven effects of placenta are-

1) Restores youth quality (prevent ageing).
2) Improves the effects of anemia.
3) Prevents and improve bloating, rashes, black spot and freckle.
4) Improves the effects of anemia.
5) Relieves the discomfort due to menopause.
6) Relieves diabetic conditions.
7) Revitalize the body during illness and after recuperation.
8) Enhances the healing of skin membrane and wounds.
9) Relieves kidney ailment
10) Relieves hypertension.
11) Relieves the inflammation for stomach and duodenum.
12) Invigorates the body.

The present study owes to probing into the wound healing property of Placentrex. Placentrex, a Proprietary drug of M/S Albert David Limited, a pharmaceutical organization of India. Placentrex, a hot extraction product of freshly collected Human Placenta, is a complex admixture of several biomolecules. The drug
Placentrex is found to be wonderfully efficacious against wound healing and is a thoroughly recommended product by physicians in many wound-healing cases. A brief description regarding the method of production of the drug “Placentrex” is given below.

**Fig 1.2: Flow sheet for Placentrex production.**

Though the drug Placentrex is most widely prescribed in wound healing cases, gynecological disorders (fallopian tubal blockage) (Sarkar et al 1976), skin
disorder (vitiligo) (Chopra et al 1996) and immune modulation (Ansari et al 1994) also find treatment with Placentrex.

Human Placental extract has been well documented as having wound healing capacity since a long time (Wu et al 2003). In fact, its usage as a wound healer finds mention in Chinese folklore as well. However, the exact molecular mechanism of placental extract on wound healing still remains elusive.

1.2. Wound Healing

The healing of an adult skin wound is a complex process requiring the collaborative efforts of many different tissues and cell lineages. The behavior of each of the contributing cell types during the different phases namely migration, matrix degradation, proliferation, matrix synthesis and contraction at the wound site are poorly understood. Wounds to skin will cause leakage of blood from damaged blood vessels (Fig 1.3, step a). The formation of a clot then serves as a temporary shield protecting the denuded wound tissue and provides a provisional matrix over which cells migrate during the repair process. The clot consists of Platelets embedded in a mesh of cross-linked fibrin derived from thrombin cleavage of Fibrinogen, Fibronectin, Vitroneectin (Fig 1.3, step b). The clot serves as a reservoir of Cytokines and growth factors that are released as activated Platelets degranulate. This cocktail of growth factors (EGF, TGFα, β, FGF, VEGF) kick-starts the wound closure process ——— initiated by the recruitment of inflammatory cells (Neutrophils, Monocytes (Fig 1.3, step c) and Macrophages (Fig 1.3, step d) to the wound site to clear up the infection by phagocytosing bacteria. Once the infiltration of the inflammatory cells has occurred, the fibroblast and keratinocyte from the adjacent sites migrate to the wound site. This migration is caused by the secretion of some fibrinolytic enzyme (Plasmin) and some extracellular matrix-degrading enzyme (Matrix metalloprotease) by the migrating cells. After migration the cells proliferate (Fig 1.3, step e) to patch up and regenerate the area (Fig 1.3, step f) (Paul Martin 1997). During the whole process,
Cell migration appears to be the key phenomena for the wound healing process, with precisely neutrophil migration to be the rate-determining step of the healing process. Neutrophil migration is reckoned to be the most crucial aspect of wound healing as because they are the first cells which migrate to the wound site to cover up the infection by virtue of their phagocytic property.

A cartoon depicting the different stages of wound healing process is enumerated below.

![A brief schematic diagram depicting different stages of cutaneous wound healing process.](Expert_Reviews_in_Molecular_Medicine_2003_Cambridge_University_Press)
1.3. Cell Migration

Cell migration is a highly integrated multistep process that orchestrates embryonic morphogenesis; contributes to tissue repair and regeneration; and drives disease progression in cancer, mental retardation, atherosclerosis, and arthritis. The migrating cell is highly polarized with complex regulatory pathways that spatially and temporally integrate its component processes.

In general, cell migration can be usefully conceptualized as a cyclic process (Lauffenburger, Horwitz, 1996). The initial response of a cell to a migration-promoting agent is to polarize and extend protrusions in the direction of migration. The protrusions can be large, broad lamellipodia or spike-like filopodia, are usually driven by actin polymerization (Fig 1.4, step 1). This cycle of polymerisation is tightly regulated by several actin-binding proteins to maintain a steady equilibrium between monomer (G actin) and polymer (F actin), with the equilibrium shifting towards F-actin, with migration. These protrusions are stabilized by adhering to the extracellular matrix (ECM) or adjacent cells via transmembrane receptors linked to the actin cytoskeleton. These adhesions serve as traction sites for migration as the cell moves forward over them, and they are disassembled at the cell rear, allowing it to detach (Fig 1.4, step 2). As new adhesion sites are formed in the direction of migration, the whole cell body translocates in the same direction with contraction at the rear side (Fig 1.4, step 3).
1.3.1. Protrusion process.

Actin filaments are intrinsically polarized with fast-growing "barbed" ends and slow-growing "pointed" ends, and this inherent polarity is used to drive membrane protrusion. However, the organization of filaments depends on the type of protrusion: In lamellipodia, actin filaments form a branching "dendritic" network, whereas in filopodia they are organized into long parallel bundles. Actin polymerization in lamellipodia is mediated by the Arp2/3 complex, which binds to the sides or tip of a preexisting actin filament and induces the formation of a new daughter filament that branches off the mother filament (Welch, Mullins, 2000 and Pollard, Borisy, 2003).

Activation of the Arp2/3 complex is localized by WASP/WAVE proteins, which are themselves activated at the cell membrane. Pushing of the membrane, the actual protrusive event is believed to occur not by elongation of the actin filament per se but by an "elastic Brownian ratchet" mechanism.
Several actin-binding proteins regulate the rate and organization of actin polymerization in protrusions by affecting the pool of available monomers and free ends (Pollard, Borisy, 2003, Remedios et al, 2003). For example, profilin prevents self-nucleation by binding to actin monomers and also serves to selectively target monomers to barbed ends. Filament elongation is terminated by capping proteins, thereby restricting polymerization to new filaments close to the plasma membrane. In addition, disassembly of older filaments, which is needed to generate actin monomers for polymerization at the front end, is assisted by proteins of the ADF/cofilin family, which sever filaments and promote actin dissociation from the pointed end.

Other proteins play supporting roles in the process as: Cortactin stabilizes branches, whereas filamin A and α actinin stabilize the entire network by cross-linking filaments (Welch and Mullins, 2000). Filopodial protrusion is thought to occur by a filament treadmilling mechanism, in which actin filaments within a bundle elongate at their barbed ends and release actin monomers from their pointed ends (Welch and Mullins, 2000). The long and unbranched filament organization is consistent with assembly occurring by elongation rather than by branched nucleation. Many proteins are enriched at filopodial tips, including Ena/VASP proteins, which bind barbed ends of actin filaments and antagonize both capping and branching, thereby allowing continuous elongation of filaments.

1.3.2. Molecular model of cell migration

Cells migrate directionally in response to a variety of chemotactic cues viz chemokines, growth factors and ECM molecules. These factors engage cell surface receptors, initiating a cascade of events, including the activation of G proteins or tyrosine kinases, the stimulation of GEFs for Rac/Cdc42, and the activation of lipid kinases (PI3K) and the subsequent recruitment of activated Rac GTPase at the leading edge of the cell. PI3 Kinase on activation forms lipid products [PI(3,4)P2/PI(3,4,5)P3] which goes onto bind the GEF of Rac and leading to
activation and translocation of Rac. On the other hand, Protein kinase C phosphorylates GEF of Rac leading to Rac activation in a PI3 Kinase independent fashion. The local activation of Rac and/or Cdc42, in concert with other regulators such as WASP/WAVE family proteins and the Arp2/3 complex, stimulates the formation of a branching actin filament network at the leading edge, which in turn induces a protrusion in the direction of migration. Cdc42 also contributes to cell polarization by mediating reorientation of the MTOC toward the cell front, leading to growth of microtubules and delivery of vesicles into this region. Adhesions transmit propulsive forces and serve as traction points over which the cell moves. The migration cycle is completed as adhesions disassemble and the rear retracts. The disassembly of adhesions is controlled by pathways that include kinases like focal adhesion kinase (FAK), ERK, Src, and the protease calpain, as well as microtubule dynamics. Retraction at the rear requires Rho kinase and is a myosin dependent process. The release of adhesions at the rear and front share some common mechanisms and are coupled to the formation of protrusions at the front.

1.3.2.1. Rho-GTPase

Rho GTPases are members of the Ras superfamily of monomeric 20-30 kDa GTP-binding proteins. Ten different mammalian Rho GTPases, some with multiple isoforms, have been identified: Rho (A, B, C isoforms), Rac (1, 2, 3 isoforms), Cdc42 (Cdc42Hs, G25K isoforms), Rnd1/Rho6, Rnd2/Rho7, Rnd3/RhoE, RhoD, RhoG, TC10 and TTF (Ridley, 2000). The most extensively characterized members are Rho, Rac and Cdc42. Rho (GTPases) are the pivotal regulators of actin cytoskeleton and control the formation of lamellipodia and filopodia.

It is not surprising, therefore, that Rho GTPases have been found to play a role in a variety of cellular processes that are dependent on the actin cytoskeleton, such as cytokinesis (Mabuchi et al, 1993, Drechsel et al, 1997) phagocytosis (Caron and Hall, 1998), pinocytosis (Ridley et al, 1992) cell migration (Nobes and Hall, 1999),

In addition to their effect on the actin cytoskeleton, they also regulate a variety of other biochemical pathways including serum response factor (SRF) and nuclear factor κB (NF-κB) transcription factors, the c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways, the phagocytic NADPH oxidase complex, G1 cell-cycle progression, the assembly of cadherin containing cell-cell contacts, secretion in mast cells and cell transformation.

Of the Rho GTPases, Rac, Cdc42, and RhoG are required for protrusion of lamellipodia and filopodia. Of Rac, Cdc42 and Rho G, Rac proteins (Rac1, 2, and 3) are perhaps the most important regulators of actin cytoskeleton remodelling. Rac2 is the predominant Rac species in human haematopoietic cells.

Each of these GTPases act as a molecular switch, cycling between an active GTP-bound, and an inactive GDP-bound, state. When bound to GTP, they are active and interact with their downstream effector proteins, which include protein kinases, lipid-modifying enzymes, and activators of the Arp2/3 complex (Etienne-Manneville and Hall, 2002).

Rho GTPases are activated by guanine nucleotide exchange factors (GEFs), which catalyses nucleotide exchange and are inactivated by GTPase activating proteins (GAPs), which stimulates GTP hydrolysis (Fig 1.5).

The Rho-family members have a unique C terminal structure of Cys-A-A-Lue/Ile (A= aliphatic amino acid) that undergoes posttranslational modifications (Alan Hall 1994). The Cys residue is either geranylated or farnesylated followed by removal of the three amino acids and carboxymethylation of the exposed Cys residue. These posttranslational modifications are essential for the activation of Rho-family members. The Rho family members are also negatively regulated by
GDI (guanine nucleotide exchange inhibitor). The GDIs carry on their action on Rho by three ways (Takai et al. 1995): (1) Rho-GDI selectively interacts with the lipid modified GDP bound form of Rho-GTPase and inhibits their conversion from the GDP-bound inactive form to GTP-bound active form. (2) Rho-GDI masks the effector domain of the Rho-family members and prevents them from interacting with their downstream effector molecules. (3) Rho-GDI inhibits the association of the lipid modified, GDP bound form of Rho family members with membranes and induces their dissociation from the membranes (Fig 1.5).

Fig 1.5: The Rho-GTPase cycle. The GTPase cycle between an active (GTP-bound) and an inactive (GDP-bound) conformation. In the active state, Rho-GTPase interacts with one of the target proteins (effectors). The cycle is highly regulated by three classes of protein: a) guanine nucleotide exchange factors (GEFs) catalyse nucleotide exchange and mediate activation; b) GTPase-activating proteins (GAPs) stimulate GTP hydrolysis, leading to inactivation; and c) guanine nucleotide exchange inhibitors (GDIs) extract the inactive GTPase from membranes.

1.3.2.2. Guanine nucleotide exchange factors (GEFs)

The GTPases can be activated by guanine-nucleotide exchange factors (GEFs) that act to accelerate nucleotide exchange by prising open the binding site of specifically the GDP bound form of the GTPases (Worthylake et al., 2000). There is a large family of Rac-GEFs. These include Vav (1, 2, 3), Tiam (1, 2), PIX (α, β), Ras-GRF (1, 2), and Sos (Manser et al., 1998; Scita et al., 1999; Stam and Collard,
Protein kinases currently seem the major direct regulators of Rac-GEF activity. For example, Vav1 can be phosphorylated and activated by Syk, Lyn and Fyn (Miranti et al., 1998, Michel et al., 1998). Similarly, Ras-GRF1 has to be tyrosine-phosphorylated to display Rac-GEF activity (Kiyono et al., 1999), and Tiam1 is phosphorylated and regulated by Ca²⁺/calmodulin-dependent protein kinase II (Fleming et al., 1999). Other regulators of Rac-GEFs, for example, phosphoinositide 3-kinases (PI3Ks) and Gβγs, largely work by affecting these phosphorylations (Kiyono et al., 1999). All the Rho family GEFs which have been identified, contain a Dbl homology (DH) domain (named for the *dbl* oncogene product identified in a diffuse B-cell lymphoma) and a pleckstrin homology (PH) domain located just C-terminal of the DH site (Zheng Y, 2001). Functionally, the DH domain is responsible for catalyzing guanine exchange activity while the PH domain appears to be involved in intracellular localization of the GEF protein and binding of lipid products of PI3K.

![Fig 1.6. The Rho-GEF family with the characteristic DH and PH domains](image-url)
Of the three mammalian isoforms of Vav (1,2,3), Vav1 is primarily expressed in hematopoietic cells, while Vav2 and Vav3 are more ubiquitously expressed (X.R. Bustelo, 2000). Vav1, Vav2 and Vav3 share identical domain structure, containing a calponin homology (CH) domain, acidic domain (Ac), a Dbl homology (GEF) domain (DH), a pleckstrin homology domain (PH) and two Src homology (SH) 3 domains that flank one SH2 domain (Turner et al 2002). The acidic domain (Ac) of Vav contains potent tyrosine phosphorylation sites, which are required for the activation of Vav and also for the binding to SH2 domains of various signaling proteins as- Lck, PI3K p85α, and PLCγ1 (Miletic et al 2006).

1.3.2.3. Phosphoinositide 3-kinase (PI3K)

Phosphoinositide 3-kinase (PI3K) family enzymes phosphorylate the phosphoinositide class of lipids at the 3-OH position of the inositol ring (Stephens et al 2000). There are three types of PI3K enzymes, class I, II and III, out of which class I is the most characterized class. Class IA enzymes consist of any one of the 'catalytic' subunits (p110α, p110β, or p110δ) complexed with any one of the 'regulatory' subunits (p85α, p85β or p55γ). Only one Class IB PI3K enzyme exists, and is made up of the p110γ catalytic and the p101 regulatory subunit. There are also three Class II PI3Ks (CIIα, CIIβ, and CIIγ) and one Class III PI3K (Vps34). The 'regulatory' p85 activity of Type 1A PI3Ks is dependent on tyrosine phosphorylation event and are activated by receptor-tyrosine kinases (growth factor receptors), antigen receptors and cytokine receptors. The p110γ activity of type IB PI3K (PI3Kγ) is activated by G protein-coupled receptors via the βγ subunits of heterotrimeric G proteins (Fig 1.7). Class II PI3Ks are thought to be activated by some tyrosine kinase receptors, GPCRs, integrins, and chemokines. The Class III PI3K appears to be constitutively activated. Upon activation, all type I PI3Ks phosphorylate phosphatidylinositol (4,5)-diphosphate (PtdIns (4,5)P₂), a constitutive membrane component, to create the lipid second messenger phosphatidylinositol (3,4,5)-triphosphate (PtdIns (3,4,5)P₃/PIP3) (and its immediate metabolic product PtdIns(3,4)P₂ /PIP2). PIP3 has been implicated in the
activation of various regulatory proteins as protein kinases, phosphatases and GEFs, which are reflected in various essential cellular functions including transcription, translation, protein synthesis, cell survival, cell cycle entry and the structure of the actin cytoskeleton (Stephens et al 2000). Mammalian neutrophils and Dictyostelium discoideum cells respond to a gradient of chemoattractants by extending actin-rich pseudopodia in the direction of highest concentration of chemoattractant (Weiner et al, 1999). This gives the cell a polarized morphology and a selective accumulation of lipid products (PIP3) of PI3-Kinase at their up-gradient edges (Wang, et al, 2002, Weiner et al, 2002).

There are many PI3K dependent Rac GEFs, which get activated by PI3K lipid product (PIP3) by virtual binding of PH domains of the GEFs. To name a few are-Vav1, Sos1, P-Rex1, SWAP-70, Tiam 1.

Fig 1.7. Class 1(A&B) PI3Ks. The p85 regulatory subunit of Type 1A are activated by upstream kinase receptors through tyrosine kinase phosphorylation. The catalytic subunit then transfers phosphate to PIP2 to generate the active second messanger PIP3. Type 1B are activated by GPCR.
1.3.2.4 Spleen tyrosine kinase (Syk)

The Syk kinases are essential for normal development and function of the immune system (Chu et al. 1998), and Syk is required for the maintenance of vascular integrity (Cheng et al. 1995). Syk contains two tandem amino-terminal SH2 domains (along with a small interdomain region A) followed by an extended interdomain region (interdomain B) before the kinase domain and it is restricted to hematopoietic cells (Chan et al., 1992; Müller et al., 1994). It is homologous to ZAP-70; overall, these two kinases (Syk and ZAP-70) share greater than 50% sequence identity.

1.3.2.4.1 SH2 domains

The SH2 domains of the two kinases are quite similar, with 56% sequence similarity. The most distinctive aspect of the tandem SH2 domains present in this family of kinases is that they bind to their targets in a co-operative manner (Hatada et al. 1995, Kurosaki et al. 1995, Ouinger et al. 1998). The N-terminal SH2 domain appears to be incomplete and requires amino acid contributions from the C-terminal SH2 domain to help complete the phospho tyrosine-binding (PTB) pocket for binding to the phosphorylated ITAM motifs (YxxL/Ix6-8YxxL/I, where x is any amino acid) (Hatada et al. 1995). Structurally, the SH2 domains of Syk and ZAP-70, and the region in between them, appear to be conserved (Fig. 1.8).

1.3.2.4.2 Interdomain A

The interdomain region (IA) between the two SH2 domain is the most highly conserved region between the two kinases, with greater than 65% of the amino acids identical between Syk and ZAP-70. A conserved tyrosine residue, Y130 in Syk and Y126 in ZAP-70, may be an important regulator of the stabilizing effect of interdomain A on the tandem SH2 domain structure (Fig. 2) (Keshvara et al. 1997). This tyrosine has been demonstrated to be an in vitro autophosphorylation site in Syk (Keshvara et al. 1997). Furthermore, mutation of this tyrosine to phenylalanine or...
glutamic acid affects the activation and receptor-binding of Syk, suggesting that this site of phosphorylation may influence the conformation of the coiled-coil structure, thereby regulating the binding of the SH2 domains to the phosphorylated ITAM motifs (Keshvara et al. 1997) (Fig 1.8).

1.3.2.4.3. Interdomain B

Following the SH2 domains and preceding the kinase domain of Syk or ZAP-70 is an extended sequence, termed interdomain B (IB), which is important in regulating the kinase activity of these proteins. These interdomain regions of Syk and ZAP-70 show about 30% sequence identity, including a number of potent tyrosine phosphorylation sites (Watts et al. 1994). An obvious difference between Syk and ZAP-70 in this region is the insertion of a 23 amino acid sequence in the Syk interdomain region that is not present in the ZAP-70 molecule. Many of the tyrosine phosphorylation sites of the interdomain region are conserved and may serve the same functions in both molecules. One of these sites appears to be a positive regulatory site, Y348 in Syk, which corresponds to Y315 in ZAP-70. Syk, Y348, has been identified as a binding site for Vav in a yeast two hybrid system (Deckert et al., 1996). Mutation of this tyrosine also results in the loss of Vav phosphorylation and Vav association with Syk. In Syk, Y348 has been demonstrated to be a site of autophosphorylation (Deckert et al. 1996, Furlong et al. 1997). Y315 of ZAP-70 is the corresponding site of phosphorylation. Mutation of this site results in decreased receptor signaling, confirming the biological importance of this conserved residue. Another site, SykY352, which corresponds to Y319 in ZAP-70, has been shown to be a site of autophosphorylation (Furlong et al. 1997) (Fig 1.8). One possible role for this site is the recruitment of the Lck SH2 domain. Y352 in Syk have also been suggested to be essential for the binding of the C-terminal SH2 domain of PLCγ1 (Law et al. 1996). Similarly, mutations in these residues decrease Lck and PLCγ1 associations.
1.3.2.4.4. Kinase domain

Overall, the kinase domains of Syk and ZAP-70 are very similar, about 60% identical. Conserved regulatory sites are found within the activation loop of the kinase domain, Y492/Y493 in ZAP-70 and Y525/Y526 in Syk. These are sites for tyrosine phosphorylation by Lck and for autophosphorylation in the case of Syk (Furlong et al. 1997, Chan et al. 1995). In ZAP-70, Y492 has been shown to be a negative regulatory site, whereas Y493 is a positive regulatory site required for ZAP-70 kinase activity (Kurosaki et al. 1995, Chan et al. 1995). Mutation of both Y525 and Y526 to phenylalanine eliminates Syk activity, indicating that these tyrosines are critical for Syk function (Kurosaki et al. 1995) (Fig 1.8).

![Diagram of Syk family kinases](image)

Courtesy (Chu et al, Immunological reviews, 1998)

**Fig 1.8. Syk family kinases.** The Syk family kinases Syk and ZAP-70 are composed of two SH2 domains at the amino-terminus of the protein (SH2-N and SH2-C) with an intervening interdomain A(ILA). A portion of the C-terminal SH2 domain is necessary to complete the N-terminal SH2 domain phosphotyrosine-binding pocket. Following the SH2 domains is an extended interdomain B (IB) and kinase domain (Kinase). Conserved tyrosine phosphorylation sites are indicated (see text).

In the case of immune response receptor signaling, Syk is reported to phosphorylate or activate several proteins as, phospholipase Cγ (Law et al., 1996), c-Cbl (Ota et al., 1996), Sht (Jabril-Cuenod et al., 1996), Vav (Teramoto et al., 1997), PI 3-kinase (Yanagi et al., 1994) and SHIP (Crowley et al., 1996). Syk
becomes activated in particular hematopoietic cells in response to ligation of the B cell receptor, the T cell receptor or several Fc receptors, and it is clearly implicated in lymphocyte development and activation (Minoguchi et al, 1994; Cambier, 1995; Cheng et al, 1995; Turner et al, 1995). In these cases, receptor engagement stimulates tyrosine phosphorylation of 'immune receptor tyrosine activation' motifs (ITAMs) in the receptor by a Src family kinase. Syk activation occurs when the tandem SH2 domains engage a dually-phosphorylated ITAM, and activation appears to occur through a chain reaction mechanism involving tyrosine phosphorylation in the activation loop of the catalytic domain by a Src kinase (Fig 1.9) (Kurosaki et al, 1995; Rowley et al 1995). Syk can also be activated with integrin adhesion receptors (Woodside et al 2001). However, the situation is little different with the integrins. Integrins, unlike the immune receptors don't have ITAM motifs. Here, the interaction is independent of tyrosine phosphorylation and of the phosphotyrosine binding function of Syks tandem SH2 domains (Woodside et al 2001).

Courtesy (Chu et al, immunological reviews, 1998)

Fig 1.9. A sequential model of Syk/ZAP-70 activation.
1.3.2.5. Leukocyte-specific protein tyrosine kinase (Lck)

The kinase p56lck (509 aa) is a T-lymphocyte-specific member of the Src family of non-receptor protein tyrosine kinase. Lck is a 56 kDa phosphoprotein expressed in variety of lymphoid and non-lymphoid cell lineages. The Lck protein resides predominantly in the cytosol where it interacts with the inner leaflet of the plasma membrane via its hydrophobic myristilated N-terminus (Resh, M.D, 1994). Lck contains myristylation sequence, unique amino-terminal regions, followed by Src homology domains SH3 and SH2, a tyrosine kinase catalytic domain, and C-terminal regulatory domain (Thomas et al 1997). The Unique region of Lck represents the domain possessing the greatest sequence diversity within this group of enzymes. This domain is thought to be involved in the interaction of the Lck with specific cellular proteins including Lck substrate (Thomas et al 1997). SH3 (Src homology 3) domain is mainly implicated in the regulation of protein-protein interactions, recognizing proline-rich region found in guanine nucleotide exchange factors and GTPase activating proteins. SH2 (Src homology 2) domain of Lck recognizes phosphorylated tyrosine residues on other proteins thereby facilitating the formation of tyrosine phosphorylation-induced multimeric complexes. The tyrosine kinase domain is the catalytic domain of Lck catalyzing the transfer of the gamma-phosphate from ATP to tyrosine residues on proteins. The catalytic domain contains a site of autophosphorylation (Tyr-394), which plays an important role in regulating the protein kinase activity (Fig 1.10).

![Fig 1.10. Lck kinase. Lck contains myristylation sequence, unique amino-terminal regions, followed by Src homology domains SH3 and SH2, a tyrosine kinase catalytic domain, and C-terminal regulatory domain](image-url)
Lck phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of the immune receptors (Iwashima et al 1994, Isakov et al 1998). The resulting phospho-ITAMs then serve as docking sites for Src homology domain 2 (SH2)-containing molecules, predominantly ZAP-70 and Syk. Only then can ZAP-70 and Syk undergo tyrosine phosphorylation, to become enzymatically active (Chan et al 1995), and further phosphorylate downstream effector molecules. Lck is known to interact with a variety of cell surface receptors as CD2 (Bell et al, 1996), CD4 (Veillette, et al 1988), CD5 (Raab et al 1994), CD8 (Veillette, et al 1988), CD44 (Ilangumaran et al 1998), CD45 (Bruyns et al, 1998), CD122 (Minami et al, 1993) and the IL-7 receptor (Page et al, 1995).


1.3.2.6. Integrins

Integrins are heterodimeric adhesion receptors formed by the non-covalent association of $\alpha$ and $\beta$ subunits. Each subunit is a type I transmembrane glycoprotein that has relatively large extracellular domains and, with the exception of the $\beta$4 subunit, a short cytoplasmic tail (Hynes, 2002). Integrins are present in all metazoans, and the number of integrins in the genome generally increases with the complexity of the organism (Hynes, 2002; Bokel and Brown, 2002), which is consistent with the central role of integrins in adhesion, migration and tissue organization. Mammals contain 18 $\alpha$ and 8 $\beta$ subunits that combine to produce at least 24 different heterodimers, each of which can bind to a specific repertoire of cell-surface, ECM or soluble protein ligands (Fig 1.11).
Cell-cell and cell-substratum adhesion is mediated by the binding of integrin extracellular domains to diverse protein ligands; however, cellular control of these adhesive interactions and their translation into dynamic cellular responses, such as cell spreading or migration, requires the integrin cytoplasmic tails. These short tails bind to intracellular ligands that connect the receptors to signalling pathways and cytoskeletal networks (Critchley, 2000; Calderwood et al., 2000; Liu et al., 2000; Brakebusch and Fassler, 2003; Giancotti and Ruoslahti, 1999; Geiger et al., 2001). Hence, by binding both extracellular and intracellular ligands, integrins provide a transmembrane link for the bidirectional transmission of mechanical force and biochemical signals across the plasma membrane.

One important mechanism by which cells regulate integrin function is through tight spatial and temporal control of integrin affinity for extracellular ligands. This is achieved by rapid, reversible changes in the conformation of the extracellular domains of the integrin heterodimer. Despite the high degree of redundancy, most integrins have specific biological functions raising the possibility of signaling differences between integrins. Integrins not only bind ligands present in the extracellular matrix such as fibronectin, laminin, collagen, vitronectin etc., certain integrins can also bind to soluble ligands such as fibrinogen or to counter-receptors such as intracellular adhesion molecules (ICAMs) on adjacent cells (C Chandra Kumar, 1998). Many of the integrins recognize the RGD (Arg-Gly-Asp) sequence in their matrix ligands (Ruoslahti, 1996). Still, they are capable of distinguishing different RGD-containing proteins such that some bind primarily to fibronectin and others to vitronectin. Integrins are also expressed in a cell-type specific manner. Examples of cell-typespecific integrins include α\textsubscript{IIb} β\textsubscript{3} in platelets, α\textsubscript{6} β\textsubscript{4} in epithelial cells, and α\textsubscript{4}β\textsubscript{2} in leukocytes. The β2 (CD18) subfamily of integrins serves a vital role in leukocyte function and the development of an effective inflammatory response in vivo (Springer, 1994). Four members of the integrin β2 subfamily have been recognized: αMβ2 (Mac-1, CD11b/CD18), αLβ2 (LFA1, CD11a/CD18), αXβ2 (p150, 95, CD11c/CD18), and αDβ2 (CD11d/CD18).
However, Mac-1 and LFA-1 are the most prominent integrins of the β2 family on the surface of inflammatory cells (Larson and Springer 1990) (Fig 1.11).

Fig 1.11. The integrin receptor family. Integrins are (αβ) heterodimers: each subunit crosses the membrane once, with most of each polypeptide (>1600 amino acids in total) in the extracellular space and two short cytoplasmic domains (20–50 amino acids). The figure depicts the mammalian subunits and their associations. 8 β subunits can assort with 18 α subunits to form 24 distinct integrins. Ligand specificity is indicated with α subunits specificity for laminins (purple) or RGD (blue). Leukocyte specific integrins are also shown.

The extracellular domains bind a wide variety of ligands, whereas the intracellular cytoplasmic domains anchor to cytoskeletal proteins. In this manner, the exterior and interior of a cell are physically linked, which allows for bidirectional transmission of mechanical and biochemical signals across the plasma membrane, and leads to a cooperative regulation of cell functions. The integrin functions are regulated by agonist and antagonist, which increase or decrease integrin affinity/avidity for adhesive ligands through a process known as “inside.out” signaling. In turn ligand occupancy and clustering of integrins trigger “outside.in.” signals that relays signal downstream to influence the cytoskeletal events.
1.3.2.6.1. "Inside-out" signaling

A central topic in the integrin research over the past decade has been the mechanism of "inside-out” activation (Liddington and Ginsberg 2002). In their resting state, integrins normally bind the molecules that activate them with low affinity. Upon stimulation, a cellular signal induces a conformational change in the integrin cytoplasmic domain that propagates to the extracellular domain. Integrins are transformed from a low- to a high affinity ligand binding state. A molecular picture has emerged for integrin “inside out” activation where a cellular signal induces the conformational change of cytoskeleton protein, talin exposing its head domain allowing it to bind to the integrin β cytoplasmic tail.

Talin is an antiparallel homodimer of two ~270 kDa subunits (Critchley, 2000). Each subunit consists of an N-terminal ~50 kDa globular head and an ~220 kDa C-terminal rod (Rees et al, 1990). Talin binds strongly to β1, β2, β3, β5 cytoplasmic tails (Horwitz et al, 1986; Knezevic et al, 1996; Pfaff et al., 1998; Sampath et al), with the integrin binding site lying with in the globular head (Calderwood et al, 1999).

The talin-cytoplasmic tail interaction unclasps the complex between the cytoplasmic tails, which then allows a conformational shift in the extracellular domain from a bent to a more extended form for high-affinity ligand binding (Takagi et al. 2002). The activated integrins may then undergo clustering whereby the transmembrane domain of each type of subunit (the α or β) interacts with itself—called homotypic oligomerization of the transmembrane domains (Li et al. 2003)(Fig 1.12).
Fig 1.12. Model for Integrin Inside-Out Activation. Cellular stimulation induces a conformational change in talin that exposes its talin head domain. The talin head domain binds to the β cytoplasmic tail, which displaces the α tail from its complex with the β tail, which in turn leads to an unclasping and a membrane associated structural change of the cytoplasmic face. The α subunit is in blue and the β subunit is in red.

The talin-integrin tail interaction is a major area of studying integrin activation. Several potential mechanism exist probing into the talin-integrin tail interaction as-

1) The protease calpain provides an in vivo mechanism for the separation of talin N- and C-terminal domains to unmask the integrin-binding site, and calpain cleavage increases talin binding to integrins in vitro (Yan et al., 2001). Talin head has a sixfold higher affinity than intact talin, probably integrin binding site is masked in intact talin (Yan et al., 2001) (Fig 1.13 A).

2) Binding of PtdIns(4,5)P2 to talin induces a conformational change that unmask the tail-binding site within the talin FERM domain and enhances its association with integrin β tails (Martel et al., 2001). Notably, talin binds to and activates one splice variant of the PtdIns(4,5)P2-producing enzyme: phosphatidylinositol phosphate kinase type Ig-90 (PIPKIγ-90) (Ling et al., 2002). Therefore, talin can stimulate PtdIns(4,5)P2 production that in turn enhance talin-integrin interactions,
which suggests that PIPKIγ-90 may positively regulate integrin activation (Fig 1.13 A).

3) Tyrosine phosphorylation of the NPxY motifs in integrin β tails by Src-family kinases leads to disruption of integrin-talin interactions (Datta et al., 2002). Integrin phosphorylation, by Src-family kinases or other kinases, may therefore be an important negative regulator of integrin activation (Fig 1.13 B).

Fig 1.13. Potential mechanisms regulating talin mediated integrin activation (A) and deactivation (B). (A) Calpain cleavage or PtdIns(4,5)P2 binding unmasks the talin binding site, potentially activating integrins. (B) Inhibition of talin binding by Src-mediated tyrosine phosphorylation of integrin NPxY motifs, and competition with other β tail-binding proteins (e.g. PTB domain proteins), or other talin-binding proteins (e.g. PIPKIγ-90), may prevent integrin-talin interactions, so inhibiting integrin activation.
1.3.2.6.2. "Outside-in" signaling

Ligand occupancy and receptor clustering initiates “outside-in” signaling that, in turn, regulates a variety of cellular responses. Briefly, when an integrin binds to the extracellular ligand, it clusters with other bound integrins, resulting in the formation of highly organized intracellular complexes known as focal adhesions that are connected to the cytoskeleton. The focal adhesions incorporate a variety of molecules, including the cytoplasmic domains of the clustered integrins, cytoskeletal proteins, and an extensive array of signaling molecules. The high local concentrations of these molecules facilitate cascades of downstream intracellular responses via protein–protein interactions, which are linked to the cytoskeleton as well as to complex intracellular signaling networks.