

***The Role of Wnt Signalling Pathway in
Mechanotransduction Pathway in SV-40 Immortalised
Human Chondrocyte Cell Lines***

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Declaration

All the work presented in this thesis has been carried out by myself. Assistance from others and any collaborations are acknowledged at the relevant places within the text.

Farinaz Afsari, August 2005.

Abbreviations

APC	adenomatous polyposis coli
ATP	adenosine triphosphate
β -TrcP	beta-transductin repeat containing protein
cAMP	cyclic adenosine monophosphate
CamKII	calcium/calmodulin dependent protein kinase II
CDK2	cyclin dependent kinase 2
CK1	casein kinase 1
CK1 α	casein kinase 1 alpha
CK1 ϵ	casein kinase 1 epsilon
CK2	casein kinase 2
Cs	chondrotin sulfate
CIB	calcium and integrin binding protein
C γ	common gamma chain
CRD	cysteine rich extracellular ligand binding domain
DAG	1,2 diacylglycerol
DEP	dishevelled-EGL-10-pleckstrin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
Dsh	dishevelled
ECM	extracellular matrix
EGF	epidermal growth factor
eIF-2B	eukaryotic protein synthesis initiation factor-2B
ER	endoplasmic reticulum
ERK	Ras extracellular signal-regulated kinase
ERK2	Ras extracellular signal-regulated kinase2
FAC	focal adhesion complex
FAK	focal adhesion kinase
FN	fibronectin
Fz	frizzled
Fzrp	frizzled related protein
GAGs	glycosaminoglycans

GAP	GTPase activity protein
GBP	glycogen synthase kinase binding protein
GDP	guanosine diphosphate
GEF	GDP/GTP exchange factor
GSK3	glycogen synthase kinase
GSK3 α	glycogen synthase kinase alpha
GSK3 β	glycogen synthase kinase beta
GTP	guanosine 5'-triphosphate
GS	glycogen synthase
HAC	human articular chondrocyte
IGF-1	insulin growth factor-1
IL-1 β	interleukin-1 beta
IL-4	interleukin-4
IL-4R α	interleukin-4 receptor alpha chain
IL-6	interleukin-6
ILK	Integrin linked kinase
IP3	inositol triphosphate
JAK	Janus kinase
JNK	c-jun N terminal kinase
Ks	keratan sulphate
LDL	low density lipoprotein
LEF	lymphoid enhancing factor
LRP5	LDL receptor related protein 5
LRP6	LDL receptor related protein 6
MAPs	microtubule associated proteins
MAPK	mitogen activated protein kinase
MAPKAPK1	MAPK activated protein kinase1
MMP	matrix metalloproteinase
MMP1	matrix metalloproteinase 1
MMP3	matrix metalloproteinase 3
MMP7	matrix metalloproteinase 7
MMP26	matrix metalloproteinase 26
mSOS	Ras guanosine 5'-triphosphate exchange factor

NF- κ B	nuclear factor kappa beta
OA	osteoarthritis
PCP	planar cell polarity
PDGF	platelet derived growth factor
PI	phosphatidylinositol
PI(3)P	phosphatidylinositol 3-phosphate
PI(4)P	phosphatidylinositol 4-phosphate
PI(3,4)P ₂	phosphatidylinositol 3,4-bisphosphate
PI(4,5)P ₂	phosphatidylinositol 4,5-bisphosphate
PI(3,4,5)P ₂	phosphatidylinositol 3,4,5-triphosphate
PI3K	phosphatidylinositol 3-kinase
PIP 5K	phosphatidylinositol-4 phosphate 5-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PP2A	protein phosphatase 2A
RA	rheumatoid artheritis
RGD	arginine, glycine, asparagines oligopeptide
RGS	regulator of G protein signalling
RT-PCR	reverse transcription polymerase chain reaction
SACs	stretch activated channels
SAPK	stress activated protein kinase
SK	Ca ²⁺ - dependent K ⁺ channels
SH2	Src homology domain
STAT	signal transducer and activator of transcription
SV40-TAg	simian virus 40 large T antigen
TAK1	transforming growth factor beta (TGF β) activated kinase
TCF	T Cell factor
TIMP	tissue inhibitors of mettaloproteinase
TIMP1	tissue inhibitors of mettaloproteinase 1
VEGF	vascular endothelial growth factor
VE cadherin	vascular endothelial cadherin

Abstract

The mechanotransduction pathway in chondrocytes is facilitated by a $\alpha 5\beta 1$ mechanoreceptor at the cell surface and involves tyrosine phosphorylation of paxillin, focal adhesion kinase and β catenin. The availability of β -catenin at the cell membrane, in the cytoplasm and in the nucleus plays a key part in the process of mesenchymal condensation during chondrogenesis, which is regulated by Wnt signalling and interaction with other signalling pathways. GSK3 β (glycogen synthase kinase 3 β), is a key mediator in the Wnt pathway and is involved in regulating β catenin cytoplasmic and nuclear distribution. Wnt binds to its receptor Frizzled (Fz) and subsequent canonical signalling leads to inhibition of GSK3 β , and cytoplasmic accumulation of β catenin. This, in turn, promotes β catenin binding to LEF/TCF transcription factors and induction of target gene expression. In the absence of a Wnt signal, GSK3 β , as a part of an axin and APC (adenomatous polyposis coli) complex, phosphorylates β catenin and induces its degradation via the ubiquitin/ proteasome pathway.

This thesis has set out to investigate whether Wnt pathway components are expressed in human chondrocyte cell lines and to explore whether the Wnt pathway plays any role in mechanotransduction pathway in chondrocytes.

Using RT-PCR, cloning, immunofluorescence and confocal microscopy it was for the first time demonstrated that the Wnt signalling components, Wnt-1, Fz-2, Fzrp and β catenin were expressed in human chondrocyte cell lines. Using confocal microscopy,

fibronectin and CD44 were identified in association with chondrocyte Wnt-Fz complexes, suggesting that they may be coreceptors necessary for transducing Wnt signals intracellularly. A kinase assay demonstrated that GSK3 β activity is increased following 40 minute of mechanical stimulation and that a phosphoinositol-3 OH-kinase (PI3K) inhibitor decreased the activity of this kinase. A Wnt agonist, Lithium, on the other hand, increased the GSK3 β activity following 20 and 60 minute of mechanical stimulation. Western blotting suggested that in this study the formation of GSK3 β / β catenin complexes were induced in the presence of Lithium and mechanical stimulation delayed this process. However, this evidence of the complex formation of GSK3 β with β catenin in Western blots was not supported by preliminary analysis of densitometric data and further investigation is required to confirm these findings.

The results indicate that Wnt signalling components are expressed in chondrocyte cell lines and may be involved in mechanical signalling in these cells. However, the induction of GSK3 β activity following mechanical stimulation was mediated by a PI3K dependent pathway rather than a Wnt pathway. This, in turn, may influence the stabilisation of GSK3 β / β catenin complex following recruitment of activated protein kinase B (PKB) by PI3K and phosphorylation of GSK3 β . These, in turn, control the cytoplasmic/nuclear distribution of β -catenin which affects the regulation of downstream target genes such as CD44, fibronectin and some metalloproteinases. CD44 and fibronectin are essential components of cartilage, which are involved in matrix assembly and the maintenance of cartilage integrity.

Chapter 1

Introduction

1.1 Structure, Composition and Function of normal human Articular Cartilage

Articular cartilage, also known as hyaline cartilage, is a smooth, shimmering dense white tissue that covers the surface of all diarthrodial joints, providing a suitable covering material for the articular ends of bones in synovial joints of the human body. Articular cartilage allows bones to move smoothly against one another, has an extremely low coefficient of friction, and is capable of bearing very large compressive loads and deformation during locomotion and exercise. The distinct characteristics of the extracellular matrix (ECM) of articular cartilage lead to protection of the underlying bone against shearing and compressive forces (Thibault *et al.*, 2002; Schumacher and Mankin., 1985) (**Fig 1.1**).

Osteoarthritis (OA) is an age related joint disorder with chronic pain. In this disease, articular cartilage is degenerated (Hattori *et al.*, 2004; Lorenz *et al.*, 2004).

Articular cartilage is composed predominantly of a hydrated extracellular matrix synthesized by the cartilage cell - the chondrocyte. The major matrix macromolecules are collagens and proteoglycans. Conversely, in osteoarthritic cartilage, structure and function of normal cartilage is disrupted and the degenerated cartilage is characterised by two phases: i) a biosynthetic phase, in which the chondrocyte cells within the cartilage seek to reconstruct the destroyed extracellular matrix. ii) a degradative phase, in which the enzymes released by chondrocytes break down the matrix. Matrix

synthesis is demolished and this, in turn, results in further increase in cartilage damage (Sandell *et al.*, 2001)

A closer insight of normal articular cartilage reveals that its components do not have a uniform distribution and arrangement, thus, they can be divided into four different zones from the surface inwards: I) a superficial (tangential) zone, II) a middle (transitional) zone, III) a radial (deep) zone and IV) a calcified zone (**Fig 1.2**).

The superficial zone is characterised by flattened chondrocytes and relatively low levels of proteoglycans. The collagen fibrils are arranged parallel to the articular surface. In the middle zone, the chondrocytes are rounded, the proteoglycans are at the highest concentration compared with the other zones, and the collagen fibrils are randomly arranged. The deep zone is characterised by collagen fibrils that are arranged perpendicularly to the underlying bone and the columns of chondrocytes are arranged along the axis of fibril orientation. The calcified zone is partly mineralised and acts as a transition between cartilage and the underlying subchondral bone (Thonar *et al.*, 1999; Poole *et al.*, 2001). The tidemark, which is situated above the subchondral bone, is a zone with projections that interdigitate with the bone. This, in turn, results in a tight anchorage of the calcified cartilage to the underlying bone.

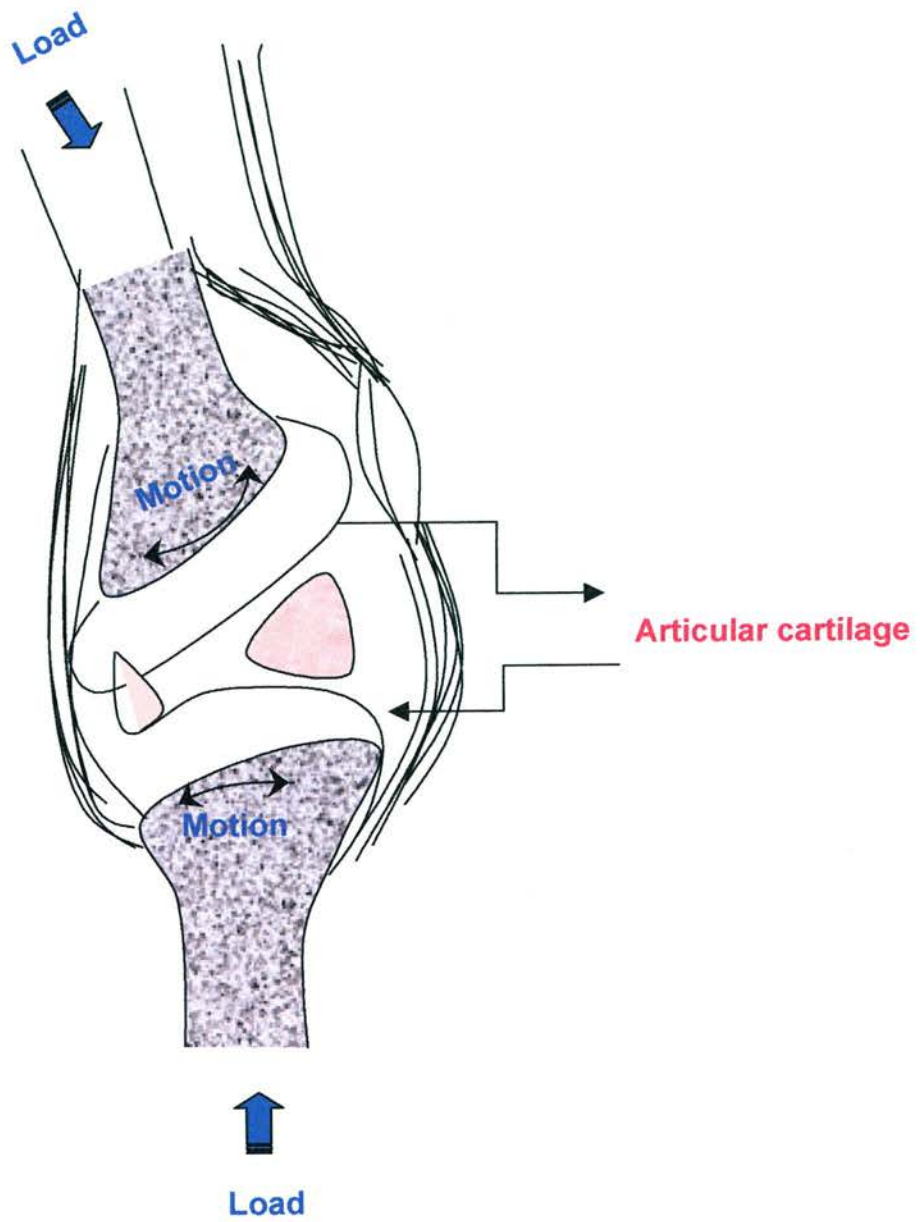
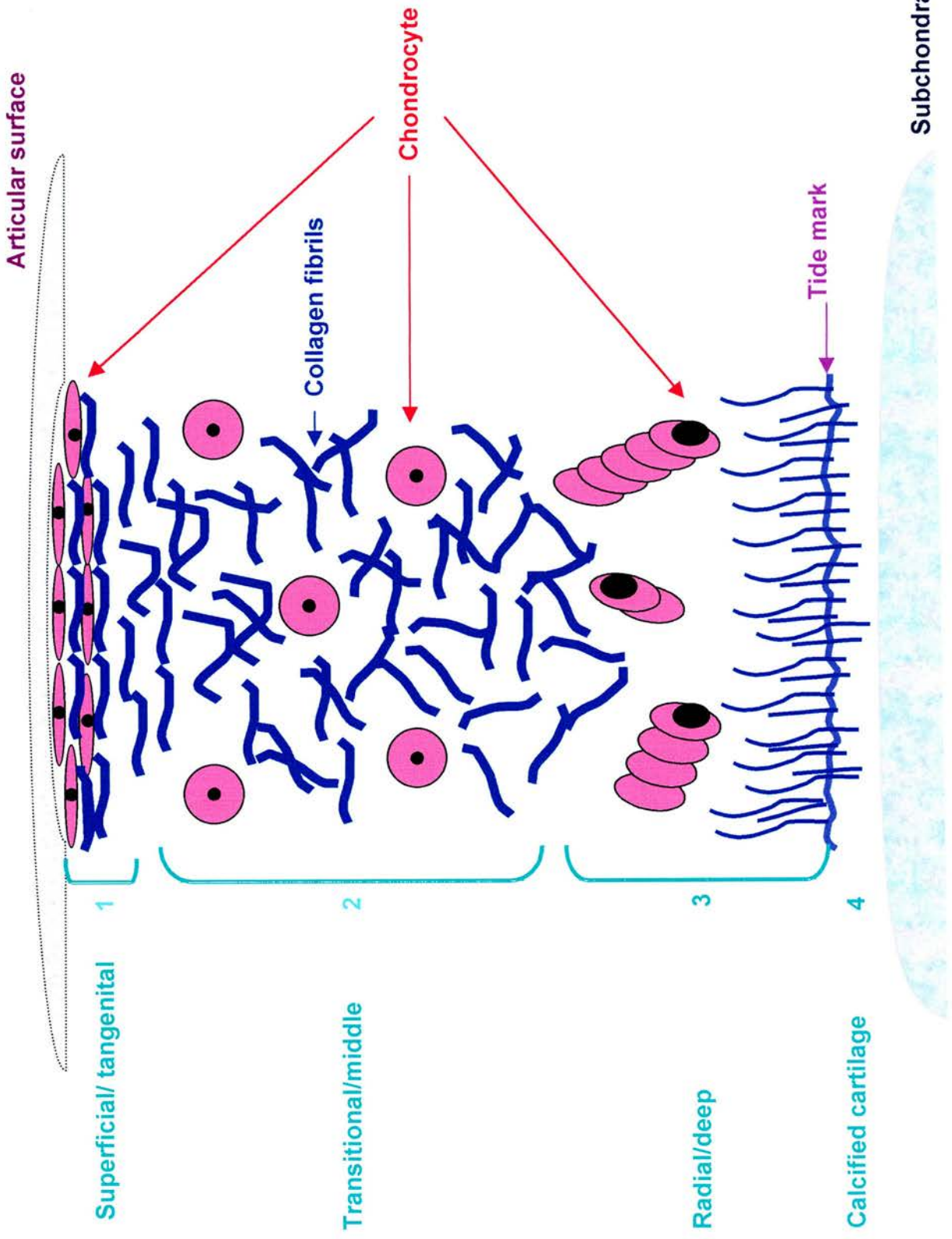


Fig 1.1: Macroscopic structure of articular cartilage within joints

Fig 1.2: Cellular and fibrillar network arrangement of normal articular cartilage in four distinct zones.



1.1.1 Cellular Components

The only cellular elements in mature articular cartilage are chondrocytes. These are able to maintain the phenotypic characteristic of cartilage under normal circumstances in spite of having no direct access to nutritional sources. This is due to cartilage being an avascular and aneural tissue. Chondrocytes are known to be metabolically very active, although their metabolism is predominantly anaerobic (Shapiro *et al.*, 1991)

Chondrocytes reveal remarkable changes in cellular density and shape across the different zones. Cell density is highest in zone I and gradually decreases through zone II to IV.

The chondrocytes in the superficial layer are disc shaped and form a layer several cells thick very close to the articular surface beneath a thin layer of matrix. As one proceeds towards the deeper layer of this zone, the chondrocytes gradually adopt a less flattened shape. In zone II the chondrocytes are more rounded, arranged in small groups and distributed randomly in the matrix. In zone III, the chondrocytes are ellipsoid and arranged in columns of 2-6 cells perpendicular to the articular surface. In the calcified zone IV, chondrocytes are lightly distributed and are surrounded by non calcified matrix (Poole *et al.*, 2001, Thonar *et al.*, 1999) (**Fig 1.2**).

In osteoarthritic cartilage, cells respond as disease progresses by proliferation, apoptosis, alteration in macromolecule synthesis and degradation, changes in chondrocyte phenotype and development of osteophytes (Sandell *et al.*, 2001)

The proliferation of chondrocytes in OA might be caused by damage to the cartilage extracellular matrix, mainly collagen network, and exposure of chondrocytes to proliferative factors in the synovial fluid. This, in turn, leads to chondrocyte clustering, which is one of the main characteristics of OA cartilage (Sandell *et al.*, 2001). Cell-cell adhesion molecules such as cadherins and β catenin and transmembrane proteins, namely, integrins may play some role in the clustering of cells in osteoarthritic cartilage.

1.1.2 Extracellular matrix (ECM) composition

The cells and matrix are structurally separate but functionally interdependent. Chondrocyte activity is necessary for the synthesis of matrix and also for its physiological responses. In turn the matrix plays an important part in maintaining the homeostasis of the cells environment. In mature articular cartilage, chondrocytes lack cell-cell contact and the ECM is responsible for mediating cell to cell communications. In addition, as the cartilage is an avascular tissue, nutrition and waste removal are achieved via diffusion through the ECM.

The composition of the ECM in adult cartilage differs in each compartment. The ECM immediately adjacent to the chondrocyte membrane, the pericellular or lamina matrix, is composed of a high quantity of large proteoglycan aggregates, which are bound to the cell surface by interacting with hyaluronate (CD44) receptors. This pericellular matrix lacks the presence of organised fibrillar collagens but type VI collagen (Keene *et al.*, 1997), decorin (Poole *et al.*, 1986) and aggrecan (Poole *et al.* 1980) are present. The territorial, or capsular, matrix which surrounds the pericellular

matrix is composed of a network of cross-linked fibrillar collagen that encloses the chondrocyte or a group of chondrocytes (the chondron). Each chondron is composed of a chondrocyte or group of chondrocytes with its/their lacunar space and a perilacunar rim. These provide mechanical support for the tissue in addition to forming the primary functional and metabolic unit responsible for cartilage matrix homeostasis. The main compartment of the ECM, furthest from the cell membrane, is the interterritorial matrix, in which the majority of collagen fibrils and proteoglycans, mainly aggrecan, are present. It has been suggested that chondrocytes actively control the metabolism of the pericellular and territorial matrix compartments but not that of the interterritorial matrix which is thought to be metabolically “inert” (Poole *et al.*, 2001). The concentration of aggrecan degradation products is highest in the interterritorial region, probably as a result of a relative deficiency in proteolysis and the preservation of degraded products that maintain their binding to hyaluronic acid (Poole *et al.*, 2001).

Collagen types II, VI, IX, XI and aggrecan are the predominant type of collagens and proteoglycans expressed in articular cartilage (Goldring *et al.*, 1994; Chen *et al.*, 2003; Caterson *et al.*, 2000; Vynios *et al.*, 2001). Type II collagen forms a network of fibrils which resists the swelling pressure generated by proteoglycans. There is a small proteoglycan, decorin which binds the collagen fibrils during their formation and reduces the final diameter of the formed collagen fibril (Pringle and Dodd., 1990). Aggrecan, with a propensity for noncovalent interaction with hyaluronic acid, forms huge aggregates that become trapped in the collagen network. The aggrecan monomer is rich in hydrophilic sulphated glycosaminoglycans (GAGs), chondroitin sulphate (Cs) and keratan sulphate (Ks) attached to a core protein. These molecules attract

water molecules and generate a distended, hydrated tissue that resists compression (Li, LP *et al.*, 2000; Thibault., 2002; Culav *et al.*, 1999). Hyaluronic acid also binds to chondrocytes through the cell surface receptor CD44, which can play a crucial role in regulating chondrocyte metabolism and ECM turnover (Chow *et al.*, 1998). There are other cell surface receptors such as annexin or anchorin CII which are involved in binding chondrocytes to type II collagen (Fernandez *et al.*, 1990; Turnay *et al.*, 1995) and receptors such as integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ can also bind type II collagen (Camper *et al.*, 1997; Salter *et al.*; 2001). Other matrix molecules expressed in cartilage include Fibrilin-1, which is responsible for the formation of the banded fibrils and microfibrillar network in pericellular sites (Keene *et al.*, 1997). There are number of proteins, matrilin-1, fibronectin and tenascin, which are absent in healthy adult cartilage but are expressed during cartilage development (Poole *et al.*, 2001). The presence of these proteins in rejuvenating cartilage is a sign of immaturity, deterioration or both in new cartilage. In addition, the proteoglycan chondroadherin binds $\alpha_2\beta_1$ integrin and induces adhesion at the chondrocyte cell surface (Camper *et al.*, 1997). Another heparin sulphate rich proteoglycan, perlecan, is essential for matrix organisation via binding to type VI collagen (Costell *et al.*, 1999; Tillet *et al.*, 1994). In addition, GLA protein prevents matrix calcification in cartilage and other tissues (Loeser *et al.*, 1992).

The collagen fibrils present in the territorial and interterritorial matrix form an extensive network throughout these regions. These fibrils differ in diameter and collagen type II forms the major part, around 90%, of the fibril. Another collagen present in the fibril is the Type IX collagen which is mainly distributed in the pericellular region of the adult cartilage and comprises around 2% of the total

collagen (Poole et al., 2001). Type XI collagen is located within and on the surface of the fibril where it influences the formation of the fibril in a way that limits the lateral expansion (Blaschke *et al.*, 2000; Mandler *et al.*, 1989). In the pericellular region there is a highly branched filamentous network of type VI collagen which binds decorin and is associated with hyaluronic acid (Bidanset *et al.*, 1992; Kielty *et al.*, 1992; McDevitt *et al.*, 1991).

In osteoarthritic cartilage, the normal process of extracellular matrix synthesis and degradation is impaired (Reginato *et al.*, 2002). Despite the initial effort of chondrocytes to reconstruct the degraded matrix, proteoglycan content of the osteoarthritic cartilage undergoes significant losses (Sandell *et al.*, 2001; Brandt *et al.*, 2003). This might be due to the high activity of 'aggrecanases', members of the ADAM (a disintegrin and metalloproteinase) family of enzymes.

Moreover, metalloproteinases (MMPs), the main matrix proteinases play some role in degrading the collagen network. In addition, the activity of MMPs is further increased as the cells are stimulated by inflammatory cytokines (Sandell *et al.*, 2001; Brandt *et al.*, 2003).

In this study, the regulatory mechanisms, responsible for expression of the cell-matrix proteins are investigated. These mechanisms are essential for maintenance of normal cartilage integrity and may be altered in osteoarthritic cartilage.

1.2 Articular cartilage and mechanical forces

The development and maintenance of articular cartilage is promoted when mechanical forces are applied within a physiological range. Formation of the articular cartilage as a load bearing surface also facilitates the resistance of this tissue to repetitive mechanical stimulation. The complex network of the cartilage extracellular matrix is responsible for the resistance of the tissue to mechanical loads. The entanglement of the extracellular matrix components resembles a 'string and balloon' model. In this model, the balloons are partly filled with water, which are represented by the proteoglycans and the strings are collagen fibers. A swelling pressure is generated as proteoglycans attract water. However, collagens oppose this pressure and a balance is reached, in which the network is not disrupted. Aggrecans contain hydrophilic GAGs, chondroitin sulphate and keratan sulphate, which are responsible for attracting water molecules and this largely regulates swelling pressure

Chondrocytes of articular cartilage are particularly sensitive to the mechanical forces and respond to them as regulatory signals. In normal joints, in the load bearing regions, the concentration of proteoglycans is more than in the non load bearing region (Urban., 1994). In addition, the chondrocytes in the load bearing region are larger and have intracellular organelles with greater volume (Eggl *et al.*, 1988).

By contrast, exposure of cartilage to abnormal loads that are outside the physiological range causes an unfavourable response in articular cartilage that leads to cartilage damage and the progression of osteoarthritis (OA). Changes in matrix composition and chondrocyte metabolism occur as the processes of degradation and repair

proceed. (Mow *et al.*, 1992; Quinn *et al.*, 1998; Millward-Sadler *et al.*, 1999; Salter and Lee., 2003; Waldman *et al.*, 2003; Demarteau *et al.*, 2003). A loss in cartilage proteoglycan concentration occurs following joint immobilisation and once the joint is remobilised the proteoglycan concentration is again gradually increased (Urban., 1994). Proteoglycan loss is related to the absence of the load (Urban., 1994). However, overloading of cartilage also leads to changes in its cellular activity and cartilage break down (Urban., 1994). Mechanical load has to be applied repeatedly in order to maintain the cartilage matrix composition and integrity.

The responses of cartilage to applied stress or strain, are known as its mechanical behaviour. Stress is measured as application of load to a defined volume or area of material; while strain is measured as changes in the volume or length of the material following exposure to finite loads. Various model systems, using cartilage from different species and joints, have been used to study the effect of mechanical forces *in vivo* and *in vitro*. Mechanical loading of articular cartilage results in the generation of compressive and shear stresses, each of which can lead to distinct biochemical outcomes. Compressive forces can be either static or dynamic (Salter and Lee 2003; Lahiji *et al.*, 2002). Cartilage exposure to dynamic loads tend to result in changes in chondrocyte extracellular matrix environment caused by an increase in hydrostatic pressure, fluid flow and matrix distortion. Dynamic force applied to joints eventually results in an increase in matrix synthesis; however, the amplitude of these stimuli determines their final effect on matrix protein synthesis (Urban *et al.*, 2000). Conversely, cartilage exposure to the static loads results in fluid expression, a rise in osmolarity and fall in pH. These changes in the extracellular matrix lead to a decrease in matrix production (Urban *et al.*, 2000). One of the major effects of mechanical load

is changes in intracellular concentrations of Na^+ , Ca^{2+} and H^+ . The major pathway, which is involved in regulation of ion transport across the membrane, is responsible for controlling cellular volume via regulating the intracellular $[\text{Na}^+]$, $[\text{Ca}^{2+}]$ and the level of pH (Wilkins *et al.*, 2000). Static loading causes fluid expression and concentrates cations in the extracellular matrix, increasing the osmolarity. Changes in extracellular composition affects the intracellular contents of $[\text{Na}^+]$ and $[\text{K}^+]$, increasing their concentration. This is due to reduction in the cell volume and opening of ion transporters (Wilkins *et al.*, 2000).

In the event of hyperosmolarity the $\text{Na}^+ / \text{K}^+ / 2\text{Cl}^-$ cotransporter is stimulated, and carries the water molecule into the cell to restore its volume. Furthermore, changes in osmolarity also have effects on the chondrocyte pH regulator $\text{Na}^+ \times \text{H}^+$ transporter. In addition, hyperosmolarity increases the activity of Ca^{2+} ATPases, which causes a decrease in intracellular Ca^{2+} concentration (Wilkins *et al.*, 2000).

Loading of the cartilage causes an increase in hydrostatic pressure, which results in fluid flow. In the event of extended loading, the fluid loss can be significant.

Eventhough, the rate at which the flow occurs is not considerably high (Hall *et al.*, 1991).

Hydrostatic pressure has also effects on membrane transporters and the intracellular and extracellular concentrations of Na^+ , K^+ , 2Cl^- and Ca^{2+} . The exposure of the cells to pressure causes an increase in Na^+ concentration as a result of the either inhibition of Na^+ / K^+ ATPase or activation of the $\text{Na}^+ / \text{K}^+ / 2\text{Cl}^-$ cotransporter. In addition, the $\text{Na}^+ \times \text{H}^+$ transporter is also activated and plays a role in regulating the load related intracellular pH (Wilkins *et al.*, 2000).

In addition to recognition of mechanical stimuli by the chondrocyte's mechanosensitive ion channels, signal transduction is also mediated by integrin transmembrane mechanoreceptors. (Wright et al., 1996, 1997). *In vitro* studies of human articular chondrocytes in culture have revealed that a variety of physiological and biochemical responses to mechanical stimuli are mediated by the integrin family of cell-matrix adhesion molecules.

1.3 Integrins and their associated signalling pathway

Anchorage of cells to ECM components or cell surface proteins is mediated by integrins. These proteins link the ECM to the intracellular cytoskeletal network and to multiple intracellular signalling pathways, the activation of which is important for cell survival, growth and differentiation (Hynes., 1999; van der Flier and Sonnenberg., 2001).

1.3.1 The integrin family of cell surface receptors

Integrins comprise a large family of glycosylated, heterodimeric transmembrane receptors. They contain a non-covalently bound α and β subunit, with each $\alpha\beta$ combination evoking individual specificity in binding and signalling properties (van der Flier and Sonnenberg., 2001). Integrins are evolutionarily conserved proteins found in many species ranging from sponges to humans. The formation of specific receptors from various α and β subunits combinations, results in a complex family of integrins. Each receptor can also have more than one function (Burke., 1999; Hynes

and Zhao., 2000). To date, 24 α and 9 β subunits have been identified in the human genome (Venter et al., 2001).

Integrins containing α_4 -, α_5 -, α_8 -, α_{11b} -, or α_V subunits bind to components of the ECM, which contain an RGD (Arg-Gly-Asp) peptide sequence, such as fibronectin and vitronectin. Other ECM components, such as laminins, contain inaccessible RGD sequences and are recognised by integrins consisting of α_3 -, α_6 -, or α_7 subunits (laminin-binding receptors). Collagen, which also contains inaccessible RGD sequences, is recognised by integrins containing α_1 -, α_2 -, α_{10} -, or α_{11} subunits (collagen-binding receptors).

$\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ are the main integrins expressed in chondrocytes (Salter *et al.*, 1992). However, as chondrocytes mature the expression of $\alpha_V\beta_5$ and $\alpha_V\beta_3$ have also been identified (Woods *et al.*, 1994, Loeser *et al.*, 2002). In cultured human and bovine chondrocytes, integrins have the same pattern of expression as that found in vivo. There is an increase in expression of β_1 integrins in OA cartilage in comparison with the normal cartilage (Loeser *et al.*, 1995). A higher level of $\alpha_2\beta_1$ and $\alpha_6\beta_1$ integrins are expressed in foetal chondrocytes and in chondrosarcoma cells compared to normal adult chondrocytes (Holmvall *et al.*, 1995; Salter *et al.*, 1995., Loeser *et al.*, 2002). The characterised receptors for type II collagen in chondrocytes are $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{10}\beta_1$ integrins (Durr *et al.*, 1993; Enomoto *et al.*, 1993; Loeser *et al.*, 1995, 2000; Camper *et al.*, 1997). In addition, $\alpha_1\beta_1$ binds type VI collagen and the cartilage matrix protein, matrilin1 (Makihira *et al.*, 1999; Loeser *et al.*, 2000). The primary chondrocyte receptor for fibronectin (FN) is the $\alpha_5\beta_1$ integrin (Loeser *et al.*, 1995).

The non-covalently associated α and β subunits consist of a large extracellular region (the ligand binding domain), a short transmembrane region and a cytoplasmic domain of varying length. Either one or both of the integrin subunits can directly interact with a number of proximal intracellular signalling components such as cytoskeletal proteins [talins, fibronectin, α -actinin], adaptor molecules [paxillin, RACK1, p130^{cas}], Ca^{2+} binding proteins [calcium and integrin binding protein (CIB), and calreticulin] and protein kinases [FAK and integrin linked kinase (ILK)] (Giancotti and Ruoslahti., 1999). Such interactions initiate multiple pathways that signal in the traditional “outside-in” signalling processes. Although once considered static adhesion molecules, integrins are quite dynamic in nature. When bound by a ligand, they may form signalling connections with intracellular components or may remain detached from the cytoskeleton and signalling pathways. Even in the absence of intracellular signalling, integrin ligation can promote the assembly and organisation of the ECM. This process appears to be controlled by factors within the cell and is referred to as “inside-out” signalling. For example the inside out signalling of $\alpha_{\text{IIb}}\beta_3$ integrin in platelets requires the activity of PKC and the Rho family of GTPases (van der Flier and Sonnenberg., 2001).

1.3.2 Integrin signalling

Binding of integrins to the ECM results in their clustering at the cell membrane and their association with cytoskeleton proteins and signalling complexes and promotes the assembly of actin filaments (**Fig 1.3**). The reorganisation of actin filaments into larger stress fibers results in more integrin aggregation, so enhancing matrix binding

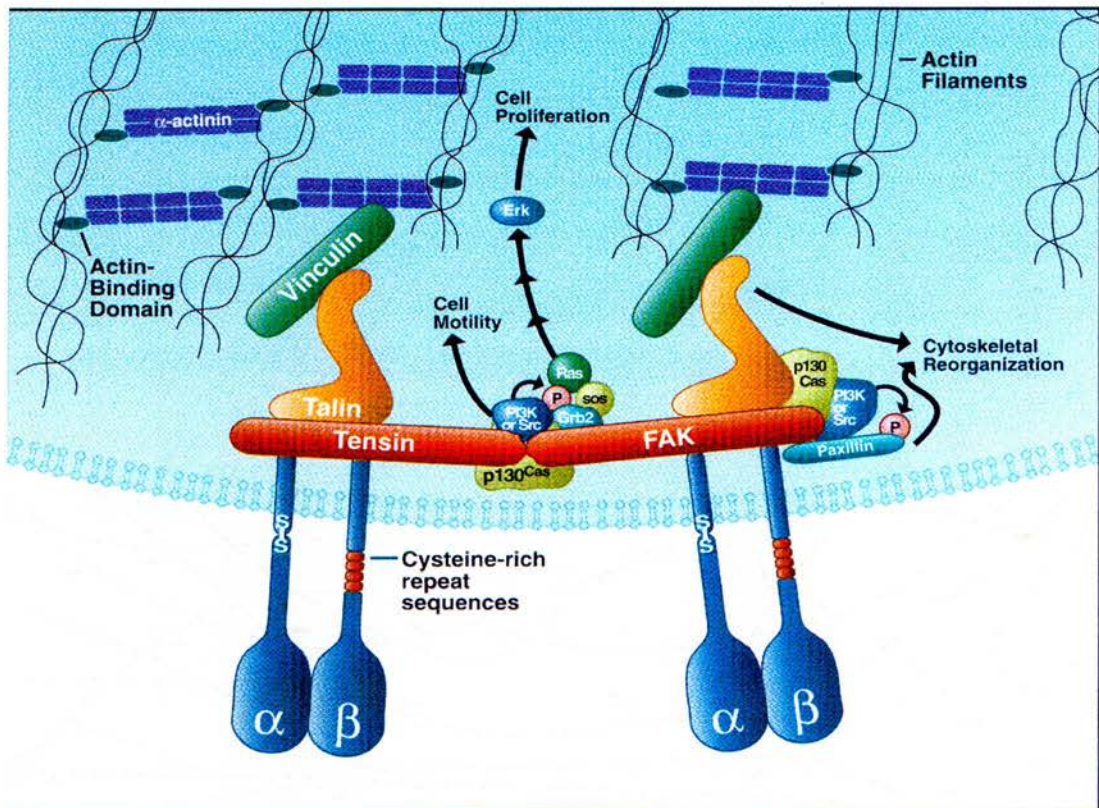


Fig 1.3: Integrins and their association with cytoskeleton proteins and signalling complexes. The noncovalently associated α and β subunits of integrins directly interact with a number of proximal intracellular signalling components such as cytoskeletal proteins (talin, fibronectin, α actinin), adaptor molecules (paxillin, RACK1, p130^{cas}), Calcium binding proteins (CIB and calreticulin), and protein kinases (FAK and ILK). Such interactions initiate multiple pathways. (adopted from <http://www.translab.com>)

and organisation via a positive feedback system and inside-out signalling. Various protein tyrosine kinases such as focal adhesion kinase (FAK) and the Src family of kinases are activated by integrins. The activation of FAK together with integrin clustering and recruitment of other signalling components, is coupled in the assembly of the focal adhesion complexes (FAC). The recruitment of FAK to the focal adhesion site occurs either by its direct interaction with an integrin β subunit or indirectly through an actin binding protein, talin, and a multi-domain protein, paxillin (Chen *et al.*, 1995; Giancotti and Ruoslahti., 1999). Activation of FAK leads to its autophosphorylation at Tyr 397 which creates a binding site for the Src homology (SH2) domain of Src or Fyn kinases (Schaller *et al.*, 1994; Schlaepfer *et al.*, 1994; Giancotti and Ruoslahti., 1999). A number of focal adhesion components are phosphorylated by Src kinase including paxillin, tensin and a docking protein p130^{Cas}, which recruits the adaptor proteins Crk and Nck (Fig 1.3) (Richardson and Parsons., 1996; Schlaepfer *et al.*, 1997).

FAK also binds to, and possibly activates, PI3K, either directly or via Src kinase (Chen *et al.*, 1996). Src phosphorylates FAK at Tyr 925, which creates a binding site for the adaptor proteins Grb2 and Ras guanosine 5' - triphosphate exchange factor mSOS (Schlaepfer *et al.*, 1994). Through these interactions FAK activates the mitogen activated protein kinase (MAPK) signalling pathway (Giancotti and Ruoslahti., 1999). Some β_1 and α_v integrins activate not only FAK but also the tyrosine kinase Fyn and the adaptor protein Shc (Wary *et al.*, 1996, 1998). This, in turn, leads to coupling of the α subunits to Fyn through a membrane adaptor protein caveolin-1. Binding of integrins to the ECM results in Fyn activation and its interaction with Shc through its SH3 domain. This, in turn, phosphorylates Shc at Tyr 317 and combines with the Grb2-mSOS complex (Wary *et al.*, 1996, 1998). The Ras

extracellular signal-regulated kinase (ERK) MAPK signalling pathway is activated after binding of integrins to the ECM, and this is facilitated by both FAK and Shc. In a number of cell types, Shc initially activates ERK to a high level followed by delayed activation of FAK, which sustains ERK activation, expression of cyclin D1 and cell cycle progression (Wary *et al.*, 1996, 1998; Schlaepfer *et al.*, 1997; Lin TH *et al.*, 1997; Pozzi *et al.*, 1998). Those integrins that do not contribute to the activation of Shc can partially activate the ERK pathway and promote cell proliferation (Wary *et al.*, 1996, 1998; Mainiero *et al.*, 1997; Pozzi *et al.*, 1998). Tyrosine phosphatases are both positive and negative regulators of the FAK and Shc pathways. Lack of the receptor-type protein tyrosine phosphatase α or the cytosolic phosphatase SHP-2 inhibits the integrin mediated ERK pathway (Oh *et al.*, 1999). The phosphatases affect both the FAK and Shc pathways and amplify them by dephosphorylating the negative regulatory site in Src-family of kinases (Giancotti and Ruoslahti., 1999). Two other protein tyrosine phosphatases, PTP-PEST and PTP-1B, inhibit FAK mediated signalling by dephosphorylating p130^{cas} (Liu *et al.*, 1998). PTEN, another cytoplasmic phosphatase, dephosphorylates the inositol lipids produced by PI3K i.e. PI(3,4,5)P₃ to PI(4,5)P₂, PI(3,4)P₂ to PI(4)P and PI(3)P to PI (Li *et al.*, 1997). Conversely, overexpression of PTEN dephosphorylates FAK and Shc and inhibits the integrin signalling pathway in the cells (Gu *et al.*, 1998, 1999).

1.3.3 Interaction of integrins with growth factor receptors

The activation of some growth factor receptors, such as the receptor for insulin, platelet derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) are optimised by appropriate cell-matrix adhesion

(Vuori *et al.*, 1994, Jones *et al.*, 1997, Soldi *et al.*, 1999). The $\alpha v\beta_3$ integrin forms complexes with insulin, PDGF and VEGF receptors and $\alpha_5\beta_1$ and other β_1 integrins interact with the EGF receptor (Miyamoto *et al.*, 1996; Schneller *et al.*, 1997; Woodard *et al.*, 1998; Moro *et al.*, 1998; Soldi *et al.*, 1999). In addition to growth factor receptors, integrins assemble in complexes with potassium- ion channels (McPhee *et al.*, 1998).

1.3.4 Integrins control cell shape, growth and survival

The contact of the cells with the ECM results in extension of filopodia. Formation of focal adhesion complexes is initiated as the integrins, which are localised at the tip of filopodia, bind to the ECM. Thus, the focal adhesion complexes with their associated actin filaments become fully developed. These events are necessary for the extension of lamellipodia and cell migration and motility (Giancotti and Ruoslahti., 1999). The regulation of cell migration is achieved by the activation of the Rho-family of GTPases (Nobes *et al.*, 1999). From the Rho family of GTPases, Cdc42 and Rac are responsible for filopodia and lamellipodia induction respectively. In addition, Rho plays a role in the induction of focal adhesion complexes and associated actin filaments. This family of Rho-GTPases regulate the assembly of the actin cytoskeleton through their interactions with downstream effectors such as the Ser-Thr kinase $p65^{\text{PAK}}$ and the enzyme phosphatidylinositol-4 phosphate 5-kinase (PIP 5-kinase) (Hall, 1998). The FAK-Src complexes regulate cell migration by inducing the disassembly of focal adhesion complexes at the trailing edge of the cell (Cary *et al.*, 1998). In addition, activation of Rho and cell proliferation are facilitated by interaction with fibronectin (FN) in the ECM (Sechler and Schwarbauer., 1998).

Thus, appropriate cellular proliferation requires coordination of ECM and cytoskeletal elements, and their physical properties are essential for cell motility, cell survival and growth (Chen *et al.*, 1997). It has been suggested that integrins and integrin-associated actin filaments may be responsible for initiation of the growth stimulatory signal mediated by Rac (Giancotti and Ruoslahti., 1999); however, the mechanism of this signalling activity is unclear. The clustering of integrins is also promoted by Rac. This establishes a positive feedback loop with further activation of Rac (Souza-Schorey *et al.*, 1998). Thus, integrins play an important role in the formation and the maintenance of tissue architecture by a coordinating control of cell shape, survival and growth.

1.3.5 Integrin signalling and mechanotransduction in chondrocytes

Activation of $\alpha_5\beta_1$ in chondrocytes leads to phosphorylation of ERK1, ERK2, MAPK, JNK and P38 (Loeser., 2002). It has also been shown that insulin growth factor-I (IGF-1) signalling interacts with integrins; in IGF-1 treated chondrocytes, the β_1 integrin subunit interacts with the IGF-1 receptor (Shakibaei *et al.*, 1999). Cytokine signalling and integrin signalling also interact in chondrocytes. Activation of $\alpha_5\beta_1$ integrin in OA chondrocytes leads to an increase in IL-1 β production (Attur *et al.*, 2000) while cooperative signalling between $\alpha_5\beta_1$ integrin and IL4 is responsible for membrane hyperpolarisation in normal chondrocytes in response to mechanical stimulation (Millward-Sadler *et al.*, 1999)

From the family of the integrin glycoproteins, $\alpha_1\beta_1$ (a collagen receptor) $\alpha_5\beta_1$

(a fibronectin receptor) and $\alpha_v\beta_5$ (a vitronectin receptor) are known to be expressed by normal and osteoarthritic adult human articular chondrocytes. The expression of integrin heterodimers in osteoarthritic adult cartilage is modified, with elevated expression of other integrins such as $\alpha_2\beta_1$ integrin (Salter et al., 2002). Activation of these key integrin mechanoreceptors leads to activation of a number of intracellular signalling cascades, which are responsible for the regulation of gene expression of matrix components necessary for the maintenance of cartilage integrity (Salter *et al.*, 2001, 2002).

When adult human articular chondrocytes (HAC) are mechanically stimulated at 0.33 Hz (2 second on/1 second off), three major cellular responses occur: I) changes in membrane potential (Wright *et al.*, 1992) II) a transient increase in protein tyrosine phosphorylation (Lee HS *et al.*, 2000) III) an alteration of the relative levels of matrix and matrix metalloprotease gene expression (Millward-Sadler *et al.*, 2000). Each of these responses has been used to study chondrocyte mechanotransduction pathways in Edinburgh.

1.4 Mechanotransduction pathways

Following mechanical stimulation multiple intracellular signalling molecules and pathways become activated. These include opening of stretch activated and calcium selective ion channels (Sachs, 1988), protein tyrosine phosphorylation (Yano *et al.*, 1996), inositol lipid metabolism (Prasad *et al.*, 1993), the activation of protein kinase C (PKC) (Millward-Sadler., 1999; Kimono *et al.*, 1996), engagement of the actin cytoskeleton and tyrosine phosphorylation of the focal adhesion complex molecules

pp125 focal adhesion kinase (FAK), paxillin and β catenin (Lee *et al.*, 2000).

Activation of these signalling molecules and their related signalling pathways lead to changes in gene expression and protein synthesis, the regulation of which are essential for the maintenance of tissue structure and function. Mechanical stimulation also alters levels of intracellular Ca^{2+} and cAMP. Integrins are associated with a focal adhesion complex that connects them to the cytoskeleton which is important in the regulation of gene expression, cell proliferation and differentiation. In mechanically stimulated cardiofibroblasts, phosphorylation of ERK-2 and JNK-1 MAP kinases is integrin dependent (Mackenna *et al.*, 1998). In HAC and bone cells subjected to cyclical mechanical strain, membrane hyperpolarisation /depolarisation responses are also integrin dependent (Salter *et al.*, 1997; Wright *et al.* 1997).

Mechanical stimulation of human chondrocytes and bone cells at 0.33 Hz (2 second on/ 1 second off) for 20 min at 37°C results in membrane hyperpolarisation as small conductance Ca^{2+} -dependent K^{+} channels (SK) are activated. However, stimulation at 0.104 Hz (2 second on/7.6 second off) results in activation of tetrodotoxin sensitive Na^{+} channels and membrane depolarisation (Wright *et al.*, 1992). By contrast mechanical stimulation of fibroblasts at 0.33Hz causes membrane depolarisation and at 0.1 Hz there is membrane hyperpolarisation (Wright *et al.*, 1992). Following mechanical stimulation both hyperpolarisation and depolarisation responses are mediated through integrins and integrin associated signalling molecules such as actin and protein tyrosine kinases (Wright *et al.*, 1996, 1997; Salter *et al.*, 1997). Signal transduction following different regimes of mechanical stimulation may be associated with a variety of integrin mechanoreceptors (Salter *et al.*, 1997).

$\alpha 5 \beta 1$ integrin, is highly expressed in human cartilage and plays an important role as the major chondrocyte mechanoreceptor. Stimulation via $\alpha 5 \beta 1$ activates a signalling pathway involving cooperation of different protein tyrosine kinases, the actin cytoskeleton and several other components associated with it. This in turn leads to activation of PLC, IP3 and the PKC pathway (Salter *et al.*, 2001). At an early stage of the mechanotransduction pathway protein tyrosine phosphorylation is important and leads to further downstream signalling events, which regulate gene expression. Following one minute of mechanical stimulation of human articular chondrocytes (HACs) at 0.33 Hz, there is a transient increase in tyrosine phosphorylation of paxillin, β -catenin and focal adhesion kinase pp125 FAK. Tyrosine phosphorylation of these three proteins is dependent on integrins and stretch activated ion channels (SACs) (Lee *et al.*, 2000). In addition, $\alpha 5$ integrin colocalises with β -catenin and pp125FAK. This is compatible with the presence of integrin- β -catenin signalling following mechanical stimulation of the chondrocyte, and activity of focal adhesion complex proteins in the mechanotransduction pathway (Lee *et al.*, 2000). There is also evidence that in the integrin linked kinase (ILK) pathway, there is an association between β -catenin and the integrin signalling mechanism (Novak *et al.*, 1998; Salter *et al.*; 2001).

1.4.1 Interleukin-4 (IL-4) and the mechanotransduction pathway

Cytokines have been shown to be involved in signalling in mechanically stimulated cells. In human bone cells stimulated at 0.33 Hz, an interleukin-1 β (IL-1 β) loop is activated (Salter *et al.*, 2000), while interleukin-6 (IL-6) plays a role in the cardiac myocyte mechanotransduction pathway (Pan *et al.*, 1999). In normal HAC, IL4 is an

active autocrine/paracrine signalling molecule in the mechanotransduction pathway (Millward-Sadler, 1999). Studies have shown the release of IL-4 following mechanical stimulation at 0.33 Hz, with subsequent signalling events downstream after initial activation of mechanoreceptors ($\alpha 5\beta 1$). These include PLC and IP3 – mediated Ca^{2+} release from endoplasmic reticulum (ER) (Millward-Sadler, 1999).

IL-4 binds to its high affinity receptor IL-4R α which leads to its heterodimerization with a second chain and its subsequent signalling. The common gamma (γ) chain is the main second chain in many cell types and this combination is known as the type I receptor (IL-4R α/γ), in the type II receptor the IL13R α subunit is associated with IL4R α . Further studies have shown that the IL-4 type II receptor is the one involved in the mechanotransduction pathway in HAC. In this signalling loop, IL-4 binds to its heterodimer receptor (IL-4R $\alpha/$ IL-13R α), then initiates a signalling cascade including PLC and IP3 mediated Ca^{2+} release which leads to activation of calcium activated potassium channels (SK), efflux of K^+ , and membrane hyperpolarisation (**Fig 1.4**).

It has been suggested that coordination between activation of integrin and IL-4 associated signalling pathways is of major importance in regulating the structure and function of the normal and diseased articular cartilage. Regulation is affected through other biochemical responses to mechanical forces such as proteoglycan synthesis (Veldhuijzen *et al.*, 1979), and alteration in the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which are involved in the pathogenesis of OA (Dean, 1991).

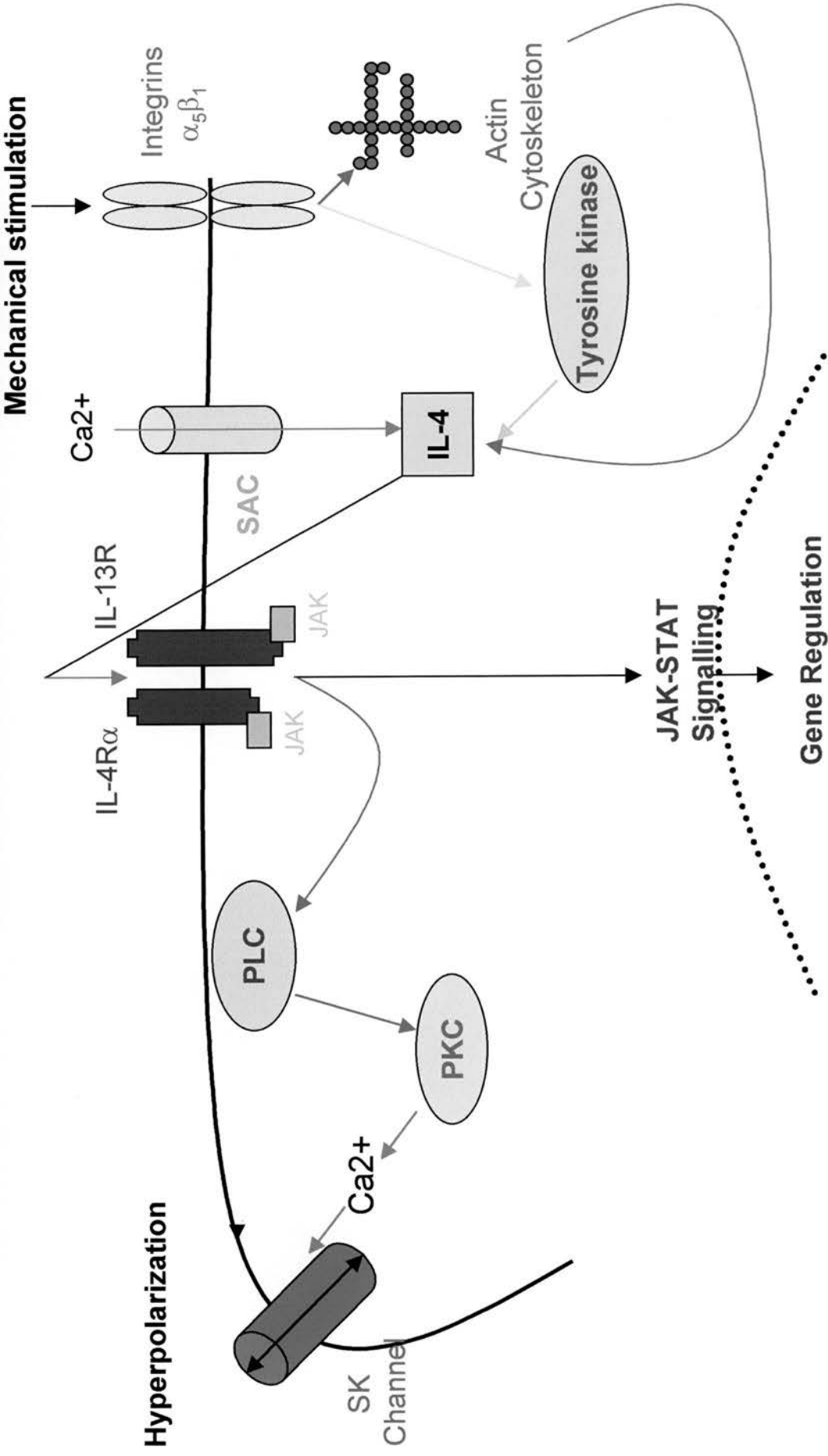


Fig 1.4 : Mechanotransduction pathway in normal human articular chondrocytes following 0.33Hz of mechanical stimulation. Tyrosine phosphorylation of protein kinases is dependent on integrins and SACs. IL-4 is released following mechanical stimulation at 0.33 Hz with subsequent activation of PLC, IP3 and PKC. This leads to activation of SK channels and membrane hyperpolarisation (Modified from Salter *et al.*, 2002).

It has been suggested that the ratio of MMPs to TIMPs is controlled by IL-4, so accounting for its chondroprotective effects (Salter et al., 2001). In addition the activity of IL-1, which stimulates the production of MMP3 and inhibits the production of proteoglycans, is suppressed by IL-4 (Shingu *et al.*, 1995; Nemoto *et al.*, 1997). In mechanically stimulated normal articular cartilage in vivo, the integrin mediated production of IL-4 is chondroprotective as it inhibits cartilage degradation and promotes matrix synthesis.

1.4.2 IL-4 and alteration of aggrecan and MMP3 gene expression in chondrocytes

In normal articular chondrocytes subjected to mechanical stimulation at 0.33Hz significant changes in aggrecan and MMP3 mRNA levels are seen within 1 hour and these return to base line levels after 24 hours. Under the same conditions there were no significant changes in tenascin, MMP1 or TIMP1 mRNA levels.

These responses were shown to involve integrins, SACs and IL-4. However, opening of SK channels which results in membrane hyperpolarisation following cyclical mechanical stimulation of chondrocytes, are not involved in the upregulation of aggrecan and MMP-3 mRNA (Millward-Sadler et al., 2000). The physiological significance of IL-4 induced membrane hyperpolarisation in normal HAC following mechanical stimulation is not yet clear; but, it may play a negative regulatory role in the control of apoptosis (Gilbert *et al.*, 1996; Wang *et al.*, 1999)

1.4.3 Mechanotransduction pathway in OA

$\alpha 5\beta 1$ integrins are the mechanoreceptors in both normal and OA chondrocytes following cyclical mechanical stimulation, and their signalling results in activation of SACs and tyrosine phosphorylation of β catenin, pp125FAK and paxillin (Lee HS *et al.*, 2000; Millward-Sadler *et al.*, 2000). However, the downstream signalling responses in chondrocytes from OA cartilage are altered. In OA chondrocytes stimulated at 0.33 Hz, tetrodotoxin sensitive Na^+ channels are activated and the cell membrane depolarises. Levels of aggrecan and MMP3 mRNA are not altered following mechanical stimulation of OA chondrocytes, suggesting a loss of chondroprotective responses (Millward-Sadler *et al.*, 2000). Moreover, the involvement of the actin cytoskeleton and PKC, which are essential for membrane hyperpolarisation in normal articular chondrocytes, are diminished in the membrane depolarisation that occurs in OA chondrocytes.

In normal and OA HAC the electrophysiological response to IL-4 differs. Furthermore, in OA HAC, IL-1 β is secreted along with IL4 (Millward-Sadler *et al.*, 2000). IL-1 β is known to regulate the expression and activity of JAK-STAT and their subsequent signalling pathways which, in turn, can modulate the IL-4 initiated intracellular signals (Salter *et al.*, 2001). An abnormal electrophysiological response to both mechanical stimulation and IL-4 application was observed in OA chondrocytes (Millward-Sadler *et al.*, 2000). Furthermore, neo-expression of molecules such as fibronectin (Chevalier *et al.*, 1996) and tenascin (Salter, 1993) in the pericellular matrix causes abnormal chondrocyte activity. Thus, abnormal responses to mechanical stimulation in OA may contribute to the progression of disease within the articular cartilage (**Fig 1.5**).

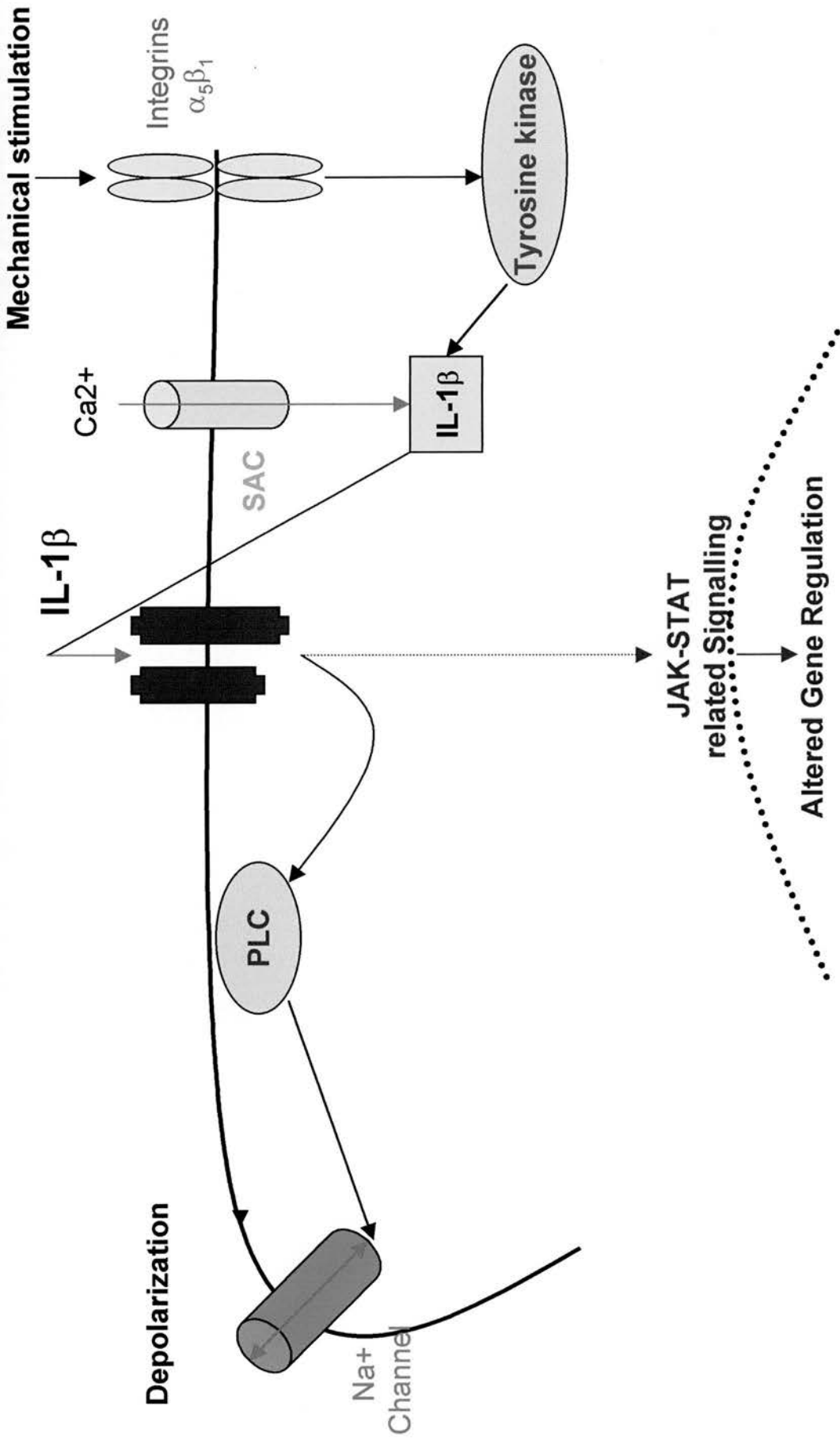


Fig 1.5 : Mechanotransduction pathway in osteoarthritic human articular chondrocytes following 0.33Hz of mechanical stimulation. Tyrosine phosphorylation of protein kinases is dependent on integrins and SACs. Mechanical stimulation of chondrocytes results in release of IL-1 β and its autocrine activity. This results in activation of tetrodotoxin sensitive Na⁺ channels and membrane depolarisation. (Modified from Salter et al., 2001).

The above studies have identified β -catenin as an integral factor in integrin mediated signalling in the mechanotransduction pathway in both normal and OA chondrocytes. However, β -catenin is also known to be involved in cadherin mediated cell-cell adhesion and in Wnt signalling pathways.

1.5 Cadherin family of cell adhesion molecules and their diversity of forms and function

Cell-cell adhesion is one of the key factors in regulating cellular and tissue morphogenesis, motility, proliferation, differentiation and survival. Cadherins are a family of glycoproteins important for stable cell-cell adhesion and tissue organisation (Kister *et al.*, 2001). The most common and ubiquitous type of intercellular adhesions essential for maintaining tissue architecture and cell polarity are the cell-cell adherens junctions (AJs). Cell-cell AJs also affect cell mobility and proliferation in different tissues. AJs are formed when the extracellular domains of classical cadherins which are expressed on the surface of neighbouring cells interact with one another. The cadherin family of proteins is divided into five subfamilies: Classical cadherins I and II, desmosomal cadherins, protocadherins and cadherin-related proteins (Koch *et al.*, 1999; Kister *et al.*, 2001). Classical cadherins, E-, N-, P- and VE- cadherins, are single span transmembrane glycoproteins, mediating Ca^{+2} dependent cell-cell adhesions through an extracellular region consisting of repeated β - barrel domains with Ca^{+2} binding sites (Angst *et al.*, 2001). E- and N- cadherins are among the best characterised cadherins, and their expression on the cell surface leads to cell sorting (Yap *et al.*, 1997b). The cytoplasmic domain of cadherins interacts with β catenin or γ catenin which in turn bind to α catenin and link the cadherin-catenin complex to the underlying actin cytoskeleton (Yap *et al.* , 1997a; Simcha *et al.*, 1998; Conacci-Sorrell *et al.* , 2002). β catenin-cadherin signalling involves the Rho family of GTPases (Angst *et al.*, 2001).

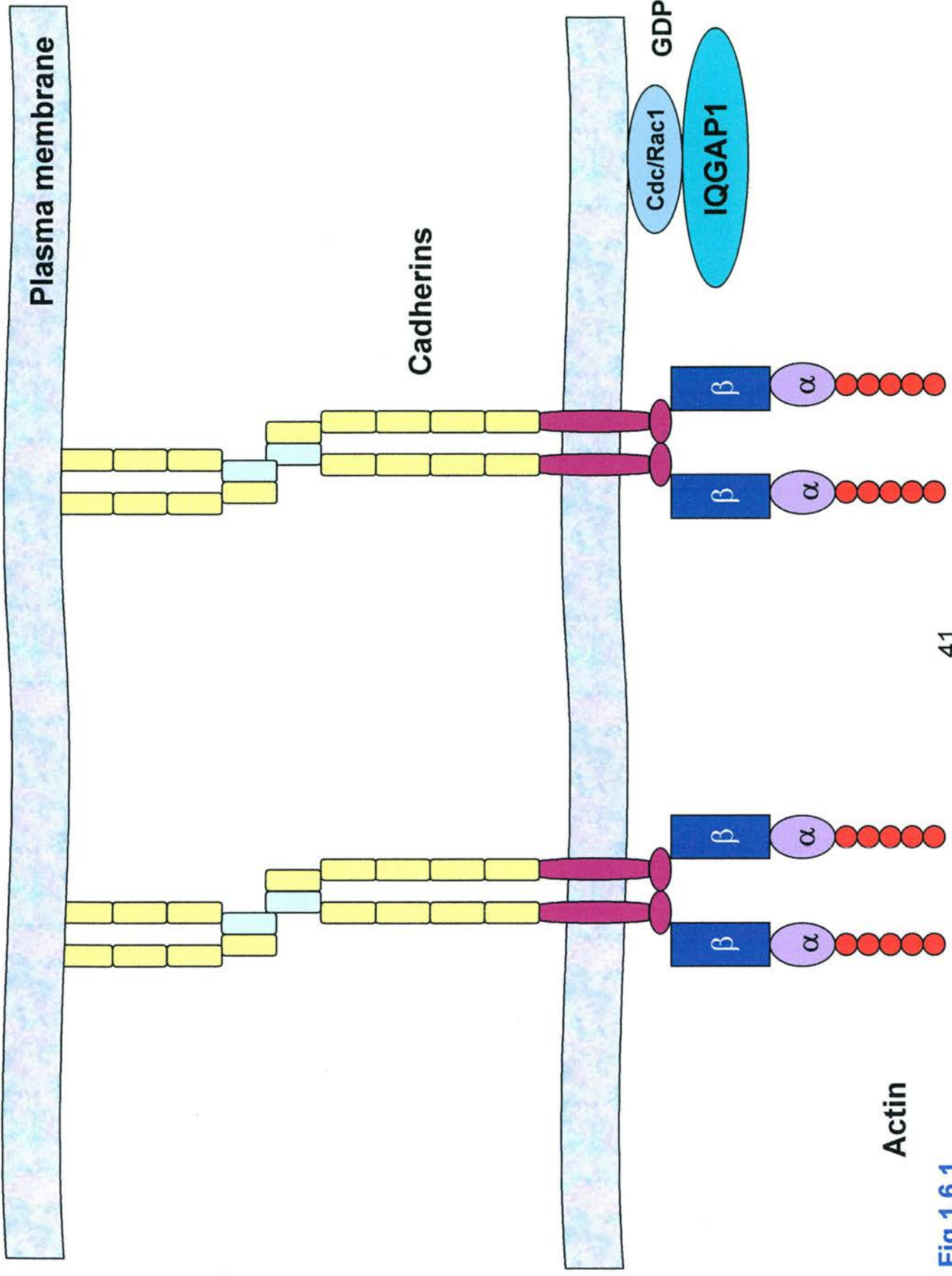
1.5.1 Rho family of GTPases and cadherin activity

The Rho family of GTPases contain GDP and GTP binding sites and exhibit GTPase activities. There are two exchangeable forms of Rho: 1) GDP bound, inactive 2) GTP bound, active. A GDP/GTP exchange factor (GEF) facilitates the conversion of the GDP bound form to the GTP bound form. This process is inhibited by a GDP dissociation inhibitor (GDI). Conversely, GTPase activity protein (GAP) converts the GTP bound form to the GDP bound form. Thus the activity of the Rho family of GTPases is regulated in a cyclical manner (**Fig 1.6**) (Bourne *et al.*, 1991; Isomura *et al.*, 1991; Takai *et al.*, 1995; Cerione *et al.*, 1996; Kaibuchi *et al.*, 1999).

It has been suggested that the activity of the Rho family of GTPases such as Cdc42, Rac1 and Rho is essential for cadherin mediated cell-cell adhesion (Kaibuchi *et al.*, 1999). It has also been shown that E-cadherin activity is directly regulated by Rac1 and indirectly regulated by RhoA and that this occurs through the rearrangement of actin filaments. The mode of action of the Rho family of GTPases (Cdc42 and Rac1) in regulating the cadherin activity is mediated through the effector protein IQGAP1, which accumulates at the cell-cell adhesion sites. It is likely that the regulation of E-cadherin activity by IQGAP1 occurs downstream of Cdc42 and Rac1. The accumulation of IQGAP1 at cell-cell adhesion sites is dependent on E cadherin and β catenin. It has been suggested that IQGAP1 interacts directly with the C-terminal region of E cadherin and β catenin both in vitro and in vivo (Kuroda *et al.*, 1998).

β catenin at its N-terminal domain (residues 120-151) interacts with α catenin and this overlaps with the IQGAP1 binding site on β catenin (residues 1-183) (Aberle *et al.*, 1994; Fukata *et al.*, 1999). IQGAP1 hinders the interaction between α catenin and β catenin and dissociates α catenin from the α - β catenin complex in vitro (Fukata *et al.*, 1999). IQGAP1 exerts its regulatory influence on E cadherin activity by dissociating the α catenin from the cadherin- catenin complex (Kuroda *et al.*, 1998). Cdc42 may exert some regulatory role on IQGAP1 activity in E cadherin -catenin complexes (Kaibuchi *et al.*, 1999). When Cdc42 is activated the inhibitory role of IQGAP1 is suppressed by preventing the interaction of IQGAP1 with β catenin and stabilising the cadherin-catenin complex.

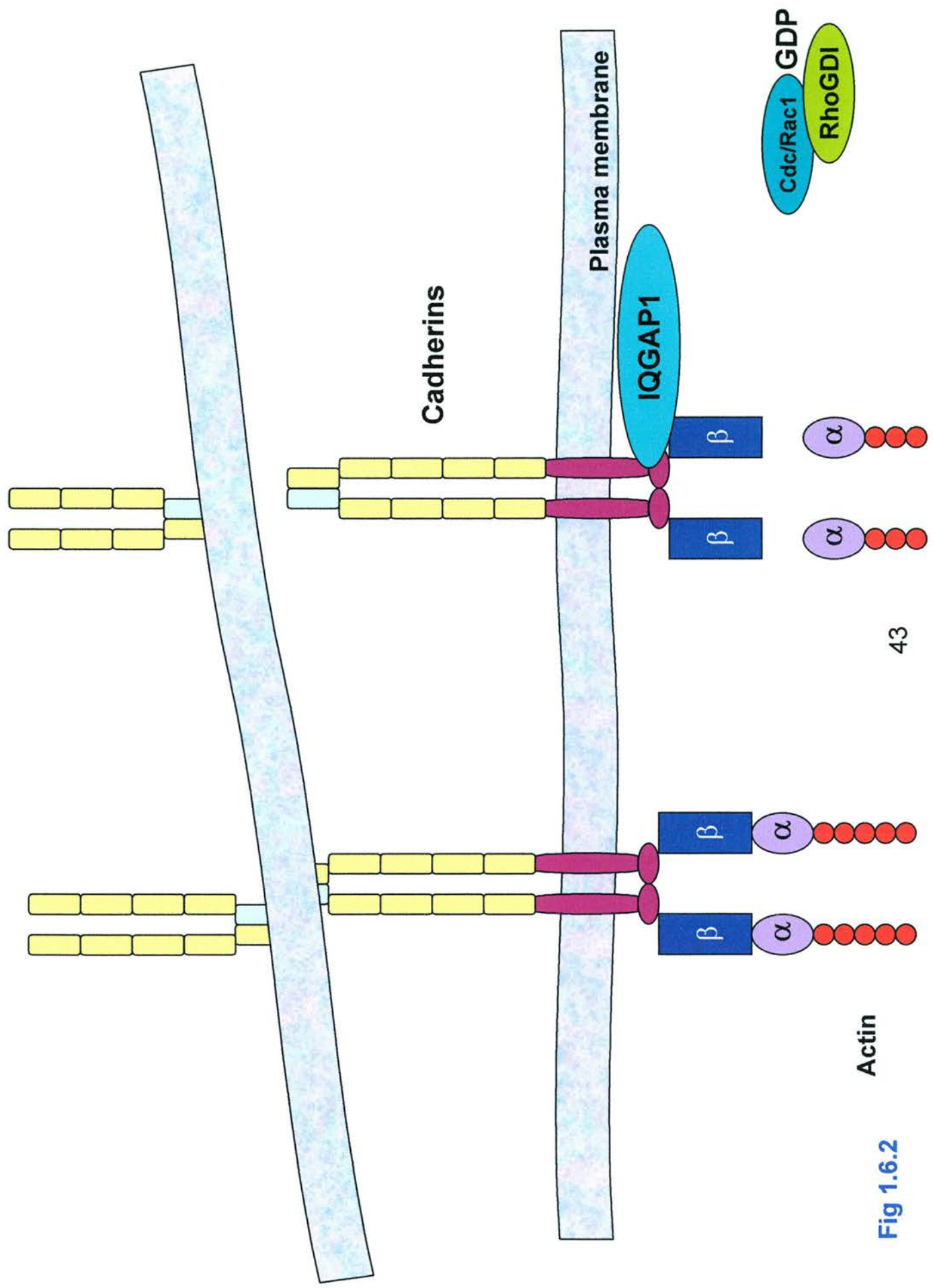
The mechanism by which cadherin mediates cell-cell contact is regulated by Cdc42, Rac1 and IQGAP1. When Cdc42 and Rac1 are GTP bound and active at cell-cell adhesion sites, they interact with IQGAP1 and prevent the binding of IQGAP1 with β catenin. This, in turn, promotes the binding of β -catenin, α catenin and cadherins, leading to stabilisation of the cadherin-catenin complex (**Fig 1.7.1**). When Cdc42 and Rac1 are GDP bound and inactive, there is no interaction between these proteins and IQGAP1. This, in turn leads to interaction of IQGAP1 with β catenin and dissociation of α catenin from the cadherin-catenin complex. This results in relatively weak cell-cell adhesion due to disruption of the interaction between E cadherin and the actin cytoskeleton (**Fig 1.7.2**). Cdc42 and Rac1 prevent IQGAP1 from disrupting the cadherin-catenin complex. This augments the linkage of E cadherin to the underlying actin cytoskeleton and results in strong cell-cell adhesion. Thus, IQGAP1 is a negative regulator and Cdc42 and Rac1 are positive regulators of cadherin adhesive activity. Induction of the activation of Rho family of GTPases, including Rho, Cdc42



Actin

Fig 1.6.1

Fig 1.6.1: The role of IQGAP1 in regulation of cadherin mediated cell-cell adhesion. Cdc42 and Rac1 interact with IQGAP1 and prevent the binding of IQGAP1 with β catenin. This leads to stabilisation of cadherin- catenin complex (modified from Kaibuchi *et al.*, 1999).



Actin

Fig 1.6.2

Fig 1.6.2: The role of IQGAP1 in the regulation of cadherin mediated cell-cell adhesion. Inactivation of Cdc42 and Rac1 results in the release of IQGAP1 and its interaction with β catenin. This causes the separation of α catenin from the cadherin-catenin complex (modified from Kaibuchi *et al.*, 1999).

and Rac is not only by cell-cell contact, but also by integrin-mediated cell matrix contact (Price *et al.*, 1998; Ren *et al.*, 1999).

It has been proposed that there is an interaction between E cadherin and some isoforms of integrins at cell-cell adhesion sites (Higgins *et al.*, 1998). Moreover, E cadherin, like growth factor receptors, induces tyrosine phosphorylation of certain proteins such as ras-GAP at cell-cell adhesion sites. IQGAP1 has an inhibitory effect on E-cadherin adhesive activity, despite being colocalised with the cadherin-catenin complex at cell-cell contact sites. However, E-cadherin forms complexes with β catenin/ α catenin or β catenin/IQGAP1 at cell-cell adhesion sites. Thus, there is a mixture of E-cadherin/ β catenin/ α catenin complexes and E cadherin/ β catenin/IQGAP1 complexes at cell-cell adhesion sites and the ratio of these determines cadherin adhesive activity (Kaibuchi *et al.*, 1999).

Another mechanism for regulation of cadherin activity is through β catenin tyrosine phosphorylation. As cadherin-mediated cell-cell adhesion is disrupted, β catenin becomes phosphorylated and α catenin dissociates from the cadherin- catenin complex (Ozawa and Kemler., 1998).

Thus one can speculate that tyrosine phosphorylation of β catenin facilitates the recruitment of IQGAP1 or GAPs to cell-cell adhesion sites. This would inhibit Cdc42 and/or Rac 1, resulting in reduced cadherin activity (Kaibuchi *et al.*, 1999).

1.5.2 Cadherins and cell-survival signalling pathway

It has been proposed that aggregation dependent cell-survival is mediated by E-cadherin. This could influence the cell-survival pathway by modulating the activity of the signalling molecules which regulate PKB function (Pece *et al.*, 1999). In epithelial MDCK cells E-cadherin homophilic cell-cell adhesion results in a remarkable increase in PI3K dependent activation of PKB and its translocation into the nucleus (Pece *et al.*, 1999). A physical association between PI3K and the E cadherin multiprotein complex at the cell-cell adhesion sites suggests a mechanism for PKB activation. The biochemical route by which E-cadherin cell-cell contacts lead to the recruitment of PI3K to cadherin complexes at the plasma membrane is facilitated by activation of specific tyrosine kinases (Pece *et al.*, 1999). Thus, E-cadherin is necessary and sufficient for inducing PKB activity at cell-cell AJs sites. The mechanism by which E-cadherin stimulates the activation of the PI3K/PKB cascade may involve activation of a tyrosine kinase. This could facilitate the recruitment of PI3K/E cadherin containing complexes to the cell membrane.

One of the first events in endothelial cell apoptosis is rapid dissociation of VE cadherin and β catenin (Brancolini *et al.*, 1997; Herren *et al.*, 1998). Studies have revealed different roles for β catenin in apoptosis. Impaired interaction of β catenin with mutated presenilin reduces the stability of free cytosolic β catenin and induces neuronal apoptosis (Zhang *et al.*, 1998). Conversely, the formation of a β catenin-TCF complex in *Drosophila* results in activation of apoptosis (Ahmed *et al.*, 1998). In vascular endothelial cells increased levels of free β catenin do not induce apoptosis (Carmeliet *et al.*, 1999). Loss of VE cadherin or lack of β catenin binding as a result

of truncation of the cytoplasmic tail causes an impaired response of endothelial cells to VEGF-A survival signals and prevents vascular development (Carmeliet *et al.*, 1999). Thus, VE cadherin/ β catenin complexes play a role in the intracellular signalling initiated by the clustering of proteins involved in VEGF-A survival signalling.

In addition to cadherin mediated signalling and its role in cell proliferation and apoptosis, Wnt mediated signalling pathways, which also regulate β catenin stability may play some roles in cell clustering and progression of OA. Thus, the mechanism by which different Wnt pathways regulate cellular responses is described and their role in chondrogenesis and cartilage development further explored.

1.6 The Wnt signalling pathway

The Wnt signalling pathway is one of many essential pathways in a variety of developmental and biological processes such as body axis formation, central nervous system formation, limb axial specification and mouse mammary gland development. There is a wide range of components involved in the transduction of Wnt signals intracellularly, but, the mechanisms involved in the activation and inactivation of Wnt pathways remain to be fully elucidated (Akiyama., 2000; Pinto *et al.*, 2003; Giles *et al.*, 2003). The Wnt proteins are a family of highly conserved, secreted, glycosylated signalling molecules, consisting of 22 members in vertebrates (**Fig 1.13**). These molecules exhibit a range of functions and regulate cell-cell interactions during embryogenesis. These functions range from patterning individual structures to fine tuning at the cellular level, and control of cell differentiation, proliferation and

survival (Church *et al.*, 2002 a, b). Insights into Wnt signalling came initially from genetic studies in *Drosophila* and *Caenorhabditis elegans* and biochemical studies of ectopic gene expression in *Xenopus* embryos. Subsequent studies of mutations of Wnt genes in mice revealed associations with developmental defects and abnormalities. The Wnt family of proteins is divided into two subgroups according to their ability to induce a secondary axis in *Xenopus* and to transform mammary epithelial cell lines. The Wnt-1 class consists of Wnt 1, -3a, -7a and 8 which play a role in the canonical Wnt pathway. This Wnt pathway is mediated through β -catenin. The Wnt-5a class of proteins includes Wnt 4, -5a and -11 proteins which activate the noncanonical Wnt pathways. The noncanonical Wnt pathways are further subdivided into the Wnt/ Ca^{2+} and Wnt/Jnk pathways. From the Wnt-5a class of proteins, Wnt 11 plays role in Wnt/Jnk signalling whilst Wnt-5a takes part in the Wnt/ Ca^{2+} pathway.

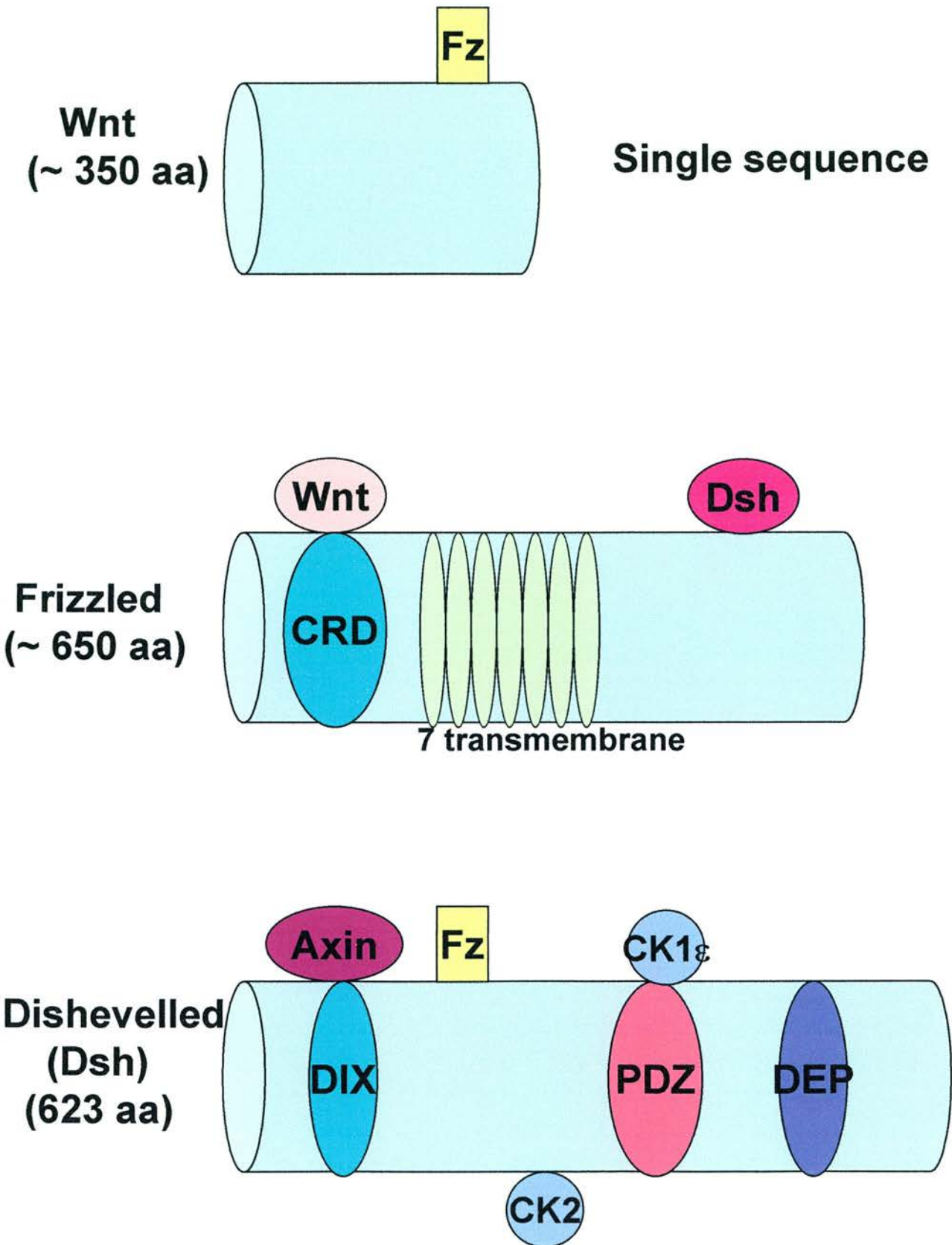
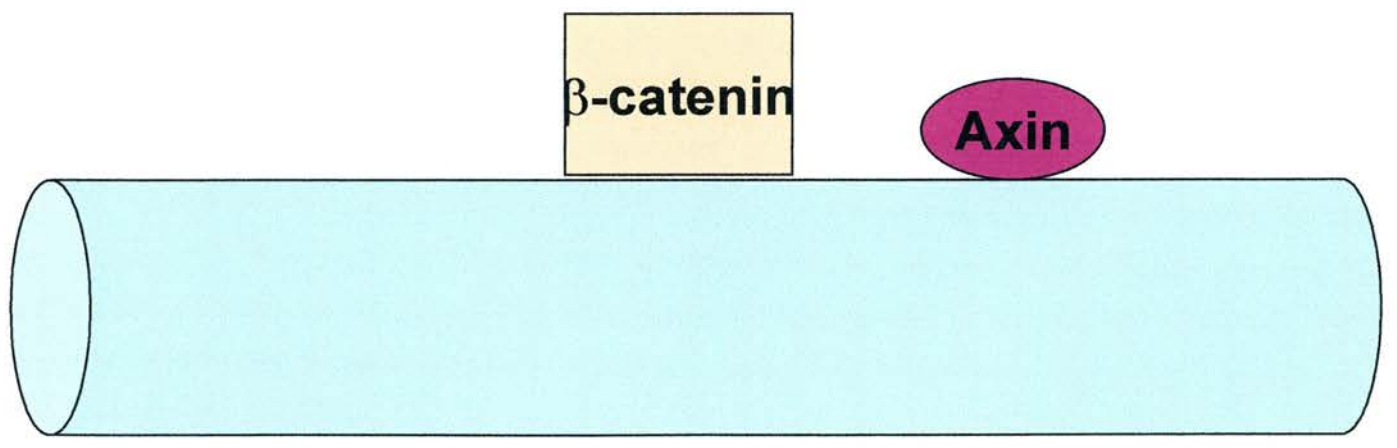
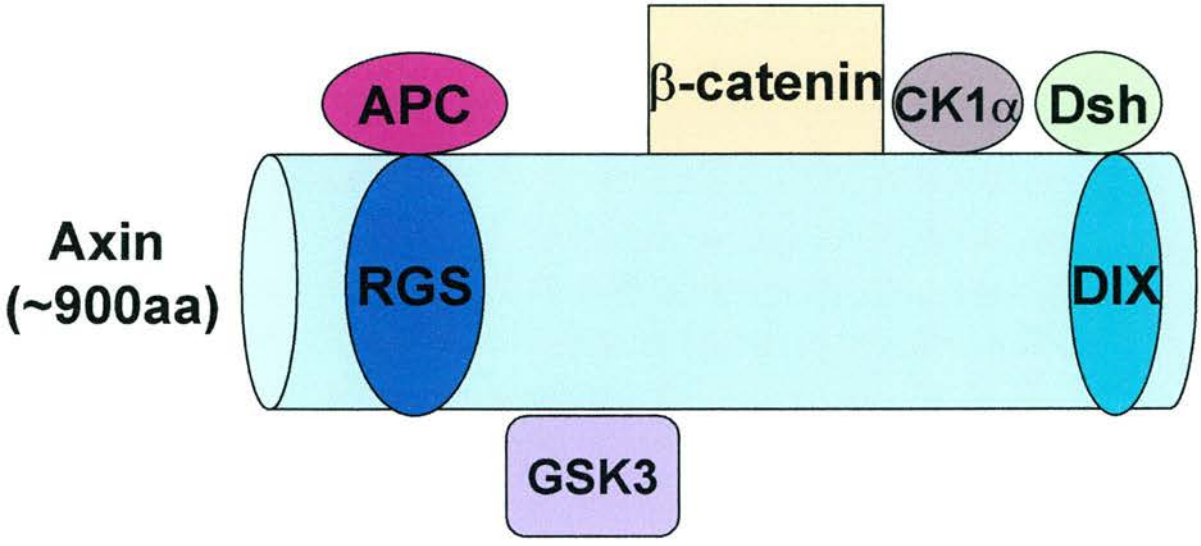


Fig 1.17: The components of the Wnt Pathway, their domains and regions of interaction with one another.



APC
(~2800aa)

Wnt proteins interact with complex receptors and transduce signals intracellularly. The specificity of these ligand –receptor complexes is determined when the Wnt protein is bound to receptors of the Frizzled (Fz) family on the cell surface. Fz receptors are a family of seven-pass transmembrane (7 TM) proteins consisting of 10 genes characterised by an N-terminal cysteine rich ligand binding domain (Bhanot *et al.*, 1996) (**Fig 1.13**). Fz receptors and a member of a single-pass transmembrane protein, low-density lipoprotein (LDL)-receptor–related protein (LRP5 and LRP6) form a complex which facilitates the transduction of Wnt signals intracellularly (Wehrli *et al.*, 2000; Tamai *et al.*, 2000; Pinson *et al.*, 2000, Logan *et al.*, 2004). For the Wnt-Fz complex to be fully functional there is a requirement for a cell surface heparan sulphate rich proteoglycan, acting as a coreceptor. One protein with this characteristic is encoded by the division abnormally delayed (dally) gene. Dally induces tissue specific effects of Wnt signalling and may play a regulatory role in the Wnt receptor complex (Tsuda *et al.*, 1999).

There are several other secreted proteins expressed during development which can bind to Wnt proteins and modulate their activity. These proteins are known as Wnt antagonists. The regulation and specificity of Wnt signals is dependent on the action of these antagonists along with the presence of different signalling receptors on the target cells (Enomoto- Iwamoto *et al.*, 2002). Cell proliferation and differentiation involves complex interplays between Wnt signalling and its modulation by frizzled related protein (Fzrp). Fzrp proteins are secretory proteins with the same homology domain as the Fz family of receptors at their N-terminus but lacking the 7 TM sequences. Thus, it is essential that a fine balance between Wnt-Fz action and Fzrp action is achieved in these cells. Whether or not all the Fzrp have antagonistic effects



on Wnt signalling activity, is not clear. It has been proposed that Fzrp either promotes Wnt secretion or functions by affecting Wnt ligand distribution (Cadigan and Nusse *et al.*, 1997).

Previous studies have shown that the classification of the Wnt family of proteins is dogmatic. There is further complexity of the canonical and noncanonical subdivisions, dependent on their Frizzled receptor profile. For example, Wnt 5a, when coinjected with Frizzled 5 (Fz 5) receptor, can induce a secondary axis in *Xenopus* whilst Wnt-5a on its own cannot (He *et al.*, 1997; Church *et al.*, 2002a). Furthermore, the Wnt-1 class does not always signal through the canonical Wnt pathway which is usually associated with Frizzled-2 and is β -catenin dependent (Kengaku *et al.*, 1998).

Members of the Wnt-5a class of proteins signal through different signalling pathways and as a result exhibit distinct effects on embryogenesis. Wnt-5a signals through both the phosphatidylinositol (PI)/ Ca^{2+} and protein kinase C (PKC) pathways (Sen; 2005). Wnt 11 signals through the JNK/MAPK pathway (Slusarski *et al.*, 1997b; Djiane *et al.*, 2000; Kuhl *et al.*, 2000 a; Tada and Smith, 2000; Church *et al.*, 2002a).

1.6.1 The canonical Wnt pathway

Secreted Wnt glycoproteins bind to a Frizzled/low density lipoprotein receptor related protein (LRP 5/6) complex along with a sulphated proteoglycan coreceptor, and transduce the signal intracellularly (Zorn 2001; Pinto *et al.*, 2003). This, in turn, activates the cytoplasmic protein Dishevelled Dsh/Dvl. The precise regulation of the phosphorylation of Dsh is not completely clear, but casein kinase 1 (CK1) and casein kinase 2 (CK2) seem to play a part (Willert *et al.*, 1997; Sakanaka *et al.*, 1999; Amit

et al., 2002). Phosphorylation and activation of Dsh/Dvl results in the inhibition of the adenomatous polyposis coli (APC)-glycogen synthase kinase (GSK3 β), axin, β -catenin multiprotein complex. This active complex is responsible for β -catenin phosphorylation and its consequent degradation by the proteasome (Giles *et al.*, 2003; Akiyama *et al.*, 2000; Logan *et al.*, 2004; Wnt genes home page: www.stanford.edu/~rnusse/wntwindow.html). It has been suggested that as Dsh /Dvl binds CK1, the priming of β -catenin is inhibited, so preventing β -catenin phosphorylation by GSK3 β indirectly (Amit *et al.*, 2002). Upon Wnt stimulation, Dsh/Dvl recruits the GSK3 binding protein (GBP) to the multiprotein complex. GBP might also play some roles in inhibiting β -catenin phosphorylation by dissociating GSK3 β from axin. Finally, LRP is responsible for sequestering axin at the cell membrane (Mao *et al.*, 2001) This is triggered as axin is removed from the destruction complex and recruited to either LRP or Fz bound Dsh (Tamai *et al.*, 2004). Protein phosphatases such as PP2A can also bind axin and dephosphorylate GSK3 β substrates such as β catenin, which in turn, regulates β catenin stability (Logan *et al.*, 2004) Each of these sequences of activation and inhibition of Wnt pathway components result in accumulation of cytosolic β -catenin. The stabilisation of β -catenin promotes its translocation into the nucleus where it binds to transcription factors which are the members of T-cell factor (TCF)/ lymphoid enhancing factor (LEF) family of DNA binding proteins. This, in turn, leads to transcription of Wnt target genes such as CD44 and fibronectin (**Fig 1.8.1**).

In the absence of a Wnt ligand, the recruitment of CK1 to the multiprotein complex is facilitated by axin, which results in the priming of β -catenin. This in turn initiates the β -catenin phosphorylation cascade, which is performed by GSK3 β . Then β -

transductin repeat-containing protein (β -TrcP) recognises the phosphorylated β -catenin, leading to degradation of β -catenin by the proteasome. The whole process results in a reduced level of cytosolic β -catenin (Giles *et al.*, 2003) (**Fig 1.8.2**).

Fig 1.8.1: The canonical Wnt pathway. (in the presence of the Wnt signal) Wnt binds to Fz, which results in phosphorylation of Dsh. This leads to inhibition of GSK3 β complex and concentration of β catenin within the cytoplasm. This results in translocation of the β catenin to the nucleus where it binds to transcription factors TCF/LEF.

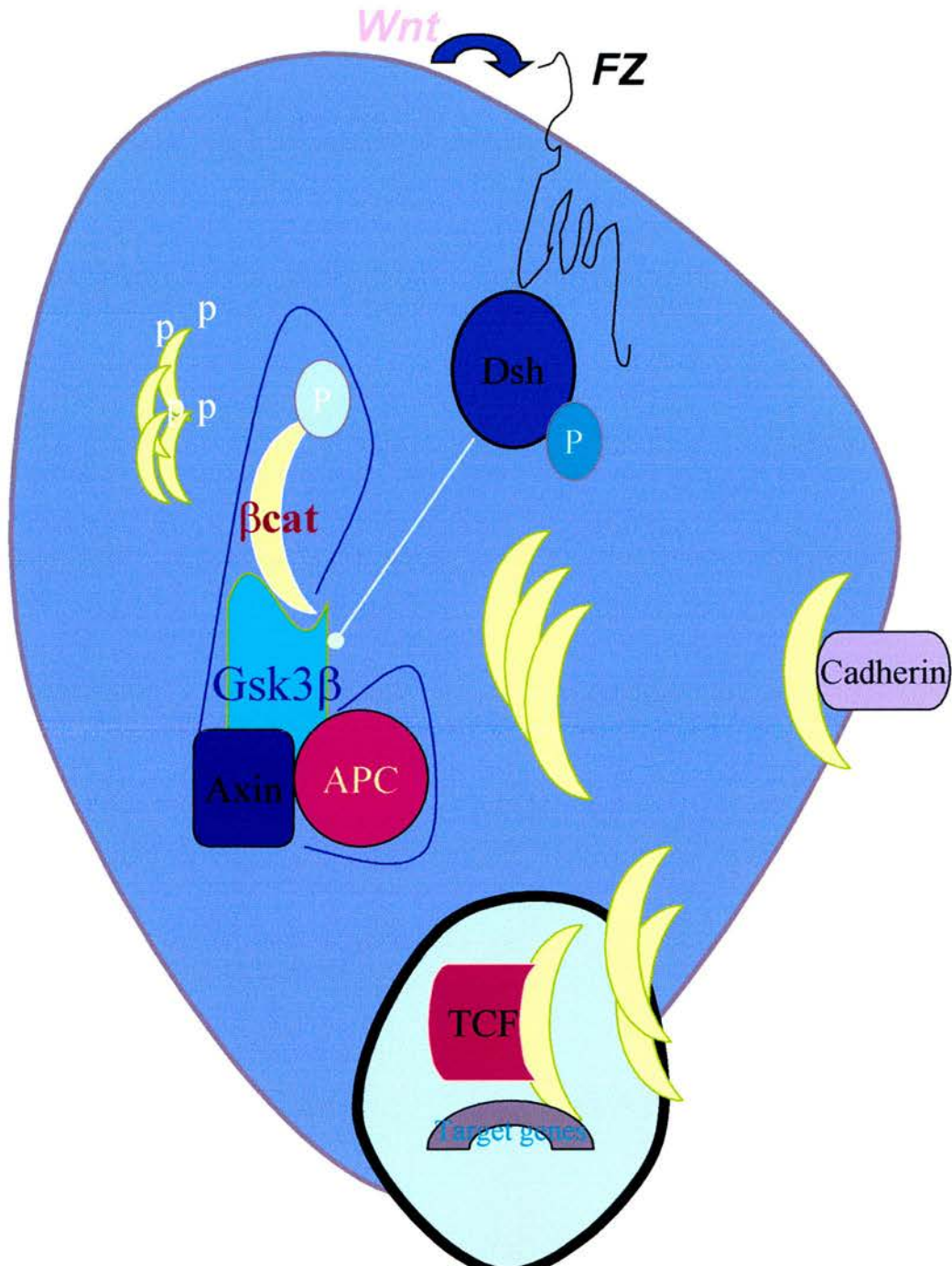
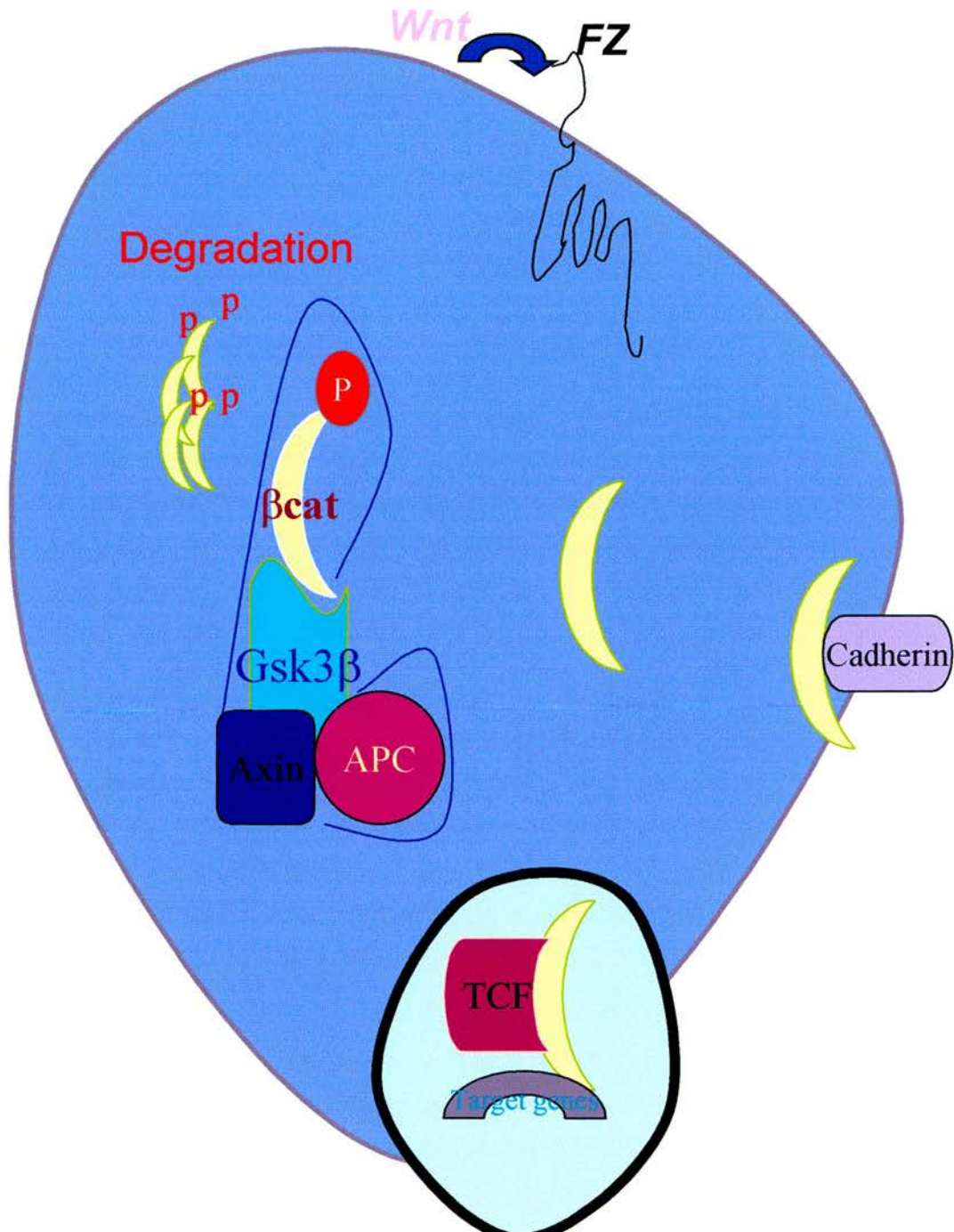


Fig 1.8.2: The canonical Wnt pathway. (in the absence of the Wnt signal). GSK3 β in complex with APC and Axin is responsible for β catenin phosphorylation and degradation through proteosome pathway.



1.6.2 The non-canonical Wnt pathways

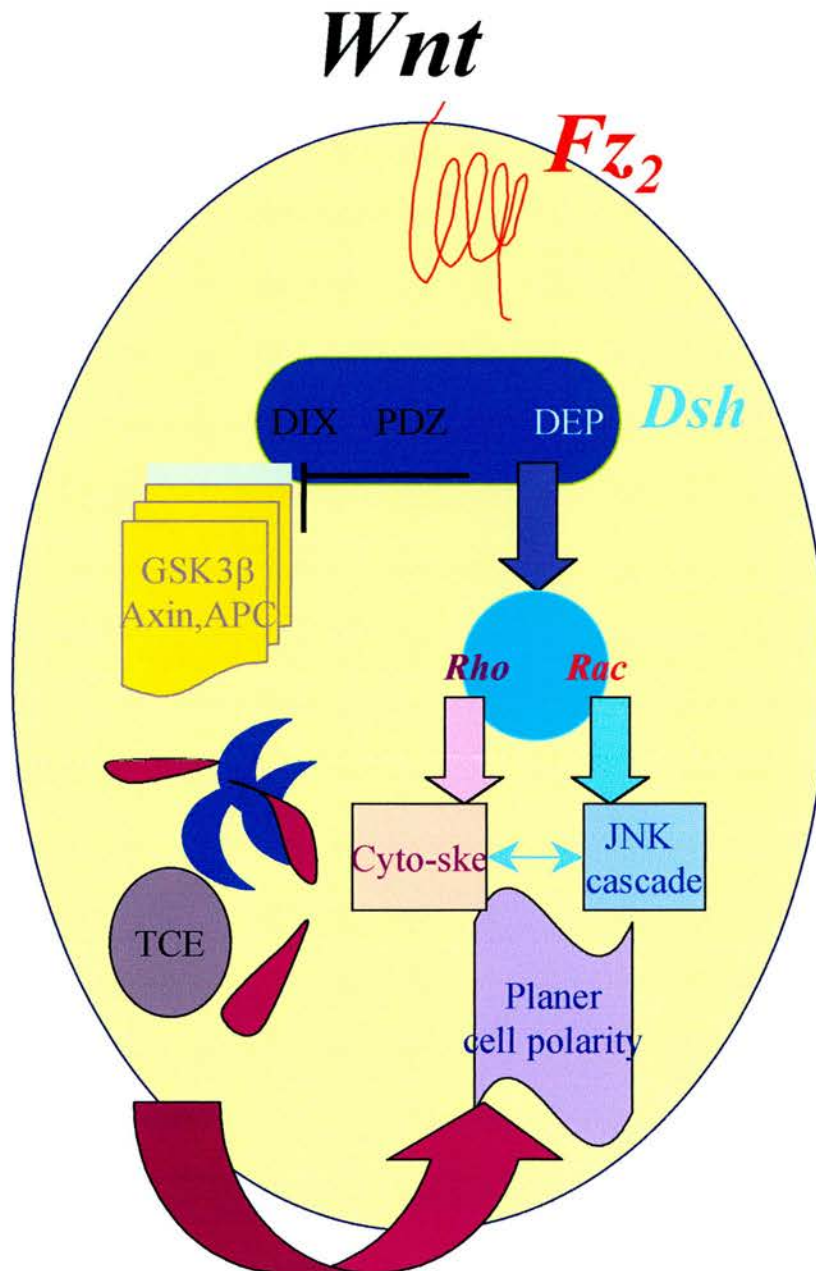
Studies carried out in zebrafish and *Xenopus*, have shown that some members of the Wnt family of proteins can signal in a β -catenin- independent manner.

In the non canonical (β -catenin- independent) Wnt pathway, the Wnt signal is transmitted intracellularly either through the JNK (c-Jun-N-terminal kinase) pathway or a Ca^{2+} dependent pathway mediated by Fz receptors (Giles *et al.*, 2003).

In the Wnt/Jnk pathway, the Wnt-5a family of proteins bind to the receptor Fz-2. This, in turn activates Dsh through a separate domain from the one that is stimulated by the Wnt-1 family of proteins (Sokol *et al.*, 2000; Wallingord *et al.*, 2000; Schwarz-Roman *et al.*, 2002). Dsh induces the activation of the Rac family of GTPases which leads to stimulation of JNK activity (Habas *et al.*, 2003). JNK activity, in turn regulates the cytoskeletal rearrangements (Sokol *et al.*, 2000; Sen., 2005). Moreover, Dsh can also control the reorganistaion of the cytoskeleton through the Rho family of GTPases. Although, activation of both Rac and Rho culminate in organisation of cytoskeleton and planer cell polarity, the stimulation of these GTPases by Dsh is mediated through two independent routes (**Fig 1.9**).

Fig 1.9: The Wnt / JNK pathway

Wnt binds to Fz-2, which activates Dsh through DEP domain. This leads to activation of Rac, which results in Jnk activity and regulation of cytoskeletal rearrangements. Dsh also activates the Rho family of GTPases, which also plays role in regulation of cytoskeletal organisation.

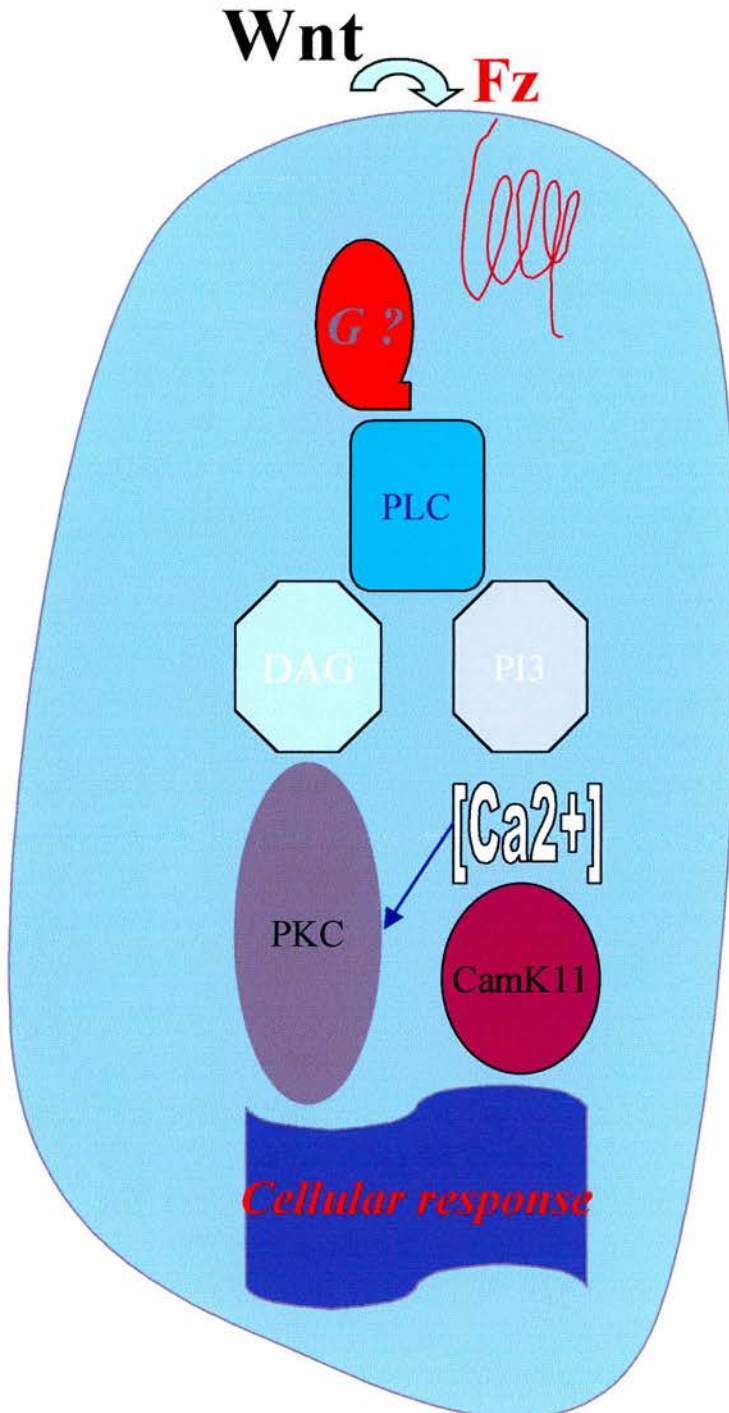


The Ca^{2+} dependent non canonical Wnt pathway, is usually mediated by Wnt-5a and not by the axis inducing Wnts (e.g Wnt-8). Activation of the Wnt/ Ca^{2+} pathway results in intracellular Ca^{2+} release and is mediated by activation of Dsh (Sen., 2005). The Wnt/ Ca^{2+} pathway is a G protein coupled pathway, although the Fz family of receptors are not directly associated with the G protein (Kuhl *et al.*, 2000b). The induction of G protein activity is preceded by activation of phospholipase C (PLC) which yields two important products: 1,2-diacylglycerol (DAG) and 1,4,5-triphosphate (IP3). Within the cytoplasm, DAG remains membrane associated whilst IP3 diffuses through the cytosol. IP3 interacts with IP3-sensitive Ca^{2+} channels in the membrane of the endoplasmic reticulum (ER), causing the release of the stored Ca^{2+} ions. At this stage protein kinase C (PKC) is activated by both DAG and Ca^{2+} . Moreover, the Ca^{2+} -sensitive enzyme Ca^{2+} -calmodulin dependent protein kinase II (CamKII) is activated by the release of Ca^{2+} ions. Eventually, the activation of these enzymes leads to various cellular responses such as activation of NF κ B(Kuhl *et al.*, 2000b; Sen., 2005) (**Fig 1.10**), (**Table 1**).

Wnt / β -catenin	Wnt/ Calcium	Wnt/Jnk
Wnt-1 family of ligands (Wnt-1, -3a, -7a and 8)	Wnt-5a family of ligands (Wnt 4, -5a and -11)	Wnt-5a family of ligands Wnt-11
Frizzled receptors (Fz-1,2)	Frizzled receptors (Fz-2,5)	Frizzled receptors (Fz-2)
Dsh (DIX,PDZ domains)	G proteins	Dsh (DEP domain)
CK1	PLC	Rac
CK2	PI3	Rho
Axin	DAG	Jnk
APC	PKC	
GSK3 β	CamKII	
β -catenin		
TCF		
LEF		

Table 1: Wnt signalling pathways and their corresponding components

Fig 1.10: The Wnt/ Ca^{2+} pathway. Wnt binds to Fz, which induces a G protein coupled pathway. This activates PLC, resulting in the yield of two products : DAG and IP3. IP3 causes the release of intracellular calcium. At this stage PKC is activated by both DAG and calcium. Calcium also activates CamKII. Activation of these kinases results in a number of cellular responses.



1.6.2.1 Interactions between Wnt/ β -catenin and Wnt/ Ca^{2+} pathways

Activation of the Wnt/ Ca^{2+} pathway triggers the release of intracellular Ca^{2+} . This Ca^{2+} discharge is induced by expression of Rat frizzled 2 (Rfz-2) but not Rat frizzled 1 (Rfz-1). Rfz-1 but not Rfz-2 activates β -catenin via the canonical Wnt pathway (Kuhl *et al.*, 2000 b). Preferential coupling of a Fz homolog to either the Wnt/ Ca^{2+} or the Wnt/ β -catenin pathway is indisputable (Ishitani *et al.*, 2003; Slusarski *et al.*, 1997a; Yang-Snyder *et al.*, 1996; Kuhl *et al.*, 2000 b; Kuhl *et al.*, 2001). Activity of the canonical Wnt pathway is also modulated by a member of Wnt-5a family of proteins via activation of the Wnt/ Ca^{2+} pathway. The downstream effects of Wnt5a and Rfz-2 which trigger intracellular Ca^{2+} release include activation of the phosphatidyl inositol pathway in a G-protein dependent manner. This, in turn, is sufficient to activate Ca^{2+} sensitive enzymes, such as PKC and Ca^{2+} /calmodulin dependent kinase II (CamKII) (Kuhl *et al.*, 2000 a,b; Kuhl *et al.*, 2001; Slusarki *et al.*, 1997 a, b; Sheldahl *et al.*, 1999; Ishitani *et al.*, 2003). It is also known that the activation of CamKII can activate the ERK/MAPK pathway in different cell types (Watt and Storm., 2001; Ishitani *et al.*, 2003). Wnt/ Ca^{2+} has an antagonistic effect on the Wnt/ β -catenin pathway (Torres *et al.*, 1996). The Wnt/ β -catenin pathway is blocked upstream of β -catenin by activation of PKC through the Wnt/ Ca^{2+} pathway and CamKII blocks the Wnt/ β -catenin pathway downstream of β -catenin by inhibition at the level of TCF/LEF transcription factors (Kuhl *et al.*, 2001; Ishitani *et al.*, 2003). Thus, finely tuned activity of the canonical and non canonical Wnt pathways is essential for appropriate biological development.

1.6.3 Intracellular components of the Wnt pathways

The members of the Wnt family of proteins bind to their receptors (Fz) with similar biochemical binding properties. These interactions lead to activation of different intracellular components of the Wnt pathway which then can be phenotypically distinguished.

1.6.3.1 Dishevelled (Dsh)

In Wnt pathways in *Drosophila*, Dishevelled (Dsh) is an intriguing protein required in two distinct signalling pathways: Wnt/JNK and Wnt/ β -catenin. Dsh is required for transmission of the Wnt signals and for the determination of epithelial planar polarity (PCP, tissue polarity) in *Drosophila*. Studies in *Drosophila* have revealed that Dsh is a highly modular protein of 623 amino acids (Klingensmith *et al.*, 1996; Boutros and Mlodzik, 1999). The Dsh protein contains highly conserved domains. At the N-terminus, there is a DIX domain (Dsh-Axin), spanning 80 amino acids, a central PDZ domain capable of binding to other receptors or forming homotypic dimers, and a C-terminus DEP domain (Dishevelled –EGL-10-pleckstrin) which is mainly found in the RGS (regulator of G protein signalling) family of proteins. Although RGS proteins are involved in G protein signalling, Dsh proteins have no apparent function in regulating the G proteins (Zeng *et al.*, 1997; Cadigan and Nusse, 1997; Ponting and Bork, 1996) (**Fig 1.13**). Transduction of signals through the Wnt/ β catenin pathway is modulated by the DIX and PDZ domains whereas the DEP domain is required for membrane localisation, activation of the JNK pathway and establishment of planar cell polarity (Axelrod *et al.*, 1998b; Rothbacher *et al.*, 1999; Boutros and Mlodzik, 1999).

1999). It is known that the DEP domain is an indispensable component for the activation of the planar cell polarity pathway in which signalling is routed via Rho/Rac and the JNK/SAPK cascade. Thus, there is involvement of Dsh in distinct Wnt signalling pathways (Aberle *et al.*, 1997; Adler *et al.*, 1992; Boutros and Mlodzik 1999; Cadigan and Nusse ., 1997).

This further confirms that Dsh can function as a binary switch between the Wnt/ β -catenin and Wnt/JNK pathways (McEwen and Peifer., 2000). Dsh controls the reorganisation of actin cytoskeleton via Rho A, Rac and the Cdc42 family of GTPases, which are the core PCP effectors, downstream of Dsh. However, activation of Rac mediated by the Wnt/Fz signalling is independent of Rho and leads to stimulation of JNK activity (Habas *et al.*, 2003). The formation of the Dsh-Rac complex induced by Wnt signals is independent of the formation of Dsh-Rho complexes.

In addition to functional roles for Dsh in the Wnt/ β catenin and PCP pathways, Dsh also functions in the Wnt/ Ca^{+2} pathway. Dsh plays role in Wnt/ Ca^{+2} signalling through activation of three effectors in this pathway: Ca^{+2} flux, PKC and calcium/calmodulin-dependent protein kinase II (CamKII). The Wnt/ Ca^{+2} and PCP pathways were discovered independently, but mechanistically they may overlap as they may both affect intracellular components through the common DEP domain on Dsh (Sheldahl *et al.*, 2003)

Cell morphology can be altered as the Wnt/ β catenin pathway targets the actin cytoskeleton via Dsh and casein kinase 1 ϵ (CK1 ϵ). Colocalisation of Dsh and CK1 ϵ with actin filaments occurs when other downstream components of the canonical Wnt pathway are absent. However, Wnt/ β catenin signals are transduced in the perinuclear region of the cell where the majority of the canonical Wnt components colocalise (Torres and Nelson., 2000). The colocalisation of Dsh and CK1 ϵ with actin fibers suggests a role for the Wnt/ β catenin pathway in altering metanephric cell morphology. It is also known that Wnt-1 signalling reduces the number and the length of actin fibers in metanephric mesenchymal cells (Torres and Nelson., 2000).

Wnt signalling modulates the actin cytoskeleton in a Dsh and/or CK1 ϵ dependent manner. This in turn mediates changes in cell shape during cell aggregation (Torres and Nelson., 2000). The regulatory role of Dsh for the organisation of actin filaments in the cytoskeleton possibly occurs via the canonical Wnt pathway, which increases aggregation of cells by enhancing cell- cell adhesion mediated by cadherins. In addition, Wnt signalling modulates integrin-mediated cell substratum adhesion via Dsh interaction with paxillin and/or ILK at focal adhesions (Bradley *et al.*, 1993; Hinck *et al.*, 1994; Torres and Nelson., 2000).

The ability of Dsh to form a complex with ILK suggests that other related signalling pathways interact with the Wnt pathways via Dsh to stimulate cell proliferation (Novak *et al.*, 1998; Wu *et al.*, 1998). Thus both β -catenin and Dsh provide possible mechanisms for cross-talk or interaction between integrin and Wnt signalling.

1.6.3.2 Glycogen synthase kinase 3 (GSK3)

Glycogen synthase kinase 3 (GSK3) is a multifunctional serine/threonine protein kinase, found in all eukaryotes. This enzyme is a key regulatory kinase in Wnt signalling pathways as well as in phospho inositide 3 (PI3) kinase dependent pathways.

GSK3 was first isolated and purified as an enzyme affecting glycogen metabolism through the phosphorylation and inactivation of the enzyme glycogen synthase (Embi *et al.*, 1980; Woodgett and Cohen, 1984; Doble and Woodgett, 2003).

There are two GSK3 isoforms, GSK3 α and GSK3 β , which are encoded by distinct genes in mammalian cells (Woodgett, 1990). These two isoforms are structurally similar but are not functionally identical (Hoeflich *et al.*, 2000). Crystallography has provided insight into the structure of GSK3 and revealed unique regulatory characteristics and a preference for primed, pre-phosphorylated substrates (Bax *et al.*, 2001; Dajani *et al.*, 2001, ter Haar *et al.*, 2001).

GSK3, like its related protein kinases CDK2, p38 γ and ERK2, undergoes phosphorylation of its residues in activation loops (the T-loops). This phosphorylation is a prerequisite for GSK3 activity (Bellon *et al.*, 1999; Brown *et al.*, 1999; Canagarajah *et al.*, 1997). In the p38 γ and ERK2 protein kinases phosphorylation of a tyrosine residue in the T-loop is required for substrate access to the catalytic site. The T-loop of GSK3 β is tyrosine phosphorylated at Y216 and the GSK3 α T-loop is tyrosine phosphorylated at Y279 with no phosphorylation on threonine.

It has been suggested that Y216/Y279 phosphorylation of GSK3 β/α might reinforce the opening up of the substrate binding site (Dajani *et al.*, 2001). Tyrosine phosphorylation of the T-loop is necessary for the phosphorylation of the substrate but it may not play a significant role in kinase activation (Dajani *et al.*, 2001; Doble and Woodgett, 2003).

Priming phosphorylation of the substrate is not strictly required for its phosphorylation by GSK3 kinases. However, if the substrate is primed then the efficiency of substrate phosphorylation by GSK3 is increased between 100 to 1000 fold (Thomas *et al.*, 1999).

Glycogen synthase (GS) is a typical primed substrate for GSK3. Prior to GS sequential multisite phosphorylation by GSK3, this substrate is phosphorylated by casein kinase II (CKII) (Fiol *et al.*, 1988; Fiol *et al.*, 1990).

The negatively charged prephosphorylated substrate binds to the positively charged pocket on GSK3. In this way the orientation of the kinase domain is optimised and the substrate is correctly situated in the enzyme's catalytic groove. In some substrates, like β catenin, the priming site is missing and the substrates possess negatively charged residues at or near the priming position, resembling phospho residues (Doble and Woodgett, 2003).

1.6.3.2.1 The effect of different stimuli on GSK3 activity

When cells are stimulated by a growth factor such as insulin, GSK3 is inactivated through a PI3K dependent pathway. PI3K phosphorylates and activates protein kinase B (PKB). This in turn, phosphorylates GSK3 α at serine 21 (Ser 21) and GSK3 β at serine 9 (Ser9) leading to their inactivation (Cross *et al.*, 1995). When GSK3 is phosphorylated and inactive, then its substrates, such as glycogen synthase and eukaryotic protein synthesis initiation factor -2B (eIF-2B), are dephosphorylated and active. This results in an increase in glycogen and protein synthesis (Cohen *et al.*, 1997).

Numerous other stimuli can also lead to phosphorylation of GSK3 β/α on Ser 9/21 with subsequent inactivation. These include the growth factors EGF and PDGF that stimulate the GSK3-inactivating kinase MAPKAP-K1 through the MAP kinases (Saito *et al.*, 1994; Brady *et al.*, 1998), Wnt growth factors (Woodgett, 1994; Haq *et al.*, 2000); cAMP activated protein kinase activators (PKA) (Fang *et al.*, 2000; Li M *et al.*, 2000; Tanji *et al.*, 2002); and PKC activators (Ballou *et al.*, 2001; Fang *et al.*, 2002).

1.6.3.2.2 The role of tyrosine and threonine phosphorylation on GSK3 activity

Phosphorylation of tyrosine residues on mammalian GSK3 is an autocatalytic event (Wang *et al.*, 1994). In unstimulated cells the GSK3 is tyrosine phosphorylated but the level of phosphorylation may not be stoichiometric (Doble and Woodgett, 2003).

A calcium sensitive, proline-rich tyrosine kinase 2 (PYK2) has been identified which may regulate the tyrosine phosphorylation of GSK3 (Doble and Woodgett, 2003), but another candidate for tyrosine phosphorylation of GSK3 is the Fyn tyrosine kinase.

In cells stimulated by insulin for 1 min, the association of GSK3 β with Fyn kinase was induced, resulting in an increase in GSK3 β Y216 phosphorylation and a transient increase in phosphorylation of the microtubule associated protein Tau (Lesort *et al.*, 1999). Longer treatment of the cells with insulin, however, resulted in GSK3 β tyrosine dephosphorylation and its serine phosphorylation, leading to an overall inhibition of GSK3 β activity (Murai *et al.*, 1996).

An additional opposing role for protein tyrosine phosphatases, which dephosphorylate GSK3 on tyrosine residues, has been recognised (Kim *et al.*, 2002). Thus a regulatory balance between tyrosine phosphorylation and dephosphorylation of the activation loop of GSK3 appears to play a significant role in the overall activity of this kinase.

1.6.3.2.3 Role of GSK3 in the Wnt pathway

One of the pathways regulated by Wnt growth factors is the canonical Wnt pathway or Wnt/ β catenin pathway (Huelsenken and Behrens, 2002; Polakis, 2000; Seidensticker and Behrens, 2000; Sharpe *et al.*, 2001).

In the Wnt/ β catenin pathway, transduction of the Wnt signal intracellularly leads to activation of the target genes such as MMP7 and CD44. Regulation of Wnt target genes is via the T-cell factor (TCF)/ lymphoid enhancer factor (LEF) transcription factors (Novak and Dedhar, 1999; Barker *et al.*, 2000; Brantjes *et al.*, 2002).

β catenin forms a complex with TCF/LEF transcription factors and activates their responsive genes.

GSK3 β maintains the same inhibitory role in the Wnt pathway as in the PI3K dependent pathways. In unstimulated cells, β catenin is phosphorylated at its N-terminal domain by GSK3 β . The phosphorylated β catenin is then targeted for ubiquitination and proteasome degradation (Dale, 1998). Wnt growth factors bind to their receptor Fz in stimulated cells which in turn leads to Dsh phosphorylation and GSK3 β inactivation through an unknown mechanism (van Noort *et al.*, 2002).

Following GSK3 β inactivation, β catenin is dephosphorylated, becomes stabilised in the cytoplasm, and then translocates to the nucleus, where it binds to TCF/LEFs and functions as a transactivator. Mutations in β catenin prevent its phosphorylation by GSK3 β and its subsequent degradation (Polakis, 2000).

In the absence of the Wnt signalling, GSK3 β phosphorylates β catenin in a complex with axin / conductin and adenomatous polyposis coli (APC) (**Fig 1.13**). This complex is referred to as a destruction complex as it leads to β catenin degradation through the proteasome pathway (Hinoi *et al.*, 2000).

APC is a tumor suppressor protein, deleted in familial adenomatous polyposis and sporadic colorectal cancers (Polakis, 1997). Axin and a related protein,

conductin / axin possess several protein- protein interaction domains and act as scaffolding proteins in the β catenin destruction complex. Axin and APC are both phosphorylated by GSK3 β . GSK3 β phosphorylates axin, increases its stability and facilitates its binding to β catenin (Ikeda *et al.*, 1998; Jho *et al.*, 1999; Yamamoto *et al.*, 1999). GSK3 β also phosphorylates APC and facilitates its binding to β catenin (Rubinfeld *et al.*, 1996). Recently, it has been suggested that β catenin is also a primed substrate for GSK3 β and a priming kinase associated with this complex is CK1 α (Amit *et al.*, 2002; Hagen *et al.*, 2002; Hagen and Vidal-Puig, 2002; Liu *et al.*, 2002; Sakanaka, 2002; Yanagawa *et al.*, 2002).

Four serine / threonine residues (S33, S37, T41 and S45) at the N-terminal of β catenin are GSK3 β phosphorylation sites. However, S45 is initially phosphorylated by CK1 α followed by the subsequent phosphorylation of T41, S37 and S33 sites by GSK3 β (**Fig 1.11**).

Thus, one can suggest that CK1 α is a negative regulator of the Wnt signalling pathway as it enhances GSK3 β activity. However, this contradicts previous work which identified CK1 as a positive regulator in the transduction of Wnt signals intracellularly (Kishida *et al* 2001; Lee *et al.*, 2001; Mckay *et al.*, 2001; Gao *et al.*, 2002). CK1 binds to and phosphorylates a range of effector proteins in the Wnt pathway namely, Dsh, APC, axin and β catenin. This indicates that CK1 can act as both positive and negative regulator of Wnt signalling (Polakis, 2002).

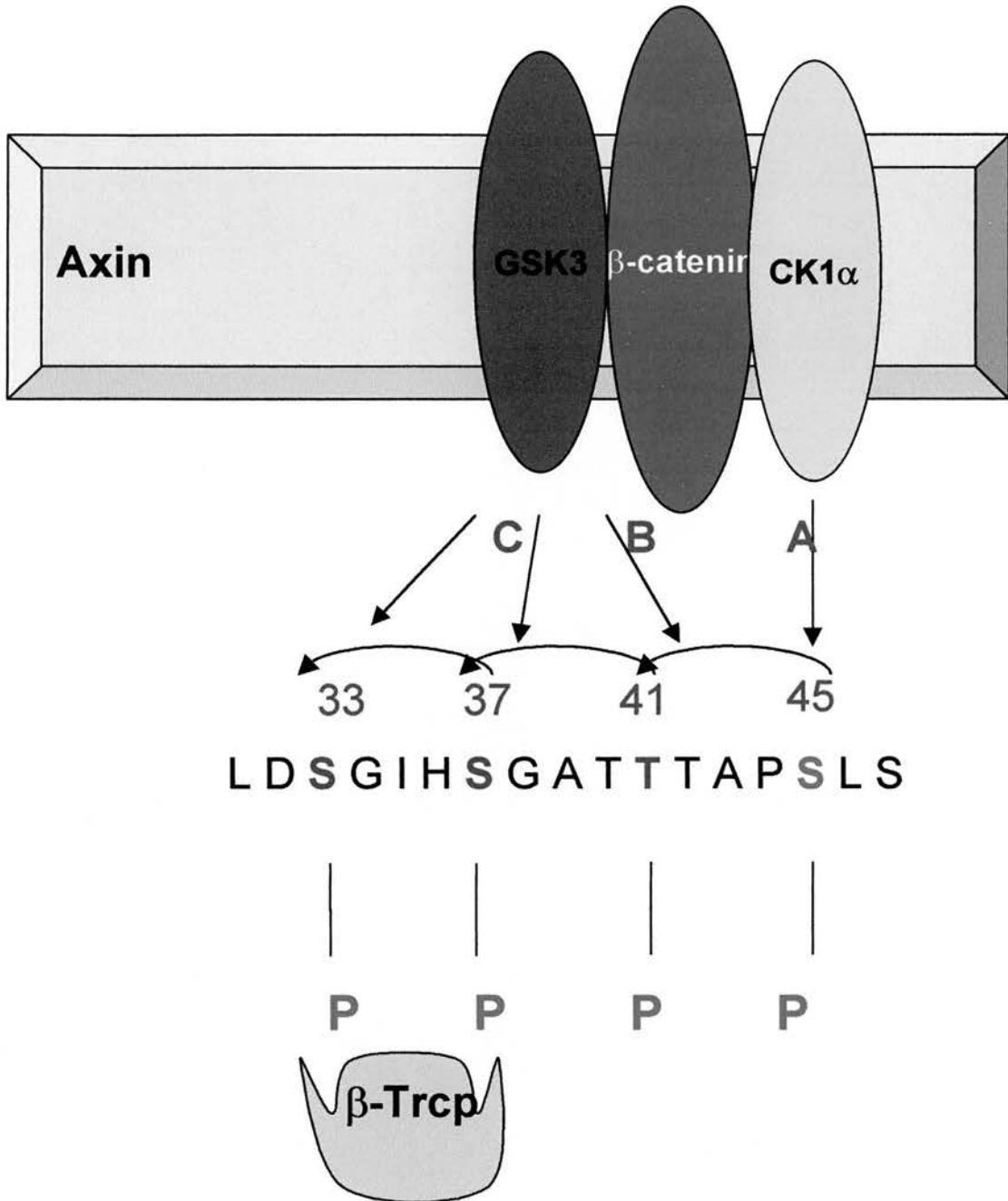


Fig 1.11: A schematic diagram for β -catenin phosphorylation and recognition by β -Trcp. β -catenin is initially phosphorylated on S45 by CK1 α which allows a further phosphorylation of β -catenin on T41, S37 and S33 by GSK3 β (modified from Liu *et al.*, 2002).

1.6.3.2 β catenin

β catenin has regulatory roles during development and a critical role in tumorigenesis. So far the role of β catenin in normal terminally differentiated mammalian tissues has not been explored (Haq *et al.*, 2003). This protein exists in two different pools in the cell, a membrane associated pool and a cytosolic one. In membranes, cadherins, with their conserved cytoplasmic domains, interact with β catenin. This in turn binds to α catenin, which is an actin binding protein that links the cadherin-catenin complex to the cytoskeleton (Sadot *et al.*, 1998). β catenin also binds to the TCF/LEF transcription factors and functions as a transactivator (Miller *et al.*, 1996). This occurs when the cytosolic pool of β catenin is stabilized and accumulates through negative regulation by GSK3 β . This, in turn, leads to β catenin translocation to the nucleus and its binding to transcription factors. Recently, it has been suggested that an additional and distinct mechanism also regulates the cytoplasmic distribution of β catenin in cardiomyocytes (Haq *et al.*, 2003). These cells were subjected to hypertrophic stress rather than Wnt stimulation. The hypertrophic stress induced the formation of the GSK3 β destruction complex without it being associated with the destabilisation of β catenin and its accumulation in the cytoplasm. In this situation GSK3 β is phosphorylated at Ser9, and inhibited following the recruitment of PKB to the axin, APC, GSK3 β complex. This, in turn, leads to β catenin stabilisation. It has also been proposed that Wnt growth factors induce the recruitment of PKB to the Axin, APC, GSK3 β complex. This recruitment was not necessary for Wnt-induced stabilisation of β catenin but it enhanced the effect of the Wnt growth factor in this process (Fukumoto *et al.*, 2001).

Thus the outcome of both hypertrophic stimuli and Wnt signalling is the stabilisation of β catenin. Although this is achieved by different routes, recruitment of PKB to the GSK3 complex may be a common path for both of these signalling mechanisms.

Expression of β catenin in cardiomyocytes in vivo and in vitro is sufficient to induce hypertrophic growth. Furthermore the activity of β catenin as a transactivator is relevant to the resultant hypertrophic response (Haq *et al.*, 2003).

1.7 Chondrogenesis and Wnt

Tissues and organs of the body are formed from a number of distinct cell types during early development.

As cells become fully differentiated, the structural and functional characteristics of the developing tissue are controlled by gene regulation. The skeletal elements of limbs are derived initially from a continuous condensation of mesenchymal cells which differentiate into two cell types: (i) chondrocytes, at the core of these condensations, forming cartilage elements and secreting and synthesizing the extracellular matrix. (ii) fibroblast-like cells at the periphery, forming a perichondrial sheath around the cartilage elements (Rooney and Archer, 1992; Hartmann and Tabin, 2000; Minina *et al.*, 2001). Cartilage elements are initially formed as the chondrocytes and perichondrial cells proliferate and new matrix is deposited. Thereafter, chondrocytes in the middle of cartilage element (diaphysis) cease to proliferate and become hypertrophic, and synthesise a distinct extracellular matrix which subsequently becomes calcified and forms bone (Poole, 1991; Rooney and Archer, 1992).

Proliferating chondrocytes continue to be observed at each terminus (epiphysis) of developing bones, with morphologically distinct populations. In the articular regions, the proliferating chondrocytes are small, spherical and tightly packed. They are located adjacent to a group of flattened radially-arranged proliferating chondrocytes (**Fig 1.12**). Proliferation and hypertrophic differentiation of chondrocytes at the ends of skeletal elements (the growth plates) results in longitudinal growth of bones. Thus, fine tuning in the regulation of the various steps of chondrocyte differentiation is necessary for steady growth and ossification of the skeletal elements that result from replacement of hypertrophic cartilage by bone (**Fig 1.12**) (Erlebacher *et al.*, 1995; Minina *et al.*, 2001).

In the process of cartilage formation a number of different markers are expressed. Initially the extracellular matrix is rich in collagen type I, hyaluronan, tenascin and fibronectin. As the mesenchymal cells differentiate into chondrocytes, significant changes in the composition of the ECM occur. Chondrocytes begin to express collagen type II, collagen type IX and XI, as well as the large chondroitin sulphate rich proteoglycan, aggrecan and link protein, while the collagen type I is no longer produced. At this stage chondrocytes with rounded morphology are trapped in their ECM. As the chondrocytes further differentiate and increase in size, collagen type X is expressed and collagen type II synthesis declines (DeLise *et al.*, 2000).

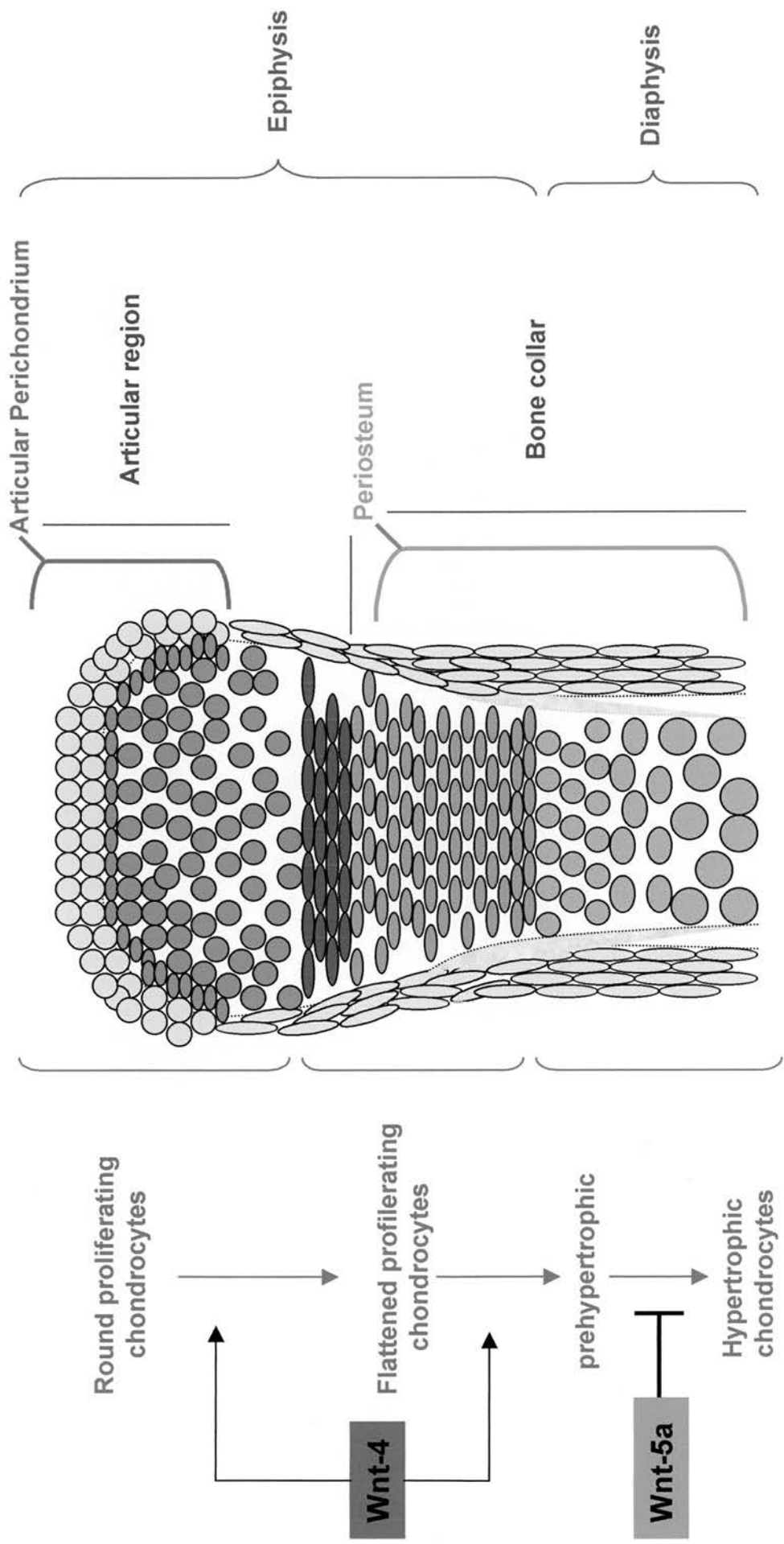


Fig 1.12: Different zones of differentiation within cartilage elements (modified from Wnt signalling during chondrogenesis; Hartmann and Tabin., 2000).

The process of mesenchymal cell condensation is an essential step in chondrogenesis and is directed by cell-cell and cell-matrix adhesion. Prior to condensation, intimate cell-cell interaction is prevented by an ECM rich in hyaluronan and collagen type I secreted by mesenchymal cells. However, as condensation proceeds, hyaluronidase activity increases and the expression of hyaluronan in the ECM is diminished (DeLise *et al.*, 2000). It has been suggested that hyaluronan is required to assist cell motility. Tight cell-cell interactions take place as the activity of hyaluronidase increases and the hyaluronan concentration decreases (Knudson *et al.*, 1987; DeLise *et al.*, 2000). As condensation proceeds and mesenchymal cells differentiate into rounded chondrocytes, collagen type IIa (an alternative spliced form of collagen II), instead of collagen type I, begins to be expressed. In addition, the production of sulfated proteoglycans such as tenascin and the chondroitin sulfate rich aggrecan are increased (DeLise *et al.*, 2000). As the expression of collagen type I switches to collagen type IIa there is also a transition from α_1 integrin to α_3 integrin expression patterns. This marks the initiation of chondrogenesis and may also induce cell differentiation (Shakibaei *et al.*, 1995). Moreover, the chondrocyte pericellular matrix, in which aggrecan is highly expressed, is anchored to the cell surface via CD44 hyaluronan receptors, which in turn direct the assembly of the chondrocyte pericellular matrix (DeLise *et al.*, 2000).

In addition to cell-cell interactions, cell-matrix interactions also play a part in mesenchymal cell condensation. Fibronectin, an ECM component, is involved in this process. Its expression increases in areas of condensation and decreases as differentiation proceeds (Kulyk *et al.*, 1989; DeLise *et al.*, 2000). The expression of fibronectin isoforms varies during chondrogenesis.

The functional role of these isoforms is to convert the mesenchyme into chondroblasts. This process may occur via $\alpha 5 \beta 1$ integrin fibronectin receptors which are associated with integrin linked kinase (ILK) (Hickok *et al.*, 1998; DeLise *et al.*, 2000). This signalling pathway is activated following fibronectin binding to the integrin. The β_1 subunit of the integrin induces the formation of focal adhesions, leading to autotyrosine phosphorylation of FAK and its activation (DeLise *et al.*, 2000). Activation of FAK may then lead to its interaction with Src homology (SH2) domains of Src or Fyn (Schaller *et al.*, 1994), or it can activate PI3K (Chen *et al.*, 1996) and regulate the mitogen activated protein kinases (MAPK), extracellular signal related kinase (ERK) and Jun N-terminal kinase (JNK) (Vuori *et al.*, 1996; Schlaepfer *et al.*, 1997). These pathways influence condensation and chondrogenesis by regulating gene transcription of ECM proteins. However, direct integrin signalling can take place via regulation of ILK. It has been suggested that when fibronectin binds to the integrin receptor, ILK serine/threonine kinase is inhibited (Hannigan *et al.*, 1996). ILK is known to inhibit GSK3 β , a component of the canonical Wnt pathway. This inhibitory effect of ILK takes place via a PI3K dependent mechanism (Delcommenne *et al.*, 1998). It has been speculated that Wnt growth factors may play significant roles during chondrogenesis. Thus, ILK activity, which is generated during fibronectin matrix assembly, may be a link between these secretory growth factors and the ECM (DeLise *et al.*, 2000)

A number of molecular signals control the initial patterning of the appendicular skeleton prior to mesenchymal differentiation (Zakany and Duboule, 1999; Hartmann and Tabin, 2001). The Wnt family of proteins are involved in regulating various aspects of chondrogenesis and limb development. Initial steps in chondrogenesis are promoted by Wnt-5a and -5b but not by Wnt 4 and -11 (Church *et al.*, 2002b). The

initiation of chondrogenesis is modulated by the effects of Wnt signalling on cell proliferation and/or adhesion. The expression of cell adhesion molecules such as NCAM, N-cadherin and β 1 integrin are increased and maintained by Wnt-1 and Wnt-7a (Stott *et al.*, 1999; Tufan and Tuan, 2001; Church *et al.*, 2002b). Wnt-5a does not seem to alter cell adhesion, and its overexpression in undifferentiated limb mesenchymal cells does not significantly affect cell proliferation (Oberlender and Tuan, 1994; Tufan and Tuan 2001; Yamaguchi *et al.*, 1999). Wnt 4 and Wnt 5b have similar functional characteristics to Wnt 5a in these cells.

Wnt-1 and -7a block chondrogenesis in vitro and in vivo (Rudnicki and Brown, 1997; Stott *et al.*, 1999; Tufan and Tuan, 2001; Church *et al.*, 2002 b). Wnt 4, -5a, -5b and -14 are expressed in the developing skeleton and exhibit distinct roles (Kawakami *et al.*, 1999; Yamaguchi *et al.*, 1999; Hartman and Tabin 2000 and 2001; Church *et al.*, 2002 b); Wnt-4 is expressed in the developing joint without having any role in the process of joint development itself. Its role is rather to promote chondrocyte maturation in adjacent cartilage (Hartmann and Tabin, 2000; Hartmann and Tabin, 2001). Wnt-4 accelerates the hypertrophic response in developing joints whilst Wnt 5a delays hypertrophy of the perichondrium (Kawakami *et al.*, 1999; Hartmann and Tabin, 2000; Church *et al.*, 2002 b). Wnt 4, -5a and -5b also influence changes in matrix molecule production and degradation during chondrogenesis. Wnt 4 modulates the rate of chondrocyte differentiation in vivo and in vitro (Hartmann and Tabin 2000; Church *et al.*, 2002b). In addition, Wnt- 4 signalling influences cartilage development in the joint (Hartmann and Tabin, 2000). Wnt-14 is expressed in the inter-zone of the developing joint mainly during the initial stages of joint development and plays a

crucial role in the formation of the synovium (Hartmann and Tabin, 2001; Church *et al* 2002b).

It has also been suggested that Wnt proteins exhibit a negative regulatory role on chondrogenesis via an undefined cellular mechanism (Hartmann and Tabin, 2001).

Chondrogenesis of mesenchymal stem cells may be initiated by Wnt 5a through PKC activation (Chang *et al.*, 1998; Yang *et al.*, 1998; Church *et al.*, 2002b). Wnt 4 signalling is mediated through β -catenin (Hartmann and Tabin, 2000) (**Table 2**).

Wnt family of proteins	Role of Wnts in skeletal development
Wnt-1	Blocks chondrogenesis in vivo and/or in vitro.
Wnt-4	Accelerates hypertrophy in developing joints. Affects changes in matrix molecule production and degradation during chondrogenesis. Modulates the rate of chondrocyte differentiation in vivo and/or in vitro. Regulates the development of cartilage elements.
Wnt-5a	Delays hypertrophy in the perichondrium.. Initiation of chondrogenesis. Affects changes in matrix molecule production and degradation during chondrogenesis.
Wnt-5b	Initiation of chondrogenesis. Affects changes in matrix molecule production and degradation during chondrogenesis.
Wnt-7a	Blocks chondrogenesis in vivo and/or in vitro.
Wnt-14	Crucial role in the initiation of formation of the synovium

Table 2: The role of the Wnt family proteins in skeletal development

1.8 Chondrogenesis and Wnt antagonists

The Wnt antagonist, Fzrp-1 is highly expressed during the early stages of chondrogenesis and at the epiphyseal and long bone anlagen (Hoang *et al.*, 1996). Variation in Fzrp-1 expression occurs during mesenchymal prechondrogenic condensation where Wnt expression is usually absent (Baranski *et al.*, 2000; Duprez *et al.*, 1999; Hoang *et al.*, 1996; Wada *et al.*, 1999). The modulation of Wnt signals by Fzrp-1 occurs at specific sites and times during skeletogenesis and may be involved in articular chondrogenesis (Enomoto- Iwamoto *et al.*, 2002). The overexpression of Fzrp-1 in chondrocytes in culture results in an increase in phenotypically immature chondrocytes and inhibits the development of mature cells. Chondrocytes overexpressing Fzrp-1 in culture are rounded with a large amount of pericellular proteoglycan-rich matrix. They have very little alkaline phosphatase activity, and barely secrete metalloproteases (Enomoto- Iwamoto *et al.*, 2002). Hence, the initial role for Fzrp-1 in limb chondrocytes is to regulate the maturation of these cells and limit their growth as they progress to mature cells. In addition, the behaviour and function of chondrocytes in different parts of the limb can be altered by Fzrp-1 (Enomoto- Iwamoto *et al.*, 2002).

1.9 Immortalised human chondrocytes cell lines

Chondrocytes are terminally differentiated cells and the only cellular component of adult articular cartilage. Chondrocytes synthesise cartilage matrix proteins as well as matrix degrading enzymes. As the capacity of mature chondrocytes to repair adult

articular cartilage is very limited, degenerative joint diseases such as osteoarthritis (OA) are characterised by progressive loss of articular cartilage.

In order to study the synthesis of cartilage matrix proteins and their degradation by proteolytic enzymes, primary cultures of human chondrocytes in monolayer culture have been widely used (Goldring, 1993). In such culture systems, chondrocytes preserve the ability to synthesise some specific cartilage matrix molecules such as collagens type II, IX and XI and the proteoglycan aggrecan. Unfortunately, following prolonged primary culture or subculture, the chondrocytes dedifferentiate and the synthesis of type II collagen is replaced by type I and III collagens (Goldring *et al.*, 1986, 1987, 1988). A number of approaches have been used in order to develop a chondrocyte cell line which is stable and able to express chondrocyte specific markers. Several immortalised chondrocyte cell lines have been produced by introduction of viral genes into nonhuman chondrocytes that result in cell lines that have high proliferative capacity whilst maintaining some characteristics of differentiated chondrocytes (Alema *et al.*, 1985; Gionti *et al.*, 1985; Horton *et al.*, 1988; Thenet *et al.*, 1992; Mallein-Gerin and Olsen., 1993).

Not all cell lines maintain the ability to produce type II collagen, although they all retain the ability to produce sulphated proteoglycans.

When simian virus 40 (SV40) large T antigen (TAg) was introduced into human articular chondrocytes, it resulted in the production of clonal lines which synthesised type I and III collagens but not type II in monolayer cell culture (Benoit *et al.*, 1995). By contrast, mouse chondrocytes are more easily immortalised and maintain a capacity to synthesise collagen type II when SV40-TAg is introduced in vitro

(Mallein-Gerin and Olsen., 1993) or in vivo (Mataga *et al.*, 1996).

The development of immortalised human chondrocyte cell lines which can be used as models for studying cartilage anabolic and catabolic responses and which maintain their differentiated phenotype is critically dependent on the tissue source and the stage of development of the cartilage from which the chondrocytes are taken.

In general, the proliferative capacity of the cell culture is maintained by expression of the SV40-Tag. This is responsible for inhibiting the p53 and RB proteins, the function of which is to maintain quiescence by hindering cell cycle progression through G1 (Fanning and Knippers., 1992). Even within a single cell population, immortalisation with SV-40 may be a consequence of more than one genetic mechanism (Duncan *et al.*, 1993). The phenotypic characteristics of immortalised cell lines are not always stable or identical. The production and secretion of matrix proteins in immortalised proliferating fibroblast monolayer cultures are greatly decreased (Rowe *et al.*, 1978; Berman and Morozevich., 1990) and SV40 immortalised cells from other sources dedifferentiate after prolonged culture (Woodworth *et al.*, 1988).

SV40-TAg-immortalised human chondrocytes are not transfected with oncogenes such as src and ras, which are required for transformation. These oncogenes have significant suppressive effects on normal collagen gene expression in both fibroblasts and chondrocytes and do not induce tumors in nude mice (Goldring *et al.*, 1994). In addition to their primary mitogenic effects immortalising components such as myc and SV40-Tag influence chondrocyte phenotype. It is therefore essential to promote culture conditions that will induce and maintain the differentiated chondrocyte

phenotype (Alema *et al* 1985; Iwamoto *et al.*, 1993). Recently, several immortalised human chondrocyte cell lines such as C20A4, T/C28I2 and T/C28a2 have been developed (Goldring *et al.*, 1994). C20A4 and T/C28I2 were derived from the costal cartilage of a 5 year old boy and a 15 year old girl respectively, using SV40-TAg (Goldring *et al.*, 1994). These cells have a proliferative phenotype as a result of continuous expression of TAg and disruption of the normal cell cycle. Endogenous synthetic activities, including matrix production, are reduced. In monolayer cultures that are serum starved or in suspension culture the rate of cell proliferation declines and the synthesis and deposition of the matrix is increased.

Proliferative stable immortalised human chondrocytes (C20/A4 and T/C28I2) express cartilage specific markers of differentiated chondrocytes under defined conditions in DMEM culture medium containing 10% FCS. C20/A4 and T/C28I2 produce the cartilage specific mRNAs encoding aggrecan, collagen types II, IX and XI, link protein and the small proteoglycans biglycans and decorin (Goldring *et al.*, 1994). In addition, very low levels of collagen type I are expressed by C20/A4 chondrocyte cell lines. These qualities would seem to make C20/A4 a good cell line for chondrocyte studies where primary tissue is difficult to obtain.

Immortalised chondrocyte cell lines are much easier to grow and maintain in culture when compared with primary chondrocyte cell cultures. In addition, C20/A4 and T/C28I2 chondrocyte cell lines maintain stable cartilage specific characteristics for a longer period. These cell lines have been shown to be useful models for studying a number of aspects of chondrocyte growth, differentiation and metabolism although

viral transformation of the chondrocytes can be associated with changes in phenotypic expression of some genes when compared with primary chondrocytes in culture.

The expression of Wnt pathway components has not previously been examined in virally transformed chondrocyte cell lines, but other cell lines have been identified as a well established tool for studying the role of Wnt pathway in number of tissues. The Fz family of receptors were found to be expressed in human embryonic stem (ES) cell lines (Sperger *et al.*, 2003). Fz receptors, which are an integral component of the Wnt/ β catenin pathway are known to delay and modulate the differentiation of ES cells in the mouse. Ricken et al., (2002) have shown expression of Wnt-2a in ovarian cancer cell lines, suggesting that the Wnt pathway may play some role in the development and function of this tissue

The chondrocyte cell lines, C20/A4 and T/C28I2, which have been used in this study, maintain the characteristic of primary chondrocytes the ability such as producing type II collagen and sulphated proteoglycans upto passage 22. In addition the mechanotransduction pathway that is mediated via $\alpha 5\beta 1$ integrin mechanoreceptor and leads to phosphorylation of β catenin, is present in C20A4 chondrocyte cell lines. Thus these cell lines are reliable model system for further studies undertaken in this thesis.

1.10 Aims of the Project

β catenin is known as an integral component of mechanotransduction and of the canonical Wnt pathway. However, its role in chondrocytes has not been identified. In this thesis, I have examined whether an interaction between mechanotransduction and Wnt pathways is required for regulation of the cytoplasmic/ nuclear distribution of β catenin. Initially, experiments were undertaken to determine whether components of the Wnt signalling pathway are present in human chondrocyte cell lines in culture. Next looked for evidence for an association of β -catenin with GSK3 β in resting cells and following mechanical stimulation. Subsequently, I studied the effects of mechanical stimulation on GSK3 β kinase activity, in order to ascertain whether Wnt signalling pathways are involved in chondrocyte mechanotransduction.

Chapter 2

Materials and Methods

All cell culture was carried out in a culture hood under sterile conditions.

All the equipment and materials, used during each experiment are listed in the Appendix I, II and III.

2.1 Cell Culture of Immortalised Human Chondrocyte Cell lines

C-20/A4 and T/C-28I2.

The immortalised human chondrocyte lines C-20/A4 and T/C-28I2, provided by Dr M. Goldring (Harvard Medical school, Boston, MA), were derived from juvenile costal cartilage from a 5-yr-old and 15-yr-old male respectively.

(Goldring et al. 1994).

C-20/A4 and T/C 28I2 (passage number between 5-20) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Foetal Calf serum (FCS) containing 100 U/ml penicillin and 100 mg/ml streptomycin in 75 cm³ cell culture flasks (Nunc). Cells were cultured for 3 days at 37⁰C in order to grow to 90% confluency. Cells were washed twice with PBS and harvested by trypsinisation with 1 ml trypsin/EDTA (1x) for 5 min. Trypsin was washed out in 10 ml of PBS and cells were pelleted by centrifugation at 700 g for 5 min. After removal of supernatant, the pellet was resuspended in DMEM /10%FCS media as appropriate. For experimental purposes, the number of cells in 1 ml of suspension was counted using a Neubauer haemocytometer and cells were seeded into 58 mm cell culture dishes (Nunc) or 24 well plates (Nunc) at a density of 4 x 10⁵ cells/ ml/plate or 0.5 x 10⁵ cells/ml/well respectively. A stock of cells was maintained for future experiments by transferring

1 ml of the cell suspension into a 75 cm³ flask containing 25 ml of media. Cells were incubated at 37⁰C and grown till confluent.

2.2 Immunofluorescence

2.2.1 Single Immunofluorescent Labelling of the C20/A4 and T/C-28I2

Chondrocyte Cell lines

C-20/A4 and T/C 28I2 (passage number between 5-20) were cultured and passaged as above. Cells were seeded onto 13mm² glass coverslips in 24 well plates at a density of 0.5×10^5 cells/ml/well and incubated at 37⁰C for a further 24 hours prior to experimentation. Cells were initially washed three times with PBS at 37⁰C and then fixed with warm (37⁰C) 4% paraformaldehyde for 5 min. The fixative was removed and the cells washed twice with PBS. For studying the intracellular proteins, cells were permeabilised with 0.1% Triton-X 100 in PBS for 4 min at room temperature. Non-specific binding was blocked by incubating the cells in 10% serum from the animal that the secondary antibody was raised in for 30 min. Excess serum was removed and cells were incubated with 1 µg/ml of primary antibody in a humidified chamber at 4⁰C overnight. The following day cells were washed 3 times with PBS, each for 5 min. An appropriate secondary antibody conjugated to fluorescein, (FITC, TRITC or Texas Red) was added to the wells and left in the dark for 45min. Cells were washed three times with PBS, each for 10 min, the PBS was removed and the cells were mounted in Vectorshield mounting medium. The edges of the coverslip were cleaned carefully with filter paper and sealed with clear nail varnish. The cells were examined with a Nikon fluorescence microscope.

As a negative control for each immunolabelling study, the primary antibody was omitted.

2.2.2 Double Immunofluorescent Labelling of the C20/A4 and T/C-28I2

Chondrocyte Cell lines for Colocalization studies by Confocal Microscopy

For double labelling and confocal studies, the first primary antibody was detected using a FITC-conjugated secondary antibody after incubation at room temperature for 45min in the dark. Cells were washed three times with PBS, each for 10 min, and incubated with a second primary antibody in a humified chamber at 4⁰C overnight. The following day cells were washed 3 times with PBS, each for 5 min. The second primary antibody was detected by a Texas red or TRITC-conjugated secondary antibody after incubation at room temperature for 45min in dark. Cells were washed three times with PBS, each for 10 min and mounted in a Vectorshield mounting medium containing DAPI.

Cells were examined with a Nikon confocal scanning microscope.

2.2.3 Triple Immunofluorescent Labelling of the C-20/A4 and T/C-28I2

Chondrocyte Cell lines for Colocalization studies by Confocal Microscopy

C-20/A4 and T/C 28I2 (passage number between 5-20) were cultured and fixed as described previously.

For the colocalisation studies non specific binding was blocked by incubating the cells in 20% goat or rabbit serum for 30 min. The excess serum was removed and cells were incubated with 1 µg/ml of anti-CD44 or anti- FN in a humified chamber

overnight at 4⁰C. Cells were washed 3 times with PBS, each for 10 min. FN or CD44 were detected using Alexa Fluor 350 goat anti-Mouse secondary antibody diluted in 20% goat serum at room temperature for 45 min in dark. Cells were washed 3 times with PBS, each for 10 min, and blocked in 20% rabbit serum for 30 min. The excess serum was removed and cells were incubated with 1 µg/ml of anti-Frizzled for 2 hours in a humified chamber at 4⁰C. Cells were washed 3 times with PBS, each for 10 min, and Fz antibody was detected by Alexa Fluor 568 rabbit anti-goat secondary antibody diluted in 20% of rabbit serum after incubation at room temperature for 45 min in the dark. Cells were washed 3 times with PBS, each for 10 min and blocked in 20% donkey serum for 30 min. The excess serum was removed and cells incubated with 1 µg/ml of anti-Wnt-1 in a humified chamber at 4⁰C overnight. The following day, cells were washed 3 times with PBS, each for 10 min, and Wnt-1 antibody was detected by Alexa Fluor 488 donkey anti-goat secondary antibody diluted in 20% of donkey serum after incubation at room temperature for 45 min in the dark. Cells were washed three times with PBS, each for 10 min, and mounted in a Vectorshield mounting medium. Cells were examined with a Nikon confocal scanning Microscope.

2.3 Cyclical Mechanical Stimulation of Human T/C-28I2 Chondrocyte Cell line

T/C 28I2 (passage number between 5-20) cells were grown on flexible, plastic 58 mm cell culture petri dishes (Nunc) and placed on top of a rubber-O ring, in a sealed pressure chamber with inlet and outlet ports. Nitrogen gas was used for pressurising the chamber and the frequency of the pressurisation was adjusted by an electronic timer attached to the inlet and outlet valves. Cyclical pressurisation resulted in deformation of the base of the petri dish and induced strain on the adherent cells.

For all experiments, a pressure of 25 kPa above atmospheric pressure at a frequency of 0.33 Hz (2 seconds on, 1 second off) was used at 37°C. This regime introduced a cyclical mechanical strain of 30,740 micro-strain to the base of the dish and its adherent cells (**Fig 2.1.1** and **Fig 2.1.2**).

2.4 Determination of Protein Concentration

The protein concentration of the samples was estimated using the method of Bradford (1976).

The concentration of each sample was measured against the BSA standards, set up in duplicate with increasing concentration from 0 to 1350 µg/ml (0, 125, 250, 375, 500, 750, 1000, 1350 µg/ml). For each protein sample, two different dilutions (1:2 and 1:5) were set up in duplicate. All the standards and diluted protein samples were prepared in the same lysis buffer as the buffer in the original protein samples.

5 µl of standards and diluted samples were transferred into a 96 well microtiter plate. Into each well, 25 µl of Reagent A+S (BioRad) was added and incubated at room temperature for 5 min, and then mixed with 200 µl of reagent B (BioRad). The plate was agitated for 15 min at room temperature and the absorbance read on a spectrophotometer at 630 nm.



Fig 2.1.2: Pressurisation chambers with inlet and outlet ports

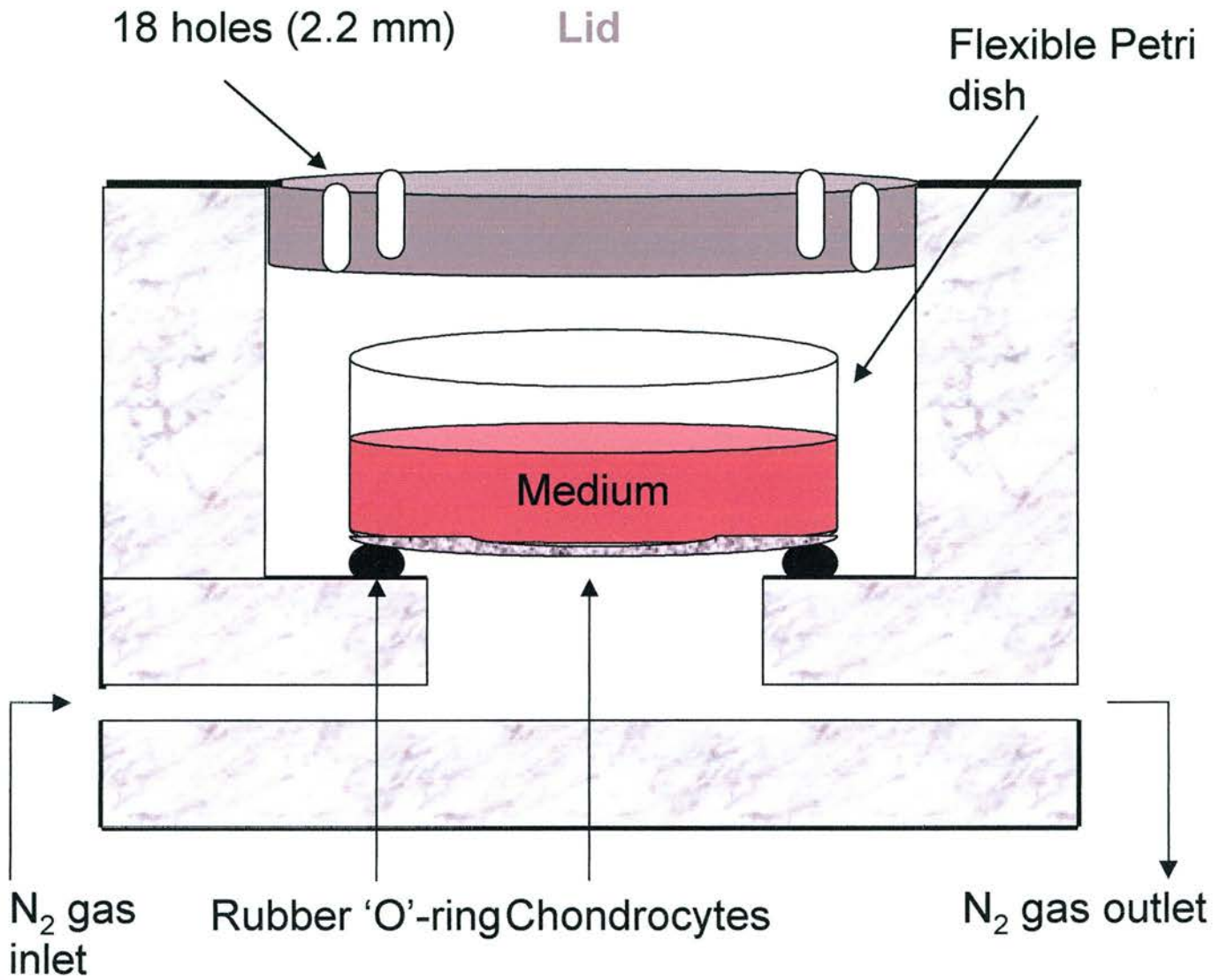


Fig 2.1.3: A pressurisation chamber used to apply strain to flexible tissue culture dishes with adherent chondrocytes.

2.5 Immunoprecipitation

Solutions are given in Appendix I

Prior to immunoprecipitation the cell lysate was precleared by incubation in a 50% (v/v) protein A-sepharose slurry with an equal volume of normal serum for 15 min at room temperature under constant rotation. Protein A-sepharose was sedimented by centrifugation at 15,000 g for 5 min. The resin containing bound IgG was resuspended in 1 ml of whole cell lysate and the mixture incubated either for 30 min at room temperature or for 2 hr at 4⁰C, with gentle agitation. The resin containing the non specifically bound proteins and any other insoluble materials was sedimented by centrifugation as above and the clear supernatant collected.

For the immunoprecipitation, 500 µg/ml of total protein in precleared lysate was collected and its volume adjusted to 1 ml with lysis buffer. The protein samples were incubated with 2µg of antibody for 1 hour at 4⁰C under constant rotation. The immune-complex formed was incubated with 30µl of protein A-sepharose for 1 hour at 4⁰C under constant rotation. The antibody/protein mix was sedimented by centrifugation as above and washed 3-4 times with lysis buffer. The immune-complexes were eluted with 25 µl of 3x Bromophenol blue sample buffer and 25µl of 10% SDS, and then stored at -70⁰C until required. For western blot analysis, the samples were boiled for 5 min at 95⁰C in order to dissociate protein/ antibody complexes. The beads were then sedimented by centrifugation as above and the proteins in the supernatants were resolved by SDS-PAGE and subsequent immunoblot analysis.

2.6 Western Blotting

2.6.1 Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Solutions are given in Appendix I

Proteins were analysed by SDS-PAGE according to the method of Laemmli, 1970. Glass gel plates (12 cm x 10 cm and 12 cm x 9 cm) and spacers were cleaned with industrial methyl alcohol (IMS). The plates with spacers in between were assembled into the clamp and held in the casting stand. The 12% (w/v) resolving gel was prepared and poured in between the plates up to 4 cm from the top edge of the smaller plate. The gel was covered with water-saturated butan-1-ol, and left for 45 minutes to polymerise. When the gel was fully set the butan-1-ol was removed and the top layer of the gel washed with distilled water and carefully dried with filter paper. The stacking gel was prepared and poured on top of the resolving gel right to the edge of the smaller plate. A comb was placed in between the plates to make the sample wells, and gel left for 30 minutes to polymerise. The comb was removed after the gel had set and the plates placed in to the Bio Rad electrophoresis apparatus, which was filled to 1/3 of its volume with electrode buffer. The central compartments of the gels were also filled to the top with electrode buffer. Prior to loading, the protein samples were mixed with 3 x sample buffer and boiled for 5 minutes. The samples were electrophoresed at 75 V through the stacking gel and 150 V through the resolving gel. The electrophoresis was terminated when the dye front was about 0.5 cm from the bottom of the resolving gel.

2.6.2 Electrophoretic Transfer of Proteins to Nitrocellulose Membranes

Prior to electrophoretic transfer of the proteins, nitrocellulose membranes were soaked in 100% methanol for 15 minutes at room temperature on a rocker plate. For each gel, two fiber pads together with four pieces of filter paper were soaked in transfer buffer.

After running the gels, the plates were removed from the apparatus. Gels were separated from the glass plates and placed onto the filter papers. The membrane was removed from the methanol and equilibrated in transfer buffer for 5 min. To ensure the correct orientation of the membrane, its top right corner was cut off before placing the membrane on the gel. The pad, filter, gel and membrane sandwich was assembled into the transfer cassette and placed in the tank according to the manufacturer's instructions (gel facing the anode panel and membrane facing the cathode panel). The tank was filled to the top with buffer and the transfer was run at 30 V at 4⁰C overnight. The following morning the voltage was increased to 60V for 1 hour before termination of electrophoresis.

2.6.3 Immunoblotting

After transfer, the membrane was washed 3-6 times in 0.1% TBST, each for 5 min and then incubated in 2% BSA blocking solution for 1 hour at room temperature with gentle agitation. The membrane was washed as above and incubated with primary antibody in TBST at 4⁰C overnight with gentle agitation. The membrane was again washed as above and incubated with HRP-secondary antibody in TBST for 1 hour at room temperature with gentle agitation.

Optimal antibody concentration was determined according to the supplier's recommendation or experimentally. The volume of solutions was sufficient to prevent the membrane from drying at any time. Prior to developing, the membrane was washed with TBST as above.

2.6.4 Development of the Blot

The protein was detected using ECL plus (Amersham Bioscience) according to the manufacturer's instructions. One ml of solution A: solution B (40:1), was poured onto the membrane and left for 1 min. The excess liquid was drained off the membrane, which was laid flat between sheets of transparent cling film. The membrane was fixed in an autoradiography cassette and X-ray films were exposed for 20 and 40 seconds. The film was developed, washed, fixed and dried at 60⁰C in a drying cabinet. For clear visualisation of signals, the blot was re-exposed for the time required. The molecular weights of the examined proteins were determined by comparison with marker proteins of known size (protein ladder) electrophoresed on the same gel. When it was necessary to study more than one protein by immunoblotting, membranes were stripped and re-probed as required

2.6.5 Stripping the membranes

The stripping buffer (Appendix I) was freshly made in a fume hood prior to each stripping procedure.

The membrane was submerged in 30 ml of stripping buffer and incubated at 55⁰C for 30 min with gentle agitation. The buffer was removed by washing the membrane

twice in large volumes of TBST, each for 10 min, at room temperature under constant rotation. The membrane was then blocked with 2 % BSA/TBST for 1 hour at room temperature under constant rotation. Following this procedure, the membrane could be reprobbed with another set of antibodies and detected as described.

2.6.6 Staining and Drying SDS Polyacrylamide Gels

When the transfer was completed, gels were removed and stained with 0.25%(w/v) Brilliant Blue R in 40% methanol with 10% (v/v) Glacial Acetic acid and incubated at room temperature for 1 hour under constant rotation. Gels were fixed in Coumassie fix for 10 min and destained with 40% (v/v) methanol with 5% (v/v) Glacial Acetic acid and 10% (v/v) Glycerol and incubated at room temperature for 1 hour under constant rotation.

The appearance of defined bands on the gels indicated the presence of proteins.

2.7 Quantification of identified proteins and data analysis

Tyrosine and serine phosphorylated GSK3 β and GSK3 β in complex with β catenin were quantified by densitometric analysis of auto radiographs using a Bio-Rad VersaDoc scanning densitometer imaging system and Quantity 1, 1-D analysis software. For imaging and analysing the density of the bands on the blot, an image of each sample was captured and presented on the computer monitor. The area of interest was defined using the computer tool bars prior to data analysis. Densitometric data were expressed as intensity/mm². The, density of each band was divided by the density of the relevant β catenin band in order to give the ratio of protein expressed

under the experimental conditions. Each experiment was carried out twice and mean of the ratios are presented in chapter 4.

2.8 Kinase Assays

2.8.1 Extraction of Cytosolic Proteins

T/C- 28I2 cell lines were seeded at a density of 4×10^5 cells/cm² /ml in 58 mm culture Petri dishes (Nunc). Cells were cultured in DMEM /10% FCS and incubated at 37⁰C for three days. Confluent cultures were incubated in serum free media for 16 hours prior to experimentation.

Cells were treated with the PI3K (Phosphatidyl Inositol 3 Kinase) inhibitor, LY294002 (10 μ M in DMSO) or the Wnt agonist, LiCl (10mM in dH₂O) for 30 min prior to mechanical stimulation. Cells were then subjected to mechanical stimulation at a frequency of 0.33Hz and pressure pulses of 0.25 atmosphere for 20, 40 and 60 min. As a control for each time point, a dish was placed in the chamber without being subjected to mechanical stimulation.

After stimulation, cells were placed on ice and washed twice with ice-cold PBS. Whole cell lysates were obtained by incubating the cells with 500 μ l ice-cold lysis buffer for 15 min. To ensure that all cells had been removed from the base of the culture dish, the dishes were scraped with a cell scraper. Cell lysates were then pipetted from the dish in to microfuge tubes. The lysates were pelleted by centrifugation for 10 min at 15,000 g at 4⁰C, the supernatants collected and the pellets discarded. Total protein concentrations of the lysates were determined by the Bradford assay (1976).

2.8.2 GSK3 β kinase assay

A kinase Assay for GSK3 β was developed by procedure modified from Haq *et al.*, 2000.

The assay of glycogen synthase kinase 3 β (GSK3 β) is based on its ability to incorporate radioactive [32 P] phosphate from γ [32 P] ATP into specific peptide substrates such as glycogen synthase (GS).



The source of the GSK3 β to be assayed determines the nature of its substrate peptide. Phospho GS peptide-2 is the peptide that is usually used for GSK3 β assays. However, GS-2 is also substrate for other kinases, hence, the immunoprecipitation of GSK3 β prior to its assay is essential.

GSK3 β generally requires substrate “priming” phosphorylation. The priming phosphate is incorporated into GSK3 β substrate peptides such as GS-2 by *in vitro* incubation with a suitable kinase such as casein kinase 2 (CK2). Synthetic primed substrates can also be obtained.

Peptides lacking this “priming” phosphate are not substrates for GSK3 β . When serine, the “priming” phosphorylated residue has been substituted with an alanine residue, it can not be phosphorylated by GSK3 β . Such a peptide can therefore be used as negative control substrate. This substrate with an alanine residue is however phosphorylated by kinases other than GSK3 β .

2.8.3 GSK3 β immunoprecipitation and the kinase assay

In this study a synthetically primed GS-2 was used as a substrate. The concentration of the substrate at which the optimal enzymatic activity of GSK3 β occurs is 50 μ M.

Prior to the kinase assay, 500 μ g/ml of total protein in the whole cell lysate was used for immunoprecipitation. The volume of samples was adjusted to 1 ml with the same lysis buffer before incubating with anti-GSK3 β (Glycogen Synthase Kinase 3 β) monoclonal antibody for 2 hr at 4 $^{\circ}$ C under constant rotation. The immune-complexes were collected after incubation with protein G- sepharose beads on a rocker plate at 4 $^{\circ}$ C overnight. The protein G-sepharose was sedimented by centrifugation as above and washed 6 times with 1 ml kinase lysis buffer and three times with 1 ml of kinase assay buffer (Appendix I). Following the last wash the beads were pelleted by centrifugation as above and the buffer was carefully removed to the meniscus of the beads.

The kinase assay reaction was then carried out by incubating the beads with GS-2 (Glycogen synthase -2) (50 μ M), cold ATP (20 μ M) and γ [32 P] ATP (4000 cpm/pmol) for 20 min at 30 $^{\circ}$ C with vigorous agitation. Samples were then spotted onto 2.5 cm 2 pieces of P81 phosphocellulose papers. The unbound 32 P was dissociated from the P81 papers by washing 3 times, each for 5 min, in 500ml of 0.5% Phosphoric acid on a rocker plate. Finally the papers were immersed in acetone and dried thoroughly before scintillation counting for measurements of radioactivity.

As a blank, a P81 paper was spotted with the reaction mix alone. The radioactivity of each sample was estimated by subtracting the radioactivity incorporated into the blank from the radioactivity incorporated into the sample.

2.9 Statistics

The sample means were compared using a Student t-test for unpaired samples.

The null hypothesis that was under examination, assumed that the means are equal for the two samples.

2.10 Total RNA Isolation

Total RNA was isolated from T/C 28I₂ and C-20A4 cell lines using Trizol reagent (Gibco). The reagent was stored at 4⁰C and brought to the room temperature prior to use.

DMEM culture medium was removed from confluent flasks of cells and the cells washed twice with PBS. Following removal of the PBS, cells were incubated with 1.2 ml of Trizol under constant rotation at room temperature for 5 min. The cells were then scraped off and transferred to 1.5 ml sterile microfuge tube, vortexed for 1 min and incubated at room temperature for 5 min. 0.2 ml of chloroform was added to each sample, vortexed for 15 sec, and incubated at room temperature for 5 min. Samples were centrifuged at 15,000 g for 15 min at 4⁰C. This resulted in separation of an upper aqueous phase containing RNA and a lower organic phase.

The upper phase with RNA was carefully transferred to a 1.5 sterile microfuge tube without disturbing the interface. The RNA was precipitated with 500 µl of

isopropanol, incubated at 4⁰C for 10-20 min, and pelleted by centrifugation at 15,000 g for 10 min at 4⁰C. The supernatant was carefully removed without disturbing the small RNA pellet, and the pellet was washed with 1 ml of 75% ethanol-DEPC (Diethyl pyrocarbonate) water, vortexed and centrifuged at 4625 g for 5 min at 4⁰C. The ethanol was carefully removed and the RNA was dried and resuspended in 50-100 µl of RNase free DEPC water.

The concentration and purity of RNA was estimated by measuring the A₂₆₀ and A₂₈₀ absorbance, using a Smart Spec 3000, BioRAD, spectrophotometer. A A₂₆₀ : A₂₈₀ ratio between 1.6- 2.0 indicated that the total RNA quality/purity was satisfactory for experimental purposes.

2.10.1 DNase Treatment of the RNA

To avoid any possible genomic contamination of the isolated RNA, each sample was DNase treated prior to first strand cDNA synthesis.

DNase reactions were carried out with 1-5 µg of total RNA in sterile 0.5 ml microfuge tubes. A maximum of 7 µl RNA was mixed with 1 µl of 10x DNase buffer, 1 U/µl DNase (Gibco), 40 U/µl RNase inhibitor (Gibco) and incubated at room temperature for 15 min. The reaction mix was heat inactivated at 70⁰C for 15 min. The DNase treated RNA was centrifuged for 30 sec and stored at -20⁰C till required for experimentation.

2.10.2 First Strand cDNA Synthesis

The whole procedure was carried out on ice.

This procedure was designed to convert 0.5-1.0 µg of DNase treated RNA into first strand cDNA.

The RNA/primer reaction mix was prepared in a sterile 0.5 ml microfuge tube by mixing 40 U/µl RNase inhibitor, 1 µg oligo dT (Adv Biothec) with 0.5-1.0 µg RNA.

The total volume of the mixture was made up to 11 µl with DEPC water. It was incubated at 70⁰C for 10 min and then placed back on ice for 5 min. cDNA synthesis proceeded after incubating the mixture with 4 µl of reaction buffer, 1 mM DTT and 1mM dNTP mix, at 42⁰C for 2 min. Superscript II enzyme 200 U/µl (Gibco) was added to the mix and incubated at 42⁰C for 1 hour. cDNA synthesis was terminated by heat inactivation at 70⁰C for 10 min. The mixture containing cDNA was then centrifuged and stored at -20⁰C till required for RT-PCR.

2.10.3 Reverse Transcriptase Polymerase Chain Reaction

Reverse transcribed cDNA was amplified by PCR. Each PCR reaction was carried out in a total volume of 50 µl containing 5 µl of cDNA, 5 µl of 10x PCR buffer, 1 µl of 10 mM dNTP mix and 3 µl of 25 mM MgCl₂. For each pair of primers (forward and reverse), 2 µl of 5 µM primer was added to the mix and its total volume made up to 49.5 µl with nuclease free water.

Just before the initiation of PCR, 0.5 µl of 10 U/ µl Taq Gold DNA polymerase was added into the mixture.

As a negative control, nuclease free water was substituted for the cDNA or the primers in the reaction mix.

The samples were briefly microfuged at 15,000 g, 4⁰C and placed in the thermocycler (PCR express, HYBAID).

A typical PCR cycling program was set as following

94 ⁰C for 3 min 1 cycle

94 ⁰C for 1.5 min
58-67 ⁰C for 1 min
72 ⁰C for 1 min } 30-45 cycles

72 ⁰C for 5 min 1 cycle

Then held at 4⁰C

Once the polymerisation program terminated, 2 µl of 0.25 % Orange-G dye was added to 20 µl of products and analysed by electrophoresis on 2% (w/v) TBE agarose minigels, run at a constant voltage of 80v for 1 hour. The gels were visualised by UV light, using Ethidium bromide (EtBr).

2.10.4 Agarose gel electrophoresis

To analyse the PCR products by electrophoresis , 2% gels was prepared by adding 2g of agarose (Gibco) to 100 ml of 1xTBE in a 200 ml conical flask and heating the mixture for 3 min at 90⁰C. The conical flask containing the molten agarose was cooled for 1 min under running tap water and then 5 µl of EtBr (10µg/ml) added and mixed thoroughly with the solution . The agarose mix was then poured into a gel

casting tray. To make the sample wells a comb was placed 2 cm from one edge of the tray. The gel was then left for 45 min at room temperature to polymerise.

2.11 Cloning

For cloning, PCR products of Frizzled-2 were generated with *Taq* polymerase, which provides them with 3' A-overhangs that increases their cloning efficiency in the TA cloning system (**Fig 2.2**).

2.11.1 Ligation

The ligation reaction was set up in a final volume of 20 μ l, in which 2 μ g of PCR product was ligated into 50 ng of pCR 2.1 vector (**Fig 2.3**). Each ligation reaction contained T4 DNA ligase (1U) in 1x ligation buffer (50 mM Tris. HCl, PH 7.4, 10 mM MgCl₂ 10 mM dithiothreitol and 1 mM ATP) and was incubated at 16 °C overnight. The control ligation reactions, in which either insert or vector was not added to the reaction mix, were carried out under the same conditions.

2.11.2 LB- Ampicillin Plates

Prior to transformation, sterile LB medium was prepared by autoclaving 2% agar medium for 15 min at 121°C 15 lb/ sq in. The medium was cooled to 55°C and 1 ml of 40 mg/ml Ampicillin and 40 mg/ml of X-Gal were added to 400 ml agar medium, before pouring into 10 cm Petri dishes. The agar was allowed to be set, then inverted and stored at 4°C.

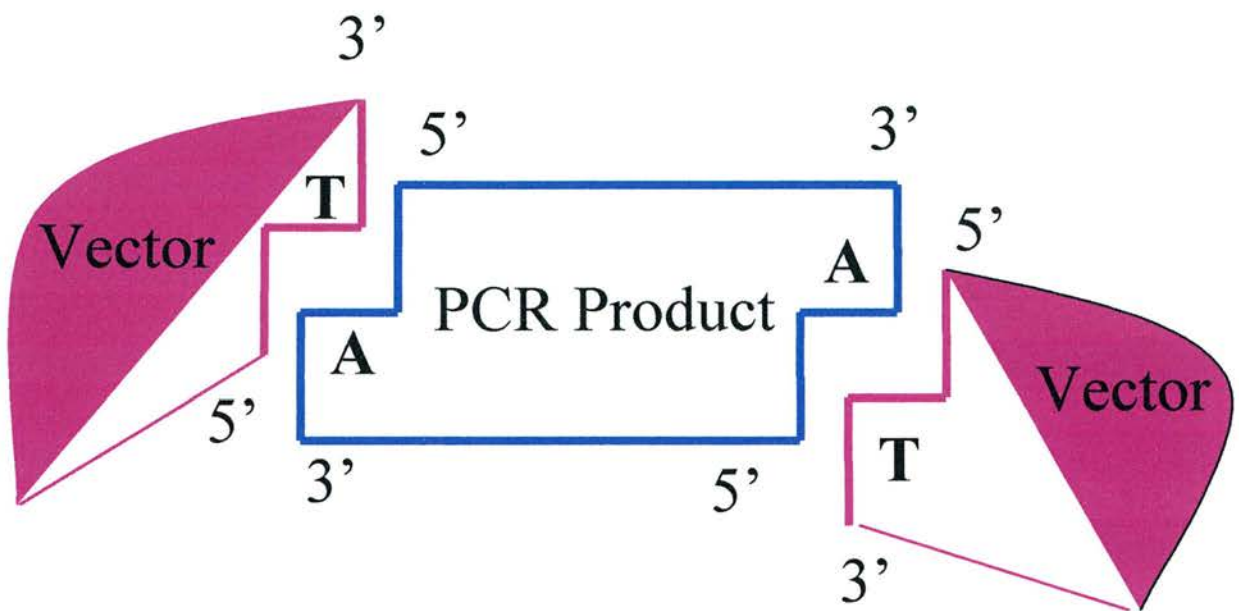
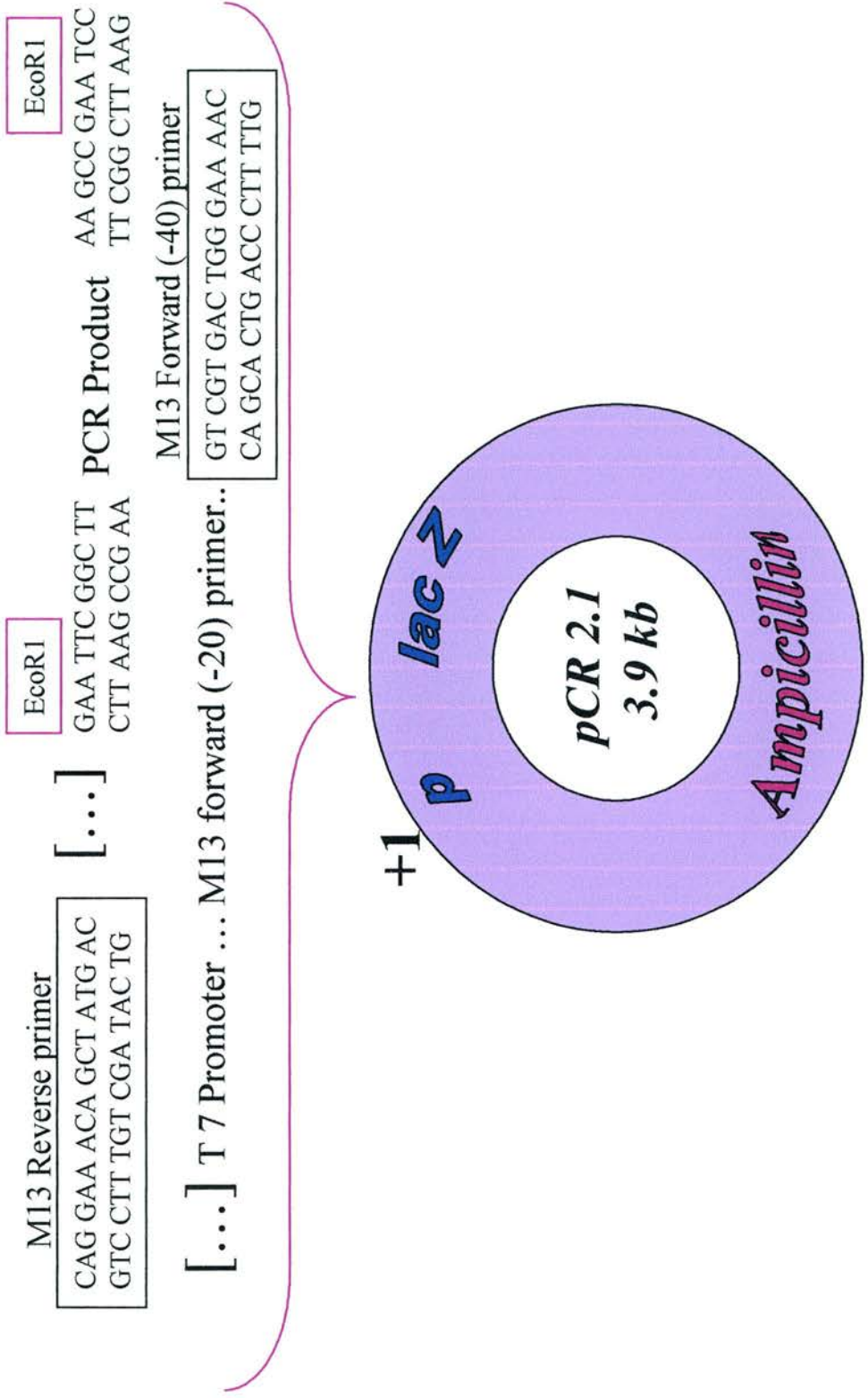


Fig 2.2: The concept behind the TA cloning method

Fig 2.3: pCR 2.1 Vector



2.9.3 Transformation

A single colony of *Escherichia. Coli XL2 (E.coli XL2)* was inoculated into 5 ml L-broth and incubated overnight at 37⁰C under constant rotation at 150 rpm. The following morning 1 ml of the culture was added to 200 ml sterile LB in a sterile flask and incubated at 37⁰C under constant rotation at 150-200 rpm. Once the culture reached an absorbance of 0.4-0.6 at 550 nm, the flask was placed on ice for 5 min. An aliquot of the bacterial culture (100 ml) was pelleted by centrifugation 1,500 g, at 4⁰C for 5 min and resuspended in 50 ml ice-cold 100 mM MgCl₂. The *E.coli* were pelleted and washed again in 100 mM MgCl₂ as above. The bacteria were pelleted again by centrifugation at 1,500 g at 4⁰C for 5 min, resuspended in 4 ml of ice cold 100 mM CaCl₂, and then incubated on ice for two hours.

The resulting competent *E.coli* was used for transformation with plasmid DNA. The transformation was carried out by mixing 10 µl of each ligation reaction with 110 µl of the competent bacteria. The vials were incubated on ice for 30 min, heat shocked for exactly 2 min at 42⁰C, and once more placed back on ice for a minimum of 15 min.

From each reaction, 100 µl of transformed *E.coli*, neat and at 1:10 dilution were spread onto separate LB-Amp agar plates. As a transformation control, 100 µl of neat competent *E.coli* were plated onto LB-Amp agar plates. Plates were incubated at 37⁰C overnight for colonies to grow.

2.9.4 Analysis of the Recombinants

To determine the presence and orientation of the insert, a single recombinant (white) colony was inoculated in to 5 ml LB containing 100 µg/ml ampicillin, and incubated

overnight at 37⁰C with agitation at 200 rpm. From the overnight cultures, 1.5 ml aliquots were transferred aseptically into sterile microfuge tubes and the *E.coli* were pelleted by centrifugation for 1 min at 15,000 g. The supernatant was removed carefully from the tube and the pellet was resuspended in 150 µl STET buffer (50 mM Tris.HCl pH 8.0, 8% sucrose (w/v), 5% Triton X-100 and 50mM EDTA), vortexing to ensure a complete suspension. Lysozyme (10 µl of 10 mg/ml fresh solution) was added to each suspension and incubated for a minimum of 10 min and a maximum of 15 min at room temperature. The lysozyme was then denatured by placing the sample in a heat block at 100⁰C, for precisely 40 s. The sample was centrifuged at 15,000 g for 10 min to pellet the debris and 120 µl of supernatant was immediately transferred into a fresh tube containing an equal volume of isopropanol and then left for 1 hour at -20⁰C. The DNA was pelleted by centrifugation at 15,000 g for 10 min and the isopropanol was removed. The pellet was washed in 500 µl of 70% ethanol, and then centrifuged as before and the ethanol was removed. A second wash using 100% ethanol was carried out after centrifugation once more at 15,000 g for 5 min. The supernatant was fully removed and the pellet was air dried by incubation at 37⁰C. Once dried, the DNA was resuspended in 16 µl of sterile water prior to analysis by restriction digestion.

2.9.5 Restriction Digests

In order to analyse the recombinant DNA in a precise manner type II restriction endonucleases were used.

A typical DNA digestion reaction was carried out in a total volume of 20 μ l. In a suitable reaction buffer, plasmid DNA (5 μ l) was incubated with 3-10 U/ μ g restriction enzyme (3-10 U/ μ g DNA) for 2 hr at 37⁰C (Appendix I).

After complete digestion of the DNA, the products were analysed on 1% agarose gels and visualised using Ethidium Bromide.

Chapter 3

Expression of components of the Wnt signalling pathway in SV-40 transformed human chondrocytes

Articular cartilage is composed of chondrocytes surrounded by a proteoglycan and collagen rich extracellular matrix.

The composition of the cartilage matrix and the effect of mechanical stimulation on maintaining the tissue integrity have been extensively studied.

Multiple intracellular signalling molecules and pathways are activated following mechanical signals imparted by stretch, pressure, tension, fluid flow, or shear stress in bone and cartilage (Wright *et al.*, 1996 and Millward-Sadler *et al.*, 1999). These include opening of stretch activated and calcium selective ion channels (Sachs, 1988), protein tyrosine phosphorylation (Yano *et al.*, 1996 and Lee *et al.*, 2000), inositol lipid metabolism (Prasad *et al.*, 1993) and activation of protein kinase C (PKC; Kimono *et al.*, 1996 and Lee *et al.*, 2002). Activation of these in turn affect the regulation of gene expression and protein synthesis that controls the maintenance of the tissue structure and function; e.g.; proteoglycan synthesis by chondrocytes (Veldhuijzen *et al.*, 1979 and Millward-Sadler *et al.*, 2000) ; and bone matrix synthesis by bone cells (Harter *et al.*, 1995).

Prior to this study, work published showed that mechanical stimulation of chondrocytes leads to the tyrosine phosphorylation of three major proteins, Focal Adhesion Kinase (FAK), β catenin and paxillin via $\alpha_5\beta_1$ integrin. This indicates a role for an integrin - β catenin signalling cascade in chondrocytes in response to mechanical stimulation (Lee *et al.*; 2000). The tyrosine phosphorylation of these

proteins was identified following mechanical stimulation of both primary chondrocyte cultures and C20/A4 chondrocyte cell line.

β catenin is also required for cell adhesion by direct association with cadherins in bone and cartilage (Monaghan *et al.*, 2001), and it is known as a key intracellular mediator of the Wnt signalling pathway (Huelsenken and Behrens., 2002).

Wnt is expressed in developing cartilage and is involved in chondrocyte maturation and differentiation (Church *et al.*, 2002b; Tufan and Tuan., 2001; Hartman and Tabin 2000 and 2001). However, little is known about Wnt in adult human articular cartilage. This study investigated whether components of the cadherin and Wnt signalling pathways are expressed in two chondrocyte cell lines, C20/A4 and T/C28I2.

3.1 Expression of cadherin, β catenin and Wnt-1 in chondrocyte cell lines.

T/C28 I 2 and C20 A4 cell lines were cultured as described in Section 2.1. The confluent cultures were labelled with anti- pan-cadherin, β catenin or Wnt-1; normal IgG was used as a negative control and CD44 as a positive control. The results are shown in **(Fig 3.1)(n>3)**. Pan-cadherin was expressed at the cell surface and cell-cell adhesions. β catenin was localized at the cell membrane, in the cytoplasm, and also in the nucleus. Wnt-1 was present as a continuous dotted line at the cell membrane. The same staining pattern was seen in both same lines.

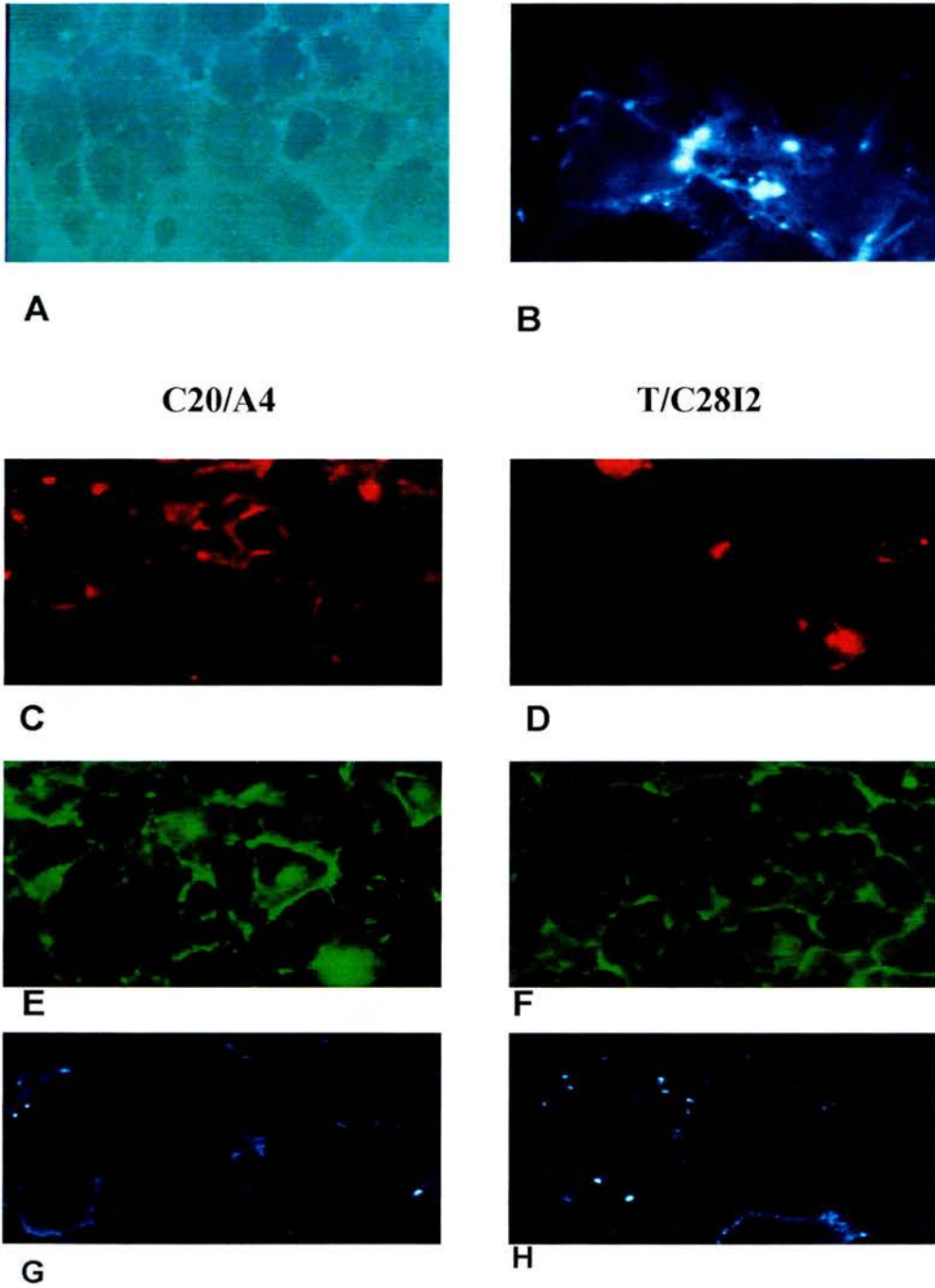


Fig 3.1: Immunofluorescence staining of C20/A4 and T/C28I2 chondrocyte cell lines .
 Controls: normal IgG as a negative control (A); CD44 as a positive control (B).
 C20/A4 cells stained for Pan-cadherin (C), β catenin (E) and Wnt-1 (G).
 T/C28I2 cells stained for Pan-cadherin (D), β catenin (F) and Wnt-1 (H).

3.2 Expression of Frizzled-2(Fz-2) by RT-PCR and cloning

Frizzled proteins are a family of ten mammalian 7TMS receptors with an extracellular Wnt-binding domain. This family of proteins share 70-90% homology in their genomic sequences. Primers were designed to the conserved regions of the Fz proteins and PCR was performed, amplifying a product of 362 bp corresponding to position 1243-1585 on human Fz mRNA sequence. The PCR products were then ligated into pCR 2.1 vector and transformed into the competent *E.coli* XL-2 cells. The recombinant white colonies were selected on LB-Amp agar plates, the DNA extracted and analysed by restriction digest using *EcoR*-1 and *Pst*-1 endonucleases. The products were then subjected to 1% agarose gels and visualised using EtBr (**Fig 3.2**) (PCR was performed more than three times and cloning was carried out once). The restriction enzyme, *EcoR*-1 cleaved the plasmid DNA on either side of the inserted PCR product, resulting in two distinct bands on the gel of 0.362 kb and 3.9 kb. These corresponded to the predicted size for vector 3.9 kb and an insert 0.362 kb which did not contain an internal *EcoR*-1 site. Digestion with *Pst*-1 resulted in a single band of 4.262 kb. As *Pst*-1 has no site on the plasmid DNA, the insert must contain a single *Pst*-1 site. Fz-2 is the only Fz receptor identified to date containing no *EcoR*-1 site and one *Pst*-1 site in the region amplified. In order to confirm that the 0.362 kb band definitely corresponds to Fz-2, the cloned inserts were gel purified and sequenced using Fz-2 primers. The sequence obtained was identical to the published sequence for human Fz-2 from the Genbank database (**Fig 3.3.1, Fig 3.3.2i and Fig3.3.2ii, Appendix IV**).

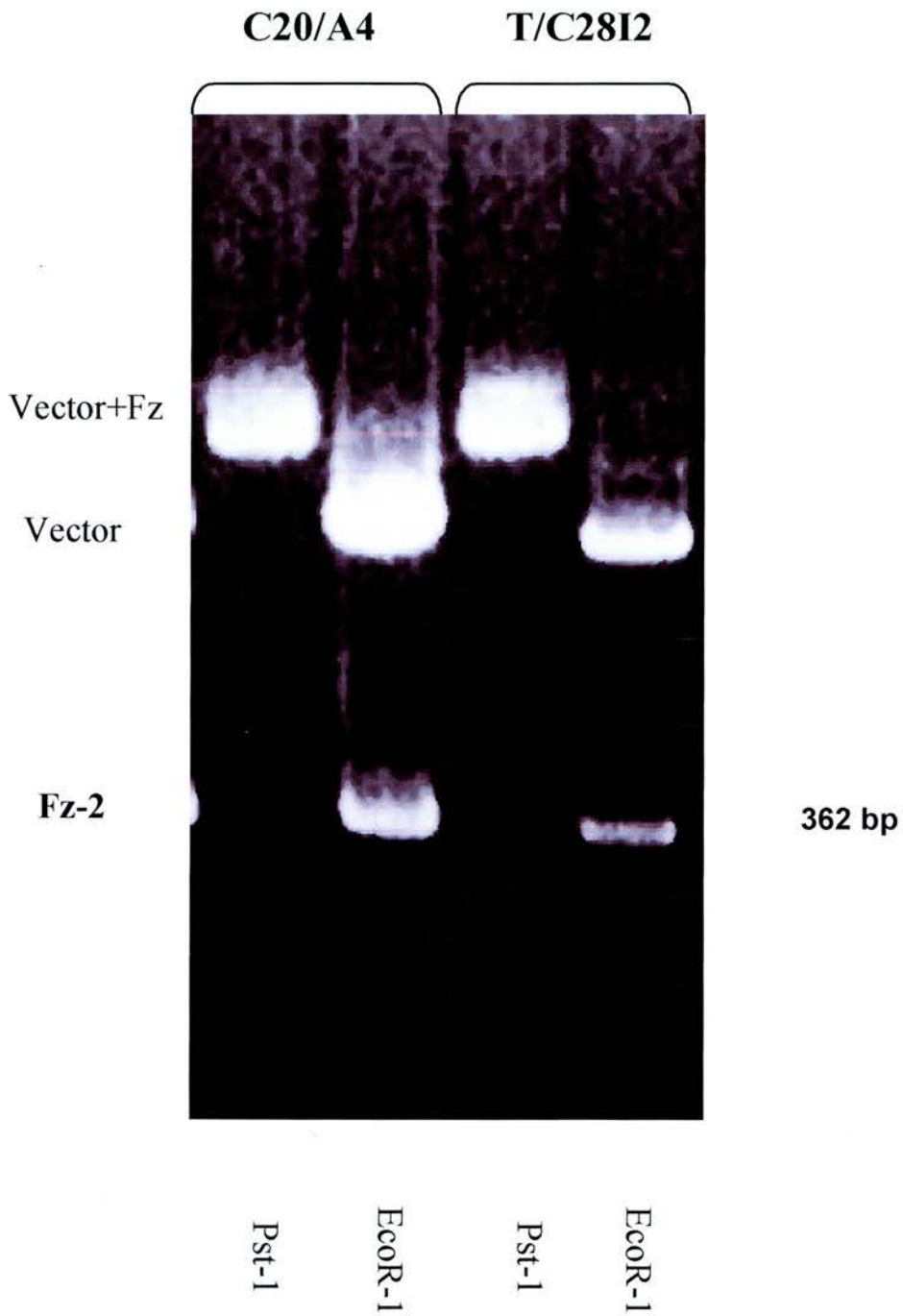


Fig 3.2: RT-PCR and cloning revealed the expression of Fz-2 in C20/A4 and T/C28I2 chondrocyte cell lines.

3.3 Expression of Frizzled related family of protein (Frzp) by RT-PCR

Recently, the Frizzled related family of proteins have been identified. These proteins share the homology domain at the N-terminus of Fz family of receptors, but lack the 7TMS sequences that are involved in Fz. Frzp are secreted and act as inhibitory proteins for Wnt signalling (Dale., 1998).

To identify whether Frzp are expressed in these chondrocyte cell lines, PCR was performed using primer sequences corresponding to position 1004-1023 and 1369-1390 on human Frzp mRNA sequence . The product was then subjected to 2% agarose gel electrophoresis and the size of the Frzp was determined by comparing its molecular weight with the standard DNA ladder (λ DNA *Hind* III digest) of known molecular weight. A product of 387bp in size was amplified, as predicted from the mRNA sequence (**Fig 3.4**)($n>3$). This product was then gel purified and sequenced using Frzp primers. The sequence obtained was identical to the published sequence for the human Frzp (**Fig 3.5.1** and **Fig 3.5.2, Appendix IV**).

3.4 Wnt receptors and coreceptors in chondrocytes

Frizzled proteins are seven-transmembrane (7TM) proteins with a cysteine rich domain (CRD) that act as receptors for Wnt proteins (Sen *et al.*, 2000).

It has been long known that Fz proteins are required as Wnt receptors for transmitting the signals intracellularly, but are not sufficient on their own. Coreceptors are required for the Wnt-Fz binding to be completely functional. Sulphated proteoglycans such as CD44 and fibronectin possibly act as coreceptors for Wnt signalling (Reichsman. *et al.*, 1996).

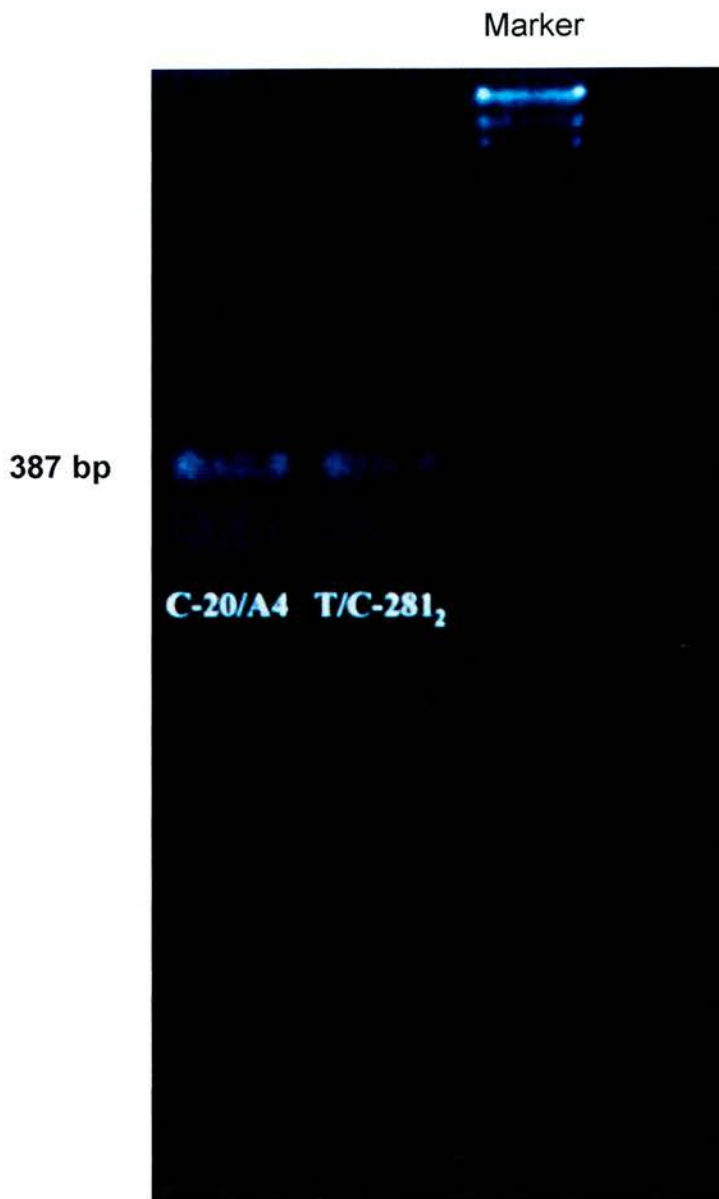


Fig 3.4: RT-PCR identified the expression of Fzrp in C20/A4 and T/C2812 chondrocyte cell lines.

To examine the hypothesis that CD44 or FN could be functioning as coreceptors for Fz receptor proteins, colocalisation studies were carried out. Chondrocyte cell lines were cultured and incubated sequentially with antibodies to CD44 or FN, Wnt-1 and Fz-1 as described in Section 2.2.3 followed by incubation with appropriate fluorescent labelled secondaries. The resultant staining was visualised using confocal microscopy. The results are shown in **(Fig 3.6)(n>3)**, Wnt-1 and Fz-1 proteins colocalise with both CD44 and FN at the cell surface and the cell-cell adhesion sites. This supports a possible role for CD44 and FN as coreceptors. Whether these interactions form functional complexes for transducing the Wnt signals intracellularly, will be a topic for further investigation.

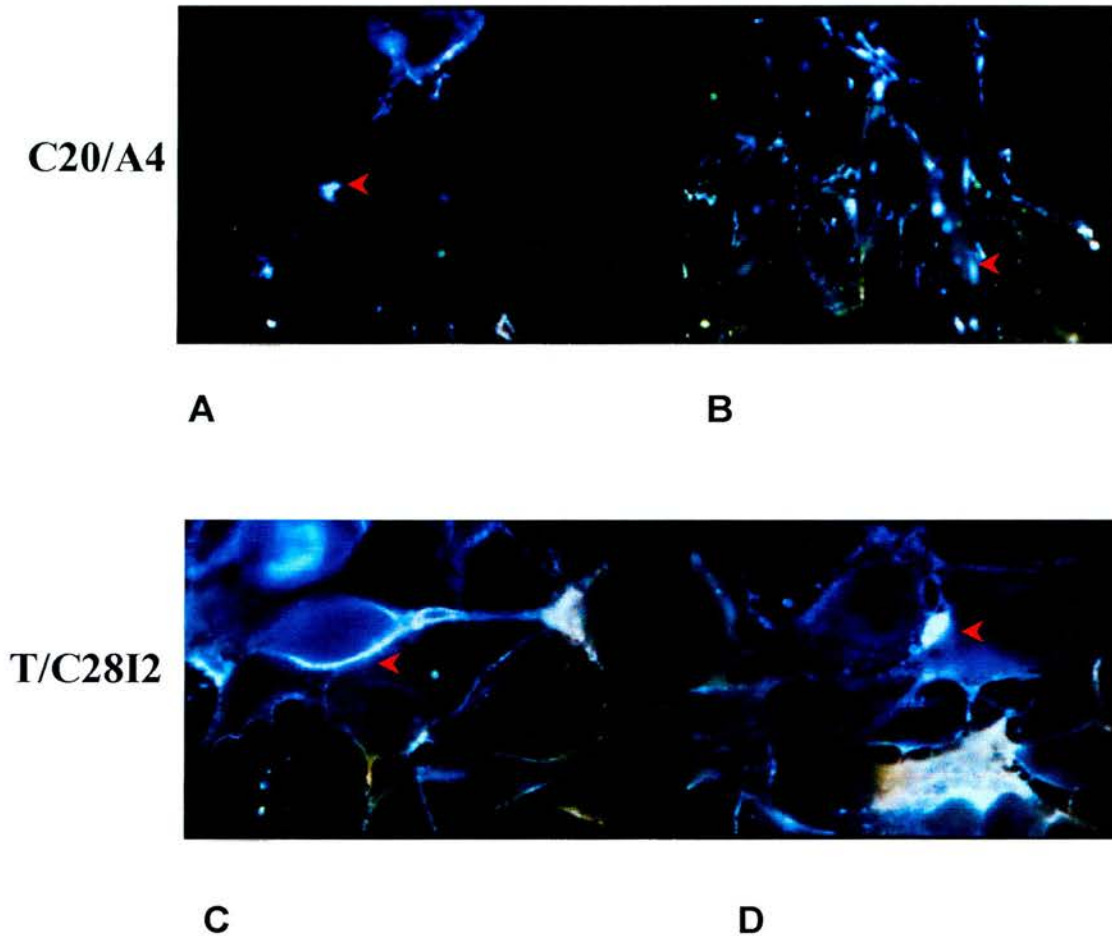


Fig 3.6: Confocal studies in c20/A4 and T/C28I2 chondrocyte cell lines. The secondary antibodies Alexa 350, Alexa 488 and Alexa 568 producing blue, green and red colours were used against CD44 or FN, Wnt-1 and Fz respectively. The combination of these colours at colocalised areas produce white colour (red arrows). Cells were viewed at magnification x 63. Colocalisation of Wnt-1, Fz and CD44 in C20/A4 (A) and T/C28I2 (C). Colocalisation of Wnt-1, Fz and FN in C20/A4 (B) and T/C28I2 (D).

Due to a technical problem the individual staining of Wnt-1, FZ, CD44 and FN could not be presented. In addition as there was a few changes in image resolution between software and platform, the scale bar could not be recorded.

3.5 Discussion

In these studies the expression of components of the Wnt signalling pathway were investigated.

The immunofluorescence studies have shown cell membrane, cytoplasmic and nuclear expression of β catenin in the chondrocytes. Association of β catenin with cadherins has been shown to be required for linking cadherin to the underlying cytoskeleton and conferring adhesiveness to the cells in a number of cell types (Sadot *et al.*, 1998). This might be crucial in OA cartilage where chondrocyte clustering is a feature.

β catenin is also a component of the Wnt pathway. Cytoplasmic distribution and nuclear localization of this protein in these chondrocyte cell lines indicates the possibility of Wnt signalling in these cells. However, β catenin is also associated with the integrin mediated signalling pathway. Thus, β catenin maybe a key point at which either the Wnt or integrin signalling pathways are stimulated, or a nodal point at which these pathways interact and facilitate further downstream signalling. These signalling pathways are responsible for the activation of target genes and for the expression and turn over of cell matrix macromolecules in chondrocyte cell lines.

Identification of protein expression of Wnt-1, one of the 22 homologs of this family of secretory glycoproteins, in these chondrocyte cell lines supports the possibility of Wnt signalling in these cells. However, a polyclonal Wnt-1 antibody was used for the confocal studies. The specificity of this antibody is not entirely certain and it is possible that the Wnt-1 polyclonal antibody could bind other members of the Wnt family of proteins in these chondrocyte cell lines. Thus, it is possible that other

members of the Wnt family of proteins may interact with Fz-2. This could result in transduction of the Wnt signals along different pathways (Toyofuku *et al.*, 2000).

Binding of the Wnt-1 protein to the cell surface or the ECM appears as a thin dotted line. Wnt is expressed in developing cartilage and is involved in chondrocyte maturation and differentiation. However, little is known about Wnt expression in adult human articular cartilage. The expression of Wnt proteins in these cell lines, in which cells are proliferating and there is a high degree of cell-cell adhesion, may have some similarity to osteoarthritic chondrocytes in cartilage, where cell clustering occurs.

Wnt proteins may act in autocrine or paracrine fashion, binding to Fz on the cell surface and transducing the signals intracellularly.

The expression of Fz -2 protein, one of the 10 homologs (isoforms) of this family of G-coupled proteins was also identified. The presence of Wnt-1 and Fz -2 could suggest that Wnt-1 and Fz-2 may function as a ligand –receptor pair in chondrocyte cell lines. There are, however, other members of the family of the Wnt proteins that bind to Fz-2 receptors and induce Wnt mediated signalling. Expression of Wnt-5a has been identified in the T/C 28I2 chondrocyte cell line in this laboratory (unpublished work). This could result in the formation of a Wnt-5a and Fz-2 ligand-receptor pair, which could transduce the Wnt signal via the Wnt/Ca²⁺ pathway which has an antagonistic effect on the Wnt/β catenin pathway. This could have significant effects on the regulatory mechanisms involved in cell proliferation and differentiation in chondrocyte cell lines. The Wnt-5a family of proteins can also bind to Fz-2 in the

Wnt/Jnk pathway and activate Dsh through a separate domain from the one that is induced by the Wnt-1 family of proteins. This, in turn can have effect on cytoskeletal rearrangement in chondrocytes rather than on their proliferation. Activation of the Wnt/Jnk pathway might also influence the activity of integrin mediated MAPK and consequently β catenin stabilisation within these cells.

The specificity of each Wnt –Fz complex is further increased by the binding of a glycosaminoglycan protein as a coreceptor (Dale., 1998). CD44 and FN are two sulphated proteoglycans that are highly expressed in articular cartilage. CD44, along with other receptors, such as integrins, facilitates the detection of the changes in the matrix components by chondrocytes. These receptors act as mechanotransducers and affect cartilage homeostasis (Knudson *et al.*, 2000, 2002). Fibronectin is another sulphated proteoglycan that plays a role in integrin signalling that can also couple with Wnt-Fz complexes and so function as a coreceptor. The colocalisation studies suggest that the formation of Wnt-1, Fz and CD44 or Wnt-1, Fz and FN complexes are involved in Wnt-1 induced stabilisation of β catenin in chondrocytes. CD44 and FN expression could however also be regulated via a Wnt/ β catenin pathway as both these glycosaminoglycan proteins are target genes in this pathway. The Wnt/ β catenin signalling pathway may be induced as the Wnt-1 family of the proteins bind to either Fz-1 or Fz-2 receptors. This, in turn, may result in stabilisation and accumulation of β catenin in the cytoplasm before its translocation to the nucleus to form a complex with TCF/LEF transcription factors and to act as transactivators of target genes such as CD44 and FN. Thus FN and CD44 may have a dual role in facilitating the signals intracellularly in chondrocytes: a) through the mechanotransduction pathway and the Wnt signalling pathway b) by mediating a

positive feedback loop through which their own expression is regulated. This, in turn, could be responsible for maintaining the integrity of cartilage composition.

Frizzled related proteins (Fzrp) are a family of secretory proteins with similarity to the cysteine rich extracellular ligand binding domains (CRD domain) of the Fz family of receptors (Bhanot *et al.*, 1996; Lin *et al.*, 1997 and Dennis *et al.*, 1999).

In this thesis expression of the Fzrp-1 in chondrocyte cell lines has been demonstrated. The specific function of this protein is not known. However, the presence of Fzrp-1, an antagonist of Wnt within these cell lines, suggests yet another level of control for transducing Wnt signals intracellularly. This regulation via Fzrp-1 occurs at the level of ligand-receptor binding, where Wnt binds to its receptor Fz. Fz receptors can be replaced by Fzrp-1, which prevents the transduction of Wnt signals intracellularly. As a result of this, the activation of target genes that are controlled by the Wnt signalling pathway can be monitored at the cell surface. This discussion emphasises tight regulatory mechanisms that are responsible for transducing Wnt signals within the cells.

It is possible that there is more than one type of Fzrp expressed by these chondrocyte cell lines, which could function to modulate Wnt mediated signalling pathways. Expression of Fzrp may be independent of the type of Wnt proteins or Fz receptors that are expressed by the cells. The selective binding of the Fzrp to specific Wnt proteins, the downregulation of Wnt activity and the further modulation of the intracellular signalling are topics for further investigation.

3.6 Conclusions

A number of studies have suggested that the presence of Fz proteins as Wnt receptors is absolutely necessary for transduction of Wnt signals during development in all cell types. It has also been proposed that either Fz-1 or Fz-2 can function independently as Wnt receptors without the need for a co receptor (Chen and Struhl., 1999). However, it is also known that heparan sulphate rich proteoglycans facilitate intracellular responses. Following transduction of Wnt signals intracellularly, Fz receptor-associated cytoplasmic components affects the cytoplasmic and nuclear distribution of β catenin and its binding to the transcription factors Tcf /Lef-1. This in turn may regulate, not only the expression of CD44 and FN, but also the activation of other target genes such as MMP7 and MMP26. MMPs are major enzymes involved in the degradation of the ECM of cartilage. In order to maintain the integrity of the normal cartilage, a fine balance between the continuous processes of production and degradation of ECM is necessary. When this dynamic equilibrium is interrupted and cartilage destruction becomes the prevalent catabolic process, then OA occurs. Thus, fine tuning of regulation of MMP production and function is essential for maintaining cartilage morphology.

Wnt signalling pathways are crucially involved in embryonic development and oncogenesis. However, it has also been proposed that the patterning and development of self-renewing adult tissues are also controlled by Wnt pathways via regulation of stem-cell compartments (Taipale and Beachy., 2001). This leads to the conclusion that expression of members of the Wnt family of proteins is not limited to the embryonic

stage of development and that they may be prolonged or reactivated following differentiation.

There is also evidence that β catenin, a key component of Wnt/ β catenin pathway, may play some role in terminally differentiated cells by regulating the growth of cardiomyocytes following hypertrophic stress (Haq *et al.*, 2003). It seems likely that Wnt signalling pathways are not only involved in chondrogenesis at the early stage of skeletal development but that they can also be expressed in fully differentiated and hypertrophic chondrocytes and they may be involved in maintaining normal skeletal growth and integrity.

Chapter 4

β catenin and GSK3 β complex formation

β catenin exists in two pools within the cell: a membrane associated and a cytosolic pool. This protein is a potential unprimed substrate for GSK3 β (Haq *et al.*, 2003). GSK3 β , when in complex with the scaffolding protein Axin and adenomatous polyposis coli (APC), phosphorylates β catenin at its N-terminal region. This phosphorylation results in β catenin being ubiquitinated and degraded by the proteasome (Aberle *et al.*, 1997; Polakis., 2001). Thus, inhibition of GSK3 β is essential for the stabilisation and accumulation of β catenin. Paradoxically, recent studies indicate that β catenin, when phosphorylated on Ser-45 by casein kinase 1 α (CK 1 α), can function as a primed substrate for GSK3 β (Liu *et al.*, 2002), leading to increased β -catenin degradation.

Increase in cytosolic accumulation of β catenin leads to its translocation into the nucleus by an unknown mechanism. There, it interacts with the T cell transcription factor (Tcf)/ lymphocyte enhancer factor (Lef), and inhibits repression of downstream genes such as c-Myc, cyclin D-1, matrix metalloproteinase-7 (MMP-7) and matrix metalloproteinase-26 (MMP-26). Thus, the interaction of β -catenin with TCF/LEF provides a transcriptional activation complex for a number of downstream target genes (Morin *et al.*, 1997; He *et al.*, 1998).

The work presented in this chapter has been designed to investigate the effect of mechanical stimulation on the formation of complexes involving β catenin and other proteins involved in Wnt signalling in the T/C28I2 chondrocyte cell line.

4.1 Does GSK3 β form a complex with β catenin?

To examine whether GSK3 β forms a complex with β -catenin and to further understand the effect of mechanical stimulation on the formation of this complex the following set of experiments were performed.

T/C2812 cells were grown to ~90% confluency in 58 mm tissue culture dishes and were subjected to 0, 1, 5 and 10 minutes of mechanical stimulation at 0.33Hz, 30,740 μ strain. In order to assess the effect of the Wnt signalling on the formation of a GSK3 β - β /catenin complex, cells were treated with Li⁺ (a Wnt agonist) before being subjected to mechanical stimulation. The cell lysates were immunoprecipitated using an anti- β catenin antibody and were analysed by western blotting, using an antibody to GSK3 β . The same blots were stripped and reprobbed with anti-phosphoserine or anti-phosphotyrosine antibodies, then stripped again and reprobbed with anti- β catenin in order to estimate the loading of the proteins on the gel and allow correction for unequal loading effects. Whole cell lysates were used as positive controls.

4.2 β catenin

All the immunoprecipitated samples, whether mechanically stimulated or not, when probed for β -catenin, showed a band approximately at 92 kDa corresponding to β -catenin. This indicates that the immunoprecipitation was successful. Moreover, the loading of the proteins was determined in these blots (**Fig 4.1**) (**Table 4.1i - 4.1iv**).

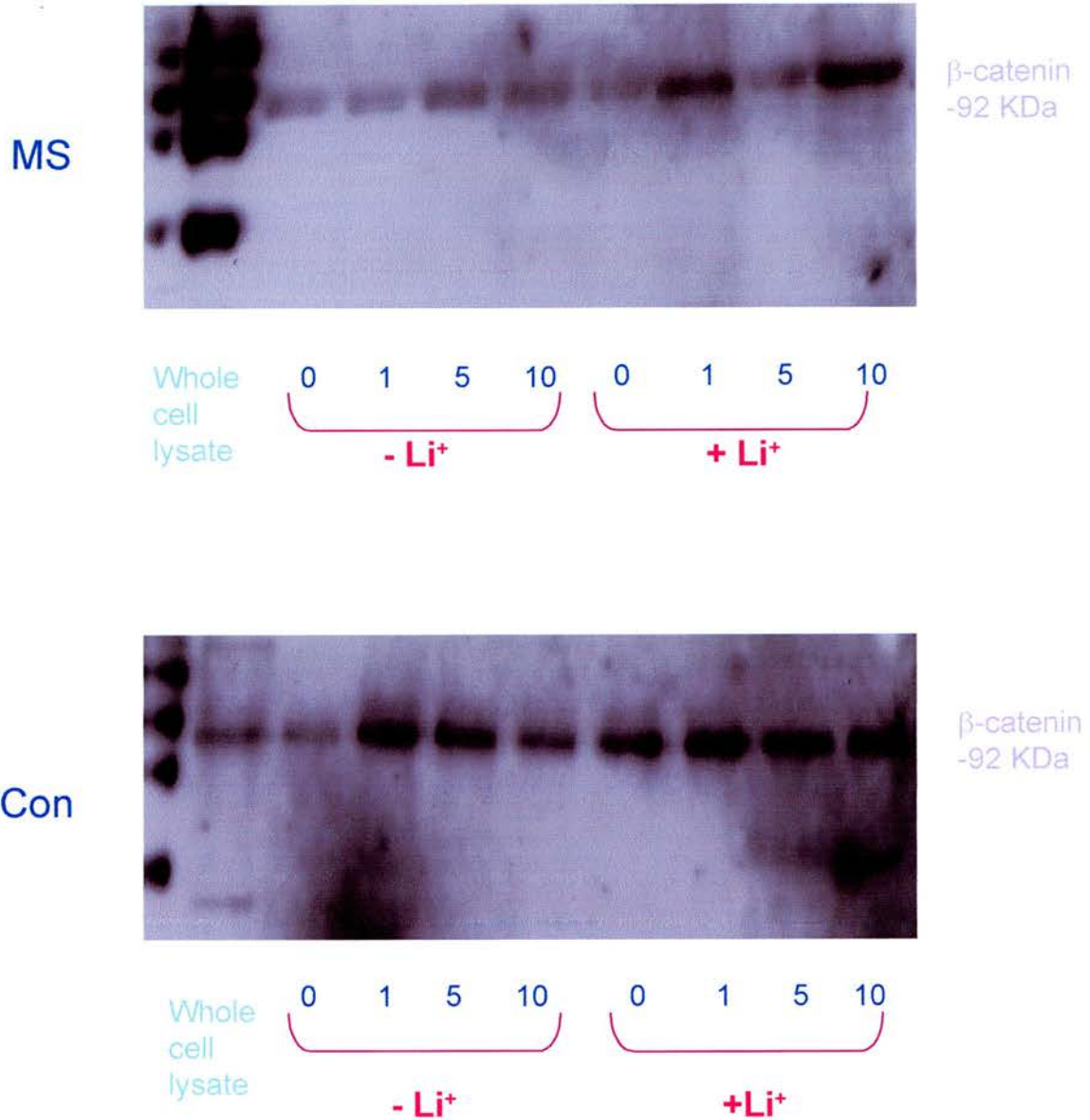


Fig 4.1: Expression of β -catenin at 92 kDa in the samples, immunoprecipitated and probed for β -catenin. Control (con): Cells were pretreated with Li⁺ and left for 1, 5, 10 minutes along with parallel controls. Mechanical stimulation (MS): Cells were pretreated with Li⁺ and then mechanically stimulated for 1,5,10 minutes along with untreated parallel controls.

Samples without Lithium treatments	Control (β catenin) Experiment 1 (Int/mm²)	Control (β catenin) Experiment 2 (Int/mm²)	β cat (1) -BK (Int/mm²)	β cat (2) -BK (Int/mm²)
Background	122981.1	161025.9		
0 minute	135108.3	306000.9	12127.23	144975.1
1 minute	183616.5	299757.5	60635.4	138731.6
5 minute	169200.8	361126.7	46219.68	200100.8
10 minute	149206.9	348713.8	26225.8	187688

Int/mm² = intensity per millimeter squared

BK= Background

Con (β cat) = control (β catenin)

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.1i: measurement of density β catenin bands in two set of experiments in resting state in which cells are not pretreated with Lithium.

Samples with Lithium treatment	Control (β catenin) Experiment 1 (Int/mm ²)	Control (β catenin) Experiment 2 (Int/mm ²)	β cat (1) -BK (Int/mm ²)	β cat (2) -BK (Int/mm ²)
Background	122981.1	161025.9		
0 minute	165565.1	355957	42584.02	194931.2
1 minute	191526.5	235337.6	68545.37	74311.7
5 minute	190819.3	248113.7	67838.16	87087.78
10 minute	207551.3	213561.9	84570.19	52536.04

Int/mm² = Intensity per millimeter squared

BK= Background

Con (β cat) = control (β catenin)

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.1ii: measurement of density β catenin bands in the resting state in two set of experiments in which cells were pretreated with Lithium.

Samples without Lithium treatments	MS (β catenin) Experiment 1 (Int/mm²)	MS (β catenin) Experiment 2 (Int/mm²)	β cat (1) -BK (Int/mm²)	β cat (2) -BK (Int/mm²)
Background	98040.37	152278.59		
0 minute	123125.91	301136.43	25085.54	148857.84
1 minute	117140.84	217767.85	19100.47	65489.26
5 minute	126321.53	211088.07	28281.16	58809.48
10 minute	135351.84	188108.48	37311.47	35829.89

Int/mm² = intensity per millimeter squared

BK= Background

MS (β cat) = Mechanically stimulated (β catenin)

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.1iii: measurement of density of β catenin bands in mechanically stimulated chondrocytes in two set of experiments in resting state in which cells are not pretreated with Lithium.

Samples with Lithium treatment	MS (β catenin) Experiment 1 (Int/mm ²)	MS (β catenin) Experiment 2 (Int/mm ²)	β cat (1) -BK (Int/mm ²)	β cat (2) -BK (Int/mm ²)
Background	98040.37	152278.59		
0 minute	136848.86	231755.08	38808.49	79476.49
1 minute	154565.22	184355.69	56524.85	32077.1
5 minute	128873.39	193504.31	30833.02	41225.72
10 minute	146612.91	168927.58	48572.54	16648.99

Int/mm² = intensity per millimeter squared

BK= Background

MS (β cat) = MS (β catenin)

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.1iv: measurement of density of β catenin bands in the mechanically stimulated chondrocytes in two set of experiments in which cells were pretreated with Lithium.

4.3 GSK3 β

Samples were pretreated with lithium and mechanically stimulated for 0, 1, 5, 10 minutes. Parallel unstimulated controls were similarly pretreated with Li⁺. Lysates were immunoprecipitated using an anti- β catenin antibody and probed for GSK3 β . The blots showed a band at approximately at 47 kDa corresponding to GSK3 β (**Fig 4.2**).

In the non-mechanically stimulated samples that were not Li⁺ treated a 47kDa band was visible at a very low level after 1 minute. GSK3 β was expressed after 5 and 10 minutes in the Li⁺ treated but not in the untreated, unstimulated samples (**Fig 4.2**).

In the unstimulated samples that were Li⁺ treated, GSK3 β was expressed after 5 minutes. The ratio of density of the expressed GSK3 β to β catenin increased when averaged over all samples (**Fig 4.3**). In the Li⁺ treated mechanically stimulated samples, a 47 kDa band was visible at low level after 1 minute of mechanical stimulation (**Fig 4.2**) but there was no expression of GSK3 β following 5 minutes of mechanical stimulation. The level of GSK3 β increased considerably after 10 minutes of mechanical stimulation. The ratio of the density of the expressed GSK3 β to β catenin after 1 and 10 minutes of mechanical stimulation was considerably increased (**Fig 4.4**).

In the samples that were not pretreated with Li⁺ there was no change in GSK3 β expression at any time point (**Fig 4.2** and **Fig 4.4**) (**Table 4.2i - 4.2viii**).

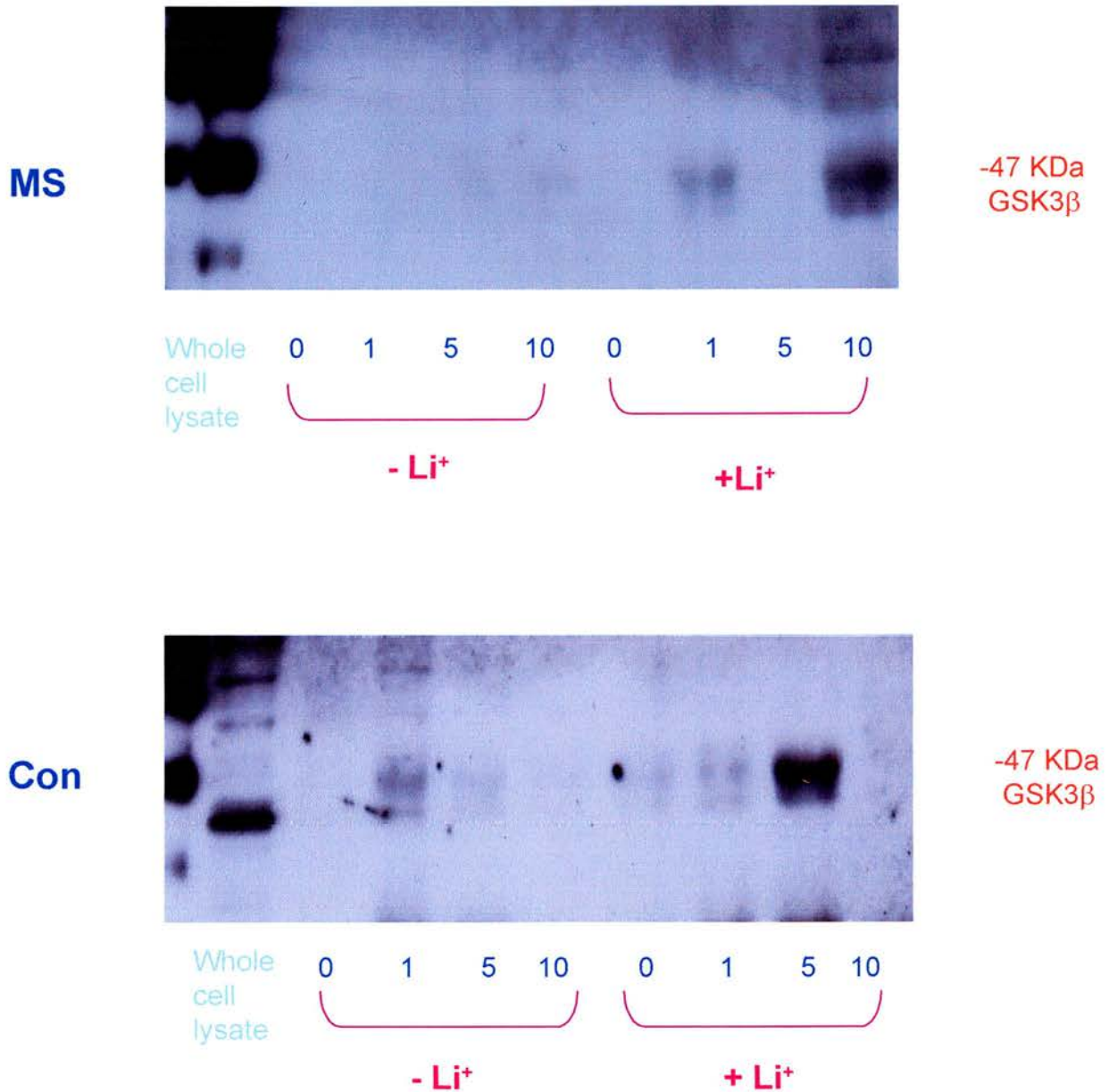


Fig 4.2: Expression of GSK3 β at 47 kDa in the samples, immunoprecipitated with anti- β catenin antibody and probed for GSK3 β . Control (con): Cells were pretreated with Li⁺ and left for 1, 5, 10 minutes along with untreated parallel controls. Mechanical stimulation (MS): Cells were pretreated with Li⁺ and then mechanically stimulated for 1,5,10 minutes along with untreated parallel controls.

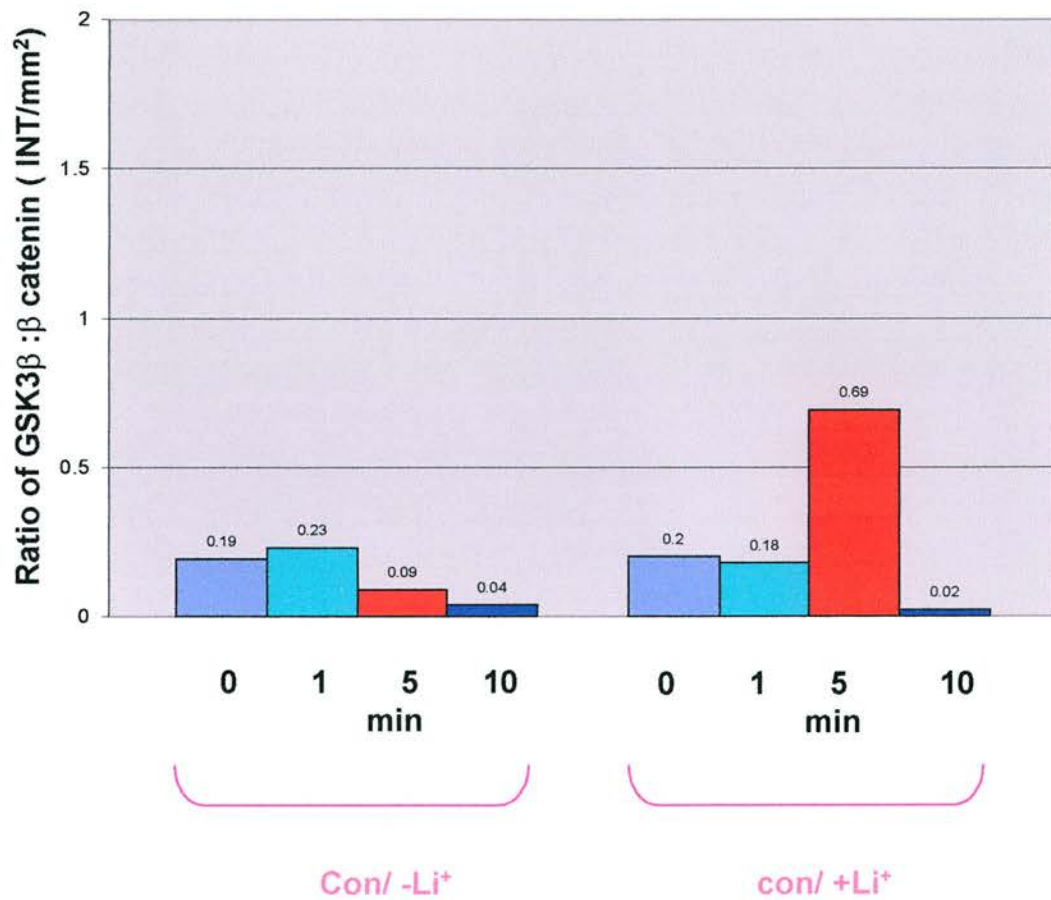


Fig 4.3: Ratio of densities of GSK3 β / β catenin in samples immunoprecipitated with anti- β catenin antibody and probed for GSK3 β . Cells were left in the resting state for 1, 5 and 10 minutes in the absence and presence of Li⁺ Mean, n=2

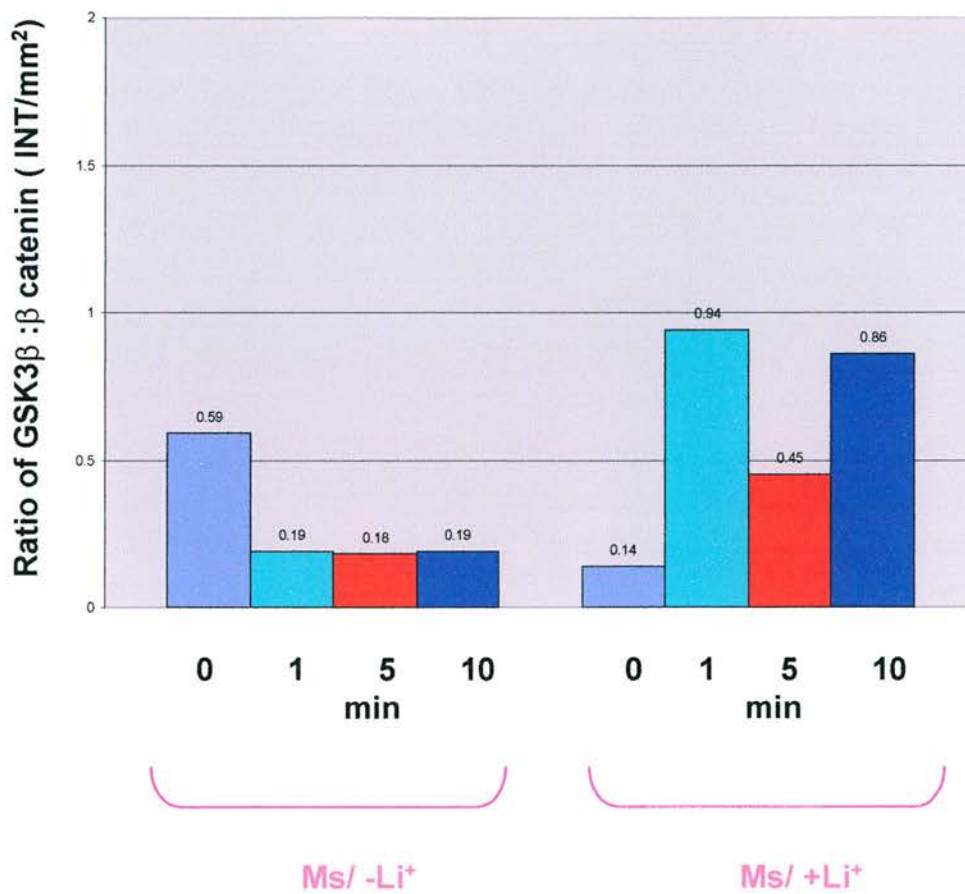


Fig 4.4: Ratio of densities of GSK3 β / β catenin in samples immunoprecipitated with anti- β catenin antibody and probed for GSK3 β . Cells were mechanically stimulated for 1, 5 and 10 minutes in the absence and presence of Li $^+$. Mean, n= 2

Samples without Lithium treatment	Control (GSK3 β) Experiment 1 (Int/mm ²)	Control (GSK3 β) Experiment 2 (Int/mm ²)	GSK3 β (1) - BK (Int/mm ²)	GSK3 β (2) - BK (Int/mm ²)
Background	102750.6	163217.6		
0 minute	106152.3	178027.9	3401.73	14810.34
1 minute	129753.2	172081.9	27002.6	8864.36
5 minute	108109.5	174139.3	5358.86	10921.69
10 minute	102160.6	180757.7	-590	17540.16

Intensity/mm² = intensity per millimeter squared

BK= Background

Con (GSK3 β) = control (GSK β)

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.2i : Measurement of density GSK3 β bands in two set of experiments in resting state in which cells were not pretreated with Lithium.

Samples with Lithium treatment	Control (GSK3 β) Experiment 1 (Int/mm ²)	Control (GSK3 β) Experiment 2 (Int/mm ²)	GSK3 β (1) - BK (Int/mm ²)	GSK3 β (2) - BK (Int/mm ²)
Background	102750.6	163217.6		
0 minute	108168.5	213905.4	5417.93	50687.87
1 minute	115974.4	174787.2	13223.79	11569.61
5 minute	194907.2	164782.8	92156.64	1565.27
10 minute	115014.8	157292	12264.19	-5925.6

Int/mm²= intensity per millimeter squared

BK= Background

Con (GSK3 β) = control (GSK β)

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.2ii : Measurement of density GSK3 β bands in two set of experiments in resting state in which chondrocytes were pretreated with Lithium.

Samples without Lithium treatment	GSK3 β : β catenin Experiment 1 (Int/mm ²)	GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN GSK3 β : β catenin from both set of experiments
0 minute	0.28	0.1	0.19
1 minute	0.45	0.06	0.225
5 minute	0.12	0.05	0.085
10 minute	-0.02	0.09	0.035

Int/mm² = intensity per millimeter squared

Table 4.2 iii : Average of the ratio of densities of GSK3 β / β catenin in two set of experiments in resting state without Lithium treatment of chondrocytes.

Samples with Lithium treatment	GSK3 β : β catenin Experiment 1 (Int/mm ²)	GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN GSK3 β : β catenin from both set of experiments
0 minute	0.13	0.26	0.195
1 minute	0.19	0.16	0.175
5 minute	1.36	0.02	0.69
10 minute	0.14	-0.11	0.015

Int/mm² = Intensity per millimeter squared

Table 4.2iv : Average of the ratio of densities of GSK3 β / β catenin in two set of experiments in resting state in the presence of Lithium.

Samples without Lithium treatment	MS (GSK3 β) Experiment 1 (Int/mm ²)	MS (GSK3 β) Experiment 2 (Int/mm ²)	MS GSK3 β (1) - BK (Int/mm ²)	MS GSK3 β (2) - BK (Int/mm ²)
Background	105292	165092.4		
0 minute	107867.3	325423.1	2575.3	160330.7
1 minute	107617.2	181560.2	2325.24	16467.82
5 minute	111475.5	173578	6183.56	8485.66
10 minute	111634	172714.9	6341.99	7622.56

Int/mm² = intensity per milimeter squared

BK= Background

MS (GSK3 β) = mechanically stimulated (GSK β)

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.2v : Measurement of density GSK3 β bands in two set of experiments in mechanically stimulated chondrocytes in which cells were not pretreated with Lithium.

Samples with Lithium treatment	MS (GSK3 β) Experiment 1 (Int/mm ²)	MS (GSK3 β) Experiment 2 (Int/mm ²)	MS GSK3 β (1) - BK (Int/mm ²)	MS GSK3 β (2) - BK (Int/mm ²)
Background	105292	165092.4		
0 minute	105796	186672.3	504.01	21579.92
1 minute	120238	217116.9	14946.01	52024.56
5 minute	106859.1	199646.8	1567.16	34554.38
10 minute	158302.6	175500.6	53010.59	10408.18

Int/mm² =intensity per milimeter squared

BK= Background

MS (GSK3 β) = mechanically stimulated (GSK β)

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.2vi : Measurement of density GSK3 β bands in two set of experiments in mechanically stimulated chondrocytes in which cells were pretreated with Lithium.

Samples without Lithium treatment	MS GSK3 β : β catenin Experiment 1 (Int/mm ²)	MS GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN GSK3 β : β catenin from both set of experiments
0 minute	0.1	1.07	0.585
1 minute	0.12	0.25	0.185
5 minute	0.22	0.14	0.18
10 minute	0.17	0.21	0.19

Int/mm²=intensity per milimeter squared

Table 4.2 vii : Average of the ratio of densities of GSK3 β / β catenin in two set of experiments in mechanically stimulated chondrocytes in the absence of lithium.

Samples with Lithium treatment	MS GSK3 β : β catenin Experiment 1 (Int/mm ²)	MS GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN GSK3 β : β catenin from both set of experiments
0 minute	0.01	0.27	0.14
1 minute	0.26	1.62	0.94
5 minute	0.05	0.84	0.445
10 minute	1.09	0.63	0.86

Int/mm²= intensity per millimeter squared

Table 4.2 viii : Average of the ratio of densities of GSK3 β / β catenin in two set of experiments in mechanically stimulated chondrocytes in the presence of lithium.

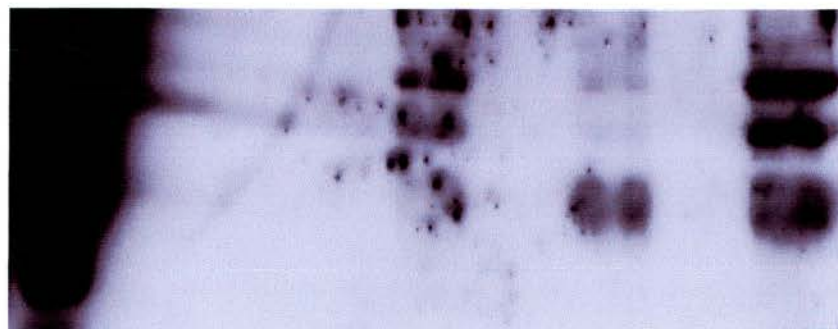
4.4 Phosphotyrosine

When whole cell lysates were immunoprecipitated using β -catenin and probed for phosphotyrosine, the blots showed 3 bands at approximately 47 kDa, corresponding to GSK3 β , 66 kDa (not identified) and 92 kDa, corresponding to β catenin (**Fig 4.5**). In the unstimulated Li⁺ treated samples, only GSK3 β tyrosine phosphorylation was upregulated following 5 minutes, but the ratio of the density of the tyrosine phosphorylated GSK3 β to β catenin was not increased (**Fig 4.6**). By 10 minutes there was no evidence of tyrosine phosphorylation of GSK3 β . However, in the Li⁺ treated mechanically stimulated samples, tyrosine phosphorylation of GSK3 β was upregulated following 1 minute of mechanical stimulation (**Fig 4.7**) decreased after 5 minutes and increased again after 10 minutes of mechanical stimulation (**Fig 4.5**). Only the 1 and 10 minute changes in phosphorylation might reach significance when corrected for β -catenin levels if more experiments were carried out (**Fig 4.7**). There was no evidence of tyrosine phosphorylation of any of the proteins following 5 minutes of mechanical stimulation but tyrosine phosphorylation of all three proteins was seen again following 10 minutes of mechanical stimulation (**Fig 4.5**). However, when averaged over all samples and corrected for β -catenin levels, there was not major increase in tyrosine phosphorylation (**Fig 4.7 and 4.9**).

In the samples that were not pretreated with lithium, and were not mechanically stimulated, immunoprecipitation with anti- β catenin antibody and probing for phosphotyrosine showed evidence of phosphorylation of three proteins (**Fig 4.5**). These proteins were tyrosine phosphorylated after 1 minute, and there was a decrease in phosphorylation after 5 and 10 minutes. However, there was a marked increase in

the ratio of densities of the tyrosine phosphorylated β catenin to β catenin at 1 and 10 minutes and decrease at 5 minutes in the samples untreated with lithium. There was a marked decrease in β catenin tyrosine phosphorylation and in the ratio of tyrosine phosphorylated β catenin to unphosphorylated β catenin at all above time points in the presence of lithium at all time (**Fig 4.5** and **Fig 4.8**). In the mechanically stimulated samples in the absence of lithium there was no tyrosine phosphorylation of these proteins after 1 or 5 minutes of mechanical stimulation but phosphorylation did occur after 10 minutes of mechanical stimulation (**Fig 4.5**). There was an increase in ratio of densities of tyrosine phosphorylated β catenin to β catenin after 1, 5 and 10 minutes of mechanical stimulation (**Fig 4.9**). (**Table 4.3i-4.3viii** and **4.4i-4.4viii**).

MS

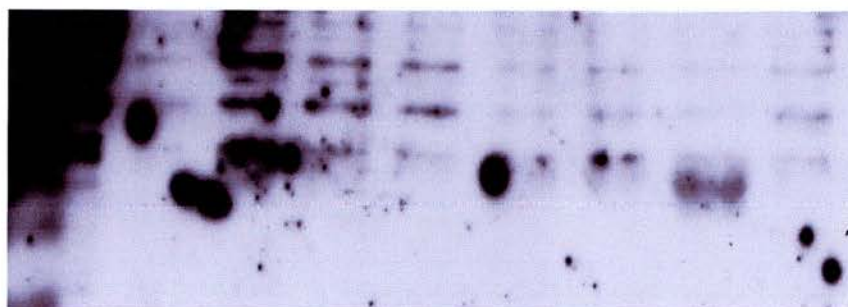


β -catenin
-92 KDa

-47 KDa
GSK3 β

Whole cell lysate 0 1 5 10 0 1 5 10
- Li⁺ + Li⁺

Con



β -catenin
-92 KDa

-47 KDa
GSK3 β

Whole cell lysate 0 1 5 10 0 1 5 10
- Li⁺ + Li⁺

Fig 4.5: Tyrosine phosphorylation of GSK3 β and β -catenin in the samples, immunoprecipitated with anti- β catenin antibody and probed for phosphotyrosine. Control (con): Cells were pretreated with Li⁺ and left for 1, 5, 10 minutes along with untreated parallel controls. Mechanical stimulation (MS): Cells were pretreated with Li⁺ and then mechanically stimulated for 1,5,10 minutes along with untreated parallel controls.

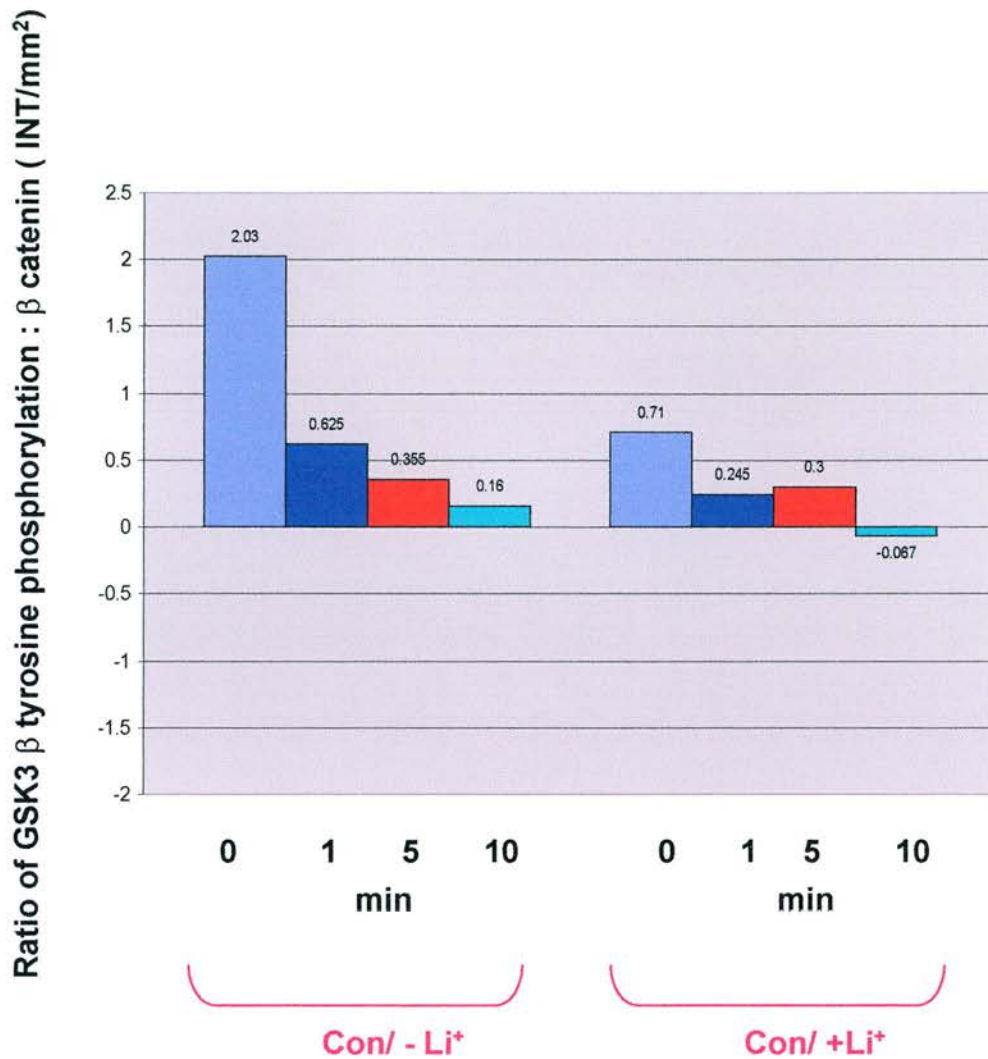


Fig 4.6: Ratio of densities of GSK3 β tyrosine phosphorylation: β catenin in samples immunoprecipitated with anti- β catenin antibody and probed for phosphotyrosine. Cells were left at the resting state for 1, 5 and 10 minutes in the absence and presence of Li^+ . Mean $n=2$

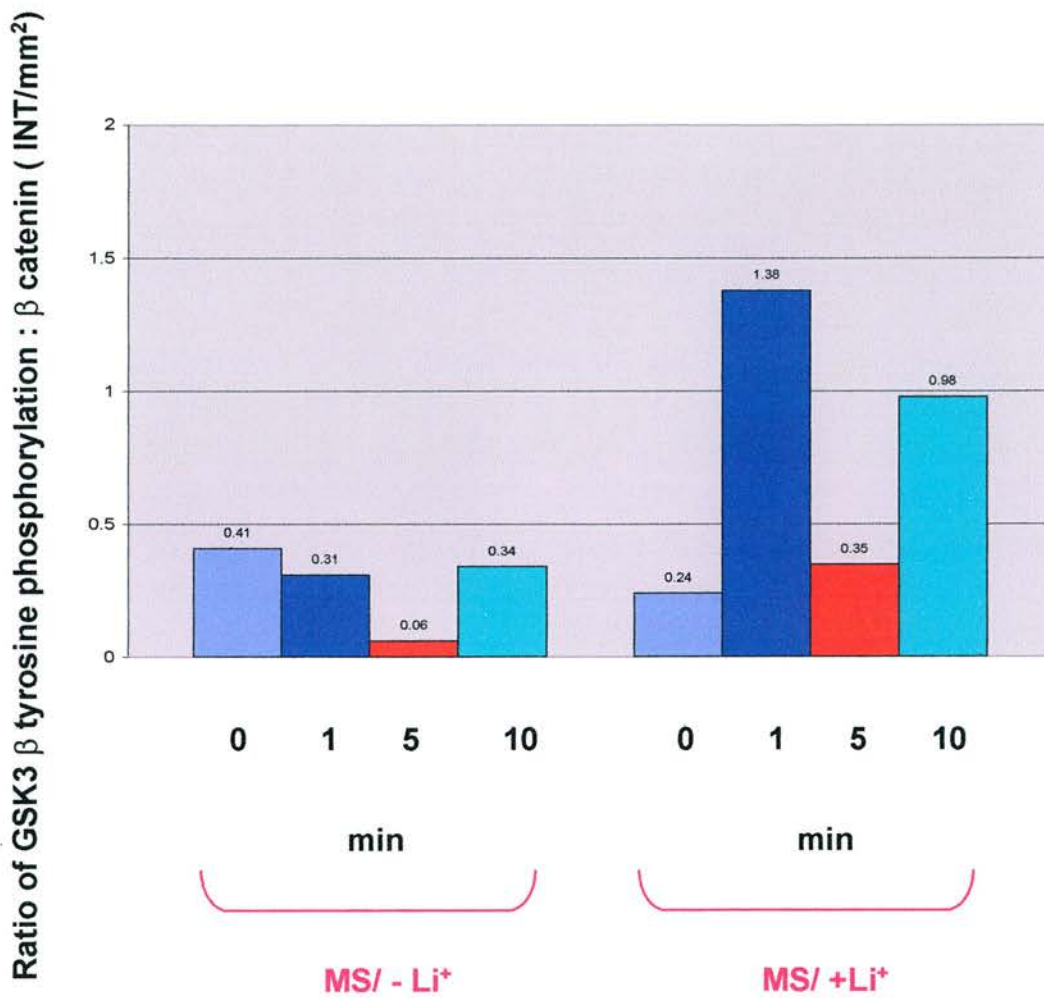


Fig 4.7: Ratio of densities of GSK3 β tyrosine phosphorylation: β catenin in samples immunoprecipitated with anti- β catenin antibody and probed for phosphotyrosine. Cells were mechanically stimulated for 1, 5 and 10 minutes in the absence and presence of Li^+ . Mean $n=2$

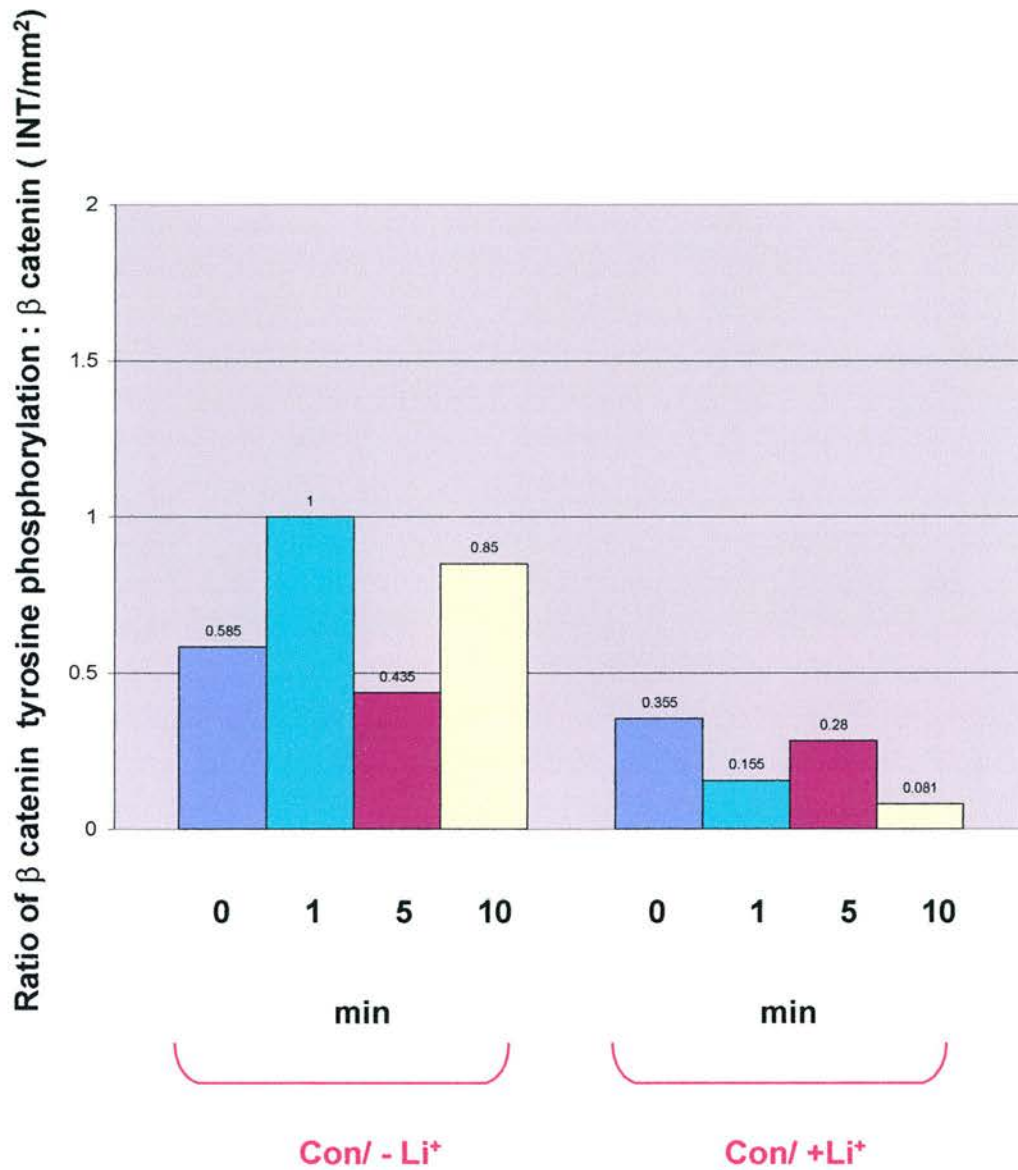


Fig 4.8: Ratio of densities of tyrosine phosphorylated β catenin: β catenin in the samples immunoprecipitated without β catenin and probed for phosphotyrosine. Cells were left at the resting state for 1, 5 and 10 minutes in the absence and presence of Li^+ . Mean $n=2$

Ratio of β catenin tyrosine phosphorylation : β catenin (INT/mm²)

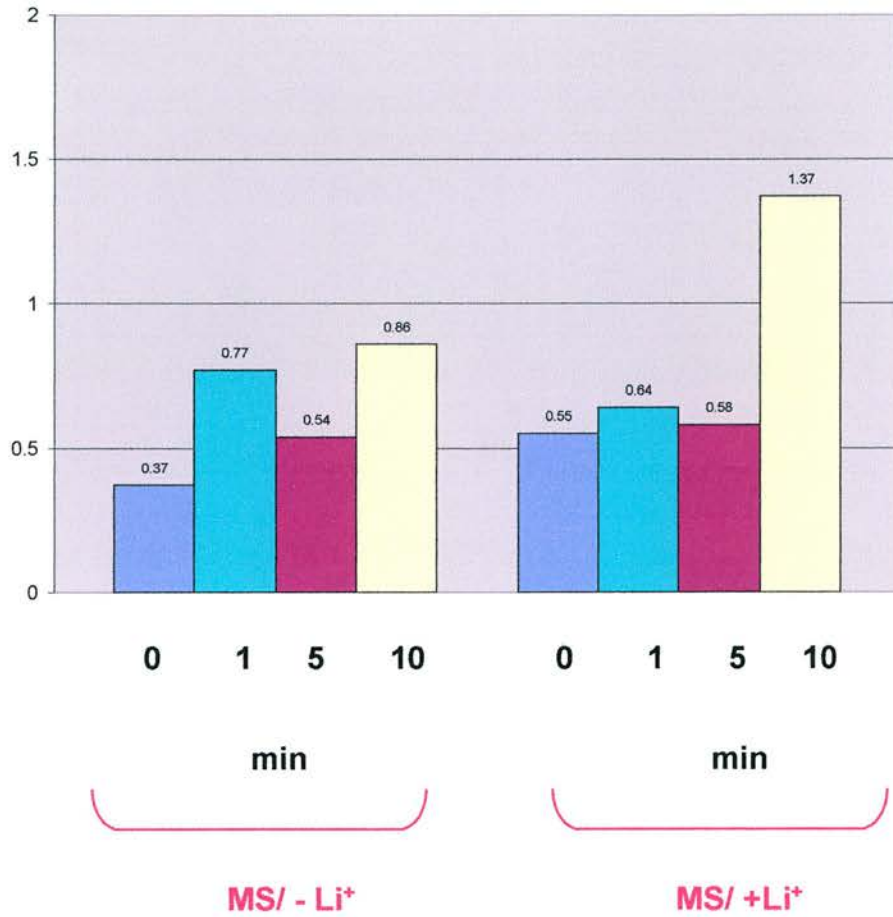


Fig 4.9: Ratio of densities of tyrosine phosphorylated β catenin : β catenin in samples immunoprecipitated without β catenin antibody and probed for phosphotyrosine. Cells were mechanically stimulated for 1, 5 and 10 minutes in the absence and presence of Li⁺. Mean n= 2

Samples without Lithium treatment	Control (YP) β catenin Experiment 1 (Int/mm ²)	Control (YP) β catenin Experiment 2 (Int/mm ²)	(YP) β catenin (1) - BK (Int/mm ²)	(YP) β catenin (2) - BK (Int/mm ²)
Background	157888.2	168270.2		
0 minute	167533.2	221348.8	9645.04	53078.53
1 minute	261491.9	208750.1	103603.8	40479.84
5 minute	192349.4	192591.3	34461.23	24321.05
10 minute	180179.5	213129.4	22291.34	44859.19

Int/mm² = intensity per millimeter squared

BK= Background

Control YP- β catenin = control tyrosine phosphorylated β catenin

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.3 i : Measurement of density of tyrosine phosphorylated β catenin bands in the resting state in two set of experiments in which chondrocytes were not pretreated with Lithium.

Samples with Lithium treatment	Control (YP) β catenin Experiment 1 (Int/mm ²)	Control (YP) β catenin Experiment 2 (Int/mm ²)	YP β catenin(1) - BK (Int/mm ²)	YP β catenin(2) - BK (Int/mm ²)
Background	157888.2	168270.2		
0 minute	174544.6	231230.6	16656.39	62960.34
1 minute	171390.8	176543.2	13502.67	8272.97
5 minute	156958.7	174393.1	-929.5	6122.87
10 minute	155705.7	173916.1	-2182.48	5645.91

Int/mm² = Intensity per millimeter squared

BK= Background

Control YP- β catenin = control tyrosine phosphorylated β catenin

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.3 ii : Measurement of density of tyrosine phosphorylated β catenin bands in the resting state in two set of experiments in which chondrocytes were pretreated with Lithium.

Samples without Lithium treatment	(YP) β catenin : β catenin Experiment 1 (Int/mm ²)	(YP) β catenin : β catenin Experiment 2 (Int/mm ²)	MEAN (YP) β catenin : β catenin from both set of experiments
0 minute	0.8	0.37	0.585
1 minute	1.71	0.29	1
5 minute	0.75	0.12	0.435
10 minute	0.85	0.85	0.85

Int/mm² = intensity per millimeter squared

YP=tyrosine phosphorylated

Table 4.3iii : Average of the ratio of densities of tyrosine phosphorylated β catenin/ β catenin in the resting state in two set of experiments in the absence of lithium.

Samples with Lithium treatment	(YP) β catenin : β catenin Experiment 1 (Int/mm ²)	(YP) β catenin : β catenin Experiment 2 (Int/mm ²)	MEAN (YP) β catenin : β catenin from both set of experiments
0 minute	0.39	0.32	0.355
1 minute	0.2	0.11	0.155
5 minute	-0.014	0.07	0.28
10 minute	-0.026	0.107	0.081

Int/mm²= Intensity per milimeter aquared

YP=tyrosine phosohorylated

Table 4.3 iv: Average of the ratio of densities of tyrosine phosphorylated β catenin/ β catenin in two set of experiments in the presence of Lithium.

Samples without Lithium treatment	MS (YP) β catenin Experiment 1 (Int/mm ²)	MS (YP) β catenin Experiment 2 (Int/mm ²)	(YP) β catenin (1) - BK (Int/mm ²)	(YP) β catenin (2) - BK (Int/mm ²)
Background	168139.3	192989.2		
0 minute	176598.1	251234.1	8458.77	58244.93
1 minute	184530.9	237803	16391.52	44814.56
5 minute	189254.9	212590.7	21115.55	19601.48
10 minute	213457.2	210725.1	45317.87	17735.95

Int/mm² = intensity per millimeter square

BK= Background

MS (YP) β catenin = mechanically stimulated, tyrosine phosphorylated β catenin

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.3 v : Measurement of density of tyrosine phosphorylated β catenin bands in two set of experiments of mechanically stimulated chondrocytes in which cells were not pretreated with Lithium.

Samples with Lithium treatment	MS (YP) β catenin Experiment 1 (Int/mm ²)	MS (YP) β catenin Experiment 2 (Int/mm ²)	(YP) β catenin (1) - BK (Int/mm ²)	(YP) β catenin (2) - BK (Int/mm ²)
Background	168139.3	192989.2		
0 minute	194083.7	226779.4	25944.32	33790.21
1 minute	200705.7	215513.2	32566.39	22524.04
5 minute	180808.5	223297.9	12669.13	30308.68
10 minute	266220	204999.2	98080.65	12010.02

Int/mm² = intensity per millimeter squared

BK= Background

MS (YP) β catenin = mechanically stimulated, tyrosine phosphorylated β catenin

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.3 vi: Measurement of density of tyrosine phosphorylated β catenin bands in two set of experiments of mechanically stimulated chondrocytes in which cells were pretreated with Lithium.

Samples without Lithium treatment	MS (YP) β catenin : β catenin Experiment 1 (Int/mm ²)	MS (YP) β catenin : β catenin Experiment 2 (Int/mm ²)	MEAN MS (YP) β catenin: β catenin from both set of experiments
0 minute	0.34	0.39	0.37
1 minute	0.86	0.68	0.77
5 minute	0.75	0.33	0.54
10 minute	1.21	0.50	0.86

Int/mm²= intensity per millimeter squared

MS=mechanically stimulated

YP=tyrosine phosphorylated

Table 4.3 vii: Average of the ratio of densities of tyrosine phosphorylated β catenin/ β catenin in two set of experiments in mechanically stimulated chondrocytes in the absence of Lithium.

Samples with Lithium treatment	MS (YP) β catenin : β catenin Experiment 1 (Int/mm ²)	MS (YP) β catenin: β catenin Experiment 2 (Int/mm ²)	MEAN MS (YP) β catenin: β catenin from both set of experiments
0 minute	0.67	0.43	0.55
1 minute	0.58	0.70	0.64
5 minute	0.41	0.74	0.58
10 minute	2.02	0.72	1.37

Int/mm²= intensity per milimeter squared

MS=mechanically stimulated

YP=tyrosine phosphorylated

Table 4.3viii: Average of the ratio of densities of tyrosine phosphorylated β catenin/ β catenin in two set of experiments in mechanically stimulated chondrocytes in the presence of Lithium.

Samples without Lithium treatment	Control (YP)GSK3 β Experiment 1 (Int/mm ²)	Control (YP)GSK3 β Experiment 2 (Int/mm ²)	(YP) GSK3 β (1) - BK (Int/mm ²)	(YP) GSK3 β (2) - BK (Int/mm ²)
Background	157888.2	167239.7		
0 minute	205967.54	182023	48079.34	14783.3
1 minute	231405.3	173449.8	73517.18	6210.1
5 minute	189382.5	173080.4	31494.38	5840.77
10 minute	164895.7	177148.9	7007.5	9909.23

Int/mm²= intensity per millimeter squared

BK= Background

Control (YP) GSK3 β = control tyrosine phosphorylated GSK3 β

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.4 i : Measurement of density of tyrosine phosphorylated GSK3 β bands in resting state in two set of experiments in which chondrocytes were not pretreated with lithium.

Samples with Lithium treatment	Control (YP)GSK3 β Experiment 1 (Int/mm ²)	Control (YP)GSK3 β Experiment 2 (Int/mm ²)	(YP) GSK3 β (1) - BK (Int/mm ²)	(YP) GSK3 β (2) - BK (Int/mm ²)
Background	157888.2	167239.7		
0 minute	199017.72	254386.1	41129.52	87146.43
1 minute	175450.4	184478	17562.25	17238.34
5 minute	191738.1	175667.1	33849.91	8427.44
10 minute	145700.9	167243.6	-12187.3	3.92

Int/mm² = intensity per millimeter squared

BK= Background

Control (YP) GSK3 β = control tyrosine phosphorylated GSK3 β

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.4ii : Measurement of density of tyrosine phosphorylated GSK3 β bands in resting state in two set of experiments in which chondrocytes were pretreated with Lithium.

Samples without Lithium treatment	(YP) GSK3 β : β catenin Experiment 1 (Int/mm ²)	(YP) GSK3 : β catenin Experiment 2 (Int/mm ²)	MEAN (YP) GSK3 β : β catenin from both set of experiments
0 minute	3.96	0.1	2.03
1 minute	1.21	0.04	0.625
5 minute	0.68	0.03	0.355
10 minute	0.27	0.05	0.16

Int/mm² = intensity per milimeter squared

YP=tyrosine phosphorylated

Table 4.4 iii: Average of the ratio of densities of tyrosine phosphorylated GSK3 β / β catenin in the resting state in two set of experiments in the absence of Lithium.

Samples with Lithium treatment	(YP) GSK3β: β catenin Experiment 1 (Int/mm ²)	(YP) GSK3β : β catenin Experiment 2 (Int/mm ²)	MEAN (YP) GSK3β: β catenin from both set of experiments
0 minute	0.97	0.45	0.71
1 minute	0.26	0.23	0.245
5 minute	0.5	0.1	0.3
10 minute	-0.14	0.007	-0.067

Int/mm²=intensity per millimeter squared

YP=tyrosine phosphorylated

Table 4.4 iv: Average of the ratio of densities of tyrosine phosphorylated GSK3β /β catenin in resting state in two set of experiments in the presence of Lithium.

Samples without Lithium treatment	MS (YP) GSK3 β Experiment 1 (Int/mm ²)	MS (YP) GSK3 β Experiment 2 (Int/mm ²)	MS (YP) GSK3 β (1) - BK (Int/mm ²)	MS (YP) GSK3 β (2) - BK (Int/mm ²)
Background	168139.3	192989.2		
0 minute	169854.2	303454.1	1714.87	110464.9
1 minute	173576.6	214476.1	5437.24	21486.95
5 minute	168203.5	199997	64.14	7007.85
10 minute	188181.3	197519.4	20042.01	4530.22

Int/mm² = intensity per milimeter squared

BK= Background

MS (YP) GSK3 β = mechanically stimulated, tyrosine phosphorylated GSK3 β

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.4 v : Measurement of density of tyrosine phosphorylated GSK3 β bands in two set of experiments in mechanically stimulated chondrocytes in which cells were not pretreated with Lithium.

Samples with Lithium treatment	MS (YP) GSK3 β Experiment 1 (Int/mm ²)	MS (YP) GSK3 β Experiment 2 (Int/mm ²)	MS (YP) GSK3 β (1) - BK (Int/mm ²)	MS (YP) GSK3 β (2) - BK (Int/mm ²)
Background	168139.3	192989.2		
0 minute	178740.3	209347.2	10600.97	16358
1 minute	222853	255460.6	54713.71	62471.42
5 minute	170351.6	215986.6	2212.24	22997.44
10 minute	235572.2	202382	67432.9	9392.79

Int/mm² = intensity per milimeter squared

BK= Background

MS (YP) GSK3 β = mechanically stimulated, tyrosine phosphorylated GSK3 β

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.4 vi : Measurement of density of tyrosine phosphorylated GSK3 β bands in two set of experiments in mechanically stimulated chondrocytes in which cells were pretreated with Lithium.

Samples without Lithium treatment	(YP) GSK3 β : β catenin Experiment 1 (Int/mm ²)	(YP) GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN (YP) GSK3 β : β catenin from both set of experiments
0 minute	0.07	0.74	0.41
1 minute	0.28	0.33	0.31
5 minute	0.002	0.12	0.06
10 minute	0.54	0.13	0.34

Int/mm² = intensity per milimeter square

YP=tyrosine phosphorylated

Table 4.4 vii: Average of the ratio of densities of tyrosine phosphorylated GSK3 β / β catenin in mechanically stimulated chondrocytes in two set of experiments in the absence of Lithium.

Samples with Lithium treatment	(YP) GSK3 β : β catenin Experiment 1 (Int/mm ²)	(YP) GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN (YP) GSK3 β : β catenin from both set of experiments
0 minute	0.27	0.21	0.24
1 minute	0.81	1.95	1.38
5 minute	0.07	0.56	0.35
10 minute	1.39	0.56	0.98

Int/mm² = intensity per milimeter square

YP=tyrosine phosphorylated

Table 4.4viii: Average of the ratio of densities of tyrosine phosphorylated GSK3 β / β catenin in mechanically stimulated chondrocytes in two set of experiments in the presence of Lithium.

5.5 Phosphoserine

Samples were pretreated with lithium and mechanically stimulated for 0, 1, 5 and 10 minutes. Parallel unstimulated controls were similarly pretreated with Li^+ . The cell lysates were immunoprecipitated using anti- β catenin antibodies and probed with anti-phosphoserine antibody. The blots showed a band at approximately 47 kDa corresponding to GSK3 β (**Fig 4.10**). In the unstimulated, lithium treated samples; there was an evidence of some serine phosphorylation of GSK3 β at 0 and 1 minute. Serine phosphorylation increased after 5 minutes and returned to basal levels at 10 minutes. However, when averaged over all samples and corrected for β -catenin levels, the increase in the density of GSK3 β serine phosphorylation after 5 minutes was very low (**Fig 4.11**). Indeed, there was evidence of a higher ratio of serine phosphorylated GSK3 β to β catenin at the 0 time point (**Fig 4.11**). This might be due to interference from overspill of the whole cell lysate samples at the 0 time point on the gel. In the mechanically stimulated samples that were lithium treated, no serine phosphorylation was detectable at the 0 time point. Following 1 minute of mechanical stimulation there was an upregulation of serine phosphorylation of the 47 kDa band. There was no serine phosphorylation of GSK3 β following 5 minutes of mechanical stimulation but phosphorylation of GSK3 β was increased again after 10 minutes of mechanical stimulation (**Fig 4.10**). However, when averaged over all samples and corrected for β -catenin, there was a marked increase in GSK3 β serine phosphorylation after 1, 5 and especially 10 minutes of mechanical stimulation (**Fig 4.12**).

In the non mechanically stimulated samples that were not lithium treated, immunoprecipitation with β catenin and probing with anti phosphoserine antibody showed evidence of increased phosphorylation of GSK3 β after 1 minute. This decreased after 5 and 10 minutes (**Fig 4.10**). When, averaged over all samples and corrected for β -catenin levels, there was a little increase in serine phosphorylated GSK3 β after 1 min. This decreased after 5 minutes and increased again after 10 minutes. However, none of these represent marked changes in serine phosphorylation of GSK3 β (**Fig 4.11**) Indeed, there was evidence of an increase in the density of GSK3 β serine phosphorylation at the 0 time point (**Fig 4.11**).

In the mechanically stimulated samples there was no serine phosphorylation of the 47 kDa band until 10 minutes of mechanical stimulation (**Fig 4.10**). However, there was a decrease in the ratio of the density of serine phosphorylated GSK3 β to β catenin after 10 minutes of mechanical stimulation in the absence of lithium when averaged over all samples (**Fig 4.12**). Again, there was evidence of GSK3 β serine phosphorylation at the 0 time point (**Fig 4.12**). (**Table 4.5i-4.5viii**).

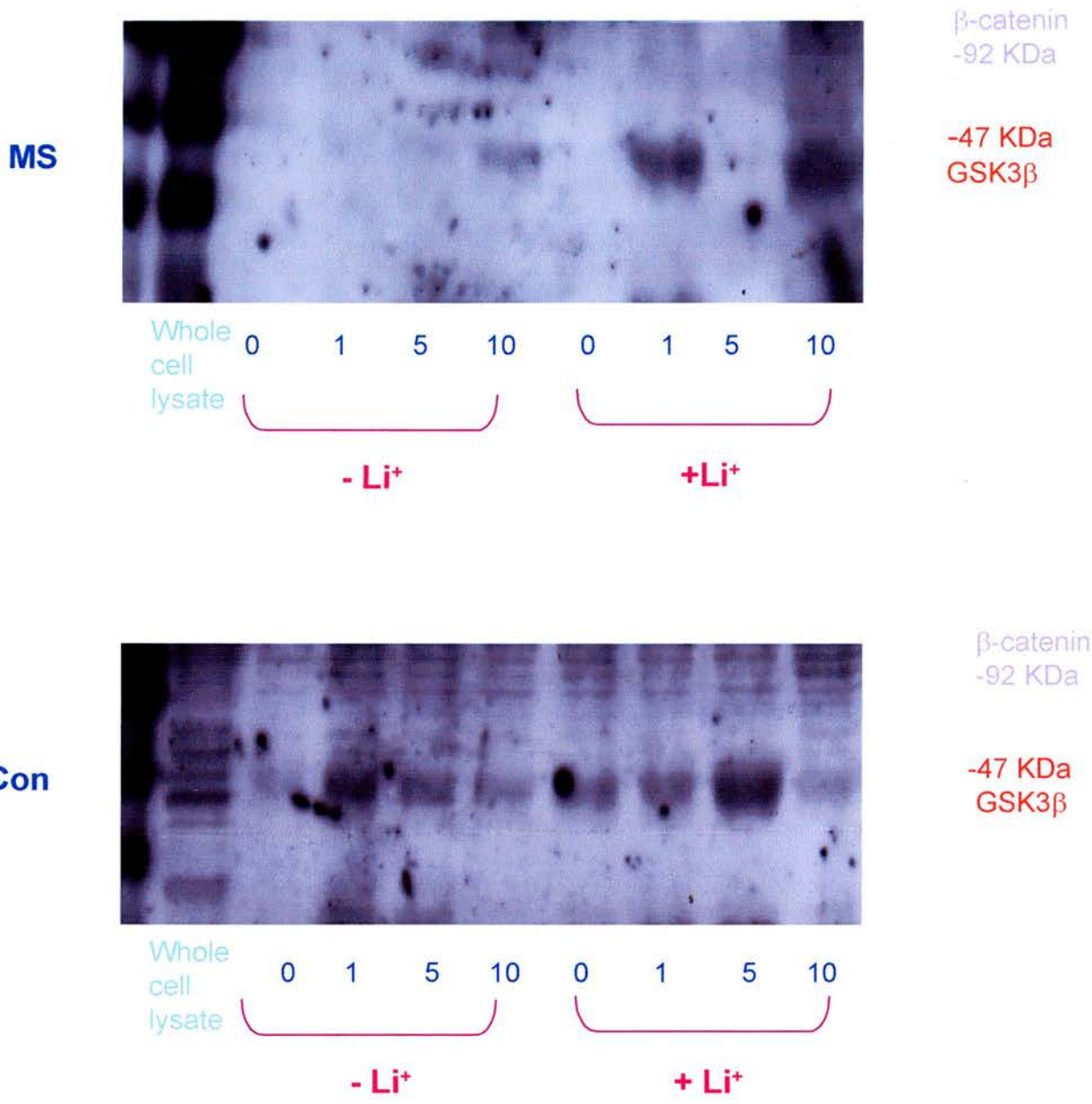


Fig 4.10: Serine phosphorylation of GSK3β in the samples, immunoprecipitated with anti-β catenin antibody and probed for phosphoserine. Control (con): Cells were pretreated with Li⁺ and left for 1, 5, 10 minutes along with untreated parallel controls. Mechanical stimulation (MS): Cells were pretreated with Li⁺ and then mechanically stimulated for 1,5,10 minutes along with untreated parallel controls.

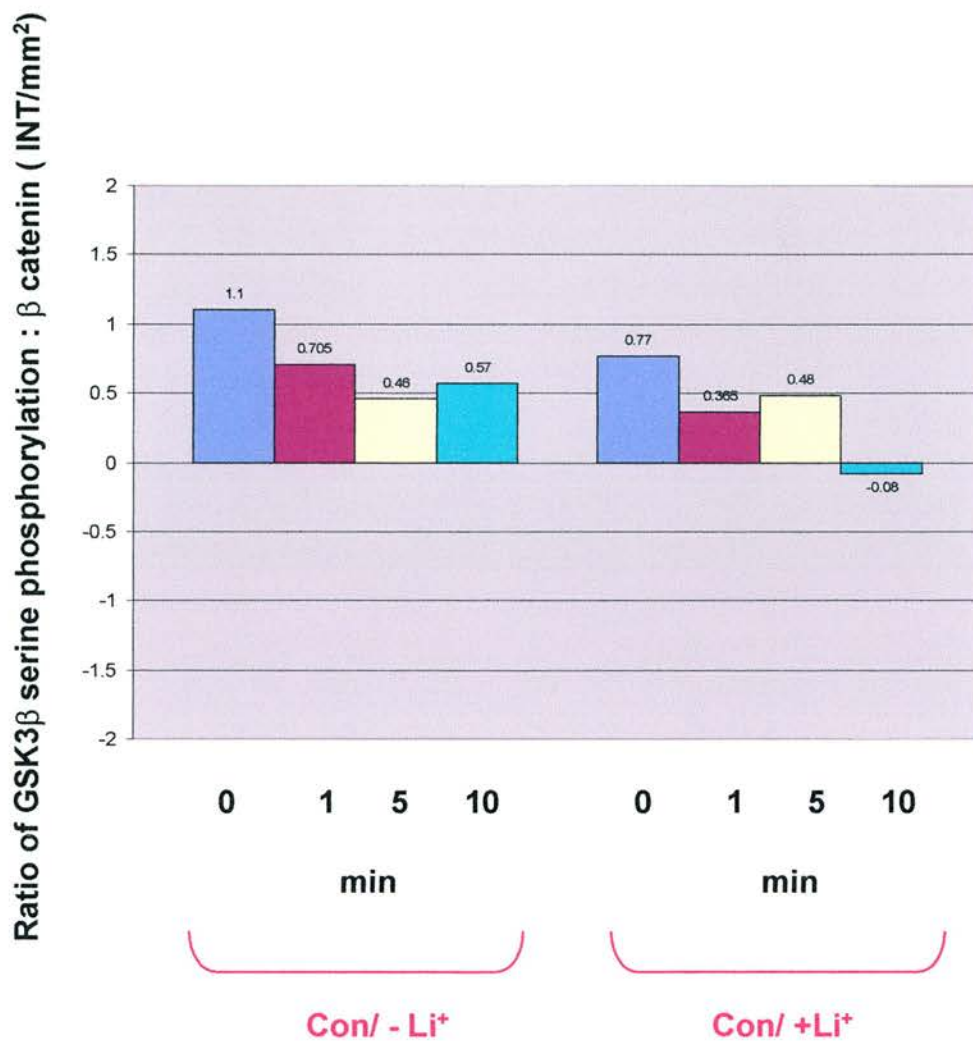


Fig 4.11: Ratio of densities of serine phosphorylated GSK3 β : β catenin in samples immunoprecipitated with anti- β catenin antibody and probed for phosphoserine. Cells were left at the resting state for 1, 5 and 10 minutes in the absence and presence of Li^+ . Mean $n=2$

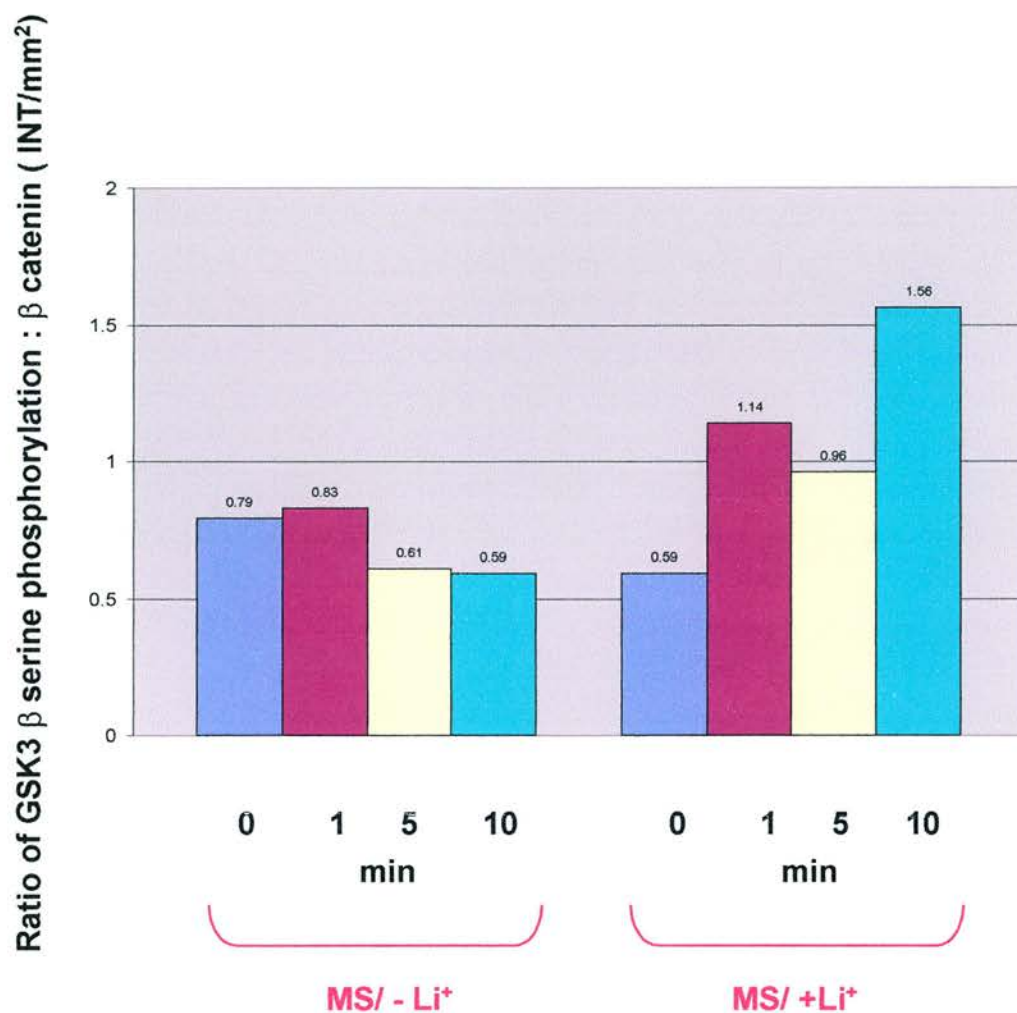


Fig 4.12: Ratio of densities of serine phosphorylated GSK3 β : β catenin in samples immunoprecipitated with anti- β catenin antibody and probed for phosphoserine. Cells were mechanically stimulated for 1, 5 and 10 minutes in the absence and presence of Li⁺. Mean n=2,

Samples without Lithium treatment	Control (Ser P) GSK3 β Experiment 1 (Int/mm ²)	Control (Ser P) GSK3 β Experiment 2 (Int/mm ²)	(Ser P) GSK3 β (1) - BK (Int/mm ²)	(Ser P) GSK3 β (2) - BK (Int/mm ²)
Background	217486.2	188664		
0 minute	240549.3	234438.3	23063.11	45774.29
1 minute	273976.7	255175.1	56490.51	66511.15
5 minute	249039.4	237661	31553.2	48997.06
10 minute	236340.2	267251.8	18854.05	78587.88

Int/mm²=intensity per milimeter squared

BK= Background

Control (Ser P) GSK3 β = control serine phosphorylated GSK3 β

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.5 i : Measurement of density of serine phosphorylated GSK3 β bands in two set of experiments in the resting state in which chondrocytes were not pretreated with Lithium.

Samples with Lithium treatment	Control (Ser P) GSK3 β Experiment 1 (Int/mm ²)	Control (Ser P) GSK3 β Experiment 2 (Int/mm ²)	(Ser P) GSK3 β (1) - BK (Int/mm ²)	(Ser P) GSK3 β (2) - BK (Int/mm ²)
Background	217486.2	188664		
0 minute	263307.9	262560	45821.74	73896.08
1 minute	247795.9	210453.7	30309.69	21789.73
5 minute	267860.1	207887.1	50373.87	19223.15
10 minute	219880.5	178799.8	2394.33	-9864.2

Int/mm²=intensity per milimeter squared

BK= Background

Control (Ser P) GSK3 β = control serine phosphorylated GSK3 β

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.5 ii : Measurement of density of serine phosphorylated GSK3 β bands in two set of experiments in resting state in which chondrocytes were pretreated with lithium.

Samples without Lithium treatment	(Ser P) GSK3 β : β catenin Experiment 1 (Int/mm ²)	(Ser P) GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN (Ser P) GSK3 β : β catenin from both set of experiments
0 minute	1.9	0.3	1.1
1 minute	0.93	0.48	0.705
5 minute	0.68	0.24	0.46
10 minute	0.72	0.42	0.57

Int/mm²=intensity per milimeter squared

Ser P=Serine phosphorylated

Table 4.5 iii : Average of the ratio of densities of serine phosphorylated GSK3 β / β catenin in the resting state in two set of experiments in the absence of Lithium.

Samples with Lithium treatment	(Ser P) GSK3 β : β catenin Experiment 1 (Int/mm ²)	(Ser P) GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN (Ser P) GSK3 β : β catenin from both set of experiments
0 minute	1.08	0.38	0.73
1 minute	0.44	0.29	0.365
5 minute	0.74	0.22	0.48
10 minute	0.02	-0.187	-0.08

Int/mm²=intensity per milimeter squared

Ser P=Serine phosphorylated

Table 4.5 iv : Average of the ratio of densities of serine phosphorylated GSK3 β / β catenin in the resting state in two set of experiments in the resting state in the presence of Lithium.

Samples without Lithium treatment	MS (Ser P) GSK3 β Experiment 1 (Int/mm ²)	MS (Ser P) GSK3 β Experiment 2 (Int/mm ²)	MS (Ser P) GSK3 β (1) - BK (Int/mm ²)	MS (Ser P) GSK3 β (2) - BK (Int/mm ²)
Background	139930.5	217754.4		
0 minute	163648.1	312876.8	23717.63	95122.45
1 minute	159266.1	260462.2	19335.61	42707.83
5 minute	164978.6	236435.4	25048.12	18681.08
10 minute	174756.4	226628.9	34825.9	8874.52

Int/mm² = intensity per millimeter squared

BK= Background

MS (Ser P) GSK3 β = mechanically stimulated, serine phosphorylated GSK3 β

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.5 v : Measurement of density of serine phosphorylated GSK3 β bands in two set of experiments in mechanically stimulated chondrocytes in which cells were not pretreated with Lithium.

Samples with Lithium treatment	MS (Ser P) GSK3 β Experiment 1 (Int/mm ²)	MS (Ser P) GSK3 β Experiment 2 (Int/mm ²)	MS (Ser P) GSK3 β (1) - BK (Int/mm ²)	MS (Ser P) GSK3 β (2) - BK (Int/mm ²)
Background	139930.5	217754.4		
0 minute	155505.4	280229.5	15574.94	62475.19
1 minute	209914.7	250640.7	69984.19	32886.35
5 minute	169870.9	256385.7	29940.38	38631.38
10 minute	223910.2	240673.3	83979.74	22918.98

Int/mm² = intensity per millimeter squared

BK= Background

MS (Ser P) GSK3 β = mechanically stimulated, serine phosphorylated GSK3 β

(1)= experiment 1

(2)= experiment 2

Li=Lithium

Table 4.5 vi : Measurement of density of serine phosphorylated GSK3 β bands in two set of experiments in mechanically stimulated chondrocytes in which cells were pretreated with Lithium.

Samples without Lithium treatment	MS (Ser P) GSK3 β : β catenin Experiment 1 (Int/mm ²)	MS (Ser P) GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN MS (Ser P) GSK3 β : β catenin from both set of experiments
0 minute	0.94	0.64	0.79
1 minute	1.01	0.65	0.83
5 minute	0.89	0.32	0.61
10 minute	0.93	0.25	0.59

Int/mm² = intensity per millimeter squared

MS= mechanically stimulated

Ser P=Serine phosphorylated

Table 4.5 vii : Average of the ratio of densities of serine phosphorylated GSK3 β / β catenin in two set of experiments in the mechanically stimulated chondrocytes in the absence of Lithium.

Samples with Lithium treatment	MS (Ser P) GSK3 β : β catenin Experiment 1 (Int/mm ²)	MS (Ser P) GSK3 β : β catenin Experiment 2 (Int/mm ²)	MEAN MS (Ser P) GSK3 β : β catenin from both set of experiments
0 minute	0.4	0.79	0.59
1 minute	1.24	1.03	1.14
5 minute	0.97	0.94	0.96
10 minute	1.73	1.38	1.56

Int/mm²=intensity per milimeter squared

MS= mechanically stimulated

Ser P=Serine phosphorylated

Table 4.5 viii : Average of the ratio of densities of serine phosphorylated GSK3 β / β catenin in two set of experiments in the mechanically stimulated chondrocytes in the presence of Lithium.

4.6 Discussion

Experiments carried out in this chapter and chapter 5 have used pressure, via a gas phase, introduced to monolayer cultured chondrocyte cell lines. This system has been applied in order to study the role of mechanical stimulation in chondrocytes.

Cyclical pressurization results in flexion of the petri dish and stretch of the attached chondrocytes. This, in turn, results in activation of Ca^{2+} dependent K^+ ion channels and membrane hyperpolarisation. In this experimental system, the effects of intermittent pressurisation are mediated by stretch activated ion channels and $\alpha 5\beta 1$ integrin chondrocyte mechanoreceptors.

Cyclical pressurisation and flexion of the petri dish results in bending of the dish and some fluid flow across the chondrocyte monolayer. This type of fluid flow can produce shear stress at the cell surface of cultured chondrocyte cell lines. Thus, the outcome of mechanotransduction pathway in normal chondrocytes, which leads to significant changes in aggrecan and MMP3 levels, may not be just due to cyclical pressure-induced strain. Fluid flow can also affect the expression of cell-matrix proteins, which are regulated by a Wnt-1 mediated signalling pathway. Fluid flow influences distribution of Wnt-1, a secretory growth factor, across the petri dish. This, in turn, causes a difference in the rate at which the transcriptional activation of Wnt mediated target genes is regulated.

4.6.1 GSK3 β / β catenin

The presence of GSK3 β / β catenin complexes following mechanical stimulation and at the basal level in the presence and absence of Lithium was examined in two set of separate experiments. **Fig 4.2 and Fig 4.3** showed that the formation of GSK3 β / β catenin complex were induced after 5 minutes in the Lithium treated samples.

However, the densitometric data in **Table 4.2 iv** demonstrated large differences in the intensity/mm² of this complex, when the two sets of experiments were compared. Large differences in GSK3 β / β catenin complex formation were also found following 1 and 10 minutes of mechanical stimulation, in the presence of Lithium **Fig 4.2 and Fig 4.4**. Further experiments need to be undertaken to determine whether these changes are real or the result of experimental variation.

If the formation of GSK3 β / β catenin complexes is conclusively established in these condotions, it is likely that this would be associated with changes in the nuclear/cytoplasmic distribution of β catenin. This, in turn, might be one of the major factors controlling the expression of the glycoproteins such as FN and CD44 in chondrocytes.

If the data presented in **Fig 4.2** was confirmed by densitometric data from a larger number of experiments, one would draw the following conclusions.

I) Formation of GSK3 β / β catenin complexes are not induced when chondrocytes are mechanically stimulated. This would suggest that integrin mediated signalling pathways are not directly involved in the formation of these complexes under these conditions. Consequently, the tyrosine phosphorylation of β catenin following

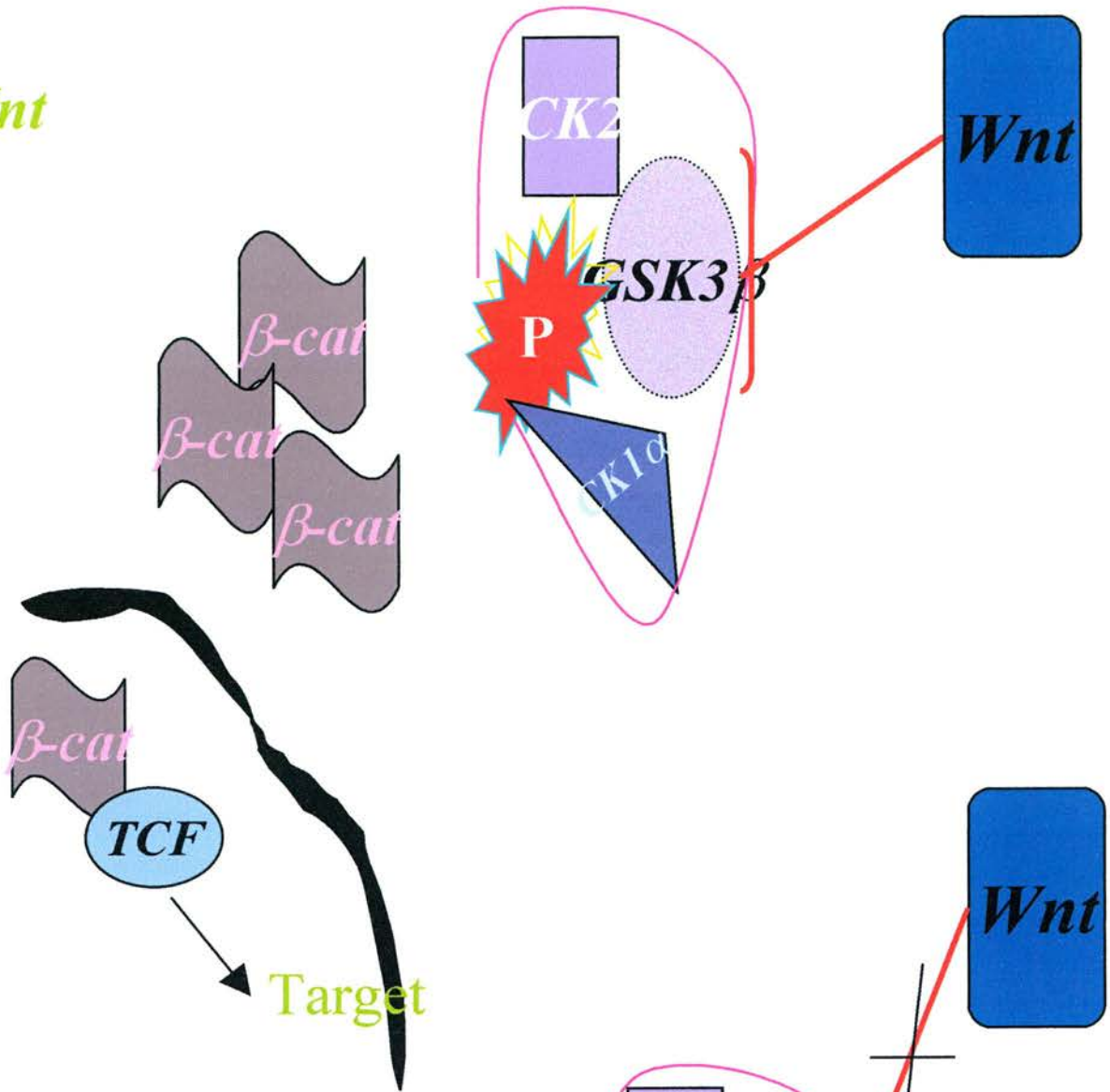
mechanical stimulation, which ultimately leads to its degradation, may not be regulated by the activity of GSK3 β . It is possible that other protein kinases, the activity of which are regulated by integrin mediated signalling pathways, are involved in β -catenin tyrosine phosphorylation. These kinases could include FAK, Fyn and Pyk2 tyrosine kinases.

II) According to previous studies (Haq *et al* 2000; Wnt genes homepage: www.stanford.edu/~rnusse/wntwindow.html)), Li⁺ inhibits GSK3 β activity, which in turn blocks GSK3 β / β catenin complex formation and β catenin phosphorylation. The unphosphorylated β catenin is dissociated from the GSK3 β , axin, APC degradation complex and accumulates within the cytoplasm (**Fig 4.13**). When chondrocytes were treated with Li⁺ (a Wnt agonist) formation of a GSK3 β / β catenin complex was induced (**Fig 4.2**). It is possible that the induction of GSK3 β / β catenin complex formation in the presence of Li⁺ may be an evidence for the activation of a Ca²⁺ dependent Wnt pathway rather than the canonical Wnt pathway in chondrocytes (**Fig 6.1**). This may occur, as a result of PKC activation, which in turn, activates PP2A phosphatases. The activation of PP2A leads to dephosphorylation and inhibition of PKB, thereby enhancing GSK3 β activity (Takana *et al.*, 2003). Activation of GSK3 β , in turn, could lead to formation of GSK3 β / β catenin complexes.

This effect of Li⁺ on PKC which leads to PKB inhibition may be independent of PI3K activity which usually leads to PKB phosphorylation and its activation.

However, induction of GSK3 β / β catenin complex formation in the presence of Li⁺ in chondrocytes, would result from activation of other pathways (**Fig 4.14**).

I) *Wnt*



~~II) *Wnt*~~

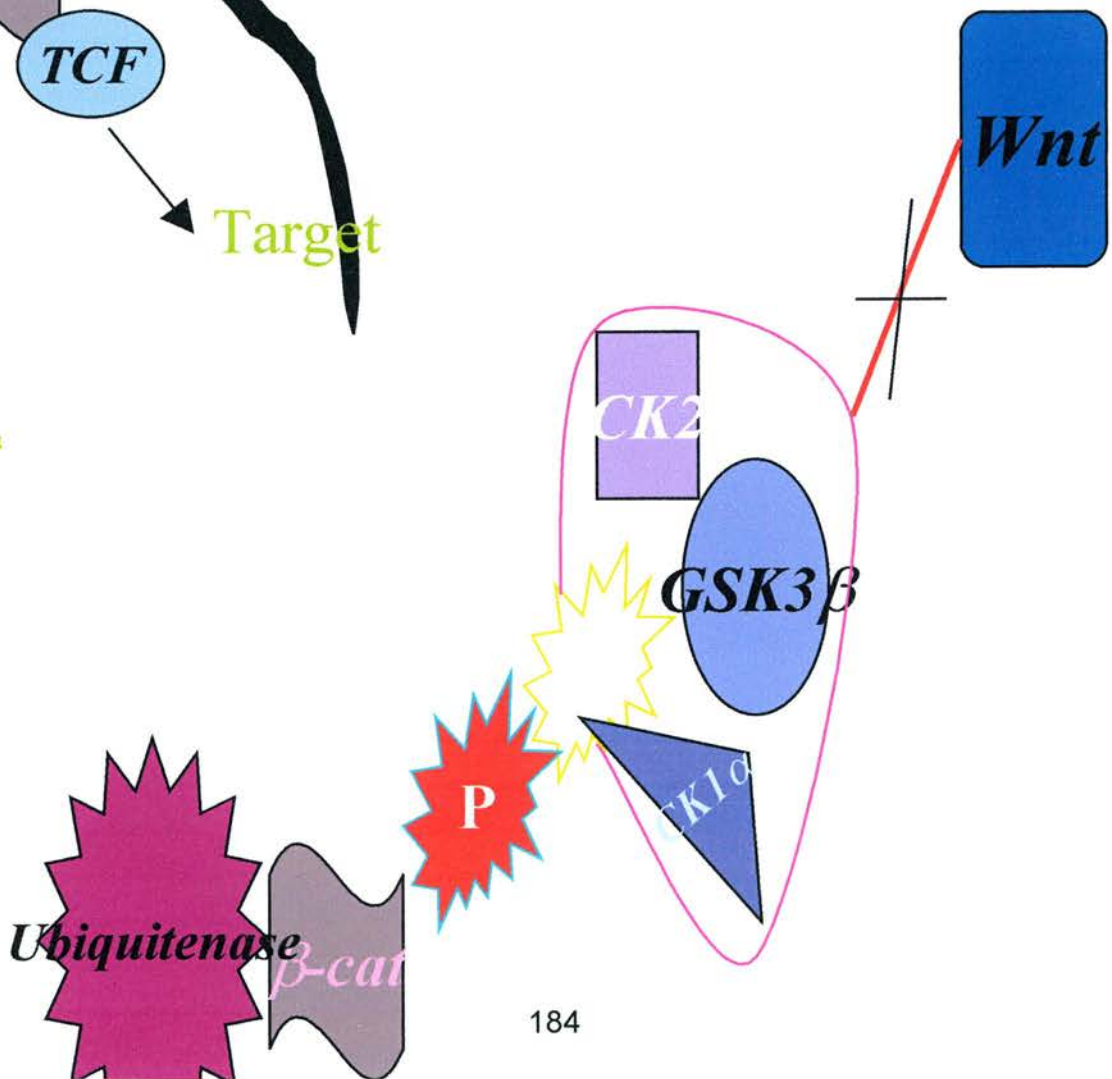


Fig 4.13

Fig 4.13: The effect of Wnt on regulating GSK3 β activity and formation of GSK3 β / β catenin complex I) In the presence of the Wnt signals, GSK3 β complex is inhibited, thus, β catenin remains unphosphorylated, accumulated in the cytoplasm and translocated in to the nucleus where it binds to the transcription factors TCF/ LEF and activates the transcription of the target genes. II) In the absence of the Wnt signals, GSK3 β complex remains activated thus it phosphorylates β catenin and facilitates its degradation by ubiquitin/proteosome pathway.

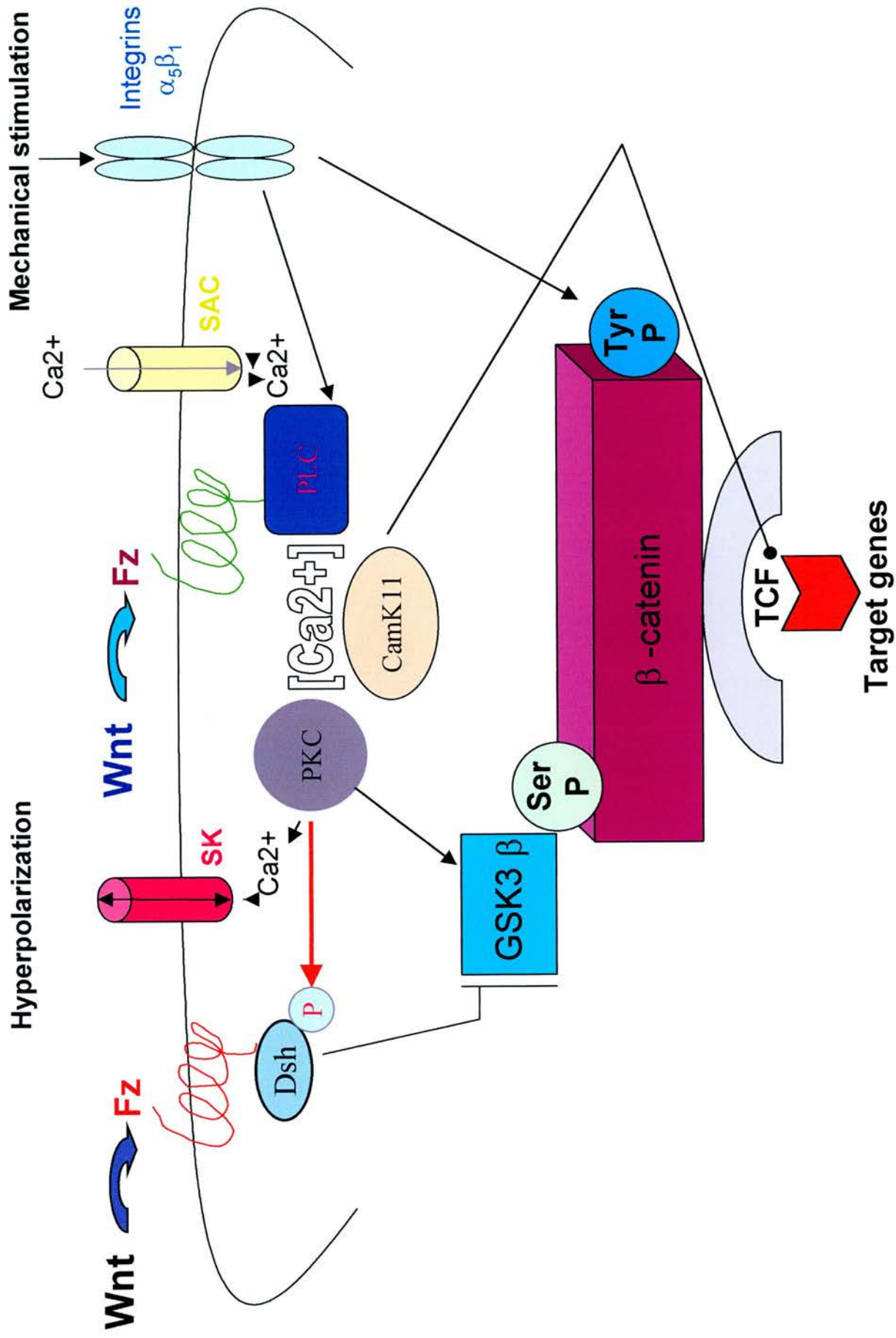


Fig 4.14: Interaction between mechanotransduction, Wnt/β catenin and Wnt/Ca²⁺ pathways

Fig 4.14: Mechanical stimulation results in activation of $\alpha 5\beta 1$ integrin and opening of SAC, which leads to activation of PLC and results in the release of intracellular calcium. Wnt/ Ca^{2+} pathway may modulate this process. Activation of PLC results in activation of PKC, which in turn results in membrane hyperpolarisation as SK channels are activated. PKC can activate GSK3 β through activation of phosphatases or inhibits GSK3 β activity by modulating Wnt/ β catenin pathway through phosphorylation of Dsh. These process of activation and inhibition of GSK3 β affects the regulation β catenin serine phosphorylation.

III) Fyn tyrosine kinase is associated with FAK, which also binds PI3K and may enhance the activity of this kinase. Activation of PI3K leads to phosphorylation and activation of PKB, which in turn, phosphorylates GSK3 β and inhibits it. Thus, significant decrease in tyrosine phosphorylation of β catenin in the Li⁺ treated cells in resting chondrocytes could be accompanied by the inhibition of GSK3 β activity through activation of Fyn /PI3K kinase. This, in turn, could inhibit the formation of GSK3 β / β catenin complexes via an integrin dependent signalling pathway (**Fig 4.8**). Serine phosphorylation of GSK3 β following 5 minutes of mechanical stimulation in the presence of Li⁺ (Wnt agonist) was significantly increased. This, in turn, resulted in depletion of GSK3 β / β catenin complexes which can be associated with the inhibition of GSK3 β via interaction between both a PI3K dependent pathway and a Wnt pathway (**Fig 4.12**).

IV) There was an increase in tyrosine phosphorylation of GSK3 β after 1 minute of mechanical stimulation and in the presence of Li⁺ (**Fig 4.9**). Phosphorylation of GSK3 β on tyrosine residues is facilitated by an integrin mediated signalling pathway and leads to GSK3 β activation (Giancotti and Ruoslahti., 1999; Doble and Woodgett., 2003). However, GSK3 β was also serine phosphorylated under the above conditions, which leads to its inhibition via a PI3K dependent pathway. In addition, GSK3 β / β catenin complexes were formed. This suggests the possibility that combined activity of an integrin mediated signalling pathway and a Wnt pathway is responsible for formation of GSK3 β / β catenin complexes and regulation of β catenin phosphorylation after 1 minute of mechanical stimulation.

Mechanical stimulation of the Li^+ treated cells caused a delay in the formation of the GSK3 β / β catenin complex when compared to unstimulated samples (**Fig 4.6 and 4.5**). This suggests that mechanical stimulation can influence the Wnt mediated signalling pathways in chondrocytes under these conditions.

4.6.2 Phosphoserine

Data presented in this study has shown no evidence of serine phosphorylation of β catenin in a chondrocyte cell line with or without mechanical stimulation in the presence or absence of Li^+ . This suggests that, under these conditions, β catenin is not serine phosphorylated, and thus can not become a primed substrate for GSK3 β . This phosphorylation is necessary for further phosphorylation and degradation of β catenin. However, according to previous studies, it is also known that a Wnt dependent pathway can regulate β catenin concentration in cells even when β catenin remains an unprimed substrate for GSK3 β (Thomas *et al.*, 1999; Frame *et al.*, 2001). Consequently, the results obtained in this study suggest that priming of β catenin may not be critical for GSK3 β / β catenin complex formation and the maintenance of β catenin cytoplasmic/nuclear distribution within the cell.

This study has further shown evidence for serine phosphorylation of GSK3 β in unstimulated chondrocytes. However, the densitometric data in **Table 4.5 iii** and **4.5 iv** suggests that there may be significant differences in the intensity/ mm^2 of serine phosphorylated GSK3 β between the two set of experiments. In order to find out if these differences are real or are attributable to experimental variation, statistical analysis of further experiments is required.

However, if the preliminary data presented in **Fig 4.10** was confirmed by densitometric data from a larger number of experiments, then the following conclusions could be drawn.

I) Pretreatment of the cells with Li^+ enhanced GSK3 β serine phosphorylation and as a consequence its inhibition. Serine phosphorylation of GSK3 β was not influenced by mechanical stimulation. However, pretreatment of the cells with Li^+ followed by mechanical stimulation led to a delay in GSK3 β serine phosphorylation. Thus, it could be speculated that serine phosphorylation of GSK3 β is mainly regulated by a Wnt dependent pathway. However, integrin mediated pathway may insert some regulatory role on the Wnt mediated serine phosphorylation of GSK3 β .

II) Moreover, phosphorylation of GSK3 β on serine residues by PI3/PKB results in its inhibition through a Wnt independent mechanism (Haq *et al.*, 2000). However, the data suggests that both the formation of GSK3 β / β catenin complexes and serine phosphorylation of GSK3 β occurred under the same conditions (**Fig 4.2** and **Fig 4.10**). Thus, one could postulate that the phosphorylation of GSK3 β at Ser-9 may be mediated by PI3/PKB activity following mechanical stimulation. This would be followed by recruitment of PKB to the GSK3 β / β catenin multiprotein complexes which are formed in the presence of the Wnt agonist Li^+ . This, in turn, results in stabilisation of β catenin in the chondrocyte cell line. Thus, the outcome of mechanical stimulation in chondrocytes is identical to the Wnt dependent signalling pathway, resulting in stabilisation of GSK3 β / β catenin complex. This suggests that a rather complicated interaction between Wnt-dependent, Wnt-independent and integrin

mediated signalling pathways is responsible for the formation of GSK3 β / β catenin complexes.

To further examine the regulatory mechanisms involved in the formation of GSK3 β / β catenin complexes and in order to distinguish between the pathways that are involved in this process, following approach could be adopted.

Immunoprecipitation and immunoblotting could be undertaken in order to detect the presence of various key signalling molecules and their interactions with the GSK3 β -axin-APC complex. In these set of experiments: i) the whole cell lysates should be collected and immunoprecipitated with anti-Frat1 antibody then immunoblotted for GSK3 β or Frat-1. ii) the whole cell lysates should be collected and immunoblotted with anti-phospho serine 9 GSK3 β or GSK3 β . iii) the whole cell lysates should be collected and immunoprecipitated with anti-axin antibody or anti-APC antibody or anti-CK1 α antibody and immunoblotted with anti- PKB antibody and anti phospho serine 473 PKB. In all these experiments the cytosolic fraction of β -catenin would be probed with anti- β -catenin to quantify its level of expression.

4.6.3 Phosphotyrosine

Previous studies in Edinburgh have shown tyrosine phosphorylation in both primary chondrocytes and the chondrocyte cell line (C20A4) immediately following mechanical stimulation at 0.33HZ, 3,700 μ strain (Lee, *et al.*, 2000).

However, this study suggests that β catenin is tyrosine phosphorylated in unstimulated cells and shows evidence that mechanical stimulation delays tyrosine phosphorylation of β catenin in the chondrocyte cell line. The densitometric data in **Table 4.3 iii** and

4.3 vii suggests that there may be significant differences in the intensity/mm² of tyrosine phosphorylated β catenin between the two set of experiments. Whether or not this is really so or whether the differences are attributable to experimental variation statistical analysis of further experiments is required.

Mechanical stimulation of the Li⁺ treated chondrocytes has no significant effect on tyrosine phosphorylation of β catenin up to 10 minutes **Fig 4.5 and Fig 4.9**. This was confirmed by densitometric data presented in **Table 4.3viii**. However, the necessity for the statistical analysis of further experiments is not ruled out. If the above pattern of β catenin tyrosine phosphorylation is further demonstrated, then it can be speculated that Wnt mediated signalling may not have a major influence on the tyrosine phosphorylation of β catenin in mechanically stimulated chondrocytes.

If the data presented in **Fig 4.5** was confirmed by densitometric data from further experiments, the following conclusions could be drawn.

I) Differences between these results and those from those in previous studies in Edinburgh, may be attributable to the use of different chondrocyte cell lines. In this study the experiments were carried out in the T/C28I2 chondrocyte cell line, while, Lee *et al* carried out their experiments in the C20/A4 chondrocyte cell line. Furthermore, the mechanical stimulation regime that was used in this study was different from the one adopted by Lee *et al*. In this study, the chondrocyte cell line was mechanically stimulated at 0.33Hz, 30,740 μ strain, while, Lee *et al*, mechanically stimulated the chondrocytes at 0.33Hz, 3,700 μ strain.

30,740 μ strain was used in this work as it was suggested that the optimal proteoglycan synthesis occurs at this strain.

II) In the cells that were subjected to a higher microstrain the hyperpolarisation response was amplified as a result of an increase in the level of intracellular Ca^{2+} concentration, which is caused by opening of mechanosensitive ion channels (Wright *et al.*, 1996). This might be required for integrin dependent tyrosine phosphorylation of β catenin following mechanical stimulation in chondrocytes.

III) Increase in activity of SAC in the chondrocytes when subjected to higher microstrain may affect transduction of integrin mediated signalling intracellularly. This may be caused by influencing the length of time at which β catenin remains tyrosine phosphorylated as a result of a greater and prolonged activation of tyrosine kinases.

Furthermore, according to **Fig 4.5 and Fig 4.7**, in this study, mechanical stimulation had no effect on tyrosine phosphorylation of GSK3 β . However, when the cells were treated with Li^+ the tyrosine phosphorylation of GSK3 β was upregulated.

Furthermore, when the Li^+ treated cells were subjected to mechanical stimulation, tyrosine phosphorylation of both GSK3 β and β catenin was further enhanced. Thus, one can speculate that Li^+ , a Wnt agonist, may induce the tyrosine phosphorylation of GSK3 β by Fyn and PYK2 tyrosine kinases, and cause an increase in the catalytic activity of GSK3 β . This, in turn, could facilitate β catenin tyrosine phosphorylation following mechanical stimulation and lead to β catenin degradation.

However, the densitometric data presented in **Table 4.4iii, 4.4iv, 4.4vii and 4.4viii**, highlights the differences between each comparable values in the two set of experiments. In order to find out whether these differences are real or due to some experimental variations, statistical analysis of further experiments is required. If this confirms the above data then it can be postulated that the tyrosine phosphorylation of GSK3 β and β catenin is predominantly regulated by integrin mediated signalling, indirectly affecting the formation of GSK3 β / β catenin complexes and the cytoplasmic/nuclear distribution of β catenin. However, a Wnt-mediated signalling pathway may also be involved in maintaining a regulatory balance between serine and tyrosine phosphorylation of GSK3 β which is crucial for its overall activity.

4.7 Conclusion

According to above data presented in **Fig 4.2, 4.5 and 4.10**, it can be concluded that serine and tyrosine phosphorylation of GSK3 β is directly related to the formation of GSK3 β / β catenin complexes in the T/C28I2 human chondrocyte cell line. GSK3 β activity and its phosphorylation at serine and tyrosine residues determine whether β catenin is in the phosphorylated or unphosphorylated state. This, in turn, modulates β catenin cytoplasmic/ nuclear distribution. Tyrosine phosphorylation of β catenin and serine phosphorylation of GSK3 β are both associated with an integrin mediated, Wnt-independent pathway. Thus, it is likely that a fine balance between Wnt and integrin mediated signalling pathways play an important role in regulating the formation of GSK3 β / β catenin complexes. As a result of this the concentration of β catenin within chondrocytes is tightly regulated, which in turn, is essential for the transcriptional regulation of the target genes such as CD44 and FN.

Chapter 5

Kinase activity

Glycogen Synthase Kinase -3 β (**GSK3 β**) is a highly conserved, multifunctional serine/threonine protein kinase, which acts as a downstream regulatory switch in numerous signalling pathways that are initiated by such diverse stimuli as Wnt growth factors, receptor tyrosine kinases and G protein coupled receptors (Doble and Woodgett., 2003; Wodarz and Nusse 1998; Dominguez and Green., 2001). GSK3 β is a key mediator of the Wnt pathway as it is involved in regulating β -catenin degradation and its cytoplasmic/nuclear distribution. GSK3 β is also a regulatory enzyme for glycogen synthesis initiated by insulin growth factors (Woodgett 1990; Welsh and Proud 1993; Dominguez and Green., 2001).

GSK3 β , unlike most protein kinases, is active in unstimulated cells and is inactivated by phosphorylation when the cells are stimulated by various factors such as insulin and Wnt mediated signalling pathways (Haq *et al.*, 2000).

In the absence of insulin growth factors, the free pool of GSK3 β phosphorylates and inactivates glycogen synthase (GS), thereby inhibiting glycogen synthesis. In the presence of insulin growth factors, the free pool of GSK3 β becomes phosphorylated at Ser-9 by protein kinase B (PKB). The phosphorylated N-terminus inhibits GSK3 β by acting as a pseudosubstrate, competing with the primed substrate (GS) for the phosphate binding site, and the catalytic site on the GSK3 β . Hence, GS is dephosphorylated, and activated, and glycogen synthesis is initiated (Frame *et al.*, 2001).

In the absence of Wnt, GSK3 β phosphorylates Axin and β catenin, triggering the stabilisation of Axin and degradation of β catenin. In the presence of Wnt, Axin is displaced from GSK3 β as a result of FRAT binding to GSK3 β . This leads to stabilisation of β catenin and its accumulation in the nucleus, where it stimulates the transcription of the Wnt target genes. Consequently, GSK3 β , a central component in various signalling pathways, is implicated in many biological processes and regulates the cross talk between several pathways through interacting with different and distinct components (Wnt genes homepage: www.stanford.edu/~rnusse/wntwindow.html).

5.1 Effect of mechanical stimulation on GSK3 β

To investigate whether GSK3 β has a role in the mechanotransduction pathway in chondrocytes, and to further understand what effect mechanical stimulation has on GSK3 β activity, a kinase assay was developed to assess kinase activity following a variety of stimuli. For the purpose of the kinase assay, lysates were prepared and immunoprecipitated using an anti-GSK3 β antibody, following stimulation the cells. They were assayed for overall enzyme activity using GS-2 peptide as the substrate. As a background control, beads were incubated with lysis buffer under the same experimental conditions as the ones that were immunoprecipitated using an anti-GSK3 β antibody, and then assayed for enzyme activity.

Initially, to assess the effect of mechanical stimulation, T/C 28I2 chondrocytes were subjected to 20, 40 and 60 minutes of mechanical stimulation at 0.33 Hz, 30,740 μ strain.

The results are shown in (**Fig 5.1**) (n=11). The resting chondrocytes showed little intrinsic kinase activity 1648.01 ± 696.53 dpm [^{32}P] incorporated / mg protein/ hour. However, in response to mechanical stimulation, kinase activity slightly increased to, 2351.81 ± 847.67 dpm [^{32}P] incorporated / mg protein/ hour after 20 minutes of mechanical stimulation; and 6564.20 ± 1729.87 dpm [^{32}P] incorporated / mg protein/ hour ($p < 0.05$) at 40 minutes before returning to near basal levels; 2704.13 ± 1371.13 dpm [^{32}P] incorporated / mg protein/ hour, after 60 min of mechanical stimulation.

5.2 Mechanisms of GSK3 β regulation

5.2.1 Role of PI3K

LY 294002, a PI3- Kinase inhibitor (Davies *et al.*, 2000), was used to investigate the role of PI3K on GSK3 β in T/C28I2 cell lines. The chondrocytes were incubated with LY294002 (10 μM in DMSO) for 30 minutes prior to mechanical stimulation, for 20, 40 or 60 minutes (**Fig 5.2**)(n=7).

In the absence of LY294002 and DMSO, basal kinase activity was 882.80 ± 522.98 dpm [^{32}P] incorporated / mg protein/ hour. Glycogen synthase (GS) in the resting state maintained very low activity as it was continuously phosphorylated by GSK3 (Halse *et al.*, 2003). In the presence of LY294002 kinase activity decreased slightly to 594.45 ± 542.39 dpm [^{32}P] incorporated / mg protein/ hour.

When chondrocytes were mechanically stimulated in the absence of LY294002, the overall kinase activity was 942.66 ± 272.53 dpm [^{32}P] incorporated / mg protein/ hour following 20 minutes of mechanical stimulation. In the presence of LY294002 the overall kinase activity was 1495.40 ± 1206.32 dpm [^{32}P] incorporated / mg protein/

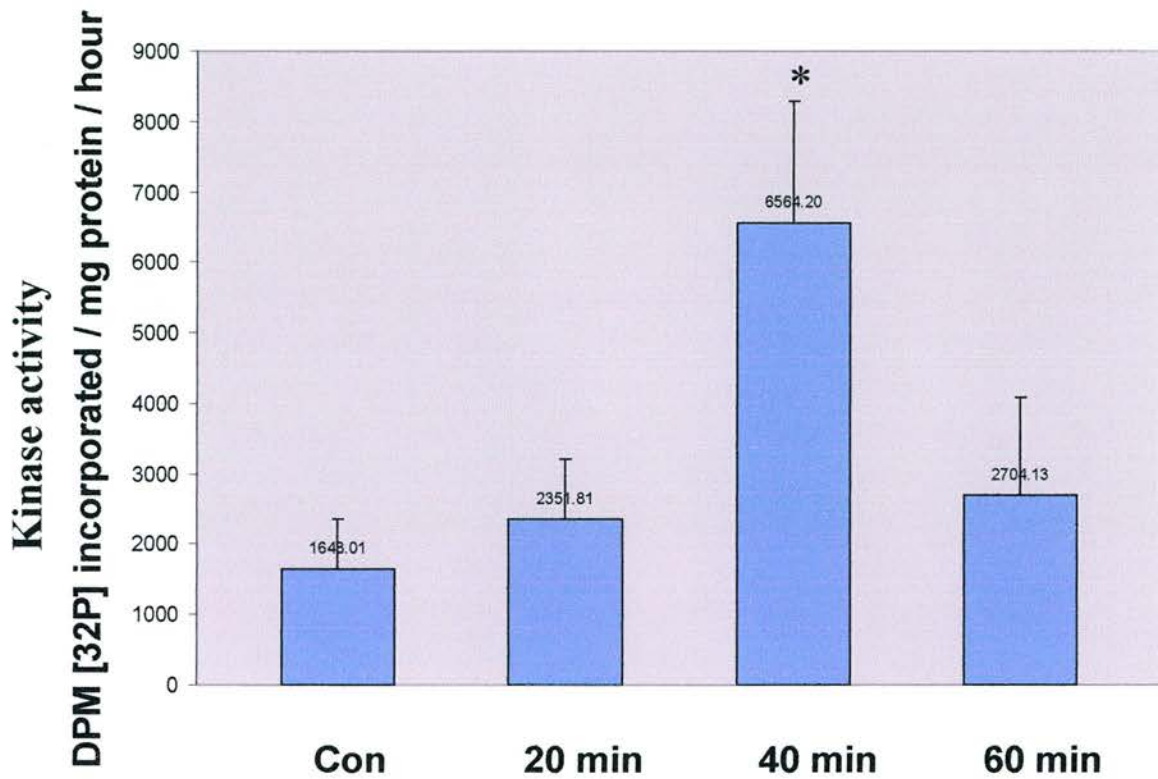


Fig 5.1: Kinase activity following cyclical mechanical stimulation for 20, 40 and 60 minutes Con: Control. mean + standard error, n = 11* P< 0.05 , Samples following 40 minutes of mechanical stimulation were compared with the control samples.

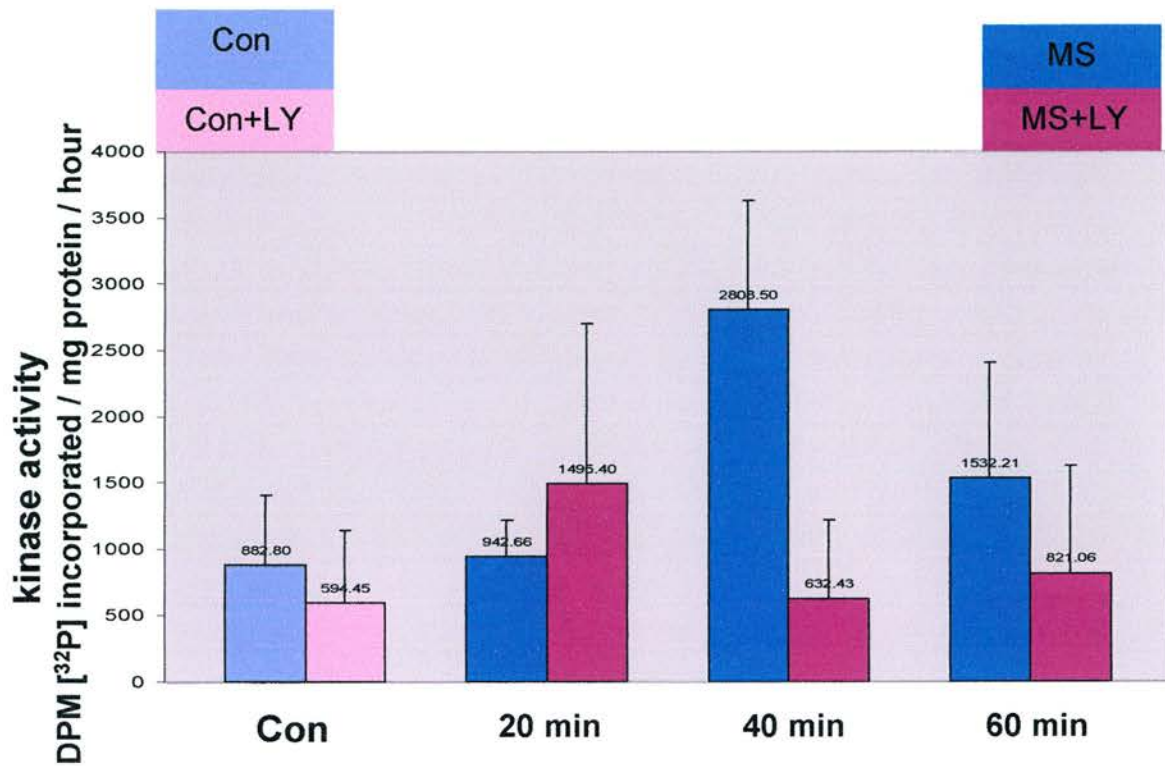


Fig 5.2: Kinase activity following 20, 40 and 60 minutes of cyclical mechanical stimulation with and without PI3K inhibitor (10 μ M LY294002). mean + standard error, n=7

hour. There was no significant change in overall kinase activity in the samples with and without treatment with LY294002 at 60 minutes. In the cells mechanically stimulated for 40 and 60 minutes in the presence of LY294002 the increase in the overall kinase activity was diminished (**Fig 5.2**). When the chondrocytes were mechanically stimulated in the absence of LY294002 the overall kinase activity was 2808.5 ± 822.76 dpm [^{32}P] incorporated / mg protein/ hour following 40 minutes of mechanical stimulation. However, in the presence of LY294002 the overall kinase activity decreased to 632.43 ± 584.12 dpm [^{32}P] incorporated / mg protein/ hour. When the chondrocytes were mechanically stimulated for 60 minutes in the absence of LY294002, the overall kinase activity was 1532.21 ± 868.91 dpm [^{32}P] incorporated / mg protein/ hour. However, in the presence of LY294002 the overall kinase activity was 821.06 ± 808.81 dpm [^{32}P] incorporated / mg protein/ hour, which represented no significant change in overall kinase activity in the samples with and without treatment with LY294002 at 60 minutes

5.2.2 Effect of Lithium

The Lithium ion (Li^+) is a Wnt agonist, and so is inhibitory to GSK3 β . Li^+ mimics the cellular responses mediated by the inhibition of GSK3 β by ILK, IGF1 and the Wnt signalling pathways (Davies *et al.*, 2000) (**Fig 5.3**).

In order to determine the effect Lithium ions on GSK3 β activity in chondrocytes, cells were serum starved overnight and treated with 10 mM Li^+ for 30 minutes prior to 20, 40 and 60 minutes of mechanical stimulation (n=3). As a control, the serum starved cells were pretreated with 10mM Li^+ for 30 minutes and then left under the same condition as the mechanically stimulated cells but without mechanical stimulation.

The cell lysates were then assayed for kinase activity using the GS-2 peptide as the substrate.

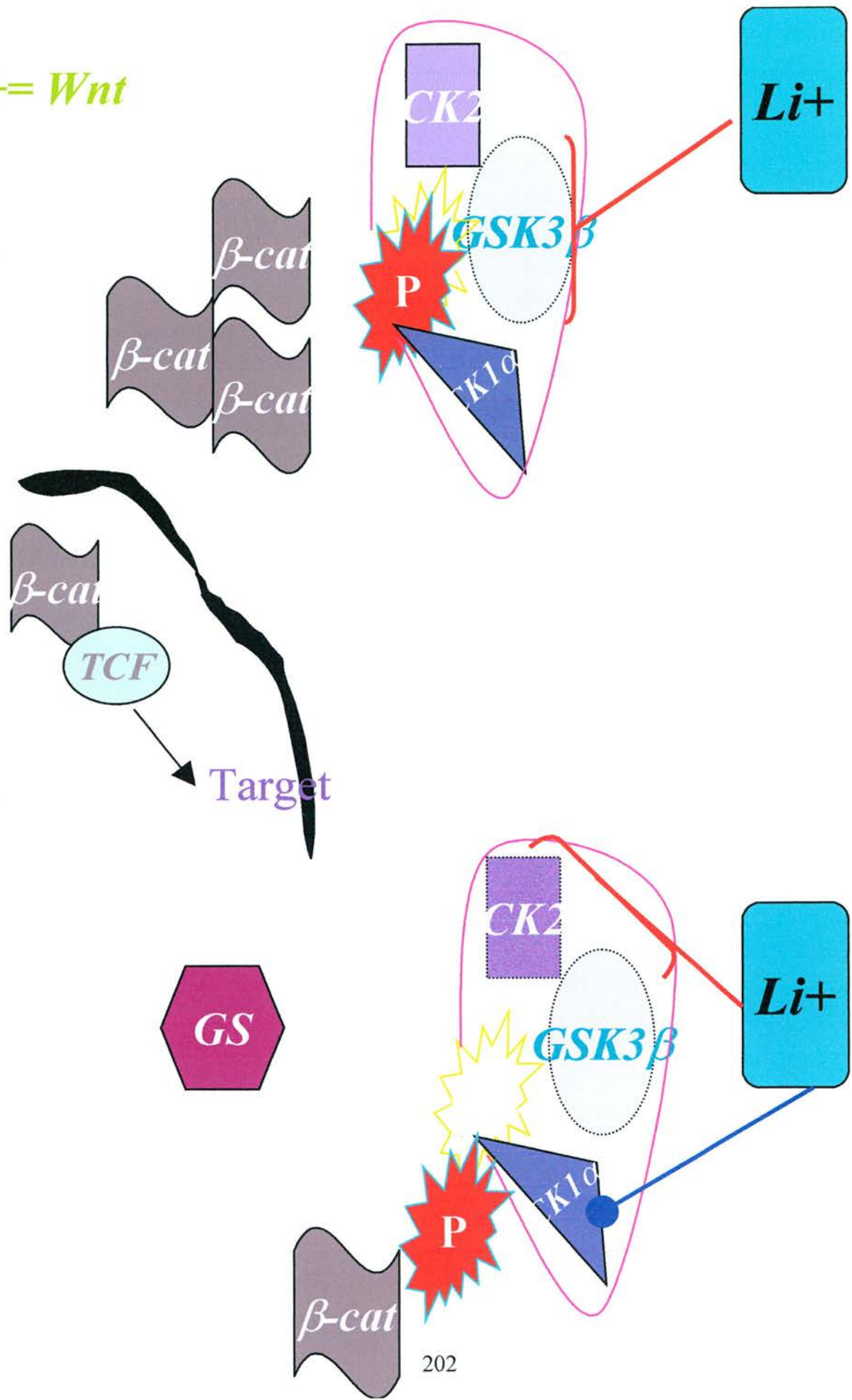
In the resting cells, treatment with Li^+ caused a significant increase in GSK3 β activity from 3829.60 ± 1918.78 dpm [^{32}P] incorporated / mg protein/ hour to 12666.14 ± 252.40 dpm [^{32}P] incorporated / mg protein/ hour ($P < 0.01$) (Fig 5.4). However, in response to mechanical stimulation in the presence of Li^+ , a biphasic pattern of GSK3 β activity was observed. After an initial peak of activity of 23004.32 ± 10532.34 dpm [^{32}P] incorporated / mg protein/ hour following 20 min of mechanical stimulation, activity declined to, 14215.98 ± 4233.27 dpm [^{32}P] incorporated / mg protein/ hour at 40 minutes and increased again to 21231.21 ± 10448.36 dpm [^{32}P] incorporated / mg protein/ hour at 60 min of mechanical stimulation (Fig 5.4). However, none of these changes reach significance when compared to their untreated parallel control, which were run under same conditions.

5.2.3 Serine-9 phosphorylation

GSK3 β usually requires a “priming” phosphorylation event in its substrates. Thus it phosphorylates a Ser/Thr residue (shown in bold type) which is N-terminal to the phosphorylated Ser/Thr residue in the sequence **Ser/Thr**-Xaa-Xaa-Xaa-Ser/Thr (P). GSK3 β does not phosphorylate peptides lacking such a “priming” phosphate.

To investigate the role of Ser-9 phosphorylation on GSK3 β activity in chondrocytes, a glycogen synthase peptide-2 (Ala-21) was substituted for the phospho glycogen synthase peptide-2. In this alternative substrate, there is an alanine substitution for Serine 9, making it unlikely that this substrate will be phosphorylated by GSK3 β . When GS-2 (Ala-21) was used as substrate, kinase activity was 8722.58 ± 2588.92 ,

•Li+= Wnt



I

II

Fig 5.3

Fig 5.3: The effect of Lithium, a Wnt agonist on GSK3 β activity.

I) In the presence of Li^+ , a Wnt agonist, the GSK3 β complex is inhibited and its substrate, β catenin remains unphosphorylated. This results in β catenin accumulation within the cytoplasm and its translocation into the nucleus. There, it binds the transcription factors TCF/ LEF and activates the transcription of the target genes. II) Li^+ is a potent inhibitor of CK2 in addition to GSK3 β . Phosphorylation of GS by CK2 is a prerequisite for phosphorylation of GS by GSK3 β . Thus, treatment of the cells with Li^+ results in the partial inhibition of the GSK3 β complex, resulting in GS remaining unphosphorylated and therefore active. However CK1 α , a primerkinase for β Catenin phosphorylation prior to its phosphorylation by GSK3 β , is induced by Li^+ .

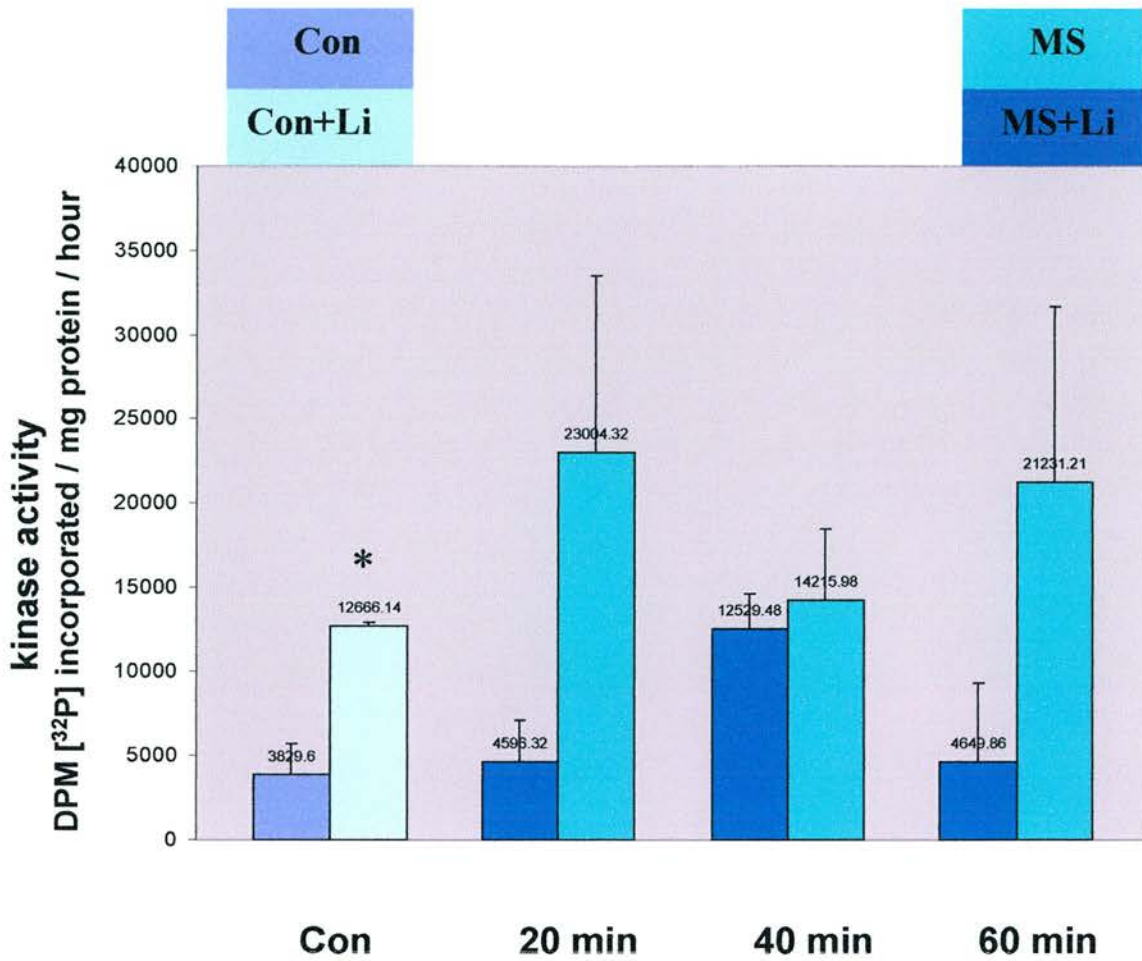


Fig 5.4: The effect of the Wnt agonist (10 mM Li⁺) on basal kinase activity (con) and following 20, 40 and 60 minutes of mechanical stimulation. mean + standard error, n =3* P<0.01 Samples, with and without lithium treatment were compared at basal level.

2249.34 ± 1374.90 and 4464.22 ± 4126.19 dpm [³²P] incorporated / mg protein/ hour at 20, 40 and 60 minutes respectively (**Fig 5.5**). However, in cells pretreated with Li⁺ and left for 20 minutes the overall kinase activity slightly decreased to 3703.56 ± 3566.18 dpm [³²P] incorporated / mg protein/ hour (**Fig 5.5**). And, in cells pretreated with Li⁺ the overall kinase activity of 10416.76 ± 1264.86 dpm [³²P] incorporated / mg protein/ hour, was restored at 40 minutes (P< 0.05) (**Fig 5.5**).

With GS-2 peptide as a substrate overall kinase activity was only 2211.96 ± 2109.90 dpm [³²P] incorporated / mg protein/ hour at 20 minutes (**Fig 5.6**). However, when the samples were treated with Li⁺ the overall kinase activity, significantly rose to 15833.87 ± 4555.47 dpm [³²P] incorporated / mg protein/ hour (P<0.05) at 20 minutes. Furthermore, in the samples without Li⁺ treatment the level of overall kinase activity slightly increased from 757.5 ± 507.39 to 8212 ± 3431.78 dpm [³²P] incorporated / mg protein/ hour at 40 minutes and from 5774.15 ± 2806.124 dpm [³²P] incorporated / mg protein/ hour at 60 minutes, (**Fig 5.6**). Treatment of the samples with Lithium was associated with a further increase in kinase activity dpm [³²P] incorporated / mg protein/ hour at 40 and 60 minutes time point, however, these changes were not significant n=3.

5.2.4 Effect of Lithium and LY294002

In order to determine the effect of the combination of Li⁺ and LY294002 on GSK3β activity in chondrocytes, cells were serum starved overnight and treated with 10μM LY294002 for 30 minutes followed by further treatment with 10mM Li⁺ for additional 30 minutes. Then the Li⁺ and LY294002 treated cells were mechanically stimulated for 20, 40, 60 minutes n=3. A set of untreated cells and a set of treated but

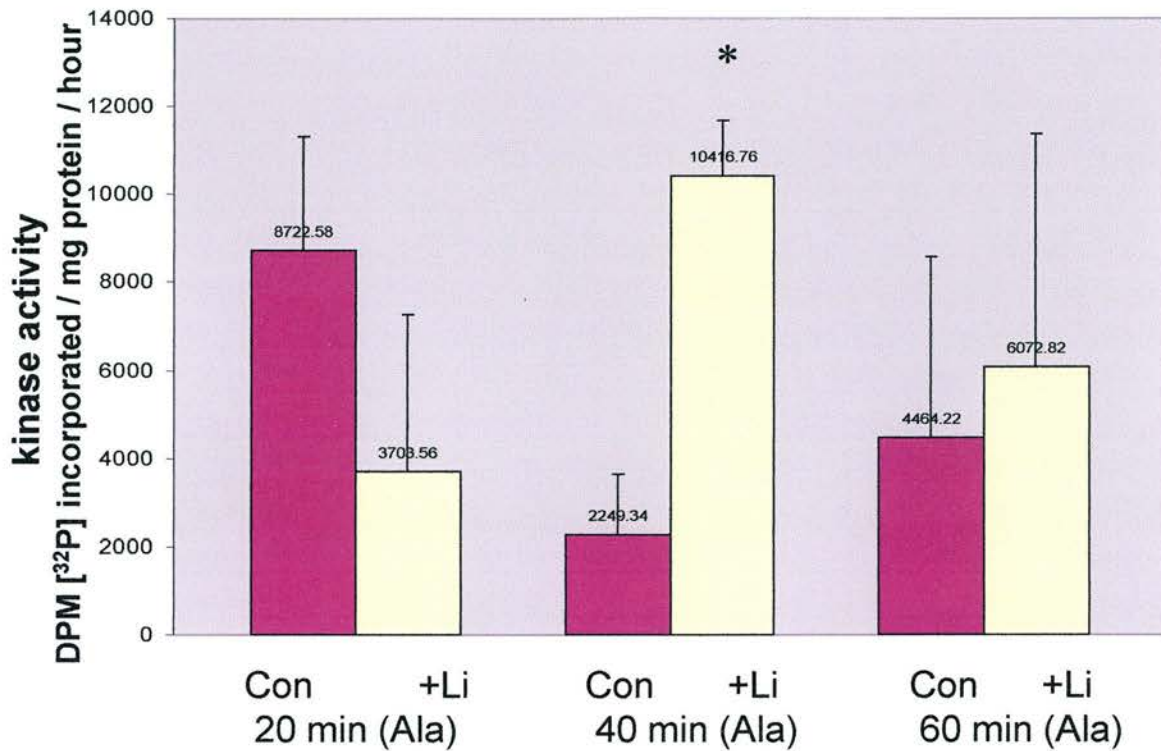


Fig 5.5: Kinase activity in the presence of 10mM lithium after 20, 40 and 60 minutes using GS-2 (Ala-21) instead of GS-2 as a substrate along with their parallel control. mean + standard error, n=3* P<0.05 Samples with and without lithium treatments compared at 40 min.

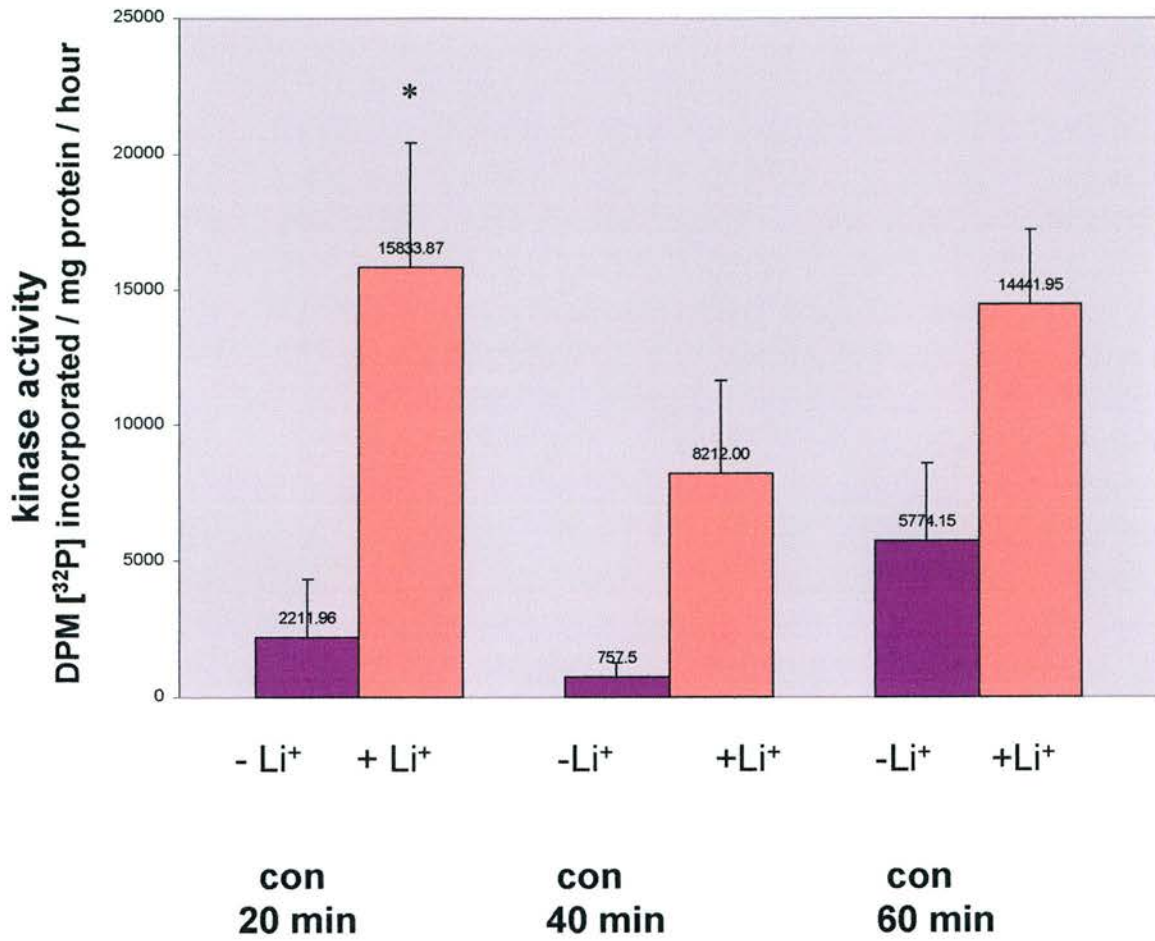


Fig 5.6: Kinase activity in the absence and presence of 10 mM Li⁺, a Wnt agonist, mean + standard error, n=5
 *P<0.05 control Li⁺ treated sample compared with control samples without Li⁺ treatment at 20 minutes.

unstimulated cells served as parallel controls. The cell lysates were assayed for kinase activity using GS-2 peptide as the substrate.

Treatment of the cells with LY294002 followed by treatment with Li^+ had no significant effect on the overall kinase activity in unstimulated cells (**Fig 5.7**). As one set of data was obtained for the each set of experiments and those data were compared with their parallel control, thus the unpaired t-test was chosen over the ANOVA to evaluate the significance of the values. Mechanical stimulation of these cells in the presence of LY294002 and also Li^+ had no significant effect on kinase activity when compared with samples not treated with these inhibitors (**Fig 5.7**). In resting chondrocytes overall kinase activity slightly increased from 4481.74 ± 1261.95 to 6011.78 ± 3115.52 dpm [^{32}P] incorporated / mg protein/ hour in the samples treated with LY294002 and Lithium, however, this is not considered as a significant rise in kinase activity. There was no significant change in overall kinase activity after 20 minutes of mechanical stimulation and only slight increase from 5994.58 ± 2428.73 to 8587.32 ± 6348.17 dpm [^{32}P] incorporated / mg protein/ hour in the LY294002 and lithium treated samples after 40 minutes of mechanical stimulation. This change also did not reach a significance. However, in the samples pretreated with LY294002 and Li^+ and then mechanically stimulated for 60 minutes a considerable decrease in overall kinase activity from 4628.2 ± 3422.80 to 345.04 ± 180.74 dpm [^{32}P] incorporated / mg protein/ hour was measured.

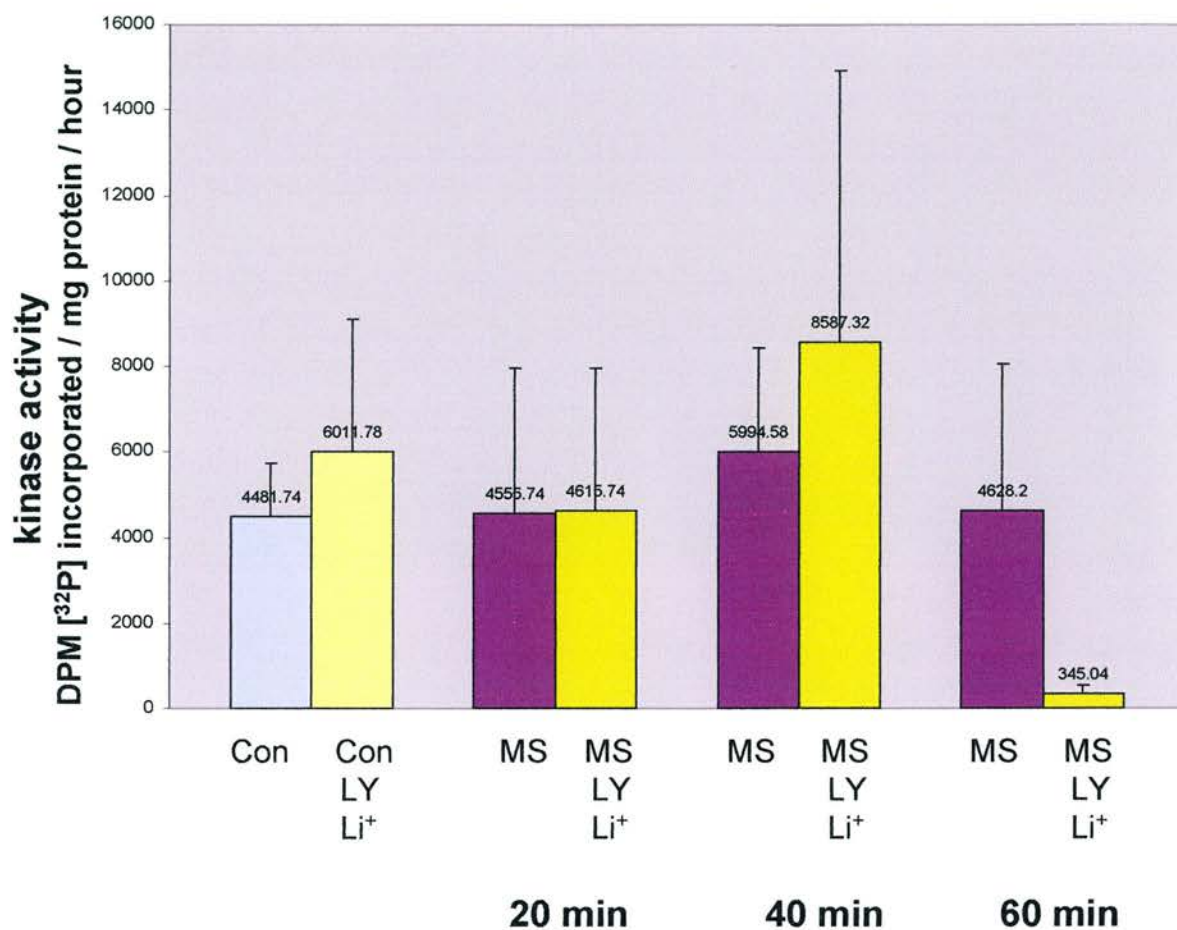


Fig 5.7: The effect of 10 mM Li⁺ and 10 μM LY294002 on kinase activity in resting controls (Con) and following 20, 40, 60 minutes of mechanical stimulation in the T/C2812 chondrocyte cell line. The cells were initially treated with LY294002 followed by treatment with Li⁺. mean + standard error, n=3

5.3 Conclusions

5.3.1 Effect of LY294002

In the presence of LY294002 there was a decrease in kinase activity at the basal level and after 40 and 60 minutes of mechanical stimulation. This could be due to inhibition of PI3K activity, as result of which PKB was also inhibited; therefore, GSK3 β remained unphosphorylated and active. This increase in activity of GSK3 β resulted in further phosphorylation of GS. As GS was phosphorylated it was inhibited, thus, incorporation of ^{32}P into GS decreased and after a certain period of time blocked. However, mechanical stimulation of the chondrocytes in the presence of Ly294002 for 20 minutes resulted in an increase in the kinase activity. This could be due to inhibition of PI3K activity, as result of which PKB was also inhibited; therefore, GSK3 β remained unphosphorylated and active.

When the cells were mechanically stimulated PKC was activated, which in turn, activated protein phosphatase 2A (PP2A) resulting in inhibition of PKB and further activation of GSK3 β (Takana *et al.*, 2003). This, in turn, might result in further phosphorylation of GS and its inhibition. In the samples pretreated with LY294002 and then mechanically stimulated, GSK3 β is activated via a PI3K dependent pathway. An increase or decrease in the dpm [^{32}P] incorporated / mg protein/ hour, was due to the effect LY294002 and mechanical stimulation on the overall activity of GS, GSK3 and CK2 (casein kinase 2), a priming kinase for GSK3 activity which could also be inhibited by LY294002. The above data showed that the overall activity of GSK3 β and GS is mediated by a PI3K dependent pathway (**Fig 5.2**).

5.3.2 Effect of GS-2(Ala-21) Substitution for GS-2 substrate

Overall kinase activity was identified despite Ala 21 substitution for Ser 9 in the substrate (**Fig 5.5**). This could be due to activity of CK2 which might have an alternative binding site on GS rather than Ser-9. Thus, the Ala-21 substitution for serine might have no effect on phosphorylation of GS by CK2. In the Lithium treated chondrocytes, Lithium as a Wnt agonist, could shift some of the CK2 activity from a PI3K mediated pathway, in which GS was a substrate, to the Wnt dependent pathway in which β catenin was a substrate and probably coimmunoprecipitated with the GSK3 β /CK2 complex. In the samples with the GS-2 (Ala-21) substrate after being left for 40 minutes, there was considerable decrease in dpm [32 P] incorporated / mg protein/ hour, which further emphasized the role of GS-2 (Ala21) substrate as a negative control in these set of experiments. This might be due to phosphorylation of GS by both CK2 and GSK3 β and its inhibition, which resulted in decrease in incorporation of 32 P in GS compared with the GS in background control (**Fig 5.5**).

In **Fig 5.6** as GS-2 peptide was a substrate very low overall kinase activity was identified. This might be due to phosphorylation of GS by both CK2 and GSK3 β and its inhibition, which resulted in decrease in incorporation of 32 P in GS compared with the GS in background control. However, treatment of the samples with lithium led to an increase in the overall kinase activity. This could be as a result of a greater involvement of CK2 in phosphorylation of β catenin via a Wnt mediated signalling pathway rather than its engagement in phosphorylation of GS via a PI3K dependent pathway. Thus, GS remains unphosphorylated and active and this increases the incorporation of 32 P into this kinase.

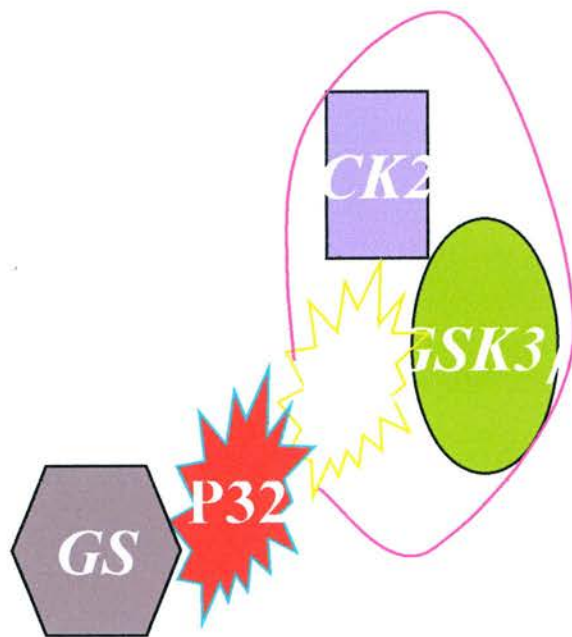
5.3.3 Effect of Lithium and LY294002

Treatment of the chondrocytes with 10 μ M LY294002 followed by 10 mM Lithium had no significant effect on the overall kinase activity at the basal level and following 20 and 40 minutes of mechanical stimulation. However, there was a major decrease after 60 minutes of mechanical stimulation (**Fig 5.7**). This could be due to interference from both the PI3K mediated signalling pathway and the Wnt mediated signalling pathway in regulating GSK3 β , GS and CK2 which resulted in less overall kinase activity, dpm [³²P] incorporated / mg protein/ hour, in the samples at this time point than the background control.

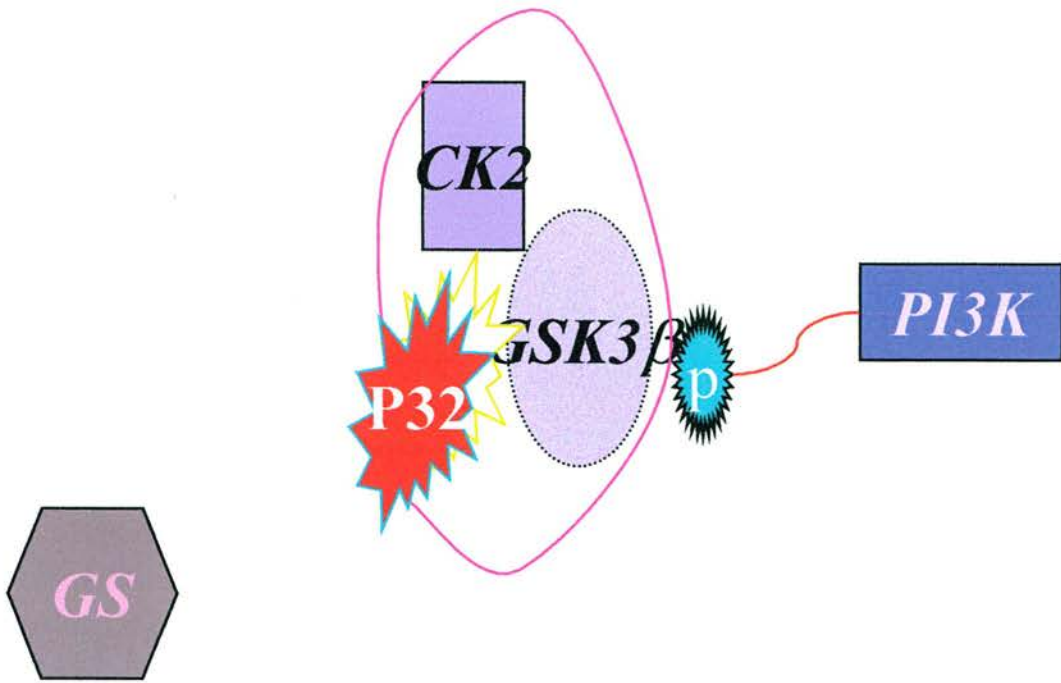
5.4 Discussion

These studies demonstrate that mechanical stimulation increased overall kinase activity. This increased activity was blocked by the PI3K inhibitor LY294002. It is known that GSK3 β is a substrate for PKB *in vivo*. As the PI3K/PKB pathway is inhibited by the LY294002 inhibitor, GSK3 β should remain in an unphosphorylated state and therefore active. Consequently, this enzyme should facilitate the phosphorylation of its priming substrate GS-2 (**Fig 5.8** and **Fig 5.9**). However, as shown in **Fig 5.2** it appears that phosphorylation of GS-2 by GSK3 β is reduced in the presence of LY294002 alone, and returns to its basal level following mechanical stimulation. This indicates that the GSK3 β was activated by a PI3K dependent pathway. However, the activity of this kinase could be influenced by other factors or other regulatory mechanisms, which result in a reduced phosphorylation of GS-2.

Fig 5.8: GSK3 β complex and the substrate binding



I) PI3K



II) Inhibition of PI3K

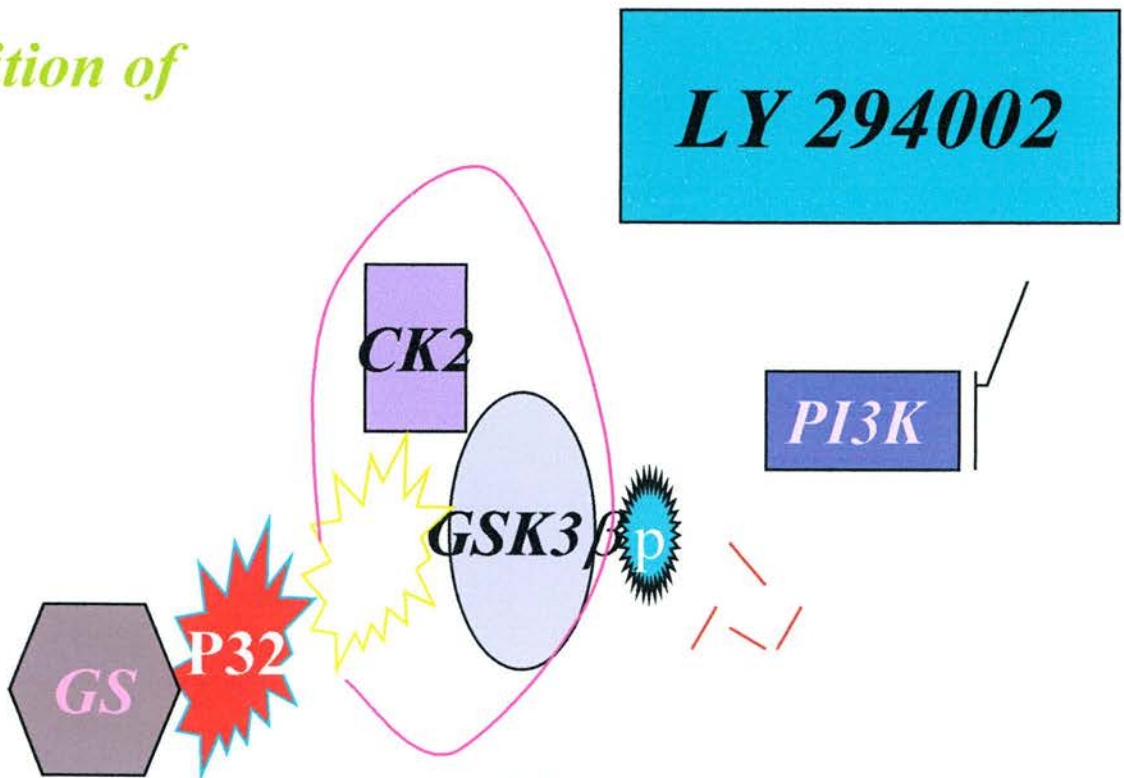


Fig 5.9

Fig 5.9: The effect of LY294002 on GSK3 β activity.

I) In a PI3K dependent pathway GSK3 β is phosphorylated by PI3K and thus inhibited, therefore its substrate, GS, remains unphosphorylated.

II) In the presence of a PI3K inhibitor, LY294002, GSK3 β remains unphosphorylated thus active and able to phosphorylate its substrate, GS.

The main mechanism for regulation of PI3K/PKB activity is phosphorylation. There are different phosphatases involved in the dephosphorylation of the Ser residues in PKB, GSK3 β and GS. Thus, in mechanically stimulated chondrocytes, the dephosphorylation of PKB on Ser 473 or GSK3 β on Ser 9 could be mediated either through activation PP2A or inhibition of PI3K. As a result of this, GSK3 β would remain unphosphorylated and active, binding and phosphorylating GS-2. These processes of phosphorylation and dephosphorylation are influenced by the presence of LY294002, which results in GSK3 β inhibition, coupled with similar activation of the relevant phosphatases.

Prior to these studies, it had been demonstrated that the PI3K /PKB /GSK3 pathway could be activated by a wide range of extracellular stimuli including integrins and insulin and that it is linked to cell survival factors and apoptosis (Lee and Juliano., 2000; Ivaska *et al.*, 2002). The activity of GSK β in these cells following mechanical stimulation may be regulated by PP2A and PKC activity. This is due to the fact that binding of α 5 β 1 integrins to FN can activate PKB, which in turn phosphorylates and inhibits GSK3 β (Ivaska *et al.*, 2002). In addition, a positive role for PP2A (serine/threonine protein phosphatase 2A) has been demonstrated in integrin mediated signalling and in various other cellular functions such as apoptosis (Ivaska *et al.*, 2002). Moreover, α 5 β 1 integrin mediated signalling in response to collagen activates PP2A, revealing an association between integrin function and this protein phosphatase (Ivaska *et al.*, 2002). It is known that mechanical stimulation of chondrocytes at 0.33Hz is mediated via the α 5 β 1 integrin receptor in chondrocytes. Thus, it is possible that GSK3 β functions as a downstream effector of an α 5 β 1 integrin mediated signalling pathway in mechanically stimulated chondrocytes.

In addition, LY294002 is also an inhibitor of casein kinase 2 (CK2), with the same potency as for PI3K. CK2 is a serine/threonine kinase which usually coimmunoprecipitates with GSK3 and its phosphorylation is a prerequisite for phosphorylation of glycogen synthase by GSK3 (Picton *et al.*, 1992 and Davis *et al.*, 2000). Therefore it is conceivable that LY294002 could block the formation of this complex which is required for the phosphorylation of GS-2 by GSK3 β in the mechanically stimulated chondrocyte cell line. In order to assess this, further experiments need to be carried out to measure the activity of CK2 in the absence and presence of LY294002. These measurements could be compared with the results obtained from the GSK3 β kinase assays. As coimmunoprecipitation of GSK3 β and CK2 is inevitable and these kinases can both phosphorylate GS-2, it would be an advantage if a substrate could be substituted for GS-2 that was more specific for GSK3 β in the kinase assay. Such a substrate would serve a diverse range of cellular functions. These would include enzymes involved in metabolism such as eIF-2B and ATP-citrate lyase, transcription factors such as c-jun and HF-AT, regulatory subunits such as the G-subunit of protein phosphatase -1 and proteins involved in the control of cell morphology such as β -catenin and MAP-1B.

5.3.1 The specificity of Lithium ion

Previous studies using Li⁺ have shown it to be a potent inhibitor of GSK3 β , but its inhibitory role is not specific to this enzyme (Syed Haq *et al.*, 2000). It is known that treatment of cells with 10 mM Li⁺ results in a more potent inhibition of GSK3 β than

of other kinases, including CK2. However, this does not rule out the possible effect of this ion, either inhibitory or otherwise, on other kinases.

Li^+ , as a Wnt agonist, would be expected to have a similar effect on intact chondrocytes as Wnt growth factors. In the Li^+ treated cells, GSK3 β , a downstream effector of Wnt stimuli, should be inactivated, resulting in an increase in the level of cytoplasmic β catenin (**Fig 5.3**). However, the data in **Fig 5.4** revealed an increase in GSK3 β activity in chondrocytes following mechanical stimulation in the presence of Li^+ . It has been proposed that Li^+ has indirect effects on the phosphatidylinositol (PI) second messenger system (Atack *et al.*, 1994, 1995a, 1995b, 1996 and 1997). More specifically, Li^+ inhibits inositol monophosphatase (IMPase), the enzyme that dephosphorylates monophosphatases (IP_1) to regenerate inositol. This, in turn, results in depletion of inositol which is required to sustain agonist stimulated activation of phospholipase C (PLC) and protein kinase C (PKC). PKC is a downstream effector of mechanotransduction pathway and is also involved in activation of tyrosine phosphatases. The tyrosine phosphorylation of GSK3 β increases its kinase activity and is required for the complete function of this enzyme. Tyrosine phosphatases dephosphorylate GSK3 β and inhibit its overall activity (Murai *et al.*, 1996). Thus, the inhibition of IMPases by Li^+ , which results in inactivation of PKC, may inhibit tyrosine phosphatases. This, in turn, leaves the tyrosine residues of GSK3 β phosphorylated and the enzyme remains activated. This may indeed be, one of the reasons for activation of GSK3 β rather than its inhibition following the treatment of the chondrocyte cell lines with Lithium. Further studies should be carried out using a PKC inhibitor prior to mechanical stimulation of chondrocytes. This would eliminate

the activity of the phosphatases and so result in more accurate estimation of GSK3 β activity.

In addition, PKC is known as a downstream effector of the Wnt/Ca²⁺ pathway which inhibits the Wnt/ β catenin pathway upstream of β catenin and GSK3 β

Thus, Li⁺ may have effect on the regulatory role of the Wnt pathways through blocking IMPase activity which is required for recycling of inositol and activation of PKC. This, in turn, may affect chondrocyte cell proliferation and differentiation through either the Wnt/Ca²⁺ or the Wnt/ β catenin pathway, or through effects on both pathways, either of which would result in the inhibition of GSK3 β .

It is also known that Li⁺ can affect cell survival by inhibiting PP2A, following apoptotic stimuli, through an effect on the PI3K/PKB/GSK3 β pathway (Mora *et al.*, 2002). In this process the inhibition of PP2A by Li⁺ prevents the dephosphorylation and inhibition of PKB, and GSK3 β remains phosphorylated and inhibited.

However, PKC can activate PP2A phosphatase, which can result in inhibition of PKB (Tanaka *et al.*, 2003), while GSK3 β remains unphosphorylated and active. Thus, treatment of chondrocytes with Li⁺ may activate PKC and PP2A phosphatases, which in turn will inhibit PKB and enhance GSK3 β activity. This process of PKB inhibition may occur independently of PI3K activity, which usually leads to PKB phosphorylation and activation, resulting in GSK3 β inhibition. This could be investigated by inhibiting PKC following treatment of the cells with Li⁺ and then examining the GSK3 β activity in chondrocytes before and following mechanical stimulation.

Thus, in summary, the biphasic pattern of GSK3 β activity following mechanical stimulation in the presence of Li⁺ in chondrocytes may result from a combination of different effects on factors involved in regulating GSK3 β activity in the conditions studied. The initial increase of GSK3 β activity following 20 minutes of mechanical stimulation in the Li⁺ treated cells may be due to the tyrosine phosphorylation of GSK3 β by Fyn kinase in association with the α 5 β 1 mechanoreceptor in the integrin signalling pathway. It may also be due to activation of PKC, which leads to activation of PP2A and inhibition of PKB, resulting in GSK3 β remaining unphosphorylated and active. This could be assessed by inhibiting PKC after treatment of the cells with Li⁺ prior to mechanical stimulation and assay of GSK3 β kinase. However, further mechanical stimulation of chondrocytes up to 40 minutes, results in GSK3 β inhibition, which may be due to activation of PKC via a Wnt mediated pathway. This, in turn, has an inhibitory effect on GSK3 β activity. Further stimulation of the chondrocytes for 60 minutes restores the GSK3 β activity. This may be due to inhibition of PKC activity through inhibition of tyrosine phosphatases or further activation of Fyn tyrosine kinase via an integrin mediated signalling pathway. Experiments using a PKC inhibitor after treating the chondrocytes with Li⁺, followed by mechanical stimulation and further treatment with a phosphatase inhibitor might help to determine the mechanism that is involved in regulating GSK3 β activity. The use of a more specific Wnt agonist and a Wnt antagonist might also lead to a more accurate assessment of GSK3 β activity.

In addition, coordinated activation of the integrin and IL-4 associated signalling pathways, which involve PLC and IP3 mediated Ca²⁺ release and activation of SK channels, are also of potential importance in chondrocytes (Salter *et al.*, 2001).

GSK3 β is a key protein kinase whose phosphorylation is regulated through number of different signalling pathways. Consequently, one can speculate that the effect of Li⁺ on GSK3 β may not necessarily result in its inhibition through the Wnt/ β catenin pathway. Li⁺ is known to have both inhibitory and stimulatory effects on PKC activity. PKC regulates the activity of GSK3 β via different mechanisms. However, PKC is not only the key downstream effector in the Wnt-mediated pathways, but also plays a part in the mechanotransduction pathway. Thus, one can speculate that GSK3 β may play some role in the mechanotransduction pathway as a downstream effector of PKC.

A fine balance in regulation of the activity of these kinases and phosphatases and coordination between the Wnt and mechanotransduction pathways may be important for maintaining cartilage integrity.

Chapter 6

Discussion

The Wnt family of proteins are fundamental players in controlling a number of developmental processes, including chondrogenesis (Church *et al.*, 2002b; Stott *et al.*, 1999; Tufan and Tuan, 2001; Hartman and Tabin 2000 and 2001).

The investigations undertaken in this thesis have focused on three main areas:

- I) The identification of components of the Wnt signalling pathway in immortalised chondrocyte cell lines.
- II) Exploration of the role of Wnt signalling pathways in immortalised chondrocyte cell lines.
- III) Examination of the interaction between the Wnt and mechanotransduction pathways in immortalised chondrocytes.

6.1 Presence of the Wnt pathways and their components in human chondrocyte cell lines

Previous studies in the Osteoarticular Research Group in Edinburgh have demonstrated that one minute of mechanical stimulation of cultured normal human articular chondrocytes at 0.33Hz and 3,700 μ strain, leads to tyrosine phosphorylation and activation of two integrin-dependent FAC components, paxillin and FAK. In addition to these proteins, β -catenin, a molecule involved in Wnt signalling and the formation of cadherin adhesion complexes, is also tyrosine phosphorylated. This suggested the possibility that the Wnt signalling pathway or catenin-cadherin

interactions might be involved in the mechanical signalling process in chondrocytes following integrin-mediated mechanotransduction.

In this study, the expression of β -catenin, pan-cadherin, Wnt-1 in immortalised human chondrocyte cell lines was examined. Previous studies had shown that N-cadherin was involved in chick embryo limb chondrogenesis, but was not expressed in an adult chick cartilage (Oberlender *et al.*, 1994). It was also known that cadherins were not expressed in normal adult human articular cartilage where there is no cell-cell contact between neighbouring articular chondrocytes *in vivo* (Lee *et al.*, 2000).

The cell lines, used in this study, were derived from costal cartilage of a 5 year old boy and a 15 year old girl who are still going through various stages of skeletal development. During these skeletal developments, chondrocyte proliferation continues at epiphysis of developing bones, resulting in an increase in cell-cell contacts where the expression of cadherin can be inevitable. It would also be interesting to see if cadherins were similarly expressed in osteoarthritic articular cartilage where chondrocyte clones or clusters are a feature. In addition, immortalised human chondrocyte cell lines were grown to 80-90% confluency in a monolayer cell culture giving a high degree of cell-cell contact. Thus, the observed expression of cadherins in these cells is not unexpected and consistent with the presence of catenin-cadherin adherens junctions and calcium dependent cell-cell adhesion (**Fig 3.1**)

Previous observations have shown that β -catenin is highly expressed in primary chondrocytes (Lee *et al.*, 2000). The role of β -catenin has been extensively studied in embryonic development and cancer biology (Wnt genes home page:

www.stanford.edu/~rnusse/wntwindow.html), but there have been few studies in terminally differentiated cells or in normal tissues of adult mammals (Haq *et al.*, 2003). β -catenin has been shown to be associated with integrin signalling and is also a major component of the Wnt signalling pathway (Lee, *et al.*, 2000; Wnt genes home page: www.stanford.edu/~rnusse/wntwindow.html). Thus, it could be a locus for interaction between the Wnt and integrin signalling pathways.

In this study in immortalised chondrocyte cell lines, the expression and colocalisation of β -catenin and cadherins at the cell surface and at the cell-cell contacts suggest a role for β -catenin in cell-cell adhesion in these cells. The detection of Wnt-1 expression at the cell surface and the expression of β -catenin in the cytoplasm and nucleus, also suggests a possible role for the canonical Wnt pathway in regulating cytoplasmic/nuclear distribution of β -catenin within these cells. The nuclear translocation of β -catenin is also induced by ILK, independently from an increase in its cytoplasmic concentration, and resulting in the enhancement of lymphoid enhancer-binding factor (LEF-1) transcriptional activity (Novak *et al.*, 1998). The nuclear localisation of β -catenin is essential in mineralizing hypertrophic chondrocytes and in ossification both *in vivo* and *in vitro* (Enomoto-Iwamoto *et al.*, 2002).

The colocalisation studies also demonstrated that CD44 and FN, two highly expressed sulphated proteoglycans in HAC, may function as Wnt coreceptors and facilitate the transduction of Wnt signals intracellularly.

In other fibroblastic cells FN expression is regulated by β -catenin/TCF (Gradl *et al.*, 1999) and the expression of CD44 is regulated through Wnt signalling pathway in

epithelial cells (Wielenga *et al.*, 1999). Thus, in immortalised chondrocytes the colocalisation of Wnt-1 and Fz with CD44 and with FN underscores the possibility of Wnt signalling in these cells. As the Wnt signals are transmitted intracellularly, they may well influence the distribution of β -catenin between the cytoplasm, the nucleus and the cell membrane. The membrane bound β -catenin is associated with cadherin and possibly with the integrin family of glycoproteins. This suggests that rather complex mechanism is required for controlling the membrane/cytoplasmic/nuclear expression of β -catenin. This may be achieved through crosstalk between the Wnt, integrin and cadherin signalling pathways, leading to appropriate gene expression of CD44 and FN in HAC. The results obtained from the GSK3 β kinase assays and the studies demonstrating GSK3 β / β catenin complexes provide some support for the hypothesis that an interaction between these pathways is required for regulating β catenin expression. Such a balanced regulatory mechanism could be a prerequisite for the regulated production of the cell-matrix proteoglycans CD44 and FN. These are essential for the assembly of focal adhesion complexes and the production and the maintenance of a normal cartilage matrix.

β -catenin may be involved in the control of the expression of fibronectin (FN), a glycoprotein which is highly expressed by chondrocytes especially in osteoarthritic articular cartilage (Heinegard *et al.*, 2003). This may occur at the level of gene transcription, where the FN gene requires the LEF/TCF family of transcription factors to be transcribed (Gradl *et al.* ,1999). α 5 β 1 integrin is a classical receptor for fibronectin in human articular chondrocytes and also functions as a mechanoreceptor (Salter *et al.*, 2001) in these cells. β -catenin could affect the rate of signal transduction following mechanical stimulation influencing the transcription of FN genes and the

synthesis of FN. Such a regulation by β -catenin could be mediated by its role in the Wnt-dependent pathway or the integrin-dependent signalling pathway, suggesting that a fine balance between these pathways may be essential for appropriate expression of fibronectin by chondrocytes. In fibroblasts, the expression of fibronectin is downregulated as cadherins are overexpressed and β -catenin is translocated to the cell membrane (Gradl *et al.*, 1999). Downregulation of FN expression probably occurs as the concentration of the cytoplasmic β -catenin decreases. In these circumstances, β -catenin is not translocated to the nucleus where normally binds to the TCF/LEF family of transcription factors and activates the transcription of FN genes. This further emphasizes the presence of cytoplasmic and nuclear pools of β -catenin within the cell. It also suggests the β -catenin pool that is associated with cadherins and has an adhesive role is separate from the pools that participate in the signalling pathways which have a regulatory role in gene transcription.

GSK3 β is a key regulatory kinase in Wnt signalling pathways and PI3K dependent pathways. This enzyme plays a major role in regulating β catenin cytoplasmic/nuclear distribution. Data from the kinase assay studies has shown that mechanical stimulation of chondrocytes results in activation of GSK3 β through a PI3K dependent pathway. However, the activity of this enzyme in the presence of Li^+ , a Wnt agonist was also increased. This emphasises that a number of pathways are involved in regulating the activity of GSK3 β in chondrocytes. In these studies it has also been shown that GSK3 β / β catenin complexes were formed in Li^+ treated, mechanically stimulated chondrocytes. This, in turn, lead to β catenin phosphorylation and its degradation through the proteasome pathway. Thus, interaction between the integrin

mediated signalling pathway and the Wnt signalling pathway are likely to be important in regulating β catenin concentrations in chondrocytes.

In this study the expression of Frizzled-2 (Fz-2) a member of the family of Fz receptors has been identified.

The presence of this receptor provides circumstantial evidence that Wnt signalling pathways may be activated in chondrocytes. Fz 2 is known to be a receptor for number of the Wnt proteins such as Wnt-1 and Wnt -5a. Both Wnt-1 and Wnt-5a are expressed in chondrocytes. The former transduce Wnt signals through the Wnt/ β catenin pathway and the latter transduce signals through the Wnt/ Ca^{2+} pathway.

Fz 2 transduces Wnt signals downstream of the Wnt/ β catenin, Wnt/ Ca^{2+} and Wnt/Jnk pathways. Thus there is a possibility that β catenin concentration within chondrocytes is regulated via an interaction between all of these Wnt-mediated signalling pathways. Furthermore, this study has shown that in the presence of a Wnt agonist, Li^+ , GSK3 β is activated and GSK3 β / β complexes are formed. However, Li^+ is known to inhibit GSK3 β activity, which in turn results in dissociation of β catenin from GSK3 β / β catenin complexes, leading to β catenin degradation. This may suggest that the formation of this complex is regulated via the Wnt/ Ca^{2+} pathway which leads to activation of PKC. PKC can either activate or inhibit GSK3 β via two different mechanisms: (i) PKC phosphorylates and activates Dsh, resulting in inhibition of GSK3 β (Kuhl et al., 2001). (ii) PKC activates PP2A, resulting in inhibition of PKB and activation of GSK3 β (Takana *et al.*, 2003). PP2A is known to play some roles in Wnt signalling pathways (Polakis., 2000). Thus, it is also possible that GSK3 β

become activated rather than inhibited in the presence of a Wnt agonist. This is a result from antagonistic activity between the Wnt/ β catenin and Wnt/ Ca^{2+} pathways.

Mechanical stimulation also enhanced GSK3 β activity. This could also be due to activation of PKC via an integrin mediated pathway. Alternatively, the Wnt/ Ca^{2+} pathway may interact with $\alpha 5\beta 1$ integrin signalling (**Fig 6.1**). This could occur as FAK binds and activates PI3K, either directly or via Src kinase. Following interaction with other adaptor proteins this could lead to activation of the mitogen-activated protein kinase (MAPK) pathway. There is some evidence that the MAPK pathway, which is composed of TAK1 MAPK kinase kinase (MAPKKK) and NLK MAPK, regulates the Wnt/ β -catenin signalling pathway. This occurs when CamKII functions as an activator of the TAK1/NLK cascade and antagonises Wnt/ β -catenin signalling. Expression of Wnt-5a modulates Wnt-1 induced transcriptional activation of target genes at the TCF level, although this has no effect on Wnt-1 mediated induction of β -catenin expression (Ishitani *et al.*, 2003). Thus, Wnt-5a can inhibit β -catenin mediated transcriptional activation via the CamKII, Tak1 and NLK MAPK pathways. This suggests possible convergence of these distinct Wnt pathways in an antagonistic manner in immortalised chondrocytes. Thus, integrin signalling may also influence the activity of the TCF/LEF family of transcription factors. This could occur via interaction of the integrin with the Wnt/ Ca^{2+} pathway. Integrin signalling augments the antagonistic effect of Wnt / Ca^{2+} on the Wnt/ β -catenin pathway (**Fig 6.1**).

These speculations about the mechanisms responsible for GSK3 β activation and β catenin phosphorylation in the mechanotransduction pathway applies in normal primary chondrocytes in which the hyperpolarisation response is PKC dependent.

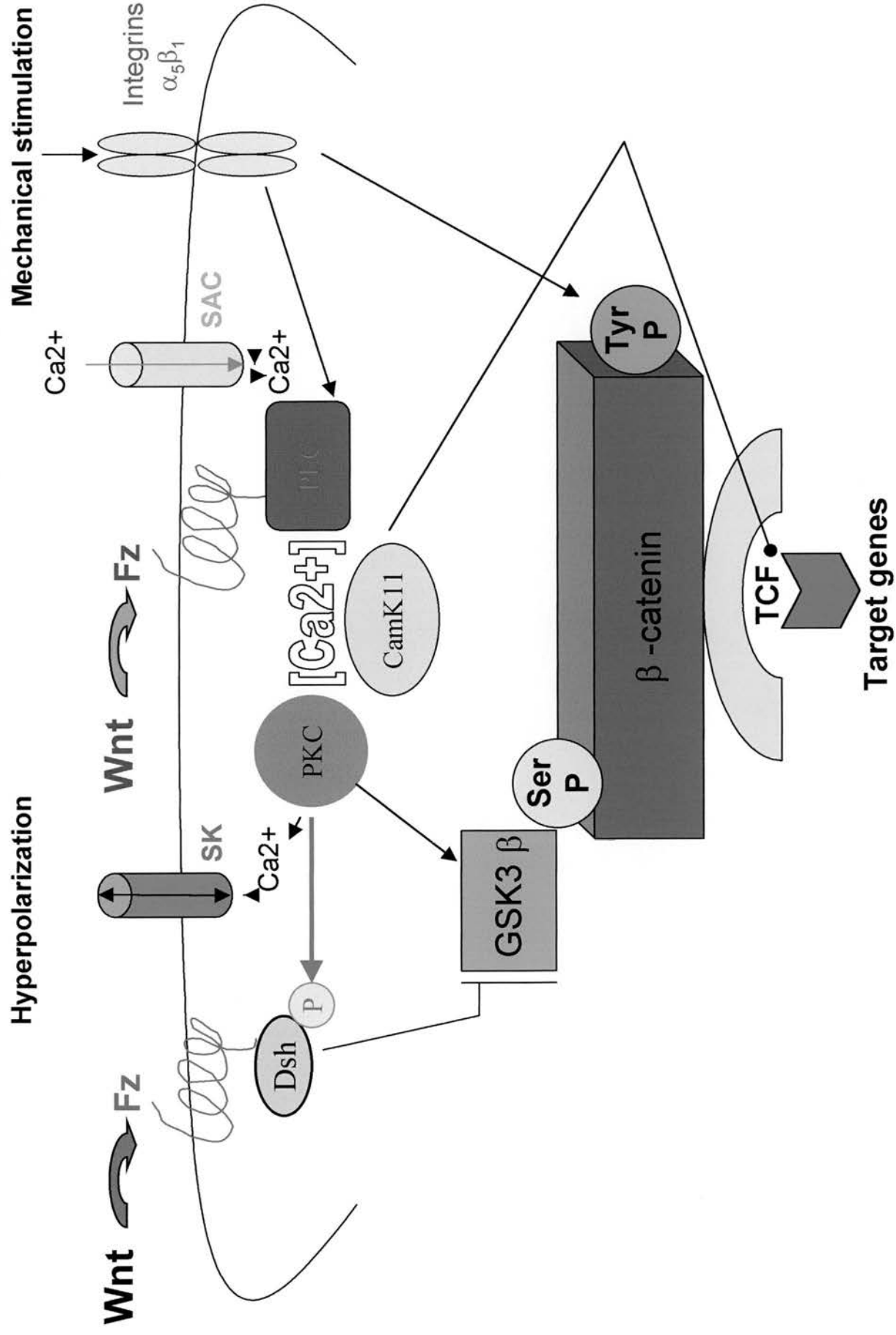


Fig 6.1: Interaction between mechanotransduction, Wnt/ β catenin and Wnt/ Ca^{2+} pathways

Fig 6.1: Mechanical stimulation results in activation of $\alpha 5\beta 1$ integrin and opening of SAC, which leads to activation of PLC and results in the release of intracellular calcium. Wnt/ Ca^{2+} pathway may modulate this process. Activation of PLC results in activation of PKC, which in turn results in membrane hyperpolarisation as SK channels are activated. PKC can activate GSK3 β through activation of phosphatases or inhibits GSK3 β activity by modulating Wnt/ β catenin pathway through phosphorylation of Dsh. These process of activation and inhibition of GSK3 β affects the regulation β catenin serine phosphorylation.

Alternative mechanisms of regulation must occur in OA chondrocytes where there is a depolarisation response to mechanical stimulation which is independent of PKC.

6.2 Frizzled related proteins and the Wnt pathways

Frizzled related protein (Fzrp) is expressed in these chondrocyte cell lines, extracted from the individuals who are still going through different stage of development.

Frizzled related proteins (Frzp) are natural Wnt antagonists, which play some roles in regulating the transduction of Wnt signals intracellularly (Dennis *et al.*, 1999). These proteins antagonise Wnt signalling through extracellular binding to Wnt growth factors, thereby blocking the binding of Wnt ligands to their Fz receptors. This, in turn, prevents transduction of Wnt signals intracellularly.

The inhibitory effect of Frzp can mediate Wnt signalling in cells during development. Frizzled related protein -1 (Frzp-1/Frzb-1), and several other Wnt proteins are expressed during skeletogenesis (Enomoto-Iwamoto *et al.*, 2002). Frzp-1/Frzb-1 is strongly expressed in the early stage of chondrogenesis and is responsible for modulation of Wnt signals at specific times and sites during skeletogenesis (Hoang *et al.*, 1996). The expression of Fzrp along with other components of the Wnt pathway in these chondrocyte cell lines emphasises that a balance between Frzp and Wnt -1 activity may be required for the regulation of the proliferation and differentiation of chondrocytes. Disruption of this balance could lead to abnormal cellular proliferation and development.

It is also known that Frzp-1/Frzb-1 interaction with Wnt-5a does not inhibit the activity of this Wnt protein. The family of frizzled related proteins also function as

Wnt chaperones for some Wnt proteins (James *et al.*, 2000). Frzp is expressed in these chondrocyte cell lines may also function as chaperones through association with Wnt-5a family of proteins.

6.3 GSK3 β , β catenin and the Wnt signalling pathway

In this study the role of Wnt signalling in chondrocyte cell lines, which were extracted from individual going through different stages of development, was examined.

Numerous previous studies have demonstrated a significant role for the Wnt family of proteins throughout chondrogenesis and skeletal development. GSK3 β may also influence the early stages of chondrogenesis and cartilage development via the Wnt signalling pathway. However, the mechanisms whereby components of the Wnt pathway affect different stages of skeletal development require further investigation.

β catenin may be involved in cartilage hypertrophy. This conclusion is drawn as the Wnt signalling and components of the Wnt pathway are involved in cardiac postnatal development (Hardiman *et al.*, 1997). It has also been suggested that the expression of β -catenin was enhanced in human cardiac hypertrophy (Rezvani *et al.*, 2000), However the mechanism by which β -catenin- LEF/TCF regulates the transcription of genes that are involved in the cell proliferation has not yet been identified.

The role of β catenin in embryonic development and cancer biology underscores its possible role in development of cartilage and chondrosarcoma cells .These studies highlight the association of β catenin with Wnt and integrin signalling pathways and its possible role in regulation of CD44, FN and metalloproteinases expression. The

above regulatory mechanism must be different between normal and diseased cartilage as the level of expression of these matrix proteins is altered. The regulatory role of Wnt signalling in level of FN and MMPs expression can be a topic of further investigation.

In the initial stages of cartilage formation the extracellular matrix is rich in fibronectin; however, the expression of this glycoprotein varies during chondrogenesis. According to these results which are obtained from immortalised chondrocyte cell lines, it can be speculated that the interaction between Wnt and integrin mediated signalling pathway may be essential in regulating the β -catenin/TCF complex formation and activating the FN genes transcription. This mechanism can be crucial in determining the amount of fibronectin synthesised during chondrogenesis and in normal cartilage. However, the interaction between these pathways in pathological conditions such as OA, where chondrocytes produce an increased amount of fibronectin, must be altered.

These studies also demonstrated that FN forms a complex with Wnt-1 and Fz, thus, may function as a coreceptor that can further facilitate the transduction of the Wnt signal intracellularly. However, the variation in the amount of synthesised fibronectin in the normal and osteoarthritic cartilage may, in turn, lead to changes in the capacity of the Wnt signal being transmitted intracellularly. This may also affect the integrin mediated signalling pathway and influence phosphorylation of FAK and β catenin in normal and OA cartilage. This, in turn, affects the cytoplasmic/nuclear distribution of β catenin and its ability in activating the gene transcription of matrix proteins in normal and OA cartilage.

As β -catenin also regulates the formation of cadherin complexes at cell-cell adhesion sites, one can speculate that β -catenin may be involved in cellular adhesion in cartilage independently from its regulatory role at the transcriptional level. One can also speculate that activation of the canonical Wnt pathway, which results in β -catenin stabilization, might simultaneously lead to cadherin-catenin stabilisation and cloning in chondrocytes. Furthermore, the stabilisation of β -catenin may result in further association of β -catenin with integrins and so influence the cell-matrix organisation and cartilage structure.

Collectively, these studies emphasise the importance of identifying the regulatory mechanisms responsible for GSK3 β activation and β -catenin cytoplasmic/nuclear distribution within the chondrocytes. The results of such investigations will be crucially important for determining the precise downstream signalling mechanisms that are involved in cartilage growth and development.

These studies can be carried on to further reveal the regulatory roles of Wnt signalling pathways in mechanotransduction pathway in normal and OA chondrocytes. This can be achieved by looking at the common downstream effectors of Wnt pathways and mechanotransduction pathway such as PKC and PI3K. This can be performed by investigating how the activation or inhibition of these effectors would influence the regulation of other components of the signalling pathways, which in turn, affects the transcription of the target genes. This should be carried out by setting up experiments such as TCF reporter (TOPflash) assay, to assess whether Wnt signalling is active and to examine its variability after mechanical stimulation. This, in turn, gives insight into

mechanism through which the target genes is transcribed. These target genes are usually the cell-matrix proteins and are responsible for maintaining cartilage integrity,

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Appendix I

Cell culture buffers and reagents

Condition media

DMEM

10% FCS

100 U/ml penicillin

100 mg/ml streptomycin

2 mM L-glutamate

Wash buffer

PBS

Immunofluorescence and confocal microscopy

Wash buffer

PBS

Immunoblotting and immunoprecipitation buffers and reagents

Lysis buffer

20 mM HEPES (pH 7.4)

50 mM β -glycerophosphate

2 mM EGTA

1% Triton X-100

150 mM NaCl

10% glycerol

Make up to 500 ml with dH₂O

Add to the Lysis buffer

1 mM PMSF

2 mM NaF

2 µg/ml aprotinin

2 µg/ml pepstatin A

10 µM E64

1mM Na₃ VO₄**12% Resolving gel: component volumes of (ml) per gel mold volume of 10 ml**

30% Acrylamide mix 4.0 ml

10% Ammonium persulphate 0.1 ml

10% SDS 0.1 ml

Temed 0.004 ml

1.5 M Tris (pH 8.8) 2.5 ml

dH₂O 3.3 ml**5% Stacking gel: component volumes of (ml) per gel mold volume of 5 ml**

30% Acrylamide mix 0.83 ml

10% Ammonium persulfate 0.05 ml

10% SDS 0.05 ml

Temed 0.005 ml

1 M Tris (pH 6.8) 0.63 ml

dH₂O 3.4 ml**Loading buffer (3x)**

75 mg Tris

0.75 g SDS

3.75 ml Mercaptoethanol

7.5 ml Glycerol

250 µl Bromophenol blue sat. sol.

25 ml H₂O

Electrode buffer (5x)

7.5 g Tris

36 g Glycine

2.5 g SDS

500 ml dH₂O

Transfer buffer

3.03 g Tris

14.4 g Glycine

200 ml Methanol

TBS (10x)

24.2g Tris

80 g NaCl

HCl (pH 7.6)

1 L dH₂O

TBST

TBS(1x)

0.1% Tween-20

Blocking buffer

2% BSA in TBST

0.2g BSA

10 ml TBST

Stripping buffer

100 mM β - mercaptoethanol

2% SDS

62.5 mM Tris pH 6.

Kinase assay buffers and reagents**Kinase Lysis buffer**

As for the reagents used in Immunoblotting lysis buffer

Kinase HEPES (10x)

200 mM HEPES (pH 7.5)

200 mM β -glycerophosphate

100 mM NaF

5 mM EDTA

5 mM EGTA

dH₂O

Kinase wash buffer

Kinase HEPES (1x)

200 mM NaCl

2mM DTT

10 mM MgCl₂

0.05% BRIJ 35

1 mM NaV

Kinase assay buffer

50 μ M Glycogen synthase peptide 2 (GS-2p)

6 mM cold ATP

100 μ M γ [32 P] ATP

Kinase Wash buffer

RT-PCR buffers and reagents**RNA extraction reagents**

1.2 ml Trizol

0.2 ml Chloroform

75% Ethanol in DEPC water

500 μ l Isopropanol

Agarose gel electrophoresis reagents

TBE buffer (10X)

2% (w/v) agarose in TBE(1x)

Tracking dye: 0.25 % Orange G

Cloning buffers and reagents**Ligation buffer**

50 mM Tris.HCl (pH 7.4)

10 mM MgCl₂

10 mM dithiothreitol

1 mM ATP

LB-Ampicillin plates

400 ml of 2% agar medium

40 mg/ml Ampicillin

40 mg/ml X-Gal

Rcombinant analysis buffer**STET buffer:**

50 m M Tris. HCl (pH 8)

8% (W/V) Sucrose

5% Triton X-100

50 mM EDTA

Appendix II

Alphabetical list of common reagents and their suppliers

Acrylamide mix; Anachem

Agarose; Sigma

Ammonium persulphate; Sigma

Ampicillin; Sigma

Aprotinin; Sigma

ATP; Amersham

ATP γ [^{32}P]; Amersham

β -glycerophosphate; Sigma

Bromophenol Blue; Sigma

BRIJ 35; Sigma

BSA; Sigma

Chloroform; Sigma

DMSO (Dimethyl sulfoxide); BDH

DTT (Dithiothreitol); Sigma

DMEM (Dulbecco's Modified Eagle's Medium); Sigma

EDTA (Ethylenediamine-tetraacetic acid); Sigma

EGTA (Ethylene glycol-bis[β -aminoethyl ether]-N, N, N' N'-tetra acetic acid); Sigma

E64 (trans-Epoxysuccinyl-L Leucylamido(4-Guanidino)-Butane); Sigma

Ethanol; BDH

Ethidium bromide; Gibco BRL

FCS (Foetal Calf Serum); Sigma

Formaldehyde; Sigma
Glycine; BDH
Glycerol; BDH
HCl; BDH
HEPES; Sigma
L-glutamate; Sigma
LiCl; Sigma
Mercaptoethanol; Sigma
Methanol; BDH
MgCl₂; Sigma
NaCl; Sigma
NaF; Sigma
NaOH; BDH
NaV; Sigma
Na₃ VO₄; Sigma
Orange G; Sigma
Penicillin; Sigma
Pepstatin; Sigma
Phosphate buffer saline (PBS); Gibco BRL
PMSF (PhenylMethylSulfonylFluoride); Sigma
Propan-2-ol; BDH
Sodium dodecyl sulphate (SDS); Sigma
Streptomycin; Sigma
Sucrose; Sigma
TBE; Invitrogen

Temed; Sigma

Tris; Sigma

Triton X-100; Sigma

Trizol; Invitrogen

Trypsin; Gibco BRL

Tween 20; Sigma

Appendix III

Antibodies

β catenin, mouse monoclonal; Transduction Laboratories

Pan-cadherin, mouse monoclonal; sigma

CD44-IM.7.8.1 rat monoclonal; F Brennan

Fibronectin (FN), mouse monoclonal; Transduction Laboratories

Frizzled (Fz), goat polyclonal; Santa Cruz

GSK3 β , mouse monoclonal; Transduction Laboratories

Wnt-1, goat polyclonal; Santa Cruz

Appendix IV

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1  gggggcgcca aggagccggg tggggggcgg cggccagcat gggccccgc agcgccctgc
61  cccgctgct gctgccgctg ctgctgctgc ccgcccggg gccggcccag ttccacgggg
121 agaagggcat ctccatcccg gaccacggct tctgccagcc catctccatc ccgctgtgca
181 cggacatcgc ctacaaccag accatcatgc ccaaccttct gggccacacg aaccaggagg
241 acgacggcct agaggtgcac cagttctatc cgctggtgaa ggtgcagtgc tcgcccgaac
301 tgcgcttctt cctgtgctcc atgtacgcac ccgtgtgcac cgtgctgtaa caggccatcc
361 cgccgtgccg ctctatctgt gagcgcgcgc gccagggctg cgaagccctc atgaacaagt
421 tcggttttca gtggcccagc cgctgcgctg gcgagcaett cccgcgccac ggcgcccagc
481 agatctcgtg cggccagaac cactccgagg acggagctcc cgcgctactc accaccgcgc
541 cgccgcggg actgcagccg ggtgccgggg gcaccccggg tggcccgggc ggcggcggcg
601 ctccccggcg ctacgccacg ctggagcacc ccttccactg cccgcgcgtc ctcaagtgct
661 catctatctt cagctacaag tttctgggcg agcgtgattg tgctgcgcc tcgcaacctg
721 cgcgcccga tggttccatg ttcttctcac aggaggagac gcgttccg cgctctgga
781 tcctcacctg gtcggtgctg tgctgcgctt ccaccttctt cactgtcacc acgtaactgg
841 tagacatgca gcgcttccgc taccagagc ggctatcat tttctgtcg ggtgctaca
901 ccattggtgc ggtggcctac atcgcgggct tcgtgctcca ggagcgcgtg gtgtgcaacg
961 agcgcttctc cgaggacggg taccgcacgg tgggtcaggg caccaagaag gagggctgca
1021 ccatctctt catgatgctc tacttcttca gcattggcag ctccatctgg tgggtcatcc
1081 tgcgctcac ctggttctcg gcagccggca tgaagtgggg ccacgaggcc atcgaggcca
1141 actctcagta cttccacctg gccgcctggg ccgtgccggc cgtcaagacc atcaccatcc
1201 tggccatggg ccagatcgac ggcgacctgc tgagcggcgt gtgcttctga ggcctcaaca
1261 gcctggaccg gcctcggggc ttctgtctag ccgcctctt cgtgtacctg ttcactcgca
1321 cgtcttctt cctggccggc ttctgtctgc tcttccgat ccgcaccatc atgaagcacg
1381 acggcaccaa gaccgaaaag ctggagcggc tcattggtcg catcggcgtc ttctccgtgc
1441 tctacacagt gcccgccacc atcgtcatcg cttgctactt ctacgagcag gccttccgcg
1501 agcactggga gcgctcgtgg gtgagccagc actgcaagag cctggccatc ccgtgcccgg
1561 cgcactacac gccgcgatg tcgcccact tcacggtcta catgatcaa tacctcatga
1621 cgctcactgt gggcatcag tcgggcttct ggatctggc gggcaagacg ctgcactcgt
1681 ggaggaagtt ctacactcgc ctaccaaca gccgacacg tgagaccacc gtgtgagga
1741 cgccccagg ccggaaccgc gcggcgttt cctccgccg gggggggcc cctacagact
1801 ccgtatttta ttttttaaa taaaaaacga tcgaaacat ttcacttta ggttgccttt
1861 taaaagagaa ctctctgcc aacacccc

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Frizzled-2 primers

Forward primer 5' CTTCGTAGGCCTCAACAGCC 3'

Reverse Primer 5' TCATGTAGACCGTGAAGTCGG 3'

Fig 3.3.1: Frizzled -2 mRNA sequence

CCTGCAGCCT	CCGGAGTCAG	TGCCGCGCGC	CGCCCGCCC	CGCCTTCCT	GCTCGCCGCA	60
CCTCCGGGAG	CCGGGGCGCA	CCCAGCCCGC	AGCGCCGCT	CCCCGCCGC	GCCGCTCCG	120
ACCGCAGGCG	GAGGGCCGCC	ACTGGCCGGG	GGGACCGGGC	AGCAGCTTGC	GGCCGCGGAG	180
CCGGGAAACG	CTGGGACTG	CGCCTTTTGT	CCCCGGAGGT	CCCTGGAAGT	TTGGCGCAGG	240
ACGCGCCGCG	GGAGGCGCGC	GAGGCAGCCC	CGACGTCGGC	GAGAACAGGG	CGCAGAGCCG	300
GCATGGGCAT	CGGGCGCAGC	GAGGGGGGCC	GCCGGGGGGC	CCTGGGCGTG	CTGCTGGCGC	360
TGGGCGCGGC	GCTTCTGGCC	GTGGGCTCGG	CCAGCGAGTA	CGACTACGTG	AGCTTCCAGT	420
CGGACATCGG	CCCCTACCAG	AGCGGGCGCT	TCTACACCAA	GCCACCTCAG	TGCGTGGACA	480
TCCCCGCGGA	CCTGCGGCTG	TGCCACAACG	TGGGTACAA	GAAGATGGTG	CTGCCCAACC	540
TGCTGGAGCA	CGAGACCATG	GCGGAGGTGA	AGCAGCAGGC	CAGCAGCTGG	GTGCCCCCTGC	600
TCAACAAGAA	CTGCCACGCC	GGGACCCAGG	TCTTCTCTGT	CTCGCTCTTC	GCGCCCGTCT	660
GCCTGGACCG	GCCCATCTAC	CCGTGTGCGT	GGCTCTGCGA	GGCCGTGCGC	GACTCGTGGC	720
AGCCCGTCA	GCAGTTCCTC	GGCTTCTACT	GGCCCGAGAT	GCTTAAGTGT	GACAAGTTC	780
CGGAGGGGGA	CGTCTGCATC	GCCATGACGC	CGCCCAATGC	CACCGAAGCC	TCCAAGCCCC	840
AAGGCACAAC	GGTGTGTCCT	CCCTGTGACA	ACGAGTTGAA	ATCTGAGGCC	ATCATTGAAC	900
ATCTCTGTGC	CAGCGAGTTC	GCACTGAGGA	TGAAAATAAA	AGAAGTGAAA	AAAGAAAATG	960
GCGACAAGAA	GATTGTCCCC	AAGAAGAAGA	AGCCCTGAA	GTTGGGGCCC	ATCAAGAAGA	1020
AGGACCTGAA	GAAGCTTGTG	CTGTACCTGA	AGAATGGGGC	TGACTGTCCC	TGCCACCAGC	1080
TGGACAACCT	CAGCCACCAC	TTCCTCATCA	TGGGCCGCAA	GGTGAAGAGC	CAGTACTTGC	1140
TGACGGCCAT	CCACAAGTGG	GACAAGAAAA	ACAAGGAGTT	CAAAACTTC	ATGAAGAAAA	1200
TGAAAAACCA	TGAGTGCCCC	ACCTTTCAGT	CCGTGTTTAA	GTGATTCTCC	CGGGGGCAGG	1260
GTGGGGAGGG	AGCCTCGGGT	GGGGTGGGAG	CGGGGGGAC	AGTGCCCGGG	AACCCGTGGT	1320
CACACACACG	CACCTGCCCTG	TCAGTAGTGG	ACATTGTAAT	CCAGTCGGCT	TGTTCTTGCA	1380
GCATTCGCCG	TCCCTTTCCT	TCCATAGCCA	CGCTCCAAC	CCCAGGGTAG	CCATGGCCGG	1440
GTAAAGCAAG	GGCCATTTCAG	ATTAGGAAGG	TTTTTAAGAT	CCGCAATGTG	GAGCAGCAGC	1500
CACTGCACAG	GAGGAGGTGA	CAAACCATTT	CCAACAGCAA	CACAGCCACT	AAAACACAAA	1560
AAGGGGGATT	GGGCGGAAAG	TGAGAGCCAG	CAGCAAAAAC	TACATTTTGC	AACTTGTGG	1620
TGTGGATCTA	TTGGCTGATC	TATGCCTTTC	AACTAGAAAA	TTCTAATGAT	TGGCAAGTCA	1680
CGTTGTTTTC	AGTCCAGAG	TAGTTTCTTT	CTGTCTGCTT	TAAATGGAAA	CAGACTCATA	1740
CCACACTTAC	AATTAAGTTC	AAGCCAGAA	AGTGATAAGT	GCAGGGAGGA	AAAGTGCAAG	1800
TCCATTATCT	AATAGTGACA	GCAAAGGGAC	CAGGGGAGAG	GCATTGCCCT	CTCTGCCAC	1860
AGTCTTTCGG	TGTGATGTTC	TTTGAATCTG	AATCAGCCAG	TCTCAGATGC	CCCAAAGTTT	1920
CGGTTCTTAT	GAGCCCGGGG	CATGATCTGA	TCCCCAAGAC	ATGTGGAGGG	GCAGCCTGTG	1980
CCTGCCTTTG	TGTCAGAAAA	AGGAAACCAC	AGTGACCTG	AGAGAGACGG	CGATTTTCGG	2040
GCTGAGAAGG	CAGTAGTTTT	CAAAACACAT	AGTTA			2075

Frizzled related protein primers

Forward primer 5' GGGGCCCATCAAGAAGAAGG 3'

Reverse primer 5' GCGGGAATGCTGCAAGAACAAG 3'

Fig 3.5.1: Frizzled-related protein mRNA sequence

