Regulation of Transforming Growth Factor-Beta signaling in Chondrocytes: Factors that regulate ALK5 versus ALK1 signaling pathways

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Table of Contents

List of Figures

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Abstract

Transforming Growth Factor-Beta (TGF-β) plays a critical role in maintenance of articular cartilage, and deregulation of its activity is implicated in osteoarthritis (OA). TGF-β signals through a pair of transmembrane serine/threonine kinases known as type I (also known as Activin-receptor Like Kinase 5, ALK5) and type II TGF-β (TβRII) signaling receptors. The TGF-β ligand binds to TβRII, which transphosphorylates the ALK5 receptor, phosphorylating Smad2/3 which subsequently bind common Smad4 (co-smad4). The Smad complexes then translocate into the nucleus to regulate gene transcription. Our previous work has shown that in chondrocytes, TGF-β transmits its signal by activating another TGF-β type I receptor, ALK1, which in turn activates Smad1/5. Our group has demonstrated that ALK5 and ALK1 pathways elicit opposing effects on chondrocyte function. The main objective of this thesis was to determine factors that regulate the balance of TGF-β/ALK5/Smad2/3 versus TGF-β/ALK1/Smad1/5 signaling pathways, and thus alter chondrocyte function. Here, we have identified hypoxia and two TGF-β co-receptors, endoglin and CD109, as factors that differentially regulate between the ALK5/Smad2/3 and ALK1/Smad1/5 signaling pathways in chondrocytes.

Articular cartilage is physiologically hypoxic, therefore chondrocytes have developed highly conserved and adaptive mechanisms to survive in this environment. Our data shows that hypoxia increases the levels of HIF-1α, ALK5, and ECM protein production while decreasing the levels of ALK1, Smad1, and endoglin. Furthermore, hypoxia decreases phospho-Smad1/5 levels while increasing phospho-Smad2/3 levels. In addition, hypoxia decreases total levels of TGF-β1 and addition of TGF-β1 decreases HIF-1α

levels in chondrocytes. Altogether, our data suggest that hypoxia differentially regulates the balance between TGF-β canonical pathway and ALK/Smad1/5 pathway and promotes chondrocytes to maintain ECM protein levels.

Our group has previously shown that endoglin, a TGF-β co-receptor, is expressed in human chondrocytes but the exact mechanisms by which endoglin regulates TGF-β signaling pathway are unknown. Here, we show that endoglin enhances TGF-β1-induced Smad1/5 phosphorylation while decreasing TGF-β1-induced Smad2/3 phosphorylation and TGF- β1-induced ECM protein production in human chondrocytes. We also observe the increased levels of endoglin in human OA compared to normal cartilage. We further investigated the role of endoglin in TGF-β signaling pathways in human chondrocytes and its role in cartilage degradation in vivo.

Our group has discovered CD109 as a novel TGF-β co-receptor that CD109 inhibits TGF-β canonical signaling pathway and reduces TGF-β-induced ECM protein synthesis in human skin cells. In this thesis, we demonstrate that CD109 promotes ALK1/Smad1/5 signaling pathway while inhibiting ALK5/Smad2/3 signaling pathway and inhibits ECM production in human chondrocytes. We demonstrate that CD109 inhibits ALK5 mRNA levels and promotes ALK5 receptor degradation in human chondrocytes. In addition, we show that CD109 promotes expression of proteases suggesting that CD109 promotes chondrocytes to exhibit OA-like characteristics.

Collectively, the results in the current thesis suggest that all three factors, hypoxia, endoglin and CD109, function as critical modulators of the ALK1/Smad1/5 versus ALK5/Smad2/3 signaling pathways in human chondrocytes and regulate chondrocyte function. Furthermore, the present results suggest that TGF-β/ALK1/Smad1/5 signaling

pathway promotes chondrocytes to exhibit OA-like characteristics. Determination of factors that differentially regulate TGF-β signaling pathways will provide useful insights to understand the cellular mechanisms by which TGF-β regulates chondrocyte function and may provide a basis for the improvement of therapeutic approaches for OA treatment.

Résumé

Le facteur de croissance transformant (TGF-β) joue un rôle essentiel dans l'entretien et la réparation du cartilage articulaire. La cascade de signalisation du TGF-β s'effectue à travers une paire de récepteurs transmembranaires de type kinase connues sous le nom de type I (ALK5) et de type II du TGF-β (TβRII) récepteur de signalisation. Le ligand TGF-β se lie au récepteur TβRII, qui effectue une transphosphorylation avec le récepteur ALK5, phosphorylant ainsi Smad2/3 qui se lient ensuite a une protéine co-Smad4 commune aux deux récepteurs. Suivant son activation, le complexe des Smad se dirige dans le noyau pour réguler la transcription. Au préalable, nos travaux ont démontré que dans les chondrocytesle TGF-β transmet son signal en activant un autre récepteur de TGF-β type I, ALK1, qui active à son tour Smad1/5. Notre groupe a donc démontré que les voies d'ALK5 et ALK1 provoquent des effets différentiels sur la fonction des chondrocytes. L'objectif principal de cette thèse est de déterminer les facteurs qui régulent l'équilibre de la voie de signalisation du TGF-β/ALK5/Smad2/3 par rapport à la voie de signalisation du TGF-β/ALK1/Smad1/5 pour comprendre comment cela modifie la fonction des chondrocytes.

Nos données montrent que l'hypoxie augmente les taux de ALK5, et la production de protéines de la matrice extracellulaire (ECM), tout en diminuant les niveaux de ALK1, Smad1, et de l'endogline. L'hypoxie diminue les niveaux de phospho-Smad1/5, tout en augmentant les niveaux de phospho-Smad2/3. Dans l'ensemble, nos données suggèrent que l'hypoxie régule l'équilibre entre la voie canonique du TGF-β et la voie alternative passant par ALK1/Smad1/5 et maintien les niveaux de protéines de l'ECM dans les chondrocytes.

Notre groupe a déjà montré que l'endogline, un corécepteur de TGF-β, est exprimée dans les chondrocytes humains, mais les mécanismes exacts par lesquels l'endogline régule la voie de signalisation du TGF-β demeurent toujours inconnus. Ici, nous démontrons que l'endogline augmente spécifiquement les niveaux de TGF-β1 qui mène à la cascade spécifique de phosphorylation des Smad1/5, tout en diminuant le TGF-β1 qui induit la phosphorylation des Smad2/3 et la production des protéines de l'ECM dans les chondrocytes humains. Nous observons également des niveaux accrus d'endogline dans l'arthrose humaine par rapport au cartilage normal.

Nous avons précédemment démontré que CD109 inhibe la voie de signalisation canonique du TGF-β et réduit la synthèse des protéines de l'ECM induite par le TGF-β dans les cellules de la peau humaine. Dans cette thèse, nous démontrons que le CD109 favorise la voie de signalisation ALK1/Smad1/5, tout en inhibant la voie de signalisation de ALK5/Smad2/3 ce qui inhibe aussi la production de l'ECM dans les chondrocytes humains. Nous démontrons aussi que CD109 inhibe les niveaux d'ARNm d'ALK5 et favorise la dégradation de la protéine ALK5 dans les chondrocytes humains. En plus, nous montrons que le CD109 favorise l'expression de protéases, suggérant que CD109 pousse les chondrocytes à présenter un phénotype d'arthrose.

En résumé, les résultats de cette thèse suggèrent que trois facteurs l'hypoxie, l'endogline et CD109, jouent un rôle important dans la modulation de la voie de signalisation ALK1/Smad1/5 par rapport à la voie de ALK5/Smad2/3 dans les chondrocytes humains et régule aussi la fonction des chondrocytes. Par ailleurs, ces résultats suggèrent que la voie de signalisation du TGF-β/ALK1/Smad1/5 favorise les chondrocytes à présenter des caractéristiques similaires à ceux retrouvé dans l'arthrose. La détermination des facteurs

qui régulent différentiellement les voies de signalisation du TGF-β fournira des indications utiles pour comprendre les mécanismes cellulaires par lesquels le TGF-β régule les fonctions des chondrocytes, ce qui pourra servir de base à l'amélioration des approches thérapeutiques pour le traitement de l'arthrose.

Contributions to Original Knowledge

This thesis has been prepared in accordance with the guidelines specified by the Faculty of Graduate and Postdoctoral Studies of McGill University. This thesis, entitled "Regulation of Transforming Growth Factor-β signaling in Chondrocytes: Factors that regulate ALK5 versus ALK1 signaling pathway" has been prepared in the manuscript-based format, and consists of an Abstract and its French translation (Résumé), four chapters entitled: (1) Introduction; (2) Hypoxia Differentially Regulates TGF-β/ALK5/Smad2/3 versus TGF-β/ALK1/Smad1/5 signaling and ECM synthesis in human chondrocytes; (3) Endoglin regulates Transforming Growth Factor-Beta signaling and ECM production in chondrocytes; (4) CD109 inhibits TGF-Beta Receptor expression, signaling and ECM protein production in human chondrocytes; and (5) General discussion. A bibliography containing all the literature referenced is provided at the end of the thesis.

I, Yoon Chi, under the direct supervision of Dr. Anie Philip, have designed the experiments, collected and analyzed the data, and framed these studies in the context of previously published literature unless otherwise stated. My contributions to original knowledge described in this thesis are as follows:

1) I have validated low oxygen tension, hypoxia, promotes TGF-β/ALK5/Smad2/3 while inhibiting TGF-β/ALK1/Smad1/5 signaling pathway in human chondrocytes

2) I have demonstrated that hypoxia enhances ECM protein synthesis in human

chondrocytes.

3) I have demonstrated that hypoxia decreases total levels of TGF-β1 and ectopic addition of TGF-β1 decreases HIF-1α levels in human chondrocytes.

4) I have shown that endoglin levels are increased in human OA cartilage compared to human normal cartilage.

5) I have demonstrated that endoglin levels are altered in subculture-induced dedifferentiated human chondrocytes.

6) I have demonstrated that endoglin promotes TGF-β/ALK1/Smad1/5 while inhibiting TGF-β/ALK5/Smad2/3 signaling pathway in human chondrocytes.

7) I have demonstrated that endoglin reduces TGF-β1 induced type II collagen and PAI-1 levels in human chondrocytes.

8) I have validated that endoglin also promotes TGF-β/Smad1/5 signaling pathway and that endoglin decreases type II collagen levels in mouse chondrocytes.

9) I have demonstrated that CD109 inhibits TGF-β type I receptor, ALK5, expression, both at mRNA and protein levels in human chondrocytes.

10) I have demonstrated that CD109 promotes receptor degradation and inhibits ALK5 expression at transcriptional level.

11) I have found that CD109 promotes TGF-β/ALK1/Smad1/5 pathway while inhibiting TGF-β/ALK5/Smad2/3 signaling pathway in human chondrocytes.

12) I have demonstrated that CD109 inhibits chondrogenic markers such as type II collagen and aggrecan, as well as PAI-1, a downstream target protein induced by the TGF-β canonical signaling pathway.

13) I have demonstrated that CD109 promotes the expression of proteases such as MMP13 and ADAMTS5 in human primary articular chondrocytes.

The data and texts from Chapters 2, 3 and 4 comprise the following manuscripts:

Chapter 3: Finnson, K.W., Parker, W.L., Chi, Y., Hoemann, C., Goldring, M.B., Antoniou, J., and Philip, A. (2010). Endoglin differentially regulates TGF-β-induced Smad2/3 and Smad1/5 signalling and its expression correlates with extracellular matrix production and cellular differentiation state in human chondrocytes. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society *18*, 1518-1527

Chapter 2: Chi, Y., Antoniou, J., and Philip, A. (2016). Hypoxia differentially regulates TGFβ/ALK5/SMAD2/3 versus TGF-β/ALK1/SMAD1/5 signaling and extracellular matrix synthesis in human chondrocytes. *To be submitted to Osteoarthritis and Cartilage*.

Chapter 4: Chi, Y., Antoniou, J., and Philip, A. (2016). CD109 inhibits TGF-beta Receptor expression, signaling and ECM protein production in human chondrocytes. *To be submitted to Osteoarthritis and Cartilage.*

All of the data presented herein are the work of Yoon Chi with the following exceptions:

1) Endoglin transfection in human chondrocytes (Fig. 3.2 and 3.3) were performed by Dr. Kenneth Finnson, a Research Associate, at Dr. Anie Philip's laboratry

2) Semi-quantitative RT-PCR (Fig. 3.4) was performed by Dr. Kenneth Finnson

3) Destabilization of medial meniscus (DMM) surgery was performed by Dr. Bertrand Lussier in collaboration with Yoon Chi and Dr. Anie Philip at the McGill Univrsity Health Centre (MUHC) Animal Facility

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Abbreviations

α2m	α-2-Macrogloblin
ActRII	Activin Type II Receptor
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
ALK	Activin Receptor-like Kinase
AP2	Adaptor Protein 2
α-sma	Alpha-smooth Muscle Actin
ATP	Adenosine Triphosphate
ATF	Activating Transcription Factor
ASPN	asporin
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
BMPRII	BMP Type II Receptor
BSA	Bovine Serum Albumin
C3	Complement 3
C4	Complement 4
C5	Complement 5
CBP	CREB Binding Protein
CCN2	CYR61/CTGF/NOV
CD	Cluster of Differentiation
CDC42	Cell division control protein 42 homolog
CDK	Cyclin Dependent Kinase
CIHR	Canadian Institutes of Health Research
Co-Smad	Common-mediator-Smad
COMP	Cartilage oligomeric matrix protein
CSC	Cancer Stem Cell
CTGF	Connective Tissue Growth Factor
DAB	3,3-Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
eNOS	Endothelial nitric oxide synthase
EPAS	Endothelial PAS domain containing protein
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum

FGF-2	Fibroblast Growth Factor 2
GAG	Glycosaminoglycan
GIPC	GAIP-interacting Protein, C-terminus
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPI	Glycophosphatidylinositol
Grb2	Growth factor receptor binding protein 2
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HHT	Hereditary Hemorrhagic Telangiectasia
HIF	Hypoxia Inducible Factor
HRE	Hypoxia responsive elements
I-Smad	Inhibitory Smad
IFN	Interferon
IGF	Insulin-like Growth Factor
Ihh	Indian hedgehog
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
LAP	Latency Associated Peptide
LLC	Large Latent Complex
LTBP	Latent TGF-beta Binding Protein
M6P	Mannose-6-Phosphate
MAD	Mothers Against Decapentalegic
MAP3K	MAP Kinase Kinase Kinase
MAPK	Mitogen Activated Protein Kinase
MCP	Monocyte Chemotactic Protein
MH	Mad Homology
miRNA	MicroRNA
MKK	MAP Kinase Kinase
MMLV	Moloney Murine Leukemia Virus
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cells
mTOR	Mammalian Target of Rapamycin
N-CAM	Neural Cell Adhesion Molecule
NES	Nuclear Export Sequence
NF-κB	Nuclear Factor-κB
NGS	Normal Goat Serum
NO	Nitric oxide
OA	Osteoarthritis
PAK	P21-activated Kinase
PAR	Protease Activated Receptor
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PMSF	Phenylmethanesulfonylfluorid
R-Smad	Receptor-regulated-Smad
RhoA	Ras homolog gene family, member A
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RUNX	Runt-related transcription factor
SARA	Smad Anchor for Receptor Activation
SBD	Smad Binding Domain
SBE	Smad Binding Element
SCC	Squamous Cell Carcinoma
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Deviation of the Mean
SF	Synovial Fluid
ShcA	Src homology 2 domain containing protein A
siRNA	Short Interfering Ribonucleic Acid
SLC	Small Latent Complex
Smurf	Smad Ubiquitylation Regulatory Factor
SnoN	Ski-novel Gene N
SNP	Single nucleotide polymorphism
Sox	Sry-related HMG box
SSc	Systemic Sclerosis
STAT	Signal Transducer and Activator of Transcription
STRAP	Serine-threonine Kinase Receptor-associated Protein
TAK	Transforming Growth Factor Beta Activated Kinase
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween
TG	Transglutaminase
TGF-β	Transforming Growth Factor Beta
TβRI	Transforming Growth Factor Beta Receptor Type 1
TβRII	Transforming Growth Factor Beta Receptor Type 2
TH2	T-Helper 2
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll-like Receptor
TNF-α	Tumour Necrosis Factor alpha
TSP	Thrombospondin
uPA	Urokinase-type Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor

WT	Wild-Type

1. Introduction

TGF-β Superfamily

1.1.1 TGF-β members

The Transforming-Growth Factor-β (TGF-β) superfamily members are highly conserved across animals and are considered to be the largest family of secreted proteins (Wrana, 2013). Over 60 members of the superfamily have been identified in multicellular organisms and over 30 members have been shown to be present in mammals (Feng and Derynck, 2005). The TGF-β superfamily can be subdivided into two major subfamilies, one comprised of TGF-β, activin, nodal, lefty, myostatin and the other consisting of bone morphogenetic protein (BMP), growth and differentiation factor (GDF), and the Müellerian inhibiting substance (MIS) (Weiss and Attisano, 2013). They regulate diverse cellular processes such as proliferation, differentiation, migration and apoptosis, as well as numerous physiological processes including embryonic development, immune responses and wound healing (Wharton and Derynck, 2009). Dysregulation of their activities contributes to numerous diseases in humans demonstrating the important and diverse roles of TGF-β superfamily in numerous physiological processes (Blobe et al., 2000).

1.1.2 TGF-β isoforms

TGF-β consists of three isoforms, TGF-β1, TGF-β2 and TGF-β3, in mammals. They share approximately 70% homology in structures and similar functions *in vitro*. However, they exhibit different functions *in vivo* which was demonstrated by TGF-β knockout mice

studies (Piek et al., 1999). For example, TGF-β1 knockout mice undergo postnatal death due to excessive inflammatory responses (Shull et al., 1992). TGF-β2 knockout mice show multiple developmental defects of tissues and organs, leading to the perinatal death (Sanford et al., 1997). TGF-β3 knockout mice exhibit delayed pulmonary development, show cleft palate malformation and die within 24 hours after birth (Kaartinen et al., 1995). The TGF-β isoforms are encoded by different genes and their functions are controlled by promoters that are differentially regulated (Roberts et al., 1991). The tissue-specific and spatio-temporal expression patterns of TGF-β isoforms indicate the important roles of each TGF-β isoform during embryonic development and tissue homeostasis (Piek et al., 1999).

1.1.3 TGF-β Synthesis and activation

TGF-β ligands are synthesized as a precursor molecule with a large amino-terminal prodomain and a more highly conserved carboxy-terminal domain that contains an active ligand region (Harrison et al., 2011). The precursor protein is proteolytically cleaved by proprotein convertases such as furin in the Golgi apparatus. This results in two homodimeric products consisting of the mature TGF-β protein (a 25kDa homodimer from the C-terminal mature peptide region) and a 65-75kDa homodimer of the N-terminal remnant from the pro-peptide, also known as Latency-Associated Peptide (LAP) (Lawrence, 1996; Munger et al., 1997). These two homodimers are non-covalently attached to each other and the complex is referred to as the Small Latent Complex (SLC), which is more stable than the bioactive TGF-β. Then, the SLC forms a covalent bond with the latent-TGF-β binding protein (LTBP), forming the large latent complex (LLC),

the most abundant secreted form. Once secreted to the extracellular matrix (ECM), the LTBP covalently binds to the ECM protein such as heparin and fibronectin by a transglutaminase (TG) which stabilizes the LLC in the ECM, sequestering and stabilizing TGF-β ligand from binding to its receptor (Annes et al., 2003). The LTBP determines the distribution and localization of TGF-β; therefore, it plays an important role in regulating TGF-β bioavailability.

The latent TGF-β can be activated by physical processes such as heat (denaturation) and extreme pH (acidification or alkalization) (Worthington et al., 2011). The latent TGF-β can be also activated by biological processes such as reactive oxygen species, proteolysis and protein-protein interactions, thrombospondin-1(TSP-1), and/or integrin activation(Worthington et al., 2011).

Integrins, a large family of cell adhesion and signaling receptors, are known to play an important role in TGF-β activation. They consist of heterodimers of 18 different α and β subunits that combine to form a family of 24 transmembrane receptors in mammals binding to different ECM proteins (Luo et al., 2007). There are two main mechanisms by which integrins activate the latent TGF-β (Wipff and Hinz, 2008). First, integrins αvβ8 and αvβ3 activate the latent TGF-β in a protease-dependent manner. They simultaneously bind to both the latent TGF-β and proteases, which leads to enzymatic cleavage of the latent complex and releases active TGF-β1. For example, αvβ8 serves as a docking protein for LAP and membrane type 1-matrix metalloproteinase (MTI-MMP or MMP14) leading to a proteolytic cleavage of the LAP and activation of TGF-β (Tatti et al., 2008). Another mechanism is through a protease-independent manner, where integrins transmit cell traction forces that ultimately change the conformation of the latent TGF-β1 complex

which eventually allows ligand activation and presentation to the receptor (Jenkins et al., 2006).

Many serine proteases including plasmin and thrombin, neutrophil elastase and mast cells chymase and tryptase, metalloproteinases such as MMP-2, -9, -13 and -14 (MT-MMP1) have been implicated in TGF-β activation in a cell-type specific manner (Jenkins, 2008). In addition, the matricelluar protein TSP-1 interacts with LAP directly which causes a conformational change in LAP that exposes the receptor binding sites of the TGF-β(Murphy-Ullrich and Poczatek, 2000).

1.2 TGF-β Receptors and signaling pathways

1.2.1 TGF-β canonical pathway

1.2.1.1 TGF-β receptor members and structures

TGF-β superfamily members transmit their signals through heteromeric complexes composed of type I and type II transmembrane receptors (also known as TβRI and TβRII respectively), which are structurally conserved serine/threonine kinases of 503 (TβRI) and 567 (TβRII) amino acids (Weiss and Attisano, 2013; Wrana et al., 1994). In mammals, there are five type II receptors and seven type I receptors also known as activin receptor-like kinases (ALKs, ALK1-7) (Moustakas and Heldin, 2009). These receptors pair up in various combinations to mediate signals for over 30 TGF-β superfamily members increasing the diversity of their effects.

The TGF-β receptors consist of a short N-terminal extracellular ligand binding domain, a single transmembrane domain, and a C-terminal cytoplasmic kinase domain that has

strong serine/threonine kinase activity along with some weak tyrosine kinase activity (Schmierer and Hill, 2006).

1.2.1.2 SMAD classes and their domains

SMADs are a highly conserved family of intracellular proteins which were identified based on the homology with drosophila MAD (Mother against Decapentaplegic) protein and Caenorhabditis elegans Sma proteins(Patterson and Padgett, 2000). There are eight different SMADs in human, six different Smads in the *C. elegans* and four in *Drosophila* (Huminiecki et al., 2009). The SMADs proteins are divided into three different subfamilies: (1) the R-SMAD (Receptor-activated SMAD), composed of SMAD2 and SMAD3 (activated by ALK5, ALK4 and ALK7) and SMAD1, 5 and 8, (activated by ALK1, 2, 3 and 6); (2) the common SMAD or co-SMAD, SMAD4, which forms a complex with the activated R-SMAD; and (3) the inhibitory SMADs or I-SMADs, SMAD6 and SMAD7, that inhibit TGF-β/BMP signaling(Derynck et al., 1996).

SMADs contain three structural domains: (1) an N-terminal Mad-homology 1 (MH1) that has DNA-binding domain and that facilitates nucleocytoplasmic shuttling of Smad complexes through its nuclear localization signals (NLSs); (2) a less conserved middle linker region that interacts with ubiquitin ligases and that plays an vital role in the cross-talk between various signaling pathways (Weiss and Attisano, 2013); and (3) a C-terminal MH2 domain that mediates Smad oligomerization and establishes signal specificity (Ten Dijke and Heldin, 2006). The I-Smads do not contain MH1 domain unlike R-Smad and Co-Smad (Heldin, 2008).

1.2.1.3 Activation of TGF-β receptors, R-Smads and SMAD nucleocytoplasmic shuttling

When bioactive, mature TGF-β ligands bind to a constitutively active kinase TβRII, the TβRII-TGF-β complex recruits TβRI. At basal state, both TβRII and TβRI exist as a monomer and ligand binding induces the dimerization(Zhang et al., 2010). The formation of the heteromeric complexes of TβRII and TβRI leads to the phosphorylation of TβRI in the Glycine-Serine (GS) region that is a 30 amino acid sequence located immediately upstream of the kinase domain (Feng and Derynck, 1997), resulting in activation of TβRI kinase activity (Massague and Gomis, 2006; Shi and Massague, 2003).

The phosphorylation of the many serine and threonine residues of the GS region of the type I receptor by TβRII is required for initiating the downstream signaling cascade and all TβRI of the TGF-β superfamily have the conserved GS region (Wieser et al., 1995).

The phosphorylation of the GS region by TβRII results in the dissociation of FKBP12 (FK506-binding protein, 12 kDa) from the GS region (Huse et al., 1999) exposing the binding site for TβRI substrates, R-Smads (Wrighton et al., 2009).

The activated R-Smad proteins bind to the co-Smad4 and the complex then translocates to the nucleus where they interact with transcription factors, various co-activators and co-repressors to regulate gene transcription (Ross and Hill, 2008; Schmierer and Hill, 2007).

The maximal nuclear localization signal of the Smad complexes is typically reached 45 minutes after ligand addition and lasts for 4 to 5hours. Depending on the SMADs and their oligomerization state, SMADs can be imported to the nucleus by importin-dependent pathways or by direct interaction with nuclear pore components (Chapnick and

Liu, 2010; Xu et al., 2002). Phosphorylation of SMAD2/3 increases their interaction with importins thus increasing their nuclear import, as compared to unphosphorylated R-SMADs (Kurisaki et al., 2001; Xiao et al., 2000).

1.2.1.4 Regulation of gene expression by SMAD complexes

Once Smad complexes translocate into the nucleus, they interact with sequence-specific DNA binding transcriptional factors, specific DNA response elements, and co-activators or co-repressors to regulate transcriptional activity (Feng and Derynck, 2005; Massague et al., 2005). Non DNA-binding transcription co-activators and co-repressors are known to recruit and interact with the basal transcription machinery to regulate the magnitude of Smad-mediated transcriptional activity (Derynck and Zhang, 2003). Transcription co-activators such as CBP/p300 activate gene transcription by bringing the DNA sequence-specific transcription factors into close proximity with the RNA polymerase II complex and/or enhancing histone acetyltransferase (HAT) activity to open chromatin structure to initiate transcription (Feng et al., 1998; Simonsson et al., 2006). Transcription co-repressors, such as c-Ski/SnoN, and Evi-1, inhibit gene transcription by disrupting the formation of Smad-coactivators complexes and/or by closing the chromatin structure though their histone deacetylase (HDAC) activity (Alliston et al., 2005; Feng et al., 2002; Wu et al., 2002).

In summary, a balance between co-repressor and co-activator activities determines Smad transcriptional responses, thereby regulating gene activation or inhibition which

eventually affect numerous aspects of regular physiological and pathological processes (Lin et al., 2008).

1.2.2 TGF-β/ALK1/Smad1/5 signaling pathway

TGF-β superfamily is able to activate TGF-β receptors to mediate diverse responses via different combinations of type I and type II receptors in a cell-specific manner allowing numerous biological responses. A specific combination of type I and type II receptors is known to activate specific groups of R-Smads (Moustakas and Heldin, 2009). In addition to TGF-β/ALK5/Smad2/3 canonical pathway, TGF-β, the prototype superfamily member, also signals through another TβRI receptor ALK1, which phosphorylates Smad-1, -5 and -8 in certain cell types, such as chondrocytes (Finnson et al. 2008) and endothelial cells (Goumans et al., 2002; Goumans et al., 2003). It has been demonstrated that the specificity of the interaction between R-Smad and type I receptor is achieved by the interaction between the L45 loop of the type I receptor, which is located just downstream of the GS motif, and the L3 loop of the R-Smads (Chen et al., 1998).

1.2.2.1 TGF-β/ALK5/Smad2/3 signaling pathway versus TGF-β/ALK1/Smad1/5 signaling pathway

The role of ALK1 in TGF-β signaling was first investigated in endothelial cells where it was suggested that TGF-β/ALK1 signaling pathway was antagonistic to TGF-β/ALK5 signaling pathway by eliciting opposing responses (Goumans et al., 2002). Despite ALK1 and ALK5's opposing effects, ALK1 requires ALK5 for the recruitment of ALK1 into a

TGF-β receptor complex and needs ALK5 kinase activity for optimal ALK1 activation (Goumans et al., 2003).

TGF-β/ALK5/Smad2/3 inhibits cell migration and proliferation, whereas TGF-β/ALK1 /Smad1/5 promotes these events in endothelial cells (Goumans et al., 2002). This balanced activity between ALK1 and ALK5 signaling pathways plays a critical role during angiogenesis (Fig.1.1). During the activation phase of angiogenesis, ALK1 signaling is upregulated, promoting endothelial cell proliferation and invasion of stroma (Blanco et al., 2005). During the resolution phase, ALK1 signaling is downregulated and endothelial cells stop proliferating, while ALK5 signaling promotes cellular differentiation and restores basal lamina (Lebrin et al., 2004). Some studies have also suggested a positive link between ALK1/Smad1/5 and fibrosis (Matsubara et al., 2006; Pannu et al., 2008), while other studies have shown pro-fibrotic effect for ALK5/Smad2/3 in the skin (Wei et al., 2011).

The cellular response to TGF-β signaling has been suggested to be determined by the ratio of ALK1 to ALK5 receptor expression at the cell surface on chondrocytes (Blaney Davidson et al., 2009). Previously, our group has also shown that both ALK1 and ALK5 are expressed in human chondrocytes and that TGF-β activates both signaling pathways and elicits opposing effects in human chondrocytes (Finnson et al., 2008). Increased ratio of ALK1 to ALK5 with increased expression of MMP13 is observed in OA human and mouse chondrocytes (Blaney Davidson et al., 2009). While TGF-β/ALK5 induces ECM protein production such as type II collagen and PAI-1, TGF-β/ALK1 pathway inhibits ECM production (Finnson et al., 2008).

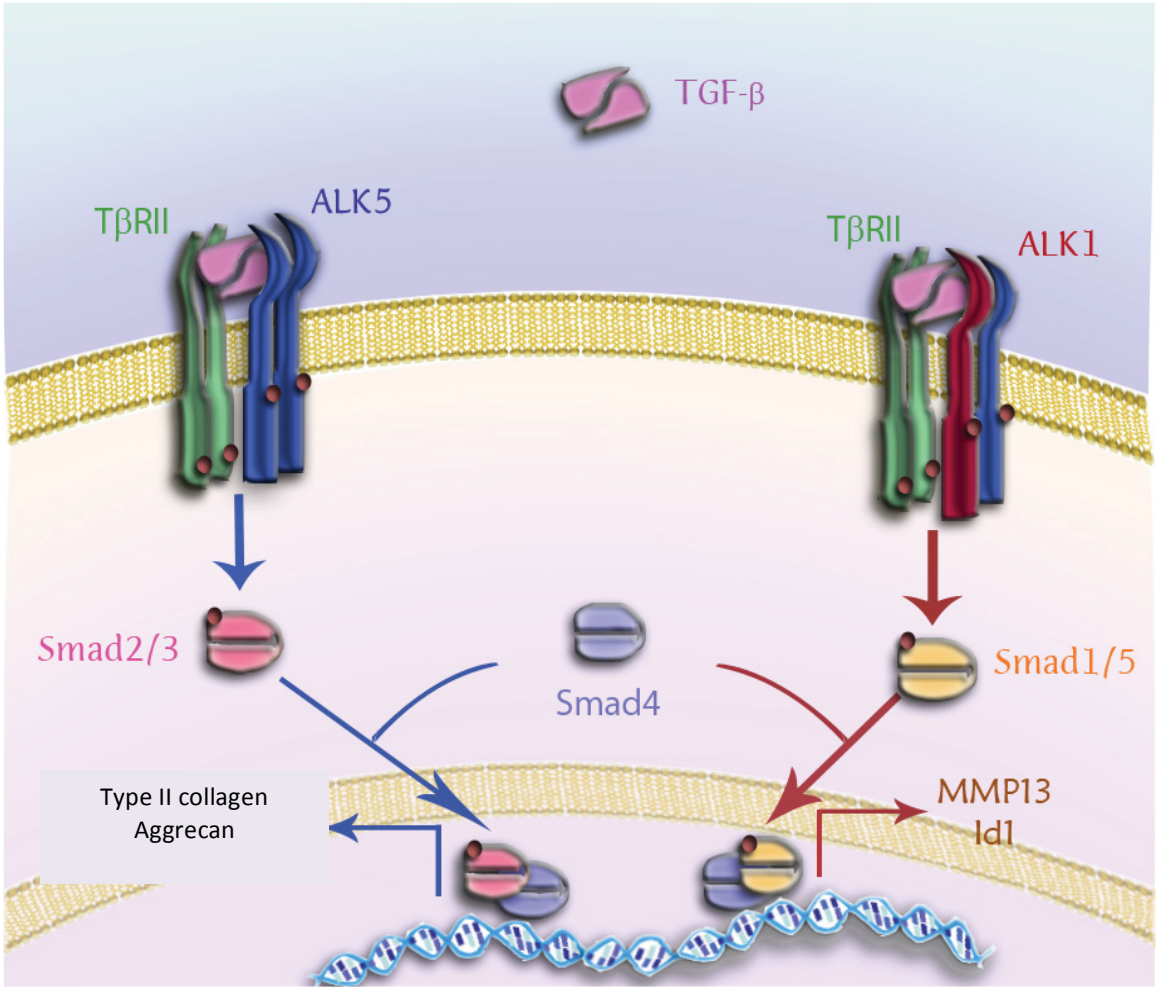

Figure 1.1: Schematic representation of the TGF-β/ALK5/Smad2/3 and TGF-β /ALK1/Smad1/5 signaling pathways in chondrocytes (Schematic diagram created by Dr. Albane Bizet)

TGF-β signals via TβRI (also termed ALK5) and TβRII. Upon transphosphorylation of ALK5 by TβRII, ALK5 phosphorylates its substrates, Smad2 and Smad3. Activation of Smad2/3 enhances ECM protein production.

Alternatively, TGF-β also signals via a different TβRI, ALK1 which results in activation of Smad1/5. Activation of Smad1/5 pathway elicits opposing cellular effects and is known to have a positive association with MMP13 expression levels in human and mouse OA chondrocytes.

1.2.3 TGF-β Non-Smad (non-canonical) pathways

In addition to the canonical TGF-β-induced SMAD pathway, TGF-β can also activate other signaling pathways in a cell-type and cell-context dependent manner. TGF-β non-canonical pathways, including that of MAPK kinases (ERK, JNK and p38), Rho-like GTPase and phosphatidylinositol-3-kinase (PI3K)/AKT (Derynck and Zhang, 2003; Moustakas and Heldin, 2005; Zhang, 2008). Activation of these non-canonical pathways can regulate TGF-β-induced cellular responses independently or by interacting with the Smad signaling pathway (Zhang, 2008).

1.3 Regulation of TGF-β signaling

1.3.1 TGF-β receptor internalization and degradation

TGF-β receptor internalization is a constitutive recycling process and an important process in maintaining TGF-β signaling. There are two internalization processes: (1) via the clathrin pathway which leads TGF-β receptors to the early endosom membranes (Tsukazaki et al., 1998) and (2) via the lipid-raft-mediated pathway through Smad7/Smurf2 containing caveolin-1 vesicles (Di Guglielmo et al., 2003).

The internalization via the clathrin pathway to early endosome membranes requires SARA (Smad anchor for receptor activation) which binds Smad2 and Smad3 through a 45-amino acid Smad-binding-domain (SBD) and then presents the Smads to the internalized receptors for phosphorylation (Wu et al., 2000). Then cPML facilitates the Smad-SARA binding and recruitment of the TGF-β receptor complexes to the early endosomes (Lin et al., 2004). SARA is known to contain a FYVE domain which

facilitates SARA localization to the early endosome membranes (Tsukazaki et al., 1998). This process is known to promote TGF-β signaling (Wrana et al., 2008).

The lipid-raft-mediated TGF-β receptor internalization pathway requires Smad7 which serves as an adaptor protein for Smurf2. The type I receptor gets ubiquitylated by the Smad7-Smurf2 ubiquitin ligase complex, resulting in lysosomal and proteasomal degradation of the receptor (Galbiati et al., 2001). Therefore, the internalization of TGF-β receptors via the lipid-raft-mediated pathway is generally known to inhibit TGF-β signaling (Wrana et al., 2008).

1.3.2 TGF-β Negative Feedback

TGF-β signaling is temporally and spatially regulated at different levels rendering tightly regulated TGF-β signaling activity in every cell type. There are several negative feedback mechanisms to terminate TGF-β signaling activity and therefore to limit the magnitude of TGF-β action in the cell.

1.3.2.1 Negative regulation by the Inhibitory Smads

The Inhibitory Smads (I-Smads) are one of the major negative regulators of the TGF-β signaling and Smad6 and Smad7 are main inhibitory Smads (Itoh and ten Dijke, 2007). Smad7 inhibits both TGF-β and BMP pathways whereas Smad6 inhibits the BMP pathway (Miyazono, 2000). TGF-β superfamily ligands induce the expression of I-Smads. For example, SMAD2/3-SMAD4 complex induces SMAD7 expression by binding to a Smad Binding element (SBE) on SMAD7 promoter (Stopa et al., 2000) while SMAD1/5-SMAD4 induces Smad6 expression (Imamura et al., 1997; Ishida et al., 2000). Also, inflammatory cytokines including IL-1, IFN-γ and TNF-α as well as EGF,

ultraviolet radiation and fluid shear stress (Afrakhte et al., 1998; Topper et al., 1997; Yan et al., 2009) can induce I-Smads expression in a cell-type-dependent manner.

I-Smads inhibit TGF-β family signalling by interacting with activated type I receptor and competing with R-Smads for activation by the receptors (Ishida et al., 2000). In addition, Smad6 interacts with phospho-Smad1 and prevents Smad1-Smad4 complex formation (Hata et al., 1998) while Smad7 interacts with SBE in the nucleus and compete with R-Smads for binding (Zhang et al., 2007). I-Smads can also inhibit TGF-β family signalling by interacting directly with Smurf E3 ubiquitin ligases and promoting the proteasomal degradation of the activated type I receptors (Ebisawa et al., 2001; Kavsak et al., 2000; Murakami et al., 2003).

1.3.3 TGF-β Regulation by co-receptors

Co-receptors are cell-surface proteins that bind the ligand and regulate the signaling of TβRI and TβRII receptors. They play an important role in regulating TGF-β signaling and actions in a cell-type dependent manner. The co-receptors are critical for normal development and adult tissue homeostasis and their aberrant expression has been reported in many pathological conditions and cancers (Bernabeu et al., 2009; Gatza et al., 2010; Hockla et al., 2010; ten Dijke et al., 2008). There are three different TGF-β co-receptors: betaglycan (type III TGF-β receptor), endoglin (CD105), and CD109.

1.3.3.1 Betaglycan (TGF-β type III receptor)

Betaglycan, also known as type III receptor, is a 200-300kDa proteoglycan that associates with TGF-β receptors (TβRI and TβRII) through homodimerization (Henis et al., 1994;

Lopez-Casillas et al., 1994). It is expressed in many cell types, but not expressed in myoblasts, endothelial and hematopoietic cells (Cheifetz et al., 1990; Ohta et al., 1987; Segarini et al., 1989). Betaglycan is known to bind to all TGF-β1, -β2 and -β3 isoforms but has a higher affinity for TGF-β2. It facilitates TGF-β binding to TβRII by presenting the ligand to the receptors. This ligand presentation by betaglycan is specifically important for TGF-β2, as TβRII does not bind TGF-β2 on its own indicating betaglycan's important role in TGF-β2 signaling (Lopez-Casillas et al., 1993).

Betaglycan knockout mice die embryonic day 13.5 due to heart defects and apoptosis in the liver, highlighting its critical role during embryonic development (Stenvers et al., 2003; Walker et al., 2011). Its structure is composed of a short cytoplasmic domain with PDZ motif, a single transmembrane domain and a large extracellular domain modified by heparin and chondroitin sulfate glycosaminoglycan (GAG) side chain (Bernabeu et al., 2009).

Although betaglycan has been known to promote TGF-β signaling, recent findings have suggested that it can also inhibit TGF-β signaling, exhibiting a dual effect on TGF-β signaling depending on the cellular context (Eickelberg et al., 2002). For example, the extracellular domain of betaglycan can be released from the cell surface upon proteolytic cleavage (Lopez-Casillas et al., 1994; Velasco-Loyden et al., 2004). The released or soluble betaglycan can bind and sequester the ligand away from the TGF-β receptors thus inhibiting TGF-β signalling (Fukushima et al., 1993). In addition, the cytoplasmic domain of betaglycan can be phosphorylated by TβRII suggesting betaglycan's role in regulating TGF-β signaling. Betaglycan phosphorylated cytoplasmic tail recruits β-

arrestin2 via PDZ domain in which promotes internalization of TβRII, thus in a ligand-independent manner (Blobe et al., 2001; Chen et al., 2003).

1.3.3.2 Endoglin (CD105)

Endoglin, also referred to as CD105, is a 180 kDa disulfided linked homodimeric glycoprotein that is expressed predominately on endothelial cells (Cheifetz et al., 1992), but also on hematopoietic cells (Rokhlin et al., 1995), immune cells (Weber et al., 2005), chondrocytes (Parker et al., 2003), fibroblasts (Rodriguez-Pena et al., 2002) and basal keratinocytes (Quintanilla et al., 2003). Endoglin shares 70% homology with betaglycan but does not have GAG chains attached on its ectodomain; it can associate with the receptors without the presence of ligand (Barbara et al., 1999). Like betaglycan, endoglin consists of a short cytoplasmic domain containing a PDZ-binding motif, a single transmembrane domain, and a large extracellular domain (Cheifetz et al., 1992). The intracellular domain of endoglin contains serine and threonine residues and some of these sites are phosphorylated by TβRII and TβRI which modulate endoglin function (Koleva et al., 2006; Ray et al., 2010). In addition, via its intracellular domain, endoglin modulates the phosphorylation state of ALK1 and ALK5 receptors, independently from TGF-β ligands (Morris et al., 2011). The human endoglin extracellular domain contains an Arg-Gly-Asp (RGD) tripeptide domain which is absent from murine endoglin (Gougos and Letarte, 1990; St-Jacques et al., 1994). There are two isoforms of endoglin arising by alternative splicing which are differentiated by the length of the intracellular domain, tissue distribution and degree of phosphorylation (Nassiri et al., 2011). L-endoglin, major isoform, contains 47 amino acids in the cytoplasmic tail and is

predominately expressed in endothelial cells, whereas S-endoglin contains only 14 amino acids (Bellon et al., 1993; Perez-Gomez et al., 2005). L-endoglin's role is more known but little is know about S-endoglin's role. It has been reported that L- and S- endoglins differentially regulate TGF-β signaling mediated by ALK5 and ALK1 showing opposing effects in myoblasts (Velasco et al., 2008).

Endoglin is unable to directly bind TGF-β on its own but it can bind TGF-β1 and TGF-β3 (but not TGF-β2) ligands in the presence of TβRII (Letamendia et al., 1998). Also, endoglin can bind activin-A, BMP-2 and BMP7 in the presence of their signaling receptors types I and II (Barbara et al., 1999). Endoglin is also known to heteromerize with betaglycan and regulate TGF-β signalling (Wong et al., 2000). The mechanism by which endoglin regulates TGF-β signaling is not completely understood but endoglin has been initially known as a TGF-β inhibitor by blocking TGF-β induced cell migration and growth in certain cell types (Letamendia et al., 1998; Li et al., 2000).

Endoglin's antagonistic role in TGF-β signaling in endothelial cells is mediated by its ability to differentially regulate the balance between the TGF-β/ALK1 and TGF-β/ALK5 signaling pathways. In these cells, endoglin is known to interact with ALK1 to promote ALK1/SMAD1/5/8 signaling and to inhibit ALK5/SMAD2/3 signaling (Blanco et al., 2005; Lebrin et al., 2004).

There is a significant correlation between endoglin expression and proliferating endothelial cells (Burrows et al., 1995). Decreased endoglin levels are shown to result in inhibition of angiogenesis *in vitro* (Li et al., 2000). These studies support that endoglin is involved in regulating angiogenesis and vascular homeostasis. Several studies also have indicated that aberrant endoglin expression is implicated in vascular related diseases.

Mutations in the gene lead to endoglin haploinsufficiency, a pathological condition known as hereditary hemorrhagic telangiectasia (HHT1, also known as Osler-Weber-Rendu disease) which is characterized by vascular dysplasia, hemorrhage in mucocutaneous tissues and visceral arteriovenous malformations (AVMs) (Haitjema et al., 1996; McAllister et al., 1994). Similarly, mutations in ALK1 receptor lead to HHT2, indicating that endoglin and ALK1 share similar functions (Sabba et al., 2007).

Furthermore, endoglin ectodomain can be cleaved at the cell surface and released as soluble endoglin. Soluble endoglin is used as a marker for preeclampsia as patients with preeclampsia show increased levels of soluble endoglin (Venkatesha et al., 2006). In addition to its involvement in vascular biology, endoglin is known to play an important role during cancer. Endoglin heterozygous mice develop malignancy in multi-stages of carcinogenesis (Quintanilla et al., 2003); endoglin is believed to be involved in cancer progression in renal carcinoma (Costello et al., 2004); and soluble endoglin level is associated with progression of squamous cell carcinoma (SCC) to a more invasive phenotype (Bernabeu et al., 2009). It was also found that endoglin is able to localize to caveolae, where it can associate with endothelial nitric oxide synthase (eNOS), which is known to regulate angiogenesis (via VEGF) (Toporsian et al., 2005). Another study has shown that caveolar localization of endoglin enhances TGF-β/ALK1 signaling whereas it promotes TGF-β/ALK5/Smad2/3 termination (ten Dijke et al., 2008). Whether endoglin and ALK1 co-localizes to caveolae to modulate TGF-β signaling pathway in endothelial cells needs to be further investigated.

1.3.3.3 CD109

Our group has initially discovered a novel 150kDa TGF-β binding protein on the cell surface of human keratinocytes that forms complexes with both TβRI and TβRII (Tam et al., 1998). This novel TGF-β binding protein, referred to as r150, showed high affinity for TGF-β1, moderate affinity for TGF-β3 but almost no affinity for TGF-β2 isoform in cell-based assays (Tam et al., 2001). Our group further demonstrated that r150 was able to directly interact with TβRI and inhibit TGF-β signaling in the presence and absence of the TGF-β ligand (Finnson et al., 2006b). Additional studies from our group have identified r150 as CD109, a GPI-anchored protein belonging to the α-2-macroglobulin/C3,C4,C5 family of thioester-containing proteins (Finnson et al., 2006b). CD109 shares several structural features with members of the α2M/complement family, including a putative bait region, a thioester signature motif and thioester reactivity defining hexapeptide (Lin et al., 2002). As a GPI-anchored protein in addition to the C-terminal GPI-anchor signal consensus sequence and a GPI-anchor cleavage site, CD109 possesses N-terminal signal sequence (Lin et al., 2002).

Our group has extensively investigated CD109's function in keratinocytes. Our *in vitro* studies have demonstrated that CD109 is a TGF-β co-receptor and that it inhibits TGF-β signaling by promoting TGF-β receptor internalization and degradation in a Smad7 and Smurf2 dependent manner through the caveolae pathway in human keratinocytes (Bizet et al., 2011; Bizet et al., 2012).

In vivo data from our group also demonstrate CD109's antagonistic effect on TGF-β signaling pathway and responses, consistent with the *in vitro* data. Transgenic mice

overexpressing CD109 in the epidermis display decreased TGF-β canonical-Smad2/3 signaling pathway and ECM synthesis during wound healing as compared to wild-type littermates (Vorstenbosch et al., 2013b). Also, these CD109 transgenic mice show more organized collagen architecture, reduced scarring, and decreased inflammatory responses in the skin during normal wound healing (Vorstenbosch et al., 2013b) and in a bleomycin-induced model of scleroderma (Vorstenbosch et al., 2013a), as compared to wild-type littermates.

CD109 is expressed in hematopoietic and mesenchymal progenitor cells, in activated T-cells and platelets, in endothelial cells and in chondrocytes (Giesert et al., 2003; Kelton et al., 1990; Murray et al., 1999; Stevens et al., 2008). It has been shown that abnormal expressions of CD109 are implicated in immune diseases, cancer, and non-cancer related skin diseases (Ertel et al., 2005; Hagiwara et al., 2010; Litvinov et al., 2011). For instance, CD109 expression was shown to be upregulated in SCC of the lung and esophagus, (Hashimoto et al., 2004), in oral cavity, uterine, cervix and endometrium, head and neck (Hagiwara et al., 2008; Hasegawa et al., 2007; Hasegawa et al., 2008; Ni et al., 2012; Sato et al., 2007; Zhang et al., 2005) and in glioblastoma (Hashimoto et al., 2004).

Our group has shown that CD109 expression is dysregulated in skin diseases other than cancers. In psoriasis, CD109 protein expression was found to be downregulated compared to normal skin (Litvinov et al., 2011). In scleroderma (SSc) skin and in SSc fibroblasts in culture, CD109 protein expression was found to be upregulated (Man et al., 2012). Loss or gain of function studies of CD109 in SSc and normal fibroblasts indicate

that CD109 inhibits ECM production in human fibroblasts (Man et al., 2012). The functional role of CD109 in tissues other than skin remains to be investigated.

1.4 Synovial joint tissue

Synovial joint tissues are comprised of articular cartilage, synovial membrane, synovial cavity, ligaments, subchondral bone and a fibrous capsule (Sharma et al., 2013). They are known to be the most common and movable type of joint in the body covering each end of the opposing skeletal bones (Pacifici et al., 2005). Articular cartilage (hyaline cartilage) covers each end of bones providing cushioning effects by absorbing shock and reducing friction during movement. Synovial fluid from synovial lining lubricates and nourishes the surrounding tissues (Pacifici et al., 2005). Ligaments hold skeletal bones in contact and fibrous tissue insulates the joint tissue (Pacifici et al., 2005).

1.4.1 Cartilage maturation and endochondral ossification

In embryogenesis, skeletal formation is from the mesoderm layer and becomes visible at around 4 weeks of gestation. Chondrogenesis is the process that results in the formation of the cartilage during the early skeletal formation and this process leads to endochondral ossification (Goldring et al., 2006). Endochondral ossification is the process of long bone formation as a result of replacement of the cartilage template. During this process, resting chondrocytes form the cartilage at the end of long bones while some chondrocytes continue to proliferate, differentiate in a highly organized fashion and are eventually replaced by bone (Zuscik et al., 2008).

Chonrogenesis begins as mesenchymal stem cells (MSC), a mesodermal-derived stem cell present in several fetal and adult tissues, recruit and migrate, proliferate and condensate. Entire processes are tightly controlled by cell to cell interaction, cell to matrix interaction, growth and differentiation factors as well as other environmental factors that regulate signaling pathways and transcription of genes in a temporal-spatial manner. Prechondrocytic mesenchymal cells start producing ECM rich in hyaluronan and collagen type I, as well as type II A collagen. The condensation process begins as hyaluronidase activity increases with an increased expression of cell adhesion molecules such as neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM) (Goldring et al., 2006). These molecules mediate cell–cell interactions (Akiyama and Lefebvre, 2011). TGF-β is one of the earliest signals in chondrogenic condensation and it stimulates the synthesis of fibronectin, which in turn regulates N-CAM (Goldring et al., 2006). The signals from cell-cell, cell-matrix and cell-cell adhesion molecule interactions activate intracellular signaling pathways that promote the differentiation of chondroprogenitor cells to fully committed chondrocytes with expression of cartilage-specific collagens II, IX, XI and aggrecan (Goldring, 2012). These cells also start expressing Sox-9, an important nuclear transcription factor in the chondrogenic program(Akiyama and Lefebvre, 2011). Sox-9 is required for the expression of type II collagen gene and other cartilage specific matrix proteins such as collagen 11a2 (Lefebvre et al., 2001). Other SOX proteins are also important in chondrocyte condensations as their activities are increased during the condensation phase in order to regulate the expressions of chondrogenic proteins such as Col9a1, aggrecan and link

protein. Their expression and activities are controlled by BMP signaling which is required for both pre-cartilaginous condensations and for the differentiation of the chondrocytes (Yoon et al., 2005). The runt-domain transcription factor, Runx2, is another well-known transcription factor that promotes chondrocyte maturation to the hypertrophic phenotype (Enomoto et al., 2000). Runx2 is expressed in all condensations (Komori, 2005), in pre-hypertrophic and less in late hypertrophic chondrocytes (Kim et al., 1999).

Immature chondrocytes undergo proliferation and the process of the endochondral ossification begins. Chondrocytes at the center of the cartilaginous bone being to mature and become prehypertrophic chondrocytes with expressing parathyroid hormone/parathyroid hormone-related peptide (PTHrP) receptor (Pthr1) and Indian hedgehog (Ihh). The pre-hypertrophic chondrocytes continue to mature and the hypertrophic chondrocytes enter into terminal differentiation by expressing osteopontin with cartilage matrix calcification, vascular invasion, and ossification. Calcified hypertrophic cartilage is eventually resorbed and replaced by bone (Yoshida et al., 2004).

1.4.2 Cartilage Structure

Chondrocytes, the only cellular component of articular cartilage, proliferate and differentiate in an organized manner which results in the formation of a growth plate with different zones of chondrocytes. These layers differ in collagen organization, proteoglycan content and chondrocyte differentiation state and morphologies (Bhosale and Richardson, 2008; Poole, 1997). The layers are composed of resting (superficial

zone), proliferating (mid-zone), hypertrophic and calcified cartilage layer (deep zone) (Yoshida et al., 2004).

First, chondrocytes display different morphologies ranging from more flatten at the surface to rounder and larger as they go into the deeper zones (Poole, 1997). Chondrocytes at the surface posses MSC-like properties (Dowthwaite et al., 2004) and produce lubricin, providing a layer of lubricants giving cartilage a smooth surface which facilitates efficient motion during joint movement (Jay and Waller, 2014). Moreover, the organization of collagen fibrillar network changes from parallel at the surface to more random arrangement in the middle zone and to radial organization in the deep zone (Goldring, 2012). The proliferating chondrocytes (mid zone) synthesize higher amount of aggrecan and other small proteoglycans. In addition, they also express type VI collagen and matrilin 1, and the cells are regulated by the PTHrP/Ihh axis (Goldring, 2012; Yoshida et al., 2004). The hypertrophic chondrocytes express type X collagen and alkaline phosphatase, and they are surrounded by calcified matrix. The calcified cartilage, the layer between nonmineralized articular cartilage and the bone, has unique matrix composition, with chondrocytes expressing hypertrophic markers and proteases involved in matrix remodeling. Matrix remodeling involving MMP 9, 13, and 14 occurs in hypertrophic cartilage zone in which VEGF-mediated vascularization happens, followed by the replacement of cartilage by bone (Onyekwelu et al., 2009). The chondrocytes in the hypertrophic cartilage exhibit characteristics that are found in both human OA and experimental OA models (van der Kraan and van den Berg, 2012). Based on these findings, it is speculated that chondrocytes lose chondrogenic phenotype as they

proliferate and differentiate to hypertrophic chondrocytes, traveling from the superficial zone to the calcified zone (hypertrophic zone).

1.4.3 Articular cartilage components and its homeostasis

Articular cartilage is an avascular, highly specialized connective tissue consisting of chondrocytes and extensive ECM proteins. ECM serves as a reservoir for growth factors and cytokines in which cells can interact. ECM proteins are predominantly composed of collagens and proteoglycans. The two main macromolecules of the tissue are collagens (types II, IX and XI) and proteoglycans (aggrecan) bearing GAG chains (Mariani et al., 2014). The strength and biological properties of articular cartilage are heavily dependent on an extensively cross-linked collagen fibrillar network. Among collagens, type II (half-life 120 years) makes up about 85- 90% of the content and provides tensile strength to the tissue (Eyre et al., 2006). Aggrecan (half-life 120 days), one of the large aggregating chondroitin sulfate proteoglycans, is the major proteoglycan of articular cartilage and has the ability to retain water in the matrix, thereby providing important biomechanical properties to the tissue (Heinegard, 2009). Other minor ECM components include versican, link-protein, cartilage oligomeric matrix protein (COMP), and fibronectin (Roughley, 2001).

In healthy adult cartilage, chondrocytes are in a quiescent phase in which there is very little turnover of collagen network and other ECM components. A fine balance between synthesis (anabolic activity) and breakdown (catabolic activity) of matrix molecules maintains cartilage integrity and homeostasis (Goldring and Marcu, 2009; Houard et al.,

2013). Factors that promote anabolic activities include TGF-β, BMPs (ex. BMP-7), and Insulin-like Growth Factor-1 (IGF-1) and they are known to stimulate ECM synthesis and inhibit proteases activities (Mueller and Tuan, 2011). On the other hand, catabolic factors include proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6, and IL-17 and catabolic mediators such as Nitric oxide (NO) and Prostaglandins (Abramson and Yazici, 2006; Mueller and Tuan, 2011). Proinflammatory cytokines enhance production of MMPs and aggrecanases (A disintegrin and metalloproteinase with thrombospondin motifs, ADAMTS), which are the key enzymes responsible for ECM degradation (Edwards et al., 2008; Kobayashi et al., 2005). Proinflammatory cytokines activate NF-κb signaling, which acts as a positive regulator of the expression of proinflammatory cytokines (Roman-Blas and Jimenez, 2006).

During degenerative joint diseases or traumatic injuries of cartilage and in aging, there is an up-regulation of catabolic pathways disrupting the balanced activities between ECM synthesis and breakdown, leading to the onset of joint tissue degenerative diseases such as OA.

1.4.3.1 Interaction of cartilage with other joint tissues (synovium and subchondral bone)

Articular cartilage is aneural and alymphatic connective tissue (Poole, 1997) that covers subchondral bone and is surrounded by synovial fluid from synovium. The close physical relationships between the cartilage and subchondral bone; and the cartilage and synovial fluid have introduced the concept of biochemical and molecular crosstalk across the joint

tissue. Since articular cartilage is not vascularized, subchondral bone and synovial tissue are the two major nutrient sources to support normal cartilage function and metabolism. Therefore, the dynamic interactions among these tissues maintain healthy joint integrity (Finnson et al., 2012; Imhof et al., 1999). Although the exact paracrine mechanisms among these tissues are yet to be determined, some studies have demonstrated that products from cartilage or subchondral bones are secreted into the joint space, in which these products travel to neighboring tissues through the synovial fluid (Westacott et al., 1997). Between articular cartilage and subchondral bone, there is a functional unit called the 'osteochondral junction' which consists of a deeper layer of non-calcified cartilage, the tidemark, calcified cartilage, the cement line and subchondral bone (Madry et al., 2010). The permeability of calcified cartilage and subchondral bone provides connecting channels between these two tissues allowing constant biomechanical and biochemical crosstalk across this region (Pan et al., 2009). For example, subchondral bone significantly influences chondrocyte survival during bovine explant culture (Amin et al., 2009) as well as the signals derived from osteoblasts and haemapoietic cells from the bone seem to regulate cartilage hypertrophy during endochondral ossification (Johnstone et al., 2000; Moreno-Rubio et al., 2010).

Besides oxygen and nutrients, the cellular elements of the synovium tissue produce lubricin and hyaluronic acid, which are responsible to reduce friction fluid, into synovial fluid (Rhee et al., 2005). Synovial fluid also acts as an important reservoir to remove metabolic waste products from chondrocytes and cartilage matrix turnover debris (Hui et al., 2012). In summary, homeostasis of a joint tissue is maintained by constant communications among articular cartilage, subchondral bone and synovial fluid.

1.5 Role of TGF-β in cartilage development, maintenance and repair

1.5.1 TGF-β in cartilage development

TGF-β plays multiple roles from early to terminal stages of cartilage development, including condensation, proliferation, and terminal differentiation of articular chondrocytes (van der Kraan et al., 2009; Wang et al., 2014).

All three TGF-β isoforms are expressed in mesenchymal condensation but their expression levels decrease at later stages of cartilage development (Pelton et al., 1991). TGF-β1 and TGF-β3 are expressed mainly in the proliferative and hypertrophic zones of cartilage in appendicular growth plates while TGF-β2 is expressed in all zones but mainly in the hypertrophic zone (Horner et al., 1998).

TGF-β is among the earliest signals facilitating chondrogenic condensation by stimulating fibronectin synthesis, which in turn regulates neural cell adhesion molecule (Goldring et al., 2006; Onyekwelu et al., 2009). TGF-β promotes chondroprogenitor cell proliferation and differentiation via its canonical signaling pathway (ALK5/Smad2/3) and activates SOX9 transcription via CREB-binding protein (CBP/p300) recruitment (Furumatsu et al., 2009; Furumatsu et al., 2005). TGF-β inhibits terminal differentiation of chondrocytes and represses chondrocytes from becoming hypertrophic chondrocytes in a Smad3-dependent manner (van der Kraan et al., 2009; van der Kraan et al., 2010; Yang et al., 2001a). TGF-β also inhibits cartilage matrix calcification and ossification.

1.5.2 TGF-β in cartilage maintenance

1.5.2.1 TGF-β in ECM synthesis and degradation

As mentioned earlier, a fine-tuned balance between ECM synthesis and degradation maintains cartilage integrity and homeostasis. TGF-β is known as one of the powerful anabolic factors as it stimulates ECM productions such as type II collagen and aggrecan and inhibits ECM breaking down enzymes such as MMPs in a Smad2/3 dependent and/or independent mechanism (Aref-Eshghi et al., 2015; Blaney Davidson et al., 2010). TGF-β also enhances production of protease inhibitors such as tissue inhibitor of metalloproteinases (TIMPs), the major inhibitor of MMP13 and ADMTS4 (Qureshi et al., 2005). TGF-β induces both TIMP-1 and TIMP-3. TIMP-3 is located in ECM and known to bind to chondroitin and sulfate. TIMP-3 also inhibits TNF-α converting enzyme (TACE), thereby inhibiting activation of TNF-α (Lee et al., 2001). Moreover, TGF-β can counteract the catabolic effects of IL-1 and TNF-α and block cartilage degradation (Blaney Davidson et al., 2007b; Brandes et al., 1991).

1.5.2.2 Interaction between ECM components and TGF-β in cartilage

The ECM of articular cartilage can be divided into the pericellular, territorial and interterritorial compartments, depending on the distance from close to further away from chondrocytes (van der Kraan et al., 2002). Chondrocytes, rather sparsely distributed in ECM, sense and respond to changes in matrix composition in order to maintain cartilage homeostasis. Therefore, there are constant feedback mechanisms between ECM components and chondrocytes, and these mechanisms are regulated by growth factors and cytokines such as TGF-β. Integrins mediate the interactions between ECM and chondrocytes (van der Kraan et al., 2002). The most abundant integrin detected on

chondrocytes is the fibronectin receptor, α5β1(Durr et al., 1993). There are other receptors such as α1β1, α2β1, α11β1 for type II and type VI collagen, αvβ3 for vitronectin and osteopontin, and α6β1 for laminin (Enomoto et al., 1993; Salter et al., 1992).

Annexin V, a member of annexins, is a type II collagen-binding molecule mainly expressed by superficial chondrocytes and the hyaluronan receptor, CD44(Ishida et al., 1997; Reid et al., 2000).

As mentioned in section 1.1.3, integrins are also involved in activation of latent TGF-β via protease-dependent and protease independent mechanisms (Wipff and Hinz, 2008). Some of the interstitial proteoglycan molecules, decorin, biglycan and fibromodulin also regulate TGF-β activity by sequestering TGF-β within ECM (Hildebrand et al., 1994).

1.5.3 Role of TGF-β in cartilage repair

Articular cartilage exhibits limited ability to self-regenerate; therefore, even minor damage induced by aging, injuries and pathological conditions lead to unsuccessful reparative processes, resulting in progressive joint degeneration (Johnstone et al., 2013). TGF-β/ALK5 activation via Smad2/3 signaling contributes to the maintenance of the stable quiescent phase of chondrocytes with synthesis of aggrecan and collagen II proteins. Smad 2 and Smad3 exert an inhibitory effect on chondrocyte hypertrophy (Yang et al., 2001a), a phenotypic hallmark of terminally differentiated chondrocytes which is observed in OA and also in aging chondrocytes (Bertrand et al., 2010). Hypertrophic

chondrocytes are associated with reduced expression of ALK5/Smad2/3 and increased MMP13 expression(van der Kraan and van den Berg, 2012).

In contrast to TGF-β/ALK5/Smad2/3 signaling pathway that blocks chondrocyte hypertrophy, TGF-β/ALK1/Smad1/5 signaling has been shown to stimulate hypertrophic differentiation by interacting with RUNX-2 (Blaney Davidson et al., 2009; Hellingman et al., 2011b). RUNX-2 is a master transcription factor involved in chondrogenic differentiation, thereby promoting chondrocyte hypertrophic differentiation and its elevated expression in OA (Orfanidou et al., 2009; Wang et al., 2004).

1.6 Crosstalk between TGF-β signaling via Smad and non-smad signaling pathways

TGF-β can also activate non-Smad signaling pathways such as TGF-β-activated kinase 1 (TAK1), a MAPK3 (ERK), MAPK kinases (p38, JNK), Rho-like GTPase signaling pathways and PI3K/Akt pathways (Zhang, 2008). TGF-β mediated non-Smad pathways in chondrocytes are poorly known, even though some studies have demonstrated that TGF-β induced non-Smad signaling pathways imply extensive crosstalks between TGF-β signaling and other signaling pathways, contributing to maintenance of cartilage function and integrity.

One of the studies has shown that TAK1 enhances production of type II collagen in adult chondrocytes without increasing transcription of the *Col2a1* gene and that this mechanism is Smad-3 independent (Qiao et al., 2005). TGF-β and BMP can activate

TAK1 which directly phosphorylates MKK 3/6, leading to the subsequent phosphorylation of p38 (Shirakabe et al., 1997). Besides the p38 pathway, TAK1 is also capable of interacting with all R-Smads (Smads 2/3 and Smads 1/5/8). *In vivo* study by *Gunnell et al*. has shown that cartilage deletion of *Tak1* using a Col2-Cre transgenic mice resulted in neonatal lethality immediately following birth, reduced chondrocyte proliferation, and delayed chondrocyte maturation within developing embryonic cartilages (Gunnell et al., 2010) highlighting TAK1's important role during chondrogenesis and cartilage maintenance.

Pro-inflammatory cytokines such as IL-1β and TNF-α activate MAPK kinase activity which decreases Smad3-Smad4 binding to DNA and thus inhibits ECM production in chondrocytes (Roman-Blas et al., 2007). In addition, ATF-2 (Activating Transcription Factor-2) cooperates with Smad3 to mediate an inhibitory effect of TGF-β on chondrocyte maturation (Ionescu et al., 2003). Moreover, PI3K, Akt, and mTOR inhibit TGF-β action to stimulate TIMP-3 expression (Beier and Loeser, 2010) which in turn increases the MMPs activities.

Both TGF-β and BMP are important as they promote early chondrogenesis (Kawamura et al., 2012). However, TGF-β and BMP seem to exert opposite effects on chondrocyte hypertrophy during the late maturation stage. The opposite roles of these two signaling pathways were demonstrated by the different phenotypes observed from *in vivo* studies. For example, mutant mice homozygous for a targeted disruption of Smad3 resulted in accelerated differentiation of chondrocytes into hypertrophy exhibiting OA-like

destruction of cartilage (Yang et al., 2001b). In addition, *in vitro* studies using Smad3-deficient chondrocytes showed enhanced BMP signaling and accelerated hypertrophic differentiation indicating that endogenous TGF-β signaling suppresses BMP signaling during chondrocyte maturation (Li et al., 2006). It was later determined that this inhibitory effect of TGF-β on BMP signaling and on chondrocyte hypertrophy was mediated by expression of SnoN (Kawamura et al., 2012). Furthermore, cartilage-specific BMP type I receptor knock out mice or double deletions of BMP-type R-Smads (Smad1 and Smad5) mice displayed severely impaired chondrogenesis emphasizing the essential role of BMP in this process (Retting et al., 2009; Yoon et al., 2005). TGF-β type I receptors activate Smad2/3 whereas BMP type I receptors phosphorylate Smad1/5. Activation of Smad2/3 and Smad1/5 signaling pathways in human chondrocytes seem to elicit opposing effects but exact mechanisms by which these two signaling are differentially regulated remain elusive.

Several studies have reported the interactions between IGF-1 and TGF-β1 in chondrocyte proliferation and differentiation (Rosselot et al., 1994; Tsukazaki et al., 1994; Yaeger et al., 1997). TGF-β increases the number of IGF-1 receptors in chondrocytes (Tsukazaki et al., 1994) while TGF-β and IGF-1 exert synergistic effects on type II collagen and aggrecan production (Yaeger et al., 1997). Similarly, both growth factors have been shown to exhibit synergistic effects to induce MSC chondrogenic differentiation (Longobardi et al., 2006; Worster et al., 2001).

There is increasing evidence suggesting that the numerous molecular events and the crosstalks among various growth factors, proinflammatory cytokines and other mediators

in the joint tissue are responsible for articular cartilage development, maintenance and degeneration leading to OA. Determination of the factors that regulate the crosstalks between TGF-β Smad and non-Smad signaling pathways in chondrocytes may provide further insights into intricate mechanisms by which TGF-β signaling pathway regulates cartilage homeostasis.

1.7 Chronic joint tissue degradation: OA

OA is the most common form of degenerative joint disease which can occur in any joint but is more common in the joints of the hand, knee, foot, spine and hip (Wieland et al., 2005). Despite unknown etiology, there are multiple risk factors for OA, including genetic predisposition, sex, trauma, obesity, inflammation, lifestyle issues, and comorbidities (Sinkov and Cymet, 2003) and it is strongly associated with increasing age (Zhai et al., 2015). OA leads to debilitating pain, substantial morbidity and disability, and limited movements that contribute to reduced social interactions which may lead to depression of a patient and a great economic burden to society (Bitton, 2009). These personal and societal burdens are expected to increase worldwide due to the increased aging population.

OA is mainly characterized by progressive degradation of cartilage, but it affects all the tissues of the joint leading to new bone formation in joint margins (osteophyte formation), changes in the subchondral bone (subchondral bone sclerosis), synovial fibrosis and thickening of the joint capsule (Bitton, 2009). Aberrant TGF-β signaling has been implicated a number of human diseases (Massague et al., 2000), including OA.

1.7.1 TGF-β signaling pathway profiles in OA patients

A recent genome-wide study has identified a correlation between the genetic variants of the TGF-β signaling pathway components in OA patients. Mutations in TGF-β1 gene are implicated in Camurati–Engelmann disease, a rare autosomal bone disorder, which is characterized by progressive cortical thickening and subsequent sclerosis of the long bones (Janssens et al., 2006). The patients with this disorder exhibit the symptoms that are similar to those in OA where bone lesions in affected joints contribute to limb pain and impaired mobility. The study conducted by *Tang et al.*, has found that active TGF-β1 was released during bone resorption and that the released TGF-β1 coordinates bone formation by promoting migration of bone marrow stromal cells (also known as bone mesenchymal stem cells) to the bone resorptive sites (Tang et al., 2009). A single nucleotide polymorphism (SNP) in TGF-β1 exon1 (T29 to C) is associated with spinal osteophyte formation in Japanese women (Yamada et al. 2000) and has been linked to decreased in bone density and fracture risk in postmenopausal Chinese women (Lau et al., 2004).

A genetic variant in asporin (ASPN), an inhibitor of the TGF-β pathway, was reported to be associated with higher susceptibility to OA in Asian and Spanish Caucasian populations (Jiang et al., 2006; Kizawa et al., 2005; Mustafa et al., 2005; Song et al., 2008). ASPN encodes a small leucine-rich ECM molecule that contains three repeat encoding for aspartic acid (D) with exon2 (Kizawa et al., 2005). The D-14 polymorphism in ASPN is more frequent in OA patients and D14 allele is highly expressed in OA

cartilage (compared to the common D13) (Kizawa et al., 2005).

Several rare mutations in SMAD3 were found in patients with aneurysm-osteoarthritis syndrome (AOS) in which patients present with early onset OA affecting primarily joints in the feet/ankle and hand/wrist but can progress to the knee, hip, facet, as well as the intervertebral disks (van de Laar et al., 2011; van de Laar et al., 2012; Wischmeijer et al., 2013). Also a SNP in the first intron of the SMAD3 has been associated with hip and knee OA in the European population (Valdes et al., 2010). Altogether, these findings emphasize the critical role of TGF-β signaling during OA development.

1.7.2 TGF-β isoforms in OA cartilage

All TGF-β isoforms are expressed in articular cartilage and are differentially expressed in OA compared to healthy, normal cartilage. Previous *in vivo* studies have demonstrated that mice deficient for genes encoding any TGF-β1 isoform show embryonic lethality and loss of TGF-β2 or TGF-β3 genes leads to numerous bone deformities including the forelimbs, hindlimbs and craniofacial bones stressing the importance of TGF-β in skeletogenesis (Dunker and Krieglstein, 2000).

Proteomic and immunohistochemical studies suggest that expression of the TGF-β isoforms (TGF-β1, -β2, -β3) are down-regulated in OA cartilage (Verdier et al., 2005; Wu et al., 2007). TGF-β1 and TGF-β2 mRNA levels are decreased in OA cartilage using an experimental model of OA (Boumediene et al., 1998). Moreover, TGF-β3 levels are

reduced in murine cartilage during OA progression in both spontaneous(STR/Ort) and collagenase-induced mouse models of OA (Blaney Davidson et al., 2006).

In contrast, other studies have reported increased levels of TGF-β1, -β2 and -β3 in human OA at both mRNA and protein levels in cartilage from advanced hip OA in comparison with normal cartilage (Pombo-Suarez et al., 2009). Their immunostaining data show that the increase in TGF-β isoforms levels is more pronounced in the deeper layer of OA cartilage and this increase is not correlated with mRNA levels of collagen and aggrecan in OA cartilage. OA chondrocytes possibly lose their abilities to respond to increased levels of TGF-β.

The discrepancies among these reports may be due to severity of OA, age, gender, genetics and mechanical factors (obesity, trauma). It is possible that the levels of TGF-β isoforms are increased in the early stages OA in an attempt to counteract the catabolic effects of proinflammatory cytokines (Pujol et al., 2008). Their levels could decrease in the mid-phase of OA, due to effects of overwhelming levels of catabolic factors and then upregulated again in the late phase as chondrocytes completely lose their abilities to respond to TGF-β.

1.7.3 Abnormal activation of TGF-β in OA cartilage

For TGF-β to be able to signal through its receptors and elicit cellular responses, it must be converted to biologically active form. TGF-β activation from its latent complexes controls TGF-β bioavailability and several studies have indicated that factors involved in latent TGF-β activation display altered expression in OA. LTBP family members are

differentially expressed in OA in which LTBP-1 and LTPB-2 are increased in human OA cartilage and in experimental models of OA (Aigner et al., 2006; Appleton et al., 2007; Wei et al., 2010). On the other hand, LTBP-3 null mice develop OA and show increased numbers of hypertrophic chondrocytes (Dabovic et al., 2002a; Dabovic et al., 2002b). The discrepant findings on increased LTBP-1/2 expression in human OA and decreased LTBP-3 expression in mice OA may be due to species specific functions of LTBP isoforms and/or their different TGF-β isoform-specificities (Annes et al., 2003).

Transglutaminase (TG) transamidation catalytic activity has been found to increase in OA joint cartilage (Rosenthal et al., 1997). Among TG family members, TG2 is expressed in hypertrophic chondrocytes and shows increased expression in knee and femoral cartilage in human OA (Heinkel et al., 2004; Johnson et al., 2001; Orlandi et al., 2009) as well as in experimental OA models (Appleton et al., 2007; Huebner et al., 2009). Whether the enhanced TG2 expression in OA correlates with increased TGF-β activation remains to be determined.

The secreted ECM protein, thrombospondin-1 (TSP-1), regulates latent TGF-β activation and increased levels of TSP-1 are expressed in response to injury (Murphy-Ullrich and Poczatek, 2000). In mild and moderate OA, TSP-1 levels are increased but its levels are decreased in severe OA (Pfander et al., 2000). Spondin-1 (another member of the TSP-1 family) has the capacity to regulate the metabolism of articular cartilage by activating latent TGF-β; it is elevated in human OA cartilage and in rat knee cartilage following surgical menisectomy (Attur et al., 2009; Karlsson et al., 2010). The increase of TSP-1

during early and mid phase of the disease may account for an adaptive response in an attempt to increase cartilage repair.

1.7.4 Aberrant TGF-β signaling activity in OA cartilage

1.7.4.1 TGF-β receptors in OA cartilage

Increasing lines of evidence indicate that TGF-β receptor expression levels are altered in OA (Blaney Davidson et al., 2005). First, TβRII levels are shown to be decreased in human OA cartilage (Verdier et al., 2005) in comparison to normal cartilage; and similarly, its mRNA levels are decreased in cultured OA primary chondrocytes as compared to normal primary chondrocytes (Dehne et al., 2009). TβRII mRNA levels in cartilage are also shown to be dramatically reduced at mid-and late stages of OA in a rabbit model (Boumediene et al., 1998).

Several *in vivo* studies also support that the loss of TβRII expression may contribute to the initiation and/or progression of OA. Chondrocyte-specific TβRII conditional knockout mice resulted in upregulation of Runx2, Mmp13, Adamts5 and Col10 expression in articular chondrocytes (Shen et al., 2013). The histological analysis showed articular cartilage degradation, increased hypertrophic chondrocyte numbers, early osteophyte formation and increased subchondral bone mass, all of which are characteristics of OA in human (Shen et al., 2013). This supports the earlier finding showing that a truncated, kinase-defective TβRII expressed in mouse skeletal tissue is associated with terminal chondrocyte differentiation and the development of OA-like features (Serra et al., 1997).

The expression levels of the TGF-β type I receptors are also altered in OA. Our group has shown that human chondrocytes also express ALK1 besides ALK5, and that both ALK5 and ALK1 are needed for TGF-β-induced Smad1/5 phosphorylation whereas only ALK5 is essential for TGF-β-induced Smad3 phosphorylation (Finnson et al., 2008). Although both ALK5 and ALK1 expression are decreased in mouse models of OA, a decrease of ALK1 expression is less than that of ALK5, resulting in an increased ratio of ALK1/ALK5 during OA which correlates with increased expression of MMP13 mRNA level (Blaney Davidson et al., 2009). Also in human OA cartilage ALK1 mRNA level is highly correlated with MMP-13 expression, whereas ALK5 mRNA level is correlated with aggrecan and collagen type II expression (Blaney Davidson et al., 2009). In addition, ALK1 has been identified as one of the upregulated genes in a mensical tear rat model of OA (Wei et al., 2010) whereas ALK5 levels are dramatically reduced in partial meniscectomy and post-surgery training rat model of OA (Gomez-Camarillo and Kouri, 2005). Together with these findings, it is suggested that the expression of TGF-β signaling receptors (TβRII and ALK5/ALK1) is differentially regulated in OA, in support of the important role they play in OA pathogenesis.

1.7.4.2 TGF-β signaling in OA cartilage

TGF-β exerts its chondroprotective effect on chondrocytes via its canonical pathway, TGF-β/Smad2/3, which prevents articular chondrocytes from undergoing terminal hypertrophic differentiation. Previous studies suggest that OA is associated with reduced TGF-β/ALK5/Smad2/3 signaling where Smad2 phosphorylation levels are reduced in cartilage during OA progression in both spontaneous- (STR/Ort) and collagenase-induced

mouse models of OA (Blaney Davidson et al., 2006). Also, Smad2 phosphorylation is decreased in cartilage in old mice compared to young mice but Smad3 phosphorylation was not examined in these models (Blaney Davidson et al., 2005).

Emerging evidence indicates that TGF-β/Smad3 also plays a critical role in the regulation of articular chondrocyte hypertrophy during OA development. Smad3 knockout mice developed spontaneous joint degeneration similar to human OA exhibiting chondrocyte hypertrophy with type X collagen expression in superficial zone, progressive loss of articular cartilage tissue and formation of osteophytes (Yang et al., 2001b). Also a recent study has reported that Smad3 phosphorylation levels are decreased in the cartilage specific Smurf-2 transgenic mice that spontaneously develop an OA-like phenotype (Wu et al., 2009). Other studies have suggested that TGF-β/Smad3 increases MMP13 proteins in cultured cartilage explants, which has been observed in other tissues (Moldovan et al., 1997). Also TGF-β/Smad3 has been shown to decrease activity of miR-140, (microRNA that is originally found in cartilage of human chondrocytes) (Tardif et al., 2013; Tuddenham et al., 2006) which inhibits expression of MMP13 and ADAMTS5, thus proteases that degrade ECM proteins (Tuddenham et al., 2006). The discrepancy in effects of TGF-β/Smad3 may be due to different experimental conditions (explants vs monolayer culture) or differences in *in vivo* and *in vitro* settings.

Our group first reported that TGF-β signals through another TβRI, ALK1, and that activation of ALK1/Smad1/5 pathway in human chondrocytes exhibits opposing effects from TGF-β/Smad2/3 pathway (Finnson et al., 2008). Since then, the hypothesis that

TGF-β shifts its pathway from Smad2/3 towards Smad1/5 in OA and this shift may play an important role in OA pathogenesis has received attention. Identification of factors regulating the ALK5/Smad2/3 and ALK1/Smad1/5 pathway may lead to the development of strategies to increase ALK5/Smad2/3 signaling while reducing ALK1/Smad1/5 signaling.

Other important TGF-β signaling regulators are Smad7/Smurf2, as they mediate TGF-β receptor degradation, an important mechanism for the termination of TGF-β signaling (Inoue and Imamura, 2008; Yan et al., 2009). IL-1β is known to increase Smad7 expression in human chondrocytes which inhibits TGF-β activity (Bauge et al., 2008; Bauge et al., 2007); and, Smad7 expression is increased in the cartilage of old mice (Blaney Davidson et al., 2005), suggesting that Smad7 is important in the progression of OA where aging is considered an important risk factor (Loeser, 2009; van der Kraan et al., 2010). However, there is no difference in Smad7 expressions between normal and OA human cartilage (Kaiser et al., 2004). On the other hand, it has been found that Smurf2 is increased in human OA as compared to normal cartilage and Smurf2-transgenic mice spontaneously develop an OA-like phenotype with decreased TGF-β signaling and increased Smad3 degradation (Wu et al., 2008).

1.7.4.3 TGF-β co-receptors in OA cartilage

The information on roles of TGF-β co-receptors, betaglycan, endoglin, and CD109, in cartilage function is limited; however, since they regulate TGF-β action, alterations in their expression levels and activities may contribute to OA progression.

We have previously shown that betaglycan is expressed in human chondrocytes and that it forms a complex not only with the signaling receptors but also with endoglin in a ligand- and TβRII-independent manner (Parker et al., 2003). Betaglycan expression levels are found to be similar in damaged and intact human OA cartilage and in a rat model of experimental OA (Appleton et al., 2007; Tsuritani et al., 2010). However, betaglycan mRNA expression levels are shown to be increased in MSC from the femur channel and in trabecular bone from the iliac crest of patients with OA compared to control group (Rollin et al., 2008; Sanchez-Sabate et al., 2009).

Our group has previously demonstrated that endoglin is expressed in human articular cartilage and in primary human articular chondrocytes (Parker et al., 2003). A large-scale gene expression study has suggested that endoglin mRNA expression is increased in human OA cartilage as compared to normal cartilage (Aigner et al., 2006) in a surgically induced model of early OA in rats [(Data from Gene Expression Omnibus (GEO), DataSet Record GDS2809, Reference Series: GSE8077] (Appleton et al., 2007). It also has been found that levels of soluble endoglin in plasma and synovial fluid correlate with primary knee OA suggesting endoglin as a biomarker for determining disease severity and that it may contribute to the pathogenesis of OA (Honsawek et al., 2009).

Our group has discovered CD109 as a novel TGF-β co-receptor inhibiting Smad2/3 phosphorylation and enhancing TGF-β receptor degradation in other cell types (Bizet et al., 2009; Bizet et al., 2012). Levels of CD109 in human OA and normal cartilage tissue are not known.

1.8 TGF-β in OA synovium and subchondral bone

Although OA has long been considered as a disorder of articular cartilage, subchondral bone plays a crucial role in the initiation and progression of OA. Osteophyte formation, a fibrocartilage-capped bony outgrowth at the margins of diarthodial joints, is well-known feature of OA (van der Kraan and van den Berg, 2007). It has been suggested that TGF-β is involved in aberrant bone remodeling and cartilage degeneration in OA as enhanced TGF-β/Smad2/3 activity in the subchondral bone has been observed (Zhen et al., 2013). Increased levels of TGF-β1 and TGF-β3 were detected in developing osteophytes and articular cartilage during murine experimental OA, and the inhibition of endogenous TGF-β prevents osteophyte formation (Scharstuhl et al., 2002). Moreover, in murine model, multiple intra-articular injections of TGF-β in joint result in osteophyte formation originated from the periosteal lining cells, located at the margin of subchondral bone (van Beuningen et al., 1994) and change in articular cartilage with strong resemblance to both experimental and spontaneous mice OA (van Beuningen et al., 2000; van Beuningen et al., 1994). Moreover, it has been demonstrated that the cells in the outer layer of osteophytes strongly express TGF-β1 in the experimental OA murine models (Blaney Davidson et al., 2007c).

There is also increasing recognition that synovial tissue contributes to OA development. Synovial lining cells are the major components in the synovial membrane besides macrophages, fibroblasts and MSCs (Fan et al., 2009). Although OA was not initially categorized as an inflammatory form of arthritis such as rheumatoid arthritis, recent

findings provide substantial evidence that synovial lining hyperplasia, infiltration of macrophages and fibrosis are also observed during OA progression (Oehler et al., 2002).

TGF-β is known as a strong inducer of synovial tissue fibrosis characterized as fibroblast proliferation and excessive accumulation of ECM proteins such as types I and III collagen (Blom et al., 2004). Elevated TGF-β activity has been detected in synovial fluid of OA patients (Blom et al., 2004) and intra-articular injection of activated recombinant TGF-β or adenovirus overexpression of active TGF-β into mouse knee exhibit substantial synovial fibrosis (Bakker et al., 2001; van Beuningen et al., 1994). These studies also show that TGF-β can also function as a chemotatic factor to recruit fibroblasts into synovial tissue to make it fibrotic. These findings indicate that TGF-β plays a critical role in synovial fibrosis in OA and contributes to OA pathogenesis.

1.9 Joint tissue environment

Articular cartilage receives oxygen and nutrients from surrounding tissues such as subchondral bone and synovia lining (Shen et al., 2014b). Chondrocytes interpret and respond to biomechanical stressors thus maintaining healthy integrity of nonvascularized tissue (Husa et al., 2010). Chondrocytes are embedded in extensive ECM with an extremely long diffusion distance from the nourishing arteries. Therefore, they have adapted highly conserved mechanisms to survive the hypoxic environment (~2-10% partial pressure of oxygen (pO_2) relative to 21% of atmospheric pO_2) (Pfander and Gelse, 2007) and adaptation to hypoxia is crucial to the survival of chondrocytes (Schipani et al.,

2001). Since oxygen and other nutrients diffuse into the tissue from the neighboring tissues, an oxygen gradient is created across cartilage tissue, from 7% O_2 (53 mm Hg) in the superficial zone to less than 2% O_2 (7.6 mm Hg) in the deep zone (Fermor et al., 2007; Silver, 1975). Oxygen tension of adult synovial fluid is 6.5 – 9.0% (50-70 mm Hg) (Fermor et al., 2010). Varying oxygen levels in joint tissue can significantly influence the metabolism of articular chondrocytes and the surrounding environment (Grimshaw and Mason, 2000).

1.9.1 HIF family and its signaling

Hypoxia-inducible factor (HIF) is a transcription factor and one of the major regulators of the hypoxic response. HIF is a heterodimer consisting of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit. Both subunits are part of the basic Helix-Loop-Helix PER-ARNT-SIM (bHLH-PAS) family of transcription factors which are important for DNA binding and dimerization (Semenza, 1999). In mammals, there are three isoforms of the α-subunit; HIF-1α encoded by *HIF1A*, HIF-2α encoded by *EPAS1* (endothelial PAS domain containing protein 1), and HIF-3α encoded by *HIF3A* (Makino et al., 2001; Semenza and Wang, 1992; Tian et al., 1997).

Mice that lack HIF-1α die around day 9 of gestation as a result of cardiac and vascular malformation (Iyer et al., 1998; Ryan et al., 1998). HIF-2 α is closely related to HIF-1 α but not in function. HIF-2α knock out mice show embryonic or perinatal lethality, but the severity of vascular defects depends on the genetic background of the mice (Nauta et al., 2014).

Under normoxic conditions, the α subunit (HIF-1α) is degraded rapidly by the ubiquitin-proteasome pathway(Huang et al., 1998). In the absence of oxygen, HIF-1α rapidly translocates into the nucleus and heteromerizes with HIF-1β (or ARNT) which subsequently binds to hypoxia responsive elements (HREs) of hypoxia-responsive genes, thus upregulating transcription of hypoxia-related genes (Hofer et al., 2001).

1.9.2 Role of hypoxia in maintaining joint tissue homeostasis

Hypoxia is a feature of a variety of normal physiological conditions including embryogenesis, high-altitude and exercise-induced stress, redox homeostasis and endothelial permeability. It regulates cell proliferation and differentiation, and also affects production of pro-inflammatory cytokines and growth factors (Etherington et al., 2002; Ten and Pinsky, 2002).

The growth plate is a constitutively avascular tissue and the HIF-1β complex is ubiquitously expressed (Rajpurohit et al., 1996; Wiener et al., 1996). Previously, *Schipani et al*. have generated cartilage specific HIF-1α knock out mice, hypoxic chondrocytes lacking HIF-1α undergo cell death, suggesting that HIF-1α is essential for hypoxic chondrocytes to survive and differentiate (Schipani et al., 2001). During endochondral ossification, chondrocytes undergo cellular proliferation and differentiation in a highly organized fashion. This process requires chondrocytes to generate enough energy through anaerobic glycolysis. The important role of HIF-1α was shown in regulating the expression of glycolytic enzymes coupled to angiogenic markers such as VEGF, and cell-cycle regulators in chondrocytes (Pfander et al., 2003). Moreover, under

hypoxia, the expression of matrix proteins such as aggrecan and type II collage is enhanced. Protein and mRNA expression levels of type II collagen and aggrecan are decreased in HIF-1α knockout chondrocytes exposed to hypoxia, underlining that hypoxia-induced matrix gene expression requires HIF-1α expression (Pfander et al., 2003). In addition, other *in vitro* studies also have shown that hypoxia is important in maintaining ECM synthesis in primary chondrocytes. For example, prolonged hypoxia treatment enhances protein and mRNA levels of proteoglycans and type II collagen in bovine primary chondrocytes (Coyle et al., 2009). Altogether, these studies indicate that hypoxia exerts profound effects on matrix deposition in human chondrocytes.

Both HIF-1α and HIF-2α are expressed in human normal and OA chondrocytes (Coimbra et al., 2004). It has been suggested that HIF-1α and HIF-2α display opposing roles in cartilage function, HIF-1α promoting cartilage homeostatic pathways while HIF-2α promoting degradative pathways that lead to OA (Husa et al., 2010; Zhang et al., 2015a). However, some studies have indicated that in healthy cartilage, HIF-2α mediates anabolic responses by directly binding to Sox-9 hence enhancing cartilage matrix genes (Lafont et al., 2007; Lafont et al., 2008; Thoms et al., 2013). Furthermore, HIF-2α has been identified as a key regulator of endochondral ossification (Saito et al., 2010). Moreover, monolayer-expanded healthy and OA chondrocytes that are redifferentiated for two weeks in pellet cultures under hypoxic (2% oxygen) conditions show increased levels of human *COL2A1* and *aggrecan* gene expression and sulfated glycosaminoglycan (sGAG) accumulation while showing lower human *COL10A1* and *COL1A1* gene expressions and less collagen I expression (Markway et al., 2013). The same study also showed lower

expression of MMPs including MMP1, MMP2, MMP3, MMP13, and MMP14, with less active MMP2 enzyme in both normal and OA human chondrocytes under hypoxia. However, the expression of aggrecanases (ADAMTS4 and ADAMTS5) is significantly decreased by hypoxia only in healthy cells. HIF-1α and HIF-2α show similar levels of increased expression in healthy and OA cells during early hypoxia but their levels decrease over time (Markway et al., 2013). The discrepancy among studies is perhaps due to different cell culture conditions and *in vitro* and *in vivo* experimental settings that may modify post-translational mechanisms of HIFs.

1.9.3 Change in oxygen tension in OA joint tissue

An increasing body of evidence suggests that low oxygen tension plays a critical role in normal fetal development but hypoxia is an important pathological stimulus which can be a feature of a variety of pathological conditions including ischemia, inflammation and carcinogenesis (Ten and Pinsky, 2002).

The mechanical loading and hypoxia are two permanent stresses that dramatically impact adult articular cartilage during progression of the OA disease. Several studies have indicated that both knee and hip OA exhibit dysfunction with vascular occlusion and reduced venous outflow that may contribute to local hypoxia making the tissue become 'hyper-hypoxic' (Chang et al., 2014; Pedersen et al., 1989). Bone ischemia is one of the features of OA joint tissue and compromised bone vasculature affects both bone and cartilage health. Hypoxia is known to have profound effects on the vasculature including alteration of vascular tone and coagulant function (Ten and Pinsky, 2002) implying that

alteration in oxygen levels during the progression of OA may impact microvasculature in subchondral bone (and/or in synovium).

Inflammatory mediators are also produced in response to inflammation or mechanical loading and they can lower the oxygen tension in the joint. In arthritic cartilage, oxygen delivery is compromised as a result of decreased capillary density indicating that pathological articular cartilage tissue (either rheumatoid or osteoarthritis) is more hypoxic than normal cartilage (James et al., 1990; Stevens et al., 1991).

In damaged cartilage, increased transcription of HIF-1α has been reported, particularly in the late-stage of the disease (Pfander et al., 2005). Also some other studies have supported the evidence by reporting a correlation between growing number of HIF-1α-positive chondrocytes during OA progression (Pfander et al., 2005) and a higher expression of HIF-1α mRNA in injured cartilage compared to uninjured cartilage (Yudoh et al., 2005). In addition to hypoxic conditions, HIF-1α expression is up-regulated by key players in cartilage damage, including IL-1β, TNF-α, reactive oxygen species and mechanical loading (Haddad and Land, 2001; Hellwig-Burgel et al., 1999; Pufe et al., 2004) highlighting that HIF-1α acts as a stress-inducible responder to catabolic modifications of OA cartilage, rather than merely as a hypoxia-inducible factor.

HIF-2α appears to be highly expressed in degenerated cartilage and strongly implicated in catabolic mechanisms leading to cartilage breakdown and endochondral bone formation, which lead to osteophyte formation, one of the typical OA outcomes

(Kawaguchi, 2008). Overexpression of HIF-2α through adenovirus system causes progressive cartilage damage directly by up-regulating the expression of various degradative enzymes, including MMP1, MMP3, MMP12, MMP13, ADAMTS4 proposing the role of HIF-2α as a catabolic inducer of OA cartilage destruction (Yang et al., 2010).

1.9.4 Hypoxia (HIF-1α) and TGF-β signaling

TGF-β and hypoxia, together and/or independently, regulate numerous cellular and biological processes involving cell-growth, differentiation, apoptosis, angiogenesis, ECM production, and embryonic development; therefore aberrant crosstalks between these two signaling pathways may lead to various pathological conditions.

Angiogenesis is one of the wound healing processes and low oxygen levels are observed in wounded or fibrotic skin in which TGF-β levels are differentially expressed (Penn et al., 2012). For instance, human dermal fibroblasts exposed to hypoxia show increased TGF-β1 mRNA levels and the condition with the increased hypoxia signaling and TGF-β levels is observed during tissue repair (Falanga et al., 1991). Also, another study has found that hypoxic conditions created in solid tumors (breast cancer) drive TGF-β production and secretion in mesenchymal stem cells (MSCs) by enhancing TGF-β promoter activity (Hung et al., 2013). The conditioned media from these MSCs significantly increased the migratory and invasive abilities of breast cancer cells (Hung et al., 2013).

In human kidney epithelial cells, TGF-β induces HIF-1α expression in a Smad3 dependent manner and Smad3 and HIF-1α are essential contributors to TGF-β1-induced collagen expression under normoxic conditions (Basu et al., 2011a). In mouse primary alveolar macrophages, TGF-β1 increases HIF-1α protein expression predominantly at the post-transcriptional level and hypoxia induced PAI-1 expression (Ueno et al., 2011). In the same study, TGF-β1 under hypoxia (<1% O_2) significantly increases PAI-1 protein levels, suggesting that the effects of TGF-β1 and hypoxia on PAI-1 expression are synergistic (Ueno et al., 2011). Moreover, hypoxia and TGF-β exert synergistic effects on expression of certain gene such as *erythropoietin* (EPO) (Sanchez-Elsner et al., 2004), *VEGF* (Sanchez-Elsner et al., 2001) *endoglin* (Sanchez-Elsner et al., 2002) and *CXC chemokine receptor 4* (CXCR4) (Dunn et al., 2009). Tumor microenvironments are known to have high levels of HIF-1α and TGF-β and hypoxia and TGF-β cooperate to modulate tumor progression (Hung et al., 2013). Another study has demonstrated that there is no synergistic effect, but hypoxia and TGF-β rather act in parallel to modulate tumor microenvironment. For example, although both TGF-β and hypoxia (HIF-1α) signaling pathways are important in bone metastases, there was no synergistic effect on promoting bone metastasis but they rather regulate a common set of tumor genes independently of each other (Dunn et al., 2009). The interaction between TGF-β and hypoxia may vary depending on severity and/or types of cancers.

It also has been shown that expression of endoglin is induced by hypoxia in endothelial cells and murine infarcted heart (Jonker and Arthur, 2002; van Laake et al., 2006). In addition, both *in vivo* and *in vitro* data have demonstrated that hypoxia increases ALK1 and Smad1/5 levels in mouse infracted ventricles and in human endothelial cells

suggesting that increased endoglin expression by hypoxia promotes ALK1/Smad1/5 signaling thereby promoting endothelial cell proliferation (Tian et al., 2010). This study supports the idea that the balance between TGF-β/ALK5/Smad2/3 and TGF-β/ALK1/Smad1/5 regulates cellular processes and a shift from TGF-β canonical signaling pathway towards the non-canonical pathway contributes to develop pathological conditions.

Based on these findings, it can be concluded that the complexity of crosstalks between TGF-β and hypoxia signaling pathways are intricate and highly context dependent.

1.10 Rational and objectives for the study

Articular chondrocyte is the only cell type responsible for maintenance of articular cartilage homeostasis. Ability of the cell to produce ECM proteins is tightly regulated by numerous growth factors and cytokines. TGF-β is one of the most powerful anabolic factors that promotes chondrocyte to produce matrix proteins and to inhibit proteases that cause ECM breakdown. Dysregulation of TGF-β signaling is linked to cartilage degeneration in OA.

In addition to the canonical type I receptor ALK5, TGF-β also transduces its signal through another type I receptor, ALK1, which phosphorylates Smad1/5. Our group has previously shown that TGF-β can activate both the ALK5/Smad2/3 and ALK1/Smad1/5 pathways in human chondrocytes and that these two signaling pathways elicit opposing effects on chondrocyte function. In addition, the ratio of ALK1 to ALK5 has been shown to be increased in OA human and mouse chondrocytes with increased expression of MMP13 (Blaney Davidson et al., 2009), suggesting that the ALK1/Smad1/5 signaling

pathway is associated with the progression of OA.

Previous studies from our group have suggested that TGF-β co-receptors endoglin and CD109 are involved in regulating between the balance of the TGF-β/ALK5/Smad2/3 and TGF-β/ALK1/Smad1/5 signaling pathways. Moreover, other studies have indicated that hypoxia, low oxygen tension, also regulates TGF-β/ALK5/Smad2/3 and TGF-β/ALK1/Smad1/5 pathways in a context dependent manner. Identification of factors that differentially regulate TGF-β signaling pathways in chondrocytes will provide insights into the mechanisms by which TGF-β controls chondrocyte homeostasis and disease conditions such as OA.

The major goal of the current thesis was to determine the factors that regulate the balance between TGF-β/ALK5/Smad2/3 and TGF-β/ALK1/Smad1/5 pathways in chondrocytes and thus regulate chondrocyte homeostasis.

The specific objectives of the thesis include; (1) To identify hypoxia, endoglin and CD109 as critical modulators of the balance between TGF-β/ALK5/Smad2/3 and TGF-β/ALK1/Smad1/5 pathways in chondrocytes; and (2) To determine functional significance of hypoxia, endoglin and CD109 in chondrocyte function.

2. Hypoxia differentially regulates TGF-β/ALK5/SMAD2/3 versus TGF-β/ALK1/SMAD1/5 signaling and extracellular matrix synthesis in human chondrocytes.

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2.1 Abstract

Objective: Transforming Growth Factor-Beta (TGF-β) plays a critical role in maintenance and repair of articular cartilage, and deregulation of its activity is implicated in osteoarthritis (OA). Articular cartilage is physiologically hypoxic and alterations in oxygen levels can regulate chondrocyte function. The purpose of the study was to determine the mechanisms by which hypoxia may modulate TGF-β signaling and action in human chondrocytes. **Methods**: The human chondrocyte cell line, C28/I2, was used as an *in vitro* model to analyze the mechanisms involved in the regulation of TGF-β signaling by hypoxia. We used a hypoxia GasPak chamber or $CoCl_2$ treatment to induce hypoxia in the cells. Cell lysates were analyzed for the expression and activation of TGF-β signaling components and production of ECM components. **Results**: Our results show that hypoxia increases the levels of HIF-1α, ALK5, type II collagen and plasminogen activator inhibitor-1 (PAI-1) while decreasing the levels of ALK1, Smad1, and endoglin (TGF-β co-receptor). Furthermore, hypoxia markedly decreases phospho-Smad1/5 levels while increasing phospho-Smad2/3 levels. In addition, hypoxia decreases total levels of TGF-β1 and addition of TGF-β1 decreases HIF-1α levels in chondrocytes. **Conclusions:** Collectively, our results have identified hypoxia as an important factor that regulates the

balance between ALK1/Smad1/5 and ALK5/Smad2/3 signaling pathways, and ECM protein production in chondrocytes.

2.2 Introduction

Adult cartilage tissue is avascular and chondrocytes are able to survive in an environment with relatively low oxygen content. Oxygen and other nutrients diffuse into cartilage from the neighboring tissues such as synovial fluid and subchondral bone, and this creates an oxygen gradient across cartilage tissue, from 7% O_2 (53 mm Hg) in the superficial zone to less than 2% O_2 (7.6 mm Hg) in the deep zone (Fermor et al., 2007; Silver, 1975). Other studies have demonstrated that varying oxygen tensions in the cartilage can significantly influence chondrocyte metabolism, proteoglycan synthesis (Grimshaw and Mason, 2000) and levels of growth factors released by the cells (Falanga et al., 1991). It has been suggested that the optimal physiological condition to maintain chondrocyte homeostasis *in vitro* is when the oxygen level is between 2 and 5% across the tissue (Coyle et al., 2009; Strobel et al., 2010). Under these conditions, hypoxia promotes chondrocytes to produce ECM proteins and helps chondrocytes to maintain their phenotype and metabolism (Shi et al., 2015). The transcription factor hypoxia-inducible factor-1α (HIF-1α) is a major mediator of the hypoxia signaling pathway.

Among the cytokines that have been studied in the context of cartilage homeostasis, TGF-β has a broad spectrum of effects with it playing a critical role in the development, growth, maintenance and repair of articular cartilage (Finnson et al., 2010a) and deregulation of its activity is implicated in OA (van der Kraan et al., 2009). In addition, it

has been reported that the crosstalks between TGF-β and hypoxia signaling pathways regulate many pathophysiological processes in various tissues (Hanna et al., 2013).

TGF-β signals through a pair of transmembrane serine/threonine kinases known as the TGF-β type II (TβRII) and type I (TβRI or activin receptor-like kinase, ALK) receptors. The ligand binds the constitutively active kinase TβRII which phosphorylates TβRI resulting in activation of TβRI kinase activity (Massague and Gomis, 2006; Shi and Massague, 2003). This in turn activates receptor-regulated Smad (R-Smad) proteins by phosphorylation, which subsequently bind to the common Smad4 (co-Smad4). The complex then translocates to the nucleus where they interact with transcription factors, various co-activators and co-repressors to regulate gene transcription and protein expression (Ross and Hill, 2008; Schmierer and Hill, 2007). Typically, TGF-β signals via the type I receptor, ALK5, to activate Smad2 and 3. However, in certain cell types, such as chondrocytes (Finnson et al., 2008) and endothelial cells (Goumans et al., 2002; Goumans et al., 2003), TGF-β also signals via ALK1 which phosphorylates Smad-1, -5 and -8 (Finnson et al., 2008; Goumans et al., 2002; Goumans et al., 2003). ALK1 activation has been shown to stimulate endothelial cell proliferation and migration during angiogenesis whereas ALK5 activation inhibits their proliferation indicating that these two pathways elicit opposing cellular responses in these cells (Goumans et al., 2003). Consistent with these results, our group has shown that these two ALK1 and ALK5 pathways are activated by TGF-β and display opposing effects in chondrocytes (Finnson et al., 2008). Others have reported that the increased ratio of ALK1 to ALK5 was correlated with increased matrix metalloproteinase-1 (MMP13) expression in human and mouse OA chondrocytes (Blaney Davidson et al., 2007a; Blaney Davidson et al., 2007b;

Blaney Davidson et al., 2009; van der Kraan et al., 2009). Thus it is possible that the activation of the ALK1 pathway promotes an OA-like phenotype in chondrocytes while the activation of the ALK5 pathway promotes the anabolic activity of TGF-β, increasing ECM protein production such as type II collagen. Determination of factors that differentially regulate the balance between ALK5/Smad2/3 and ALK1/Smad1/5 signaling pathways will provide insights into the cellular mechanisms by which TGF-β regulates chondrocyte function. In the current study, we identify hypoxia as a potential regulator in TGF-β signaling pathways by showing that hypoxia enhances ALK5/Smad2/3 pathway while diminishing ALK1/Smad1/5 signaling pathway and decreasing endoglin levels. Moreover, we demonstrate that hypoxia enhances the production of ECM proteins such as type II collagen and PAI-1 and exhibits a negative correlation with TGF-β1 levels.

2.3 Materials and Methods

Cell culture

The C28/I2 was developed from chondrocytes isolated from juvenile human costal cartilage and immortalized by retroviral infection of the SV40 large T antigen (Finger et al., 2003; Goldring et al., 1994). The cells were grown in monolayers in Dulbecco's modified Eagle's medium (DMEM)/F12 (Wisent, QC) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2mM L-glutamine and fungizone and maintained at 37°C in a humidified incubator under 5% CO_2. The medium was changed every 3 days. The cells were cultured in T75 flasks. Once grown to confluence, they were passaged at a ratio of 1:10.

Primary osteoarthritic (OA) articular chondrocytes

Articular cartilage samples were collected from medial and lateral femoral condyles obtained at hip joint surgery from adult patients diagnosed as having OA. All procedures were approved by the Research Ethics Board of the McGill University Health Center and all cartilage samples were obtained with informed consent. The cartilage was rinsed with cold phosphate buffered saline (PBS), minced and then incubated for 1 h at 37°C with 1 mg/ml pronase followed by overnight digestion at 37°C with 1 mg/ml collagenase (Sigma Aldrich, Oakville, ON). The suspension was passed through a 70 mm cell sieve and chondrocytes were collected by 10 min centrifugation (1,500 x g), washed with PBS and plated at a density of $2.5x10^5$ cells/cm^2. At passage 2, the cells were used for experiments.

Hypoxia experiment

Chondrocytes (C28/I2 and primary chondrocytes) were plated into 6-well plates at a density of $2.5x10^5$ cells/well with complete media. Next day, the cells were washed with PBS twice; serum-free media was added to the each well for overnight serum-starvation. The fresh serum-free media was added to each well of the plate and the plates were put into a GasPak chamber with Sachets (BD Bioscience, Canada) and the chamber was stored in the cell culture incubator. The GasPak chamber creates the hypoxic condition in which the chamber environment brings oxygen level down to less than 2% two hours after the sachets are added to the chamber. Cells were exposed to the hypoxic condition for 24 hours or 2 hours. Alternatively, we chose 1 hour of Cobalt Chloride treatment ($CoCl_2$, Sigma-Aldrich) for studying Smads phosphorylation levels since Smads

phosphorylation is a transient process (Feng and Derynck, 2005). The cells for the control group were cultured in the cell culture incubator under 20% O_2 (normoxia) or no $CoCl_2$ was added. The optimal $CoCl_2$ was selected according to the literature (200uM) (Duval et al., 2009).

TGF-β treatment

Chondrocytes were washed with PBS and serum-starved overnight. Next day, they were treated for 45 minutes with 0-15 pM TGF-β1 (Genzyme, Framingham, MA) under serum-free conditions.

Preparation of cell lysates

Cell lysates from the C28/I2 cells were prepared in Nonidet-P40 (NP40) buffer **[**150 mM sodium chloride**,** 1.0% NP-40**,** 50 mM Tris, pH 8.0] containing the protease and phosphotase inhibitor mixture (1mM sodium fluoride, 1mM PMSF, 1mM Orthovanadate and Phosphatase Inhibitor Cocktail Tablet; Roche). Cell lysates from human OA primary articular chondrocytes were prepared in RIPA buffer (radioimmunoprecipitation assay buffer) **[**25 mM Tris-HCl pH 7.6, 10% glycerol, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS] containing the inhibitor mixture as above. Cell debris were removed by centrifugation. Protein concentration was determined by Lowry protein assay (Biorad) and Prism software, and the samples were prepared in 5X sample buffer.

Western blot analysis

Cell lysates were separated by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Fisher Scientific). The membranes were blocked with Tris-buffered saline-Tween20 (TBST) containing 5% milk for 1 hour at room temperature and incubated with primary antibodies at 4°C overnight. The membranes were washed 3 times for 10 minutes each and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Cell Signaling) for 1 hour at room temperature (RT). The signals on the membranes were detected using enhanced chemiluminescence (ECL) system (GE Healthcare, Canada). The primary antibodies that were used for this study include pSmad1 (9511), pSmad2 (3101), pSmad3 (9520), Smad2 (3103, Cell Signaling, 1:1000); ALK5 (Santa Cruz, 1:1000); ALK1 (R&D system; 1:500); HIF1-α (BD Biosciences, 1:1000); Smad1 (Zymed 1:1000); Smad 3 (Abnova; 1:1000); PAI-1 (BD Biosciences, 1:1000); type II collagen (Chemicon, 1:1000); and endoglin (Santa Cruz P4A4, 1:1000). The membranes were stripped and then reprobed with β-actin (Santa Cruz, TX 1:1000) or GAPDH (Cell Signaling, 1:1000) antibodies as a loading control.

Enzyme-linked immunosorbent assay (ELISA)

The total level of TGF-β1 was measured using ELISA (eBioscience). Briefly, conditioned media was collected from the C28 cells that were incubated in the hypoxic conditions for 24 hours (GasPak chamber) or normoxia (as a control group). The 96-well plate (provided by the manufacturer) was coated with the coating buffer followed by blocking for one hour at the RT. To activate latent TGF-β1 to the biologically active form, the conditioned media was heated at 70°C for 10 minutes. Then the samples were

added to the wells and the plate was incubated overnight at 4°C. The remaining steps were performed according to the manufacturer's protocol.

TGF-β specific antibody array

Cell lysates were prepared after 24 hours of hypoxia treatment using the GasPak chamber and analyzed for TGF-β specific antibody array (Full Moon Biosystems, CA). All the steps were preceded as indicated in the manufacturer's protocol. Protein samples were biotinylated and then coupled with the provided slide. Each slide had 176 TGF-β signaling related protein antibodies embedded with 6 replicates for each protein. The prepared slides were sent to the company for imaging scan and further data analysis.

Statistical analysis

Means calculated from replicates of three or more independent experiments were used for statistical analysis. A two-tailed Student t-test was used to determine statistical significance between two groups. A value of $p<0.05$ was considered significant.

2.4 Results

Hypoxia differentially regulates TGF-β type I receptors, ALK5 and ALK1.

To determine the effect of low oxygen tension on the expression of TGF-β type I receptors, ALK5 and ALK1, chondrocytes were incubated in the hypoxic GasPak chamber (O_2 content less than 2%) for 24 hours under serum-free conditions followed by western blot analysis. The Figure 2.1 (A,B) shows that hypoxia increases ALK5 levels while decreasing ALK1 levels when compared to normoxic conditions ($p <0.05$). The

increased levels of HIF-1α in cells incubated in the GasPak chamber system confirm that hypoxia signaling pathway is induced using this method. Next, we studied the effect of hypoxia on the expression of the TGF-β downstream components. As shown in the Figure 2.1 (C and D), hypoxia decreases Smad1 levels ($P<0.05$) when compared to normoxic conditions. However, Smad2 levels do not show any significant differences between hypoxic and normoxic conditions.

Hypoxia differentially regulates TGF-β/Smad1/5 and TGF-β/Smad2/3 signaling pathways and decreases endoglin levels.

To determine whether low oxygen tension differentially regulates TGF-β signaling pathways, the cells were incubated in the GasPak chamber for two hours or treated with $CoCl_2$ (0-200uM) for one hour. Cobalt is a transition metal which induces hypoxia by stabilizing HIF-α, more specifically by inhibiting the interaction between HIF-α and von Hippel-Lindau protein (Yuan et al., 2003). The shorter duration of hypoxia treatment was used to enable the detection of transient activation of Smad proteins. Figure 2.2 A demonstrates that hypoxic conditions induced by $CoCl_2$ treatment decreases the phosphorylation of Smad1 while increasing the phosphorylation of Smad3 with the increased levels of HIF-1α in a dose-dependent manner. The Smad2 phosphorylation levels are moderately increased but did not reach statistical significance. Hypoxia induced using the GasPak chamber for two-hours also resulted in a significant decrease in Smad1 phosphorylation and a moderate but not statistically significant increase in Smad2 and Smad3 phosphorylation levels. The levels of total Smad1, Smad2, and Smad3 remain unchanged under these conditions.

Endoglin is a TGF-β co-receptor that has been shown to promote TGF-β/ALK1/Smad1/5 signaling pathway during endothelial cell proliferation (Lebrin et al., 2004). We examined whether hypoxia modulates endoglin expression. In contrast to the effect of hypoxia in endothelial cells, we observed that hypoxia decreases the levels of ALK1, Smad1 (Fig. 2.1) and Smad1 phosphorylation (Fig. 2.2 B-E). We observed a significant decrease in endoglin levels under hypoxia in human chondrocytes (Fig. 2.2 F and G, $p<0.05$). This is in line with the decreased ALK1/Smad1 pathway activation that we observed in Fig. 2.1 and 2.2.

In order to confirm our results on the effect of hypoxia on TGF-β Smad signaling pathways shown in Fig 2.2, we performed a TGF-β–specific phospho-antibody array in chondrocytes. Six phospho-proteins showing the highest fold changes under hypoxia when compared to normoxic conditions as detected using the phospho-antibody array is shown in Fig. 2.3 A. Analysis of the data as a ratio of the phospho-protein/non-phospho-protein is shown in Fig. 2.3 B. Together these data confirm that hypoxia decreases phospho-Smad1 levels and the ratio of phospho Smad1/total Smad1 while increasing phospho-Smad 2 levels and the ratio of phospho-Smad2/total Smad2. Other proteins showing marked alterations under hypoxic conditions include PAK3 (p21 protein (Cdc42/Rac)-activated kinase 3), Myc, AKT and LIMK1 (LIM domain kinase 1) (Fig. 2. 3).

Hypoxia differentially regulates ECM proteins.

Next, we investigated whether hypoxia differentially regulates expression of the ECM proteins such as type II collagen and PAI-1. The C28/I2 human chondrocytes were incubated in the GasPak chamber for 24 hours under serum-free conditions followed by determination of type II collagen and PAI-1 using anti-type II collagen and anti-PAI-1 antibodies. Result shown in Figure 2.4 demonstrates that hypoxia significantly increases type II collagen and PAI-1 levels ($p<0.05$).

Hypoxia differentially regulates TGF-β signaling components in human primary articular OA chondrocytes.

So far, we have used C28 cell line to study the effect of hypoxia on TGF-β signaling components. We next used human primary articular chondrocytes to confirm the physiological relevance of our results obtained with the cell line. Results shown in Fig. 2.5 A demonstrate that primary articular OA chondrocytes exposed to the hypoxia for 24 hours under serum-free conditions show decreased ALK1 and Smad1 levels and increased ALK5 levels when compared to normoxic conditions (Fig. 2.5 A). There was no significant difference in the level of total Smad2 between hypoxia and normoxia. As expected, hypoxia increased HIF-1α levels (Fig. 2.5 A). Importantly, hypoxia enhances the expression of type II collagen and PAI-1 (Fig. 2.5 B) while decreasing endoglin levels (Fig. 2.5 C) in human primary OA chondrocytes.

Hypoxia decreases total TGF-β1 levels and TGF-β1-induced Smad1/5 phosphorylation in human chondrocytes.

Our results so far showed that hypoxia differentially regulates TGF-β signaling pathways and ECM protein expressions in human chondrocytes. The effect of hypoxia on TGF-β1 levels in human chondrocytes has not been reported, and we therefore examined whether hypoxia modulates TGF-β1 levels in human chondrocytes. We collected the conditioned media from the C28/I2 cells grown under hypoxic or normoxic conditions and analyzed them using ELISA. Our results demonstrate that hypoxia significantly decreases the total levels of TGF-β1 compared to the normoxic conditions in human chondrocytes (Fig. 2.6 A). Importantly, hypoxia induced by $CoCl_2$ also attenuates the dose-dependent TGF-β1-induced Smad1 phosphorylation levels in human chondrocytes (Fig. 2.6 B)

Ectopic addition of TGF-β1 decreases HIF-1a levels in human chondrocytes.

We also examined whether TGF-β1 reciprocally modulates HIF-1α levels in human chondrocytes. We pre-treated the C28 human chondrocytes with $CoCl_2$ for one hour under serum-free conditions in the absence of and TGF-β1 treatment for 45 minutes. Our data indicate that TGF-β1 inhibits HIF-1α expression in a dose-dependent manner in human chondrocytes (Fig. 2.6 C and D).

2.5 Discussion

TGF-β is a multifunctional growth factor that plays a critical role in maintaining cartilage homeostasis. Aberrant TGF-β signaling has been implicated in diseases associated with cartilage, such as OA (Shen et al., 2014c). Our group has previously shown that in addition to the TGF-β canonical ALK5/Smad2/3 pathway, chondrocytes express and

activate another TGF-β type I receptor, ALK1 which activates Smad1/5 pathway, and that the TGF-β/ALK5 and TGF-β/ALK1 pathways exert opposing effects in chondrocytes (Finnson et al., 2008). It has been suggested that there is a shift from the canonical TGF-β signaling pathway to TGF-β/Smad1/5 signaling pathway during cartilage degeneration (Bush and Beier, 2013). Factors that may regulate this shift in the balance between ALK5/Smad2/3 and ALK1/Smad1/5 signaling pathways in human chondrocytes remain to be determined. In the current study, we show that hypoxia increases ALK5 and Smad2/3 phosphorylation levels while decreasing the levels of ALK1, Smad1, phospho-Smad1/5 phosphorylation and endoglin in human chondrocytes. Moreover, we also show that hypoxia enhances ECM protein production and decreases total levels of TGF-β1 in these cells. Furthermore, ectopic addition of TGF-β1 reciprocally decreases HIF-1α levels in human chondrocytes. Taken together, our results show that hypoxia is a critical regulator of TGF-β signaling pathways and ECM protein production in human chondrocytes.

Our results showing that hypoxia increases ALK5 protein levels and Smad2 and 3 phosphorylation levels while significantly decreasing the levels of ALK1, Smad1 and phospho-Smad1/5 indicate that hypoxia promotes ALK5/Smad2/3 pathway while inhibiting ALK1/Smad1/5 pathway in human chondrocytes. The opposing effects of hypoxia on the ALK5 versus the ALK1 signaling pathways emphasizes the notion that these two signaling pathways are differentially regulated in human chondrocytes under hypoxic condition (Finnson et al., 2008).

Our previous work has demonstrated that endoglin, a TGF-β co-receptor, promotes TGF-β1-mediated ALK1/Smad1/5 signaling pathway while inhibiting TGF-β1-induced

ALK5/Smad2/3 signaling pathway in human chondrocytes (Finnson et al., 2010a) and establishing that endoglin is a regulator of TGF-β signaling pathways in human chondrocytes. In the current study, our finding that hypoxia decreases endoglin levels while also decreasing the levels of ALK1, Smad1 and phospho-Smad1/5 corroborates our previous findings that endoglin and ALK1 act in the same pathway in human chondrocytes (Finnson et al., 2010a). This is contrary to the results obtained in endothelial cells. *Tian et al*. have demonstrated that hypoxia promotes ALK1/Smad1/5 pathway and increases endoglin expression in mouse and human endothelial cells (Tian et al., 2010). Interestingly, *Sanchez-Elsner et al.* have reported that TGF-β and hypoxia together enhance endoglin expression at the transcriptional level (Sanchez-Elsner et al., 2002). In contrast to these results, our data indicating a negative effect of hypoxia on ALK1/Smad1/5 signaling pathway in human chondrocytes may be attributable to cell type specific differences and cellular context.

Our data obtained using phospho-antibody array support the notion that hypoxia promotes TGF-β/ALK5/Smad2/3 pathway and inhibits TGF-β/ALK1/Smad1/5 pathway in human chondrocytes. Moreover, the array data show that hypoxia modulates the levels of other non-Smads TGF-β related signaling proteins in human chondrocytes. PAK3 shows significantly increased levels and LIMK1 shows significantly decreased levels under hypoxic conditions. PAK3 and LIMK1 are both known to be involved in cytoskeletal reorganization in which hypoxia signaling pathway is also involved (Liang et al., 2011; Ning et al., 2004; Rey and Semenza, 2010; Strobel et al., 2010). Their function or roles in modulating TGF-β pathways in human chondrocytes is poorly understood.

Our finding that hypoxia significantly increases the levels of ECM proteins such as type

II collagen and PAI-1 suggests that low oxygen tension provides more favorable condition for chondrocytes to maintain function *in vitro*. This is consistent with previous reports by *Strobel et al.* and *Coyle et al.* which show that culturing human chondrocytes in 2-5% O_2 enhances their anabolic activities while reducing catabolic activities in the cells (Coyle et al., 2009; Strobel et al., 2010) resulting in an increase in ECM proteins. Our previous work has shown that TGF-β canonical pathway through ALK5/Smad2/3 promotes chondrocytes to enhance ECM synthesis and that ALK1/Smad1/5 pathway inhibits ECM protein production (Finnson et al., 2008). This is consistent with the report that activation of the ALK5/Smad2/3 pathway suppresses chondrocyte differentiation (Yang et al., 2001b). Conversely, other studies have suggested that the activation of ALK1 signaling pathway promotes chondrocytes to exhibit OA-like features with the activation of ALK1/Smad1/5 pathway promoting chondrocyte hypertrophy and increasing expressions of MMP13, type X collagen, and alkaline phosphatases (Hellingman et al., 2011a). Moreover, an increase in the ALK1/ALK5 ratio has been suggested to be associated with the elevated MMP-13 expression in OA in humans and mice (Blaney Davidson et al., 2009). Our present data showing that hypoxia decreases ALK1/Smad1/5 pathway while promoting ALK5/Smad2/3 pathway and increasing ECM protein levels emphasize that lower oxygen tension promotes chondrocytes to maintain homeostasis by favoring TGF-β canonical pathway.

Validation of our findings using the human chondrocyte cell line, C28, was obtained with human primary articular OA chondrocyte. Our data using the primary cells show that hypoxia increases the levels of ECM proteins and ALK5 while decreasing the levels of ALK1 and endoglin in human primary chondrocytes are in congruence with our findings

in C28 cells and unequivocally establish that hypoxia provides more optimal conditions for chondrocytes to function as seen by an increased production of ECM proteins.

Our results demonstrating that hypoxia decreases total levels of TGF-β1 in human chondrocytes is in stark contrast to the previous studies which demonstrate a positive effect of hypoxia on TGF-β levels. Furthermore, we observe a decrease in TGF-β1-induced Smad1/5 phosphorylation in human chondrocytes. *Falanga et al.* have reported that human dermal fibroblasts exposed to hypoxia shows increased mRNA levels of TGF-β1 (Falanga et al., 1991) and *Hung et al.* have shown that hypoxia enhances the secretion of TGF-β1 in mesenchymal stem cells during breast cancer progression (Hung et al., 2013). However, our results in chondrocytes are consistent with the only other study showing that hypoxia decreases TGF-β1 levels in chondrocytes (Edip, 2013). The conflicting reports in the effect of hypoxia on TGF-β1 levels is likely due to cell-type specific differences, although experimental conditions cannot be excluded at the times.

We show, for the first time, that addition of TGF-β1 decreases HIF-1α levels in human chondrocytes under hypoxia. This is in contrast to other studies where it has been shown that TGF-β1 increases HIF-1α protein levels and the synergistic effect of HIF-1α on TGF-β signaling in other cell types such as cancer cells and epithelial cells (Basu et al., 2011b; McMahon et al., 2006; Ueno et al., 2011). The discrepancy may be due to the different cell types and/or experimental conditions such as duration of hypoxia. Further studies are needed to provide insights into the crosstalk between TGF-β and hypoxia signaling pathways in human chondrocytes under healthy and pathological conditions.

In summary, we identify hypoxia as an important factor in the differential regulation of ALK5/Smad2/3 versus ALK1/Smad1/5 signaling pathways in human chondrocytes.

Additionally, our data showing hypoxia increasing ALK5/Smad2/3 pathway and enhancing ECM proteins support the notion that maintaining human chondrocytes in low oxygen tension provides more optimal conditions than normoxic conditions for chondrocyte function. Identification of factors such as hypoxia as regulator of distinct TGF-β signaling pathways in chondrocytes will provide more insights into the mechanisms by which TGF-β regulates chondrocyte function and may lead to strategies for therapeutic interventions to enhance cartilage repair.

2.6 Role of the funding source

This study was supported by CIHR Grants MOP13732 and MOP 69060 (to AP) and the Fonds de recherche du Québec en Sante and the Arthritis Society scholarships (to YC).

2.7 Contribution

Yoon Chi contributed to the conception and design, collection and assembly of the data, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content and final approval of the article.

John Antoniou contributed to the provision of study materials, collection of the data, critical revision of the article for important intellectual content and final approval of the article.

Anie Philip contributed to the conception and design of the study, obtaining funding, the assembly of the data, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content and final approval of the article.

2.8 Conflict of interest

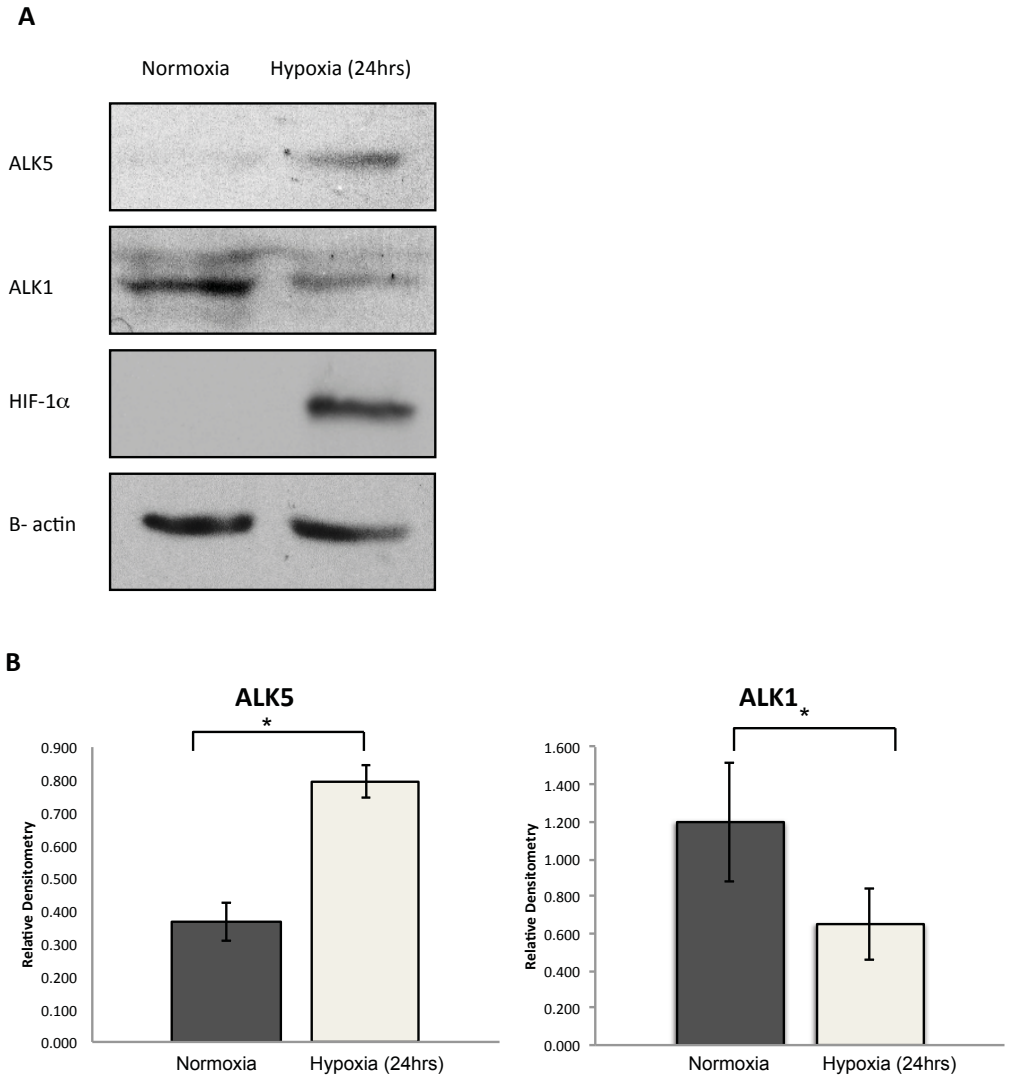
The authors state that they have no conflicts of interest.

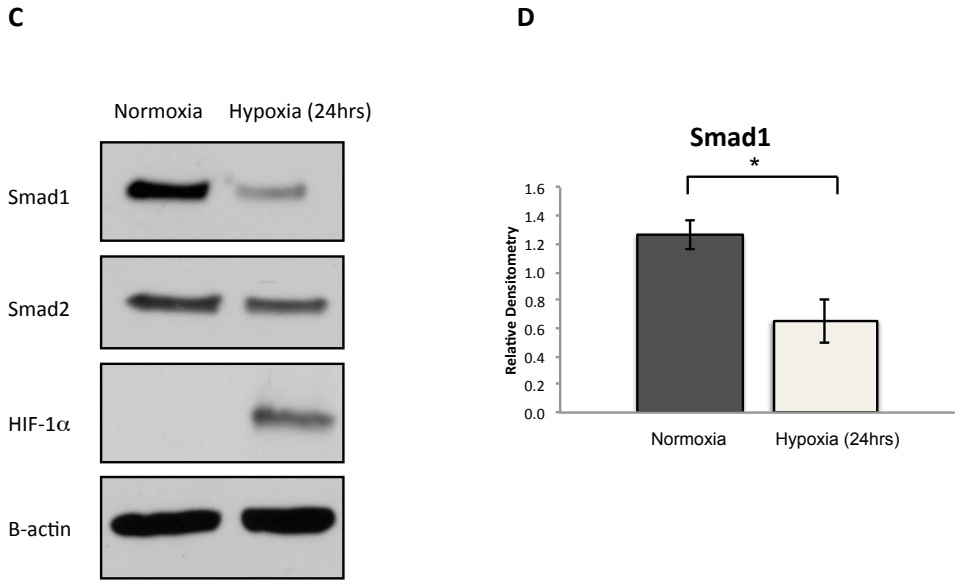
2.9 Acknowledgment

We thank our post-doctoral fellow, Dr. Priyanka Sehgal for her constructive feedback and in-depth comments on an earlier version of the manuscript that greatly improved the manuscript.

2.10 Figures

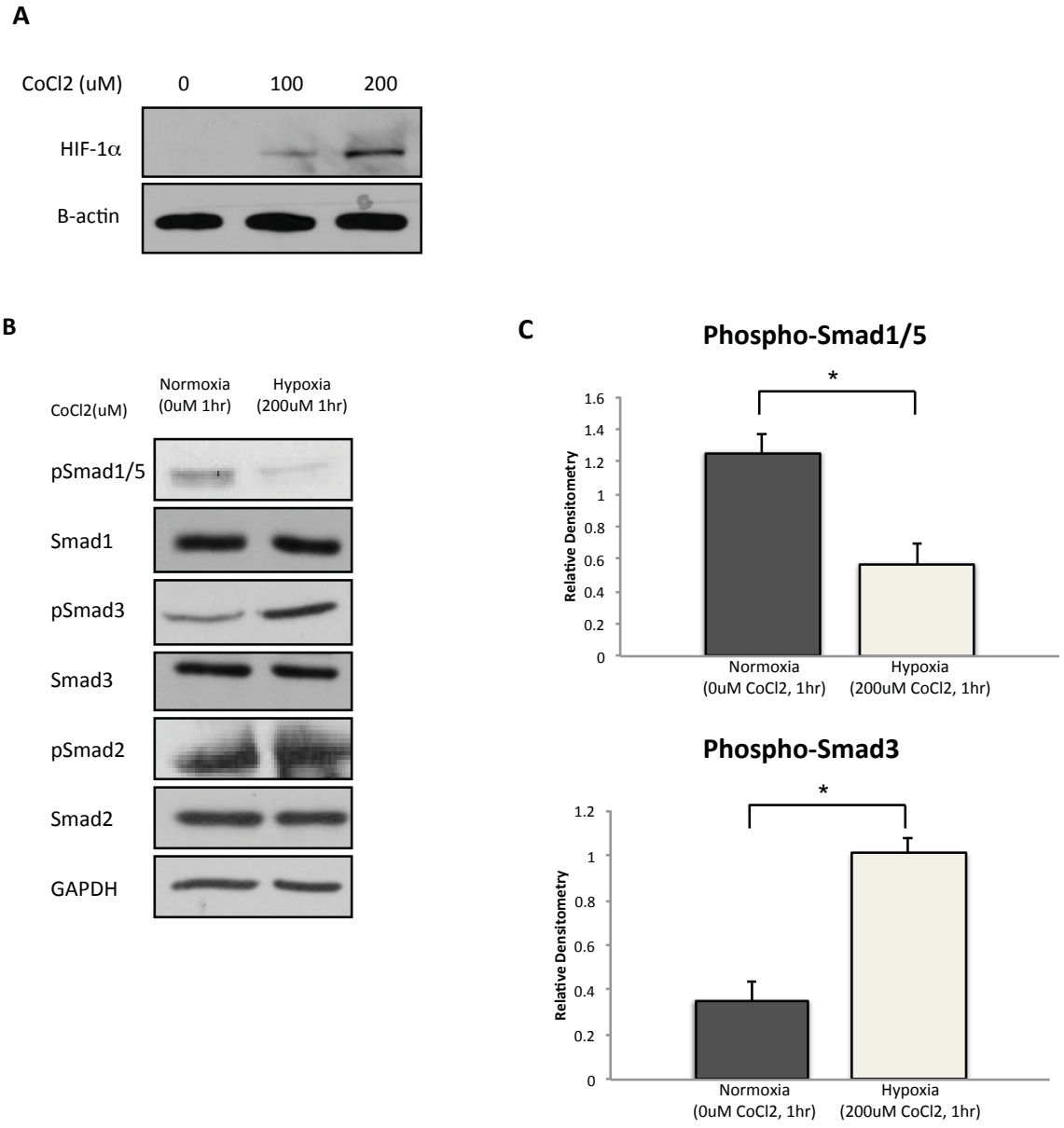
Fig. 2.1: **Low oxygen tension differentially regulates TGF-β type I receptors and TGF-β downstream components**.

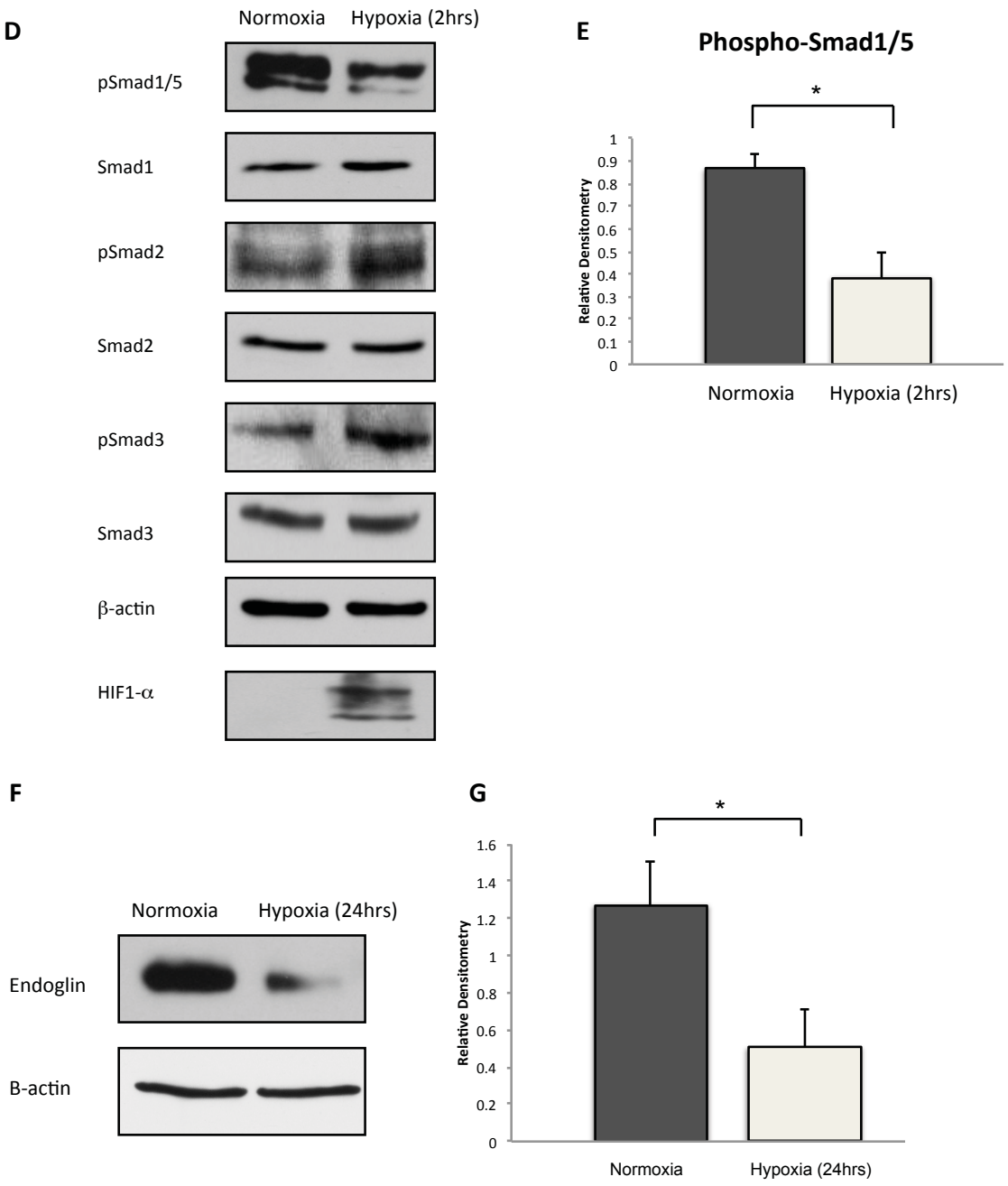




The C28/I2 cells were serum-starved overnight and then put in the GasPak chamber for 24 hours (hypoxia; O_2 less than 2%) or they were grown in serum-free media in the 37°C humidified incubator under 5% CO_2 as a control group (normoxia). Cell lysates were prepared and analyzed by western blot using **(A)** anti-ALK5, anti-ALK1 and anti-HIF1α; **(B)** Densitometry of ALK5 and ALK1 protein levels; **(C)** anti-Smad1, anti-Smad2 and anti-HIF1α; **(D)** Densitometry of Smad1 protein levels. Beta-actin was used as a loading control to confirm the equal amount of the protein in each lane. Results shown are representative of four independent experiments (* $p < 0.05$).

Fig. 2.2: **Low oxygen tension differentially regulates SMAD3 and SMAD1/5 activity.**

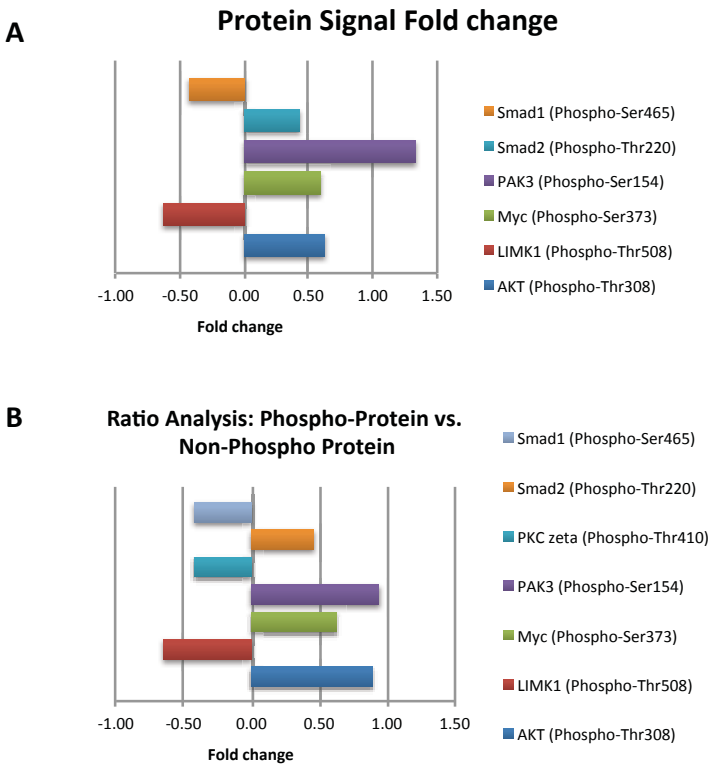




In order to study transient activity of Smads phosphorylation, we used one-hour cobalt chloride ($CoCl_2$) treatment and two-hour GasPak chamber treatment to induce hypoxia. **(A)** The C28/I2 cells were treated with $CoCl_2$ at 100uM or 200uM under serum-free conditions for one hour. The cells that were treated with 0uM were considered as a control group (normoxia). Cell lysates were prepared and analyzed by western blot with

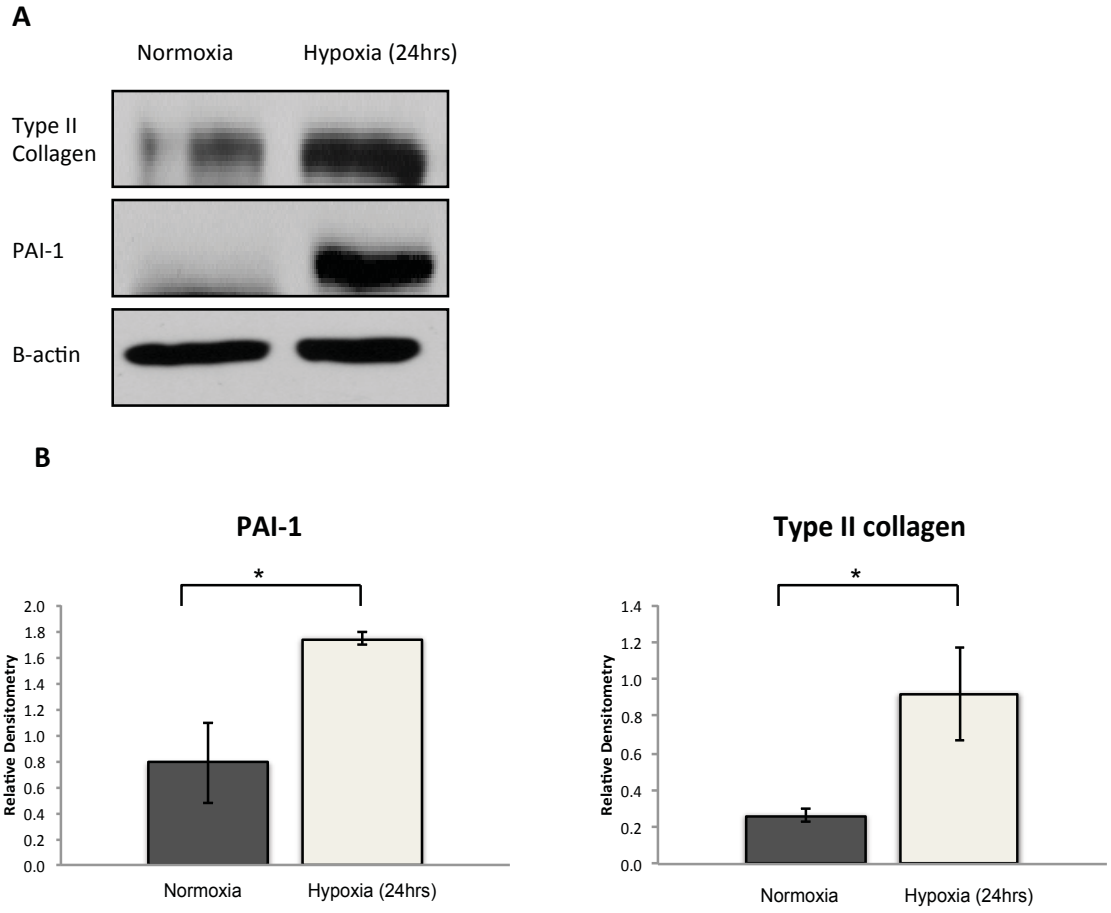
anti-HIF-1α. **(B)** The C28/I2 cells were pre-treated with $CoCl_2$ at 200uM (hypoxia) or 0uM (normoxia, control group) for one hour under serum-free conditions. **(C)** Densitometry of pSmad1/5 and pSmad3 from (B). **(D)** The cells were put in the GasPak chamber for 2 hours (hypoxia; O_2 less than 2%) under serum-free conditions or grown in the 37°C humidified incubator under 5% CO_2 as a control group (normoxia). Cell lysates were analyzed by western blot with anti-phosphoSmad1/5, anti-phosphoSmad3 and anti-phosphoSmad2 as well as their respective total Smads antibodies. **(E)** Densitometry of pSmad1/5 from (D). **(F)** The C28/I2 cells were grown in the GasPak chamber for 24 hours (hypoxia; O_2 less than 2%) under serum-free conditions. Cell lysates were prepared and analyzed by western blot for endoglin. **(G)** Densitometry of endoglin levels. Beta-actin or GAPDH was used as a loading control to confirm equal amount of protein in each lane. Results shown are representative of minimum three independent experiments (* $p < 0.05$).

Fig. 2.3: Hypoxia differentially regulates TGF-β related signaling proteins.



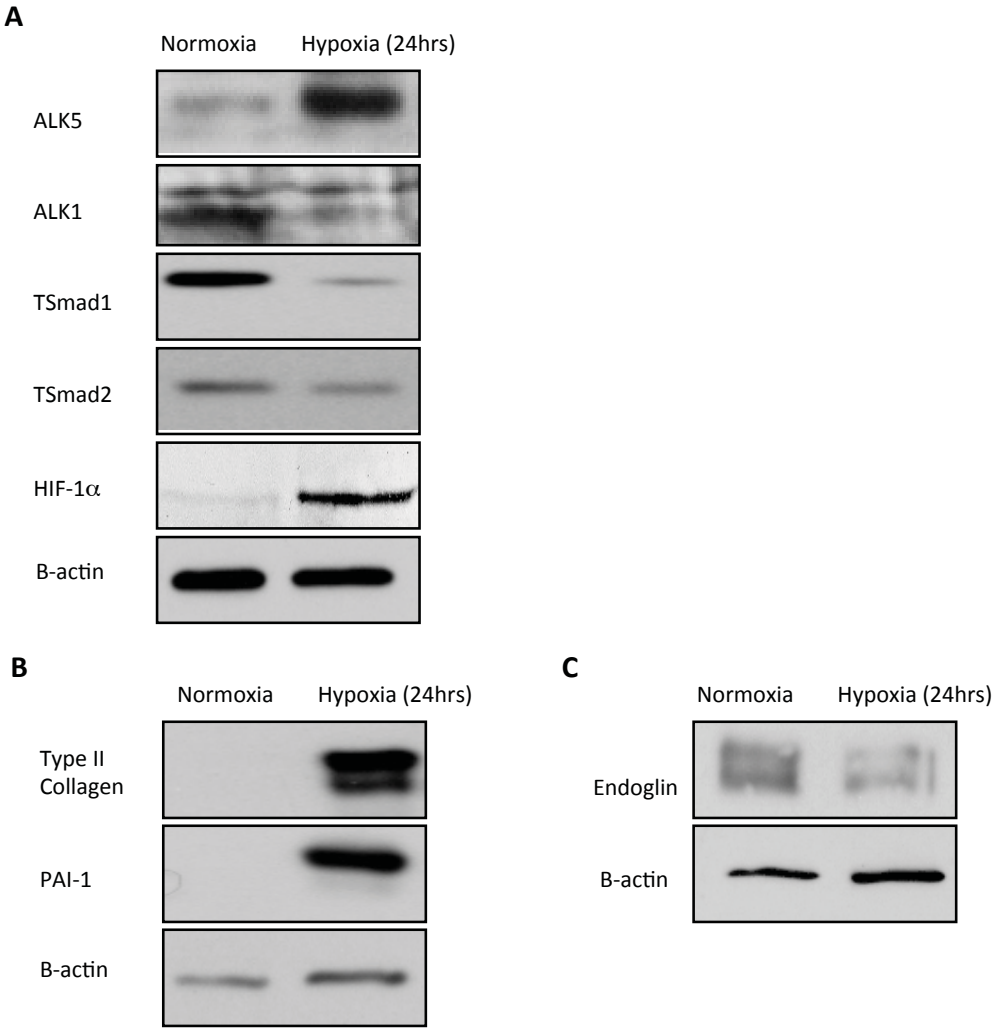
The C28/I2 cells were serum-starved overnight followed by hypoxia treatment for 24 hours using the GasPak chamber. Cell lysates were prepared and added to the slides that contain 174 TGF-β related phospho-specific antibodies. The TGF-β signaling phospho-specific antibody array was performed as described in the manufacturer's protocol (Full-moon biosystems, Inc). **(A)** The phosphorylated proteins with highest fold changes are selected. **(B)** The ratio of each phosphorylated to non-phosphorylated protein was analyzed for all proteins and the ones showing the highest fold changes are shown.

Fig. 2.4: **Hypoxia increases levels of TGF-β induced ECM proteins in human chondrocytes.**



The C28/I2 cells were serum-starved overnight and were put in the GasPak chamber for 24 hours (hypoxia; O_2 less than 2%) or grown in serum-free media in the 37°C humidified incubator under 5% CO_2 as a control group (normoxia). Cell lysates were prepared and analyzed by western blot using **(A)** anti-type II collagen and anti-PAI-1. (**B)** Densitometry of PAI-1 and type II collagen levels. Beta-actin was used as a loading control to confirm the equal amount of the protein in each lane. Results shown are representative of four independent experiments (* $p < 0.05$)

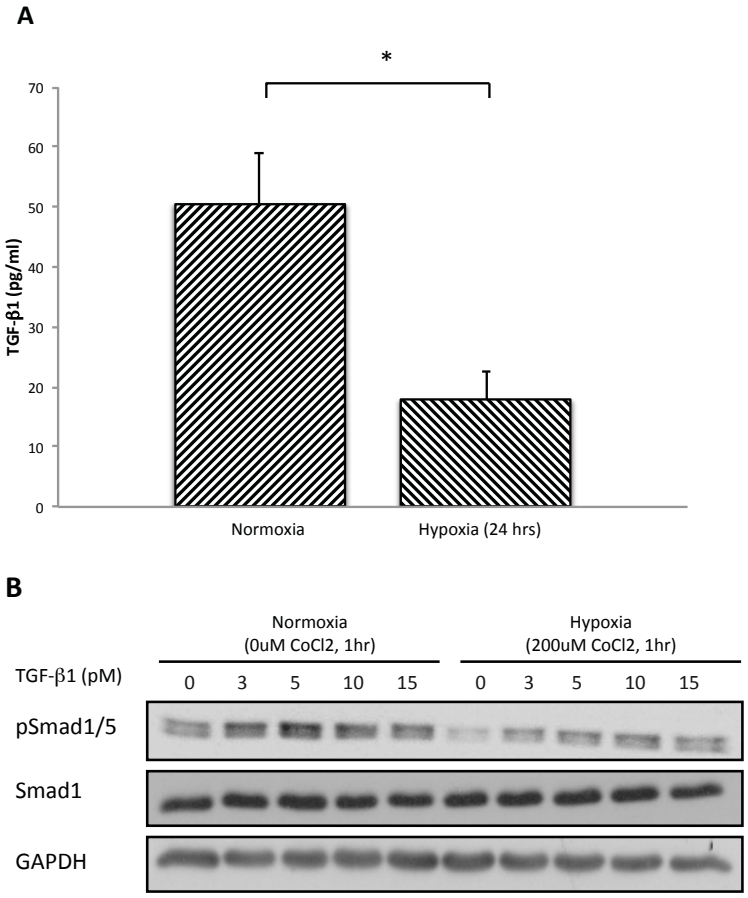
Fig. 2.5: Hypoxia differentially regulates the TGF-β signaling components and ECM proteins in human primary articular chondrocytes.

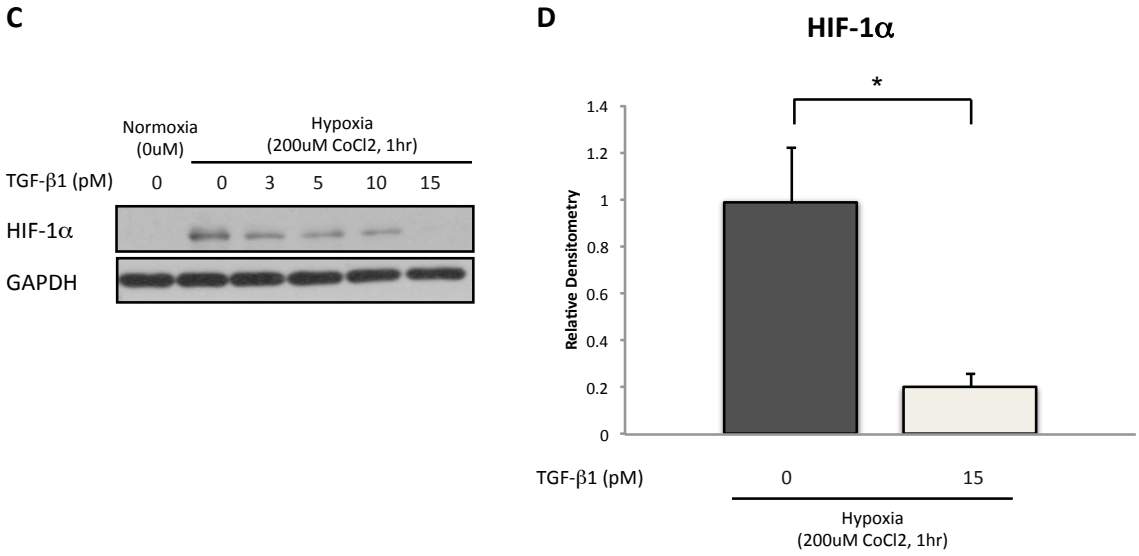


Human primary OA chondrocytes were serum-starved overnight and incubated in the GasPak chamber for 24 hours (hypoxia; O_2 less than 2%) or grown in serum-free media in the 37°C humidified incubator under 5% CO_2 as a control group (normoxia). Cell lysates were prepared and analyzed by western blot using **(A)** anti-ALK5, anti-ALK1, anti-HIF1α, anti-Smad1, anti-Smad2 antibodies; **(B)** anti-type II collagen, anti-PAI-1 antibodies and **(C)** anti-endoglin antibody. Beta-actin was used as a loading control to

confirm the equal amount of protein in each lane. Results shown are representative of three independent experiments (three different patients/biological samples).

Fig. 2.6: Hypoxia decreases TGF-β1 levels, TGF-β1-idncued Smad1/5 phosphorylation and ectopic addition of TGF-β1 decreases HIF-1α levels in human chondrocytes.





(A) The C28/I2 cells were serum-starved overnight and were grown in the GasPak chamber for 24 hours (hypoxia) or they were grown in serum-free media in the 37°C humidified incubator under 5% CO_2 as a control group (normoxia). The conditioned media was collected and analyzed for ELISA. Results shown are representative of four independent experiments. **(B)** The C28 cells were pre-treated with $CoCl_2$ at 200uM (hypoxia) for one hour under serum-free conditions followed by TGF-β1 treatment (0-15pM) for 45 minutes. The levels of phosphorylated Smad1 and total Smad1 were analyzed by western blot with anti-phospho-Smad1 and anti-Smad1 antibodies. **(C)** The levels of HIF-1α were analyzed by western blot with anti-HIF1α antibody and **(D)** densitometry of HIF-1α levels from (C). GAPDH was used as a loading control to confirm the equal amount of protein in each lane. Results shown are representative of three independent experiments (* $p < 0.05$).

Bridging statement for the next chapter

In the previous manuscript, we have demonstrated that low oxygen tension (hypoxia) differentially regulates the balance between TGF-β/ALK5/Smad2/3 canonical pathway and ALK1/Smad1/5 signaling pathways. Our data showing that hypoxia enhances ECM protein production indicate that hypoxia provides more favorable condition for chondrocytes to maintain homeostasis. Furthermore, we have demonstrated that hypoxia decreases endoglin levels and TGF-β1 levels in human chondrocytes establishing hypoxia as a critical modulator of TGF-β signaling pathways in human chondrocytes.

Endoglin is a TGF-β co-receptor that is predominately expressed in dividing endothelial cells. Our group has previously shown that endoglin is expressed in human chondrocytes and that it binds to both ALK5 and ALK1 receptors in chondrocytes. The second part of the thesis was aimed at determining a functional role of endoglin in human chondrocytes and its modulation of TGF-β signaling pathway in chondrocytes. We further compared the levels of endoglin between human OA and normal cartilage. Moreover, we examined role of endoglin during cartilage degeneration using an animal model. The data using human chondrocytes presented in the following chapter were published under the title of *"Endoglin differentially regulates TGF-β-induced Smad2/3 and Smad1/5 signaling and its expression correlates with extracellular matrix production and cellular differentiation state in human chondrocytes"* in the journal of *Osteoarthritis and Cartilage* in 2010 where I am one of the co-authors.

3. Endoglin regulates Transforming Growth Factor-Beta signaling and ECM production in chondrocytes

3.1 Introduction

Osteoarthritis (OA) is a chronic degenerative joint disorder which is characterized by destruction of articular cartilage, alterations of subchondral bone and synovial fibrosis (Goldring and Goldring, 2010). The appearance of fibrillations, matrix depletion, cell clusters, and changes in matrix composition in OA reflect the aberrant behavior of chondrocytes (Tetlow et al., 2001). There are multiple risk factors for OA including joint instability, obesity, hereditary contributions, mechanical factors and aging (Shane Anderson and Loeser, 2010). Transforming Growth Factor-Beta (TGF-β) is a pleiotropic cytokine that plays a critical role in the development, growth, maintenance and repair of articular cartilage (Finnson et al., 2010b) and deregulation of TGF-β activity is implicated in OA (van der Kraan et al., 2009).

TGF-β signals through a pair of transmembrane serine/threonine kinases known as the type II (TβRII) and type I (TβRI or activin receptor-like kinase, ALK5) TGF-β receptors. Upon ligand binding to TβRII, a constitutively active kinase, it phosphorylates TβRI resulting in activation of TβRI kinase activity (Massague and Gomis, 2006; Shi and Massague, 2003). This activates receptor-regulated intracellular Smad proteins (R-Smad2 and 3) by phosphorylation, which subsequently binds to the common Smad4 (co-SMAD4). The complex then accumulates in the nucleus where they interact with transcription factors, various co-activators and co-repressors to regulate gene expression (Ross and Hill, 2008; Schmierer and Hill, 2007). Typically, TGF-β signals through the

type I receptor, ALK5, to activate the R-Smads Smad2/3. However, in certain cell types, such as chondrocytes and endothelial cells, TGF-β signals not only through ALK5, but also through ALK1 which phosphorylates Smad-1, -5 and -8 (Finnson et al., 2008; Goumans et al., 2002; Goumans et al., 2003). Also, ALK5 is required for ALK1 signaling upon TGF-β binding to ALK5, ALK5 forms a complex with ALK1, activating ALK1/Smad1/5 pathway.

Endoglin is a 180 kDa homodimeric disulfide-linked single-pass transmembrane glycoprotein. It is a TGF-β co-receptor that is highly expressed in actively proliferating endothelial cells (Nassiri et al., 2011). Endoglin binds TGF-β1 and TGF-β3 with high affinity in the presence of TβRII but does not bind TGF-β2 isoform (Bernabeu et al., 2009). In endothelial cells, endoglin is involved in angiogenesis by modulating the balance between TGF-β/ALK1 and TGF-β/ALK5 signaling pathways. Activation of the ALK1/Smad1/5 pathway has been shown to stimulate endothelial cell proliferation and migration, whereas activation of the ALK5/Smad2/3 pathway inhibits this process. During angiogenesis the TGF-β/ALK1/Smad1/5 signaling activity is increased whereas TGF-β/ALK5/Smad2/3 activity is diminished with endoglin promoting the TGF-β/ALK1/Smad1/5 signaling pathway (Lebrin et al., 2004). Mutations in endoglin and ALK1 genes have been shown to lead to an autosomal dominant vascular disease known as hereditary hemorrhagic telangiectasia, HHT1 and HHT2, respectively. The similar clinical features between HHT1 and HHT2 indicate that endoglin and ALK1 share similar functions in the cell (Sabba et al., 2007).

Our group has previously shown that endoglin is expressed in human chondrocytes and that complexes with both TβRI receptors (ALK5 and ALK1) in these cells (Parker et al.,

2003). Our laboratory has also previously reported that TGF-β signals through both ALK5 and ALK1 receptors in human chondrocytes and that activation of ALK5 and ALK1 signaling pathways elicit opposing effects (Finnson et al., 2008). However, the functional significance of endoglin expression and its modulation of TGF-β signaling pathways in chondrocytes are unknown. The current study establishes endoglin as a regulator of TGF-β/Smad signaling pathways and ECM production in human chondrocytes and suggest that endoglin may represent a potential marker for chondrocyte dedifferentiation and/or phenotype in OA.

3.2 Materials and methods

Cell culture

Primary chondrocytes were isolated from human osteoarthritic (OA) articular cartilage obtained intraoperatively from adult arthroplasty specimens or from adult cartilage obtained from traumatic open joint injuries with no evidence of degenerative joint disease (normal cartilage, N). All procedures were approved by the Research Ethics Board of the McGill University Health Center and all cartilage samples were obtained with informed consent. Once received, the cartilage was rinsed with cold phosphate buffered saline (PBS), minced and then incubated for 1 hour at 37°C with 1 mg/ml pronase (Sigma) followed by overnight digestion at 37°C with 1 mg/ml collagenase (Sigma). Next day, the suspension was passed through a 70 mm cell sieve and chondrocytes were collected by 10 min centrifugation (1,500 x g), washed with PBS twice and plated at a density of $2.5x10^5$ cells/cm^2.

Once the primary chondrocytes reached passage 4, they were put in the 3-dimensional (3-

D) alginate culture system to promote chondrocyte redifferentiation (De Ceuninck et al., 2004). Briefly, chondrocytes ($4x10^6$ cells/ml) were suspended in a 1.2% alginate solution and passed drop-wise through a 25-gauge needle into a 102 mM CaCl2 solution resulting in the formation of alginate beads. The beads were washed with 0.15 M NaCl and transferred to culture flasks with serum-containing media. The alginate beads were cultured for 21 days and the media was changed every third day. Alginate beads were depolymerised with 55 mM sodium citrate/ 0.15 M NaCl and the recovered chondrocytes washed in PBS and resuspended in serum-containing media, were plated ($2.5x10^5$ cells/cm^2) and grown to confluence.

Subculture-induced dedifferentiation was performed by serially passaging confluent chondrocytes at a 1:2 split ratio for up to 12 passages. Cells at passage 1-2 are designated as **'**early passage**'** and cells at passage 10-12 are designated as **'**late passage**'**. Chondrocyte morphology was visualized by phase contrast microscopy and images were captured with a digital camera (Finnson et al., 2010a).

Transient transfection

Chondrocytes were transiently transfected with endoglin-specific siRNA or (control siRNA) in order to knockdown endoglin expression. For C28 cells, lipofectamine 2000 (Invitrogen) transfection reagent was used according to the manufacturer's instruction.

Human primary articular chondrocytes were transiently transfected with endoglin-specific siRNA or (control siRNA) by using a calcium phosphate precipitation method for cells in suspension as described previously (Qureshi et al., 2008). Briefly, cells were detached by 0.05% trypsin–EDTA treatment, then stopped by adding DMEM/F12-10%

FBS followed by centrifugation, and 2.5 X 10^5 cell aliquots suspended in 70 ul of ODN/siRNA (250 nM)–calcium phosphate precipitate (2.2 M CaCl2 and 2 Hepes-buffered solution [HBS]) for 30 min with gentle shaking every 5 min. Then, 1 ml of DMEM/F12-10% FBS was added to the pellet and plated in the serum-containing medium for 5 to 6h at 37°C for adherence. After that, medium was removed and the fresh serum containing media was added to the cells, which were maintained at 37°C in the incubator for 24 hours.

Cartilage extraction

Total protein was extracted from human articular cartilage as described previously (Hoemann et al., 2002). Briefly, cartilage tissue was pulverized under liquid nitrogen using a biopulverizer (Biospec Products Inc., Bartlesville, OK) and then homogenized in GuCl extraction buffer [4 M GuCl/50 mM Tris, pH 7.5 and 1 mM ethylenediaminetetraacetic acid (EDTA)]. Samples were vortexed at 4°C for 30 min, centrifuged at 15,000 x g for 10 min at 4°C and the supernatants were collected and precipitated with 5 vol 100% ethanol at 20°C. Pellets were washed with 75% ethanol, dried, and resuspended in 8 M urea. Samples containing equivalent amounts of protein were analyzed by western blot using an anti-endoglin (Santa Cruz, P4A4, 1:1000) antibody. Membranes were stained with Ponceau to confirm equal protein loading.

Western blot analysis

Cell lysates were separated by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Fisher Scientific). The membranes were blocked

with Tris-buffered saline-Tween20 (TBST) containing 5% milk for 1 hour at room temperature and incubated with primary antibodies at 4°C overnight. The membranes were washed 3 times for 10 minutes each and were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Cell Signaling) for 1 hour at room temperature. The signals on the membranes were detected using enhanced chemiluminescence (ECL) system (GE Healthcare, Canada). The membranes were stripped and then reprobed with β-actin antibody (Santa Cruz, TX) as a loading control.

RNA extraction and RT-PCR (Human chondrocytes)

Total RNA was extracted from primary articular chondrocytes using the RNeasy mini kit (Qiagen, Mississauga, ON) and reverse transcribed using moloney murine leukemia virus (MMLV)-RT and oligo-dT primer (Invitrogen). PCR was performed using human endoglin (forward primer, 5`- CCAAGACCGGGTCTCAAGAC-3`; reverse primer, 5`-CTGGGTCGAGTGGAGGACTG -3`) (Bellon et al., 1993) and human GAPDH (forward primer, 5`-GAAGGTGAAGGTCGGAGTC-3`; reverse primer, 5`-GAAGATGGTGATGGGATTTC-3`) primers (Yoshimura et al., 2003) and Taq polymerase (Invitrogen) with an initial denaturation step of 3 min at 94°C, followed by 26 cycles (exponential phase) of denaturation at 94°C (30 s), annealing at 55°C (45 s), and extension at 72°C (60 s). PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining (Finnson et al., 2008).

Generating endoglin heterozygote mice (*Eng*$^{+/-}$)

Endogin heterozygote (*Eng*$^{+/-}$) mice containing only one allele of endoglin were

generated as previously described (Bourdeau et al., 1999) (obtained from Dr. Letarte from University of Toronto, Toronto, Ontario). Congenic *Eng*$^{+/-}$ and their respective littermate controls were successfully generated by backcrosses to C57BL/6 mice (Jackson Laboratory). The routine genotyping was performed to differentiate between *Eng*$^{+/-}$ and their WT littermates. Briefly, the tail clips of each mouse were collected at the time of weaning. DNA was extracted using a HotSHOT method (hot sodium hydroxide and tris) as previously described (Truett et al., 2000) followed by PCR reaction. PCR products were resolved by agarose gel electrophoresis and visualized by SYBR safe DNA gel stain (Life Technologies).

The Facility Animal Care Committees (FACCs) and other animal ethics subcomittees of the McGill University and the McGill University Health Care (MUHC) Research Institute approved all animal procedures and protocols.

Immunohistochemistry (Human Cartilage)

Human cartilage tissue was fixed in a periodate-lysine-paraformaldehyde solution (McLean and Nakane, 1974) and embedded in a solution containing two parts 20% sucrose phosphate buffer to one part O.C.T. embedding medium (Tissue-Tek, Miles, USA). Cryostat sections (8 um) were obtained, fixed in 4% paraformaldehyde, washed in PBS and treated with chondroitinase ABC for 1 hour at 37°C. The sections were washed in PBS, blocked for 1 hour at room temperature with 5% normal goat serum and then incubated overnight at 4°C with anti-endoglin (Dako, SN6h, 1:100) antibody. Sections were washed in PBS and incubated at room temperature for 30 min with a HRP-conjugated anti-mouse secondary antibody. Immune complexes were detected using the

Vectastain ABC kit according to the manufacturers' instructions (Vector Laboratories, Burlington, ON).

Destabilization of Medial Meniscus (DMM) Surgery

Eng$^{+/-}$ mice and WT littermates at the age of 14-week were subjected to the DMM surgery to induced OA as described previously (Glasson et al., 2007). Briefly, after isoflurane anaesthesia, the medial meniscotibial ligament (which anchors the medial meniscus to the tibial plateau) was transected, displacing medial meniscus and resulting in the free movement of medial meniscus medially. A sham operation (an arthrotomy without the transection of the medial meniscotibial ligament) was performed in the right knee joint of the control group (Zhang et al., 2015b). At 14 weeks post-surgery, the mice were sacrificed and right knee joints of each mouse were collected for histological assessment and biochemical studies.

Histology (Mouse knee joint)

Mouse knee joints were dissected and fixed overnight in Tissue-Fix (Chaptec, Montreal, Quebec, Canada), decalcified for 1.5-2 hours with RDO Rapid Decalcifier (Apex Engineering, Plainfield, Illinois, USA). The decalcified knee joints were washed with PBS twice followed by embedding in paraffin and sectioning. Sections were cut (5um) and deparaffinised in xylene followed by a graded series of alcohol washes for further staining processes.

For Safranin-O/Fast Green staining, the sections were stained according to the manufacturer's protocol (Sigma–Aldrich, Oakville, Ontario, Canada). Osteoarthritis

Research Society International (OARSI) histological scoring was used to assess cartilage integrity of the samples (Glasson et al., 2010). The stained slides were evaluated blindly and independently by two individuals with expertise in evaluating slides using OARSI scoring system.

Immunohistochemistry for mouse cartilage tissue

For mouse cartilage IHC studies, we used the Universal Dako Labelled Streptavidin-Biotin-2 System, Horseradish Peroxidase (LSAB2 System, HRP; DAKO, Burlington, ON, Canada) and followed their recommended instructions. The prepared tissue sections were deparaffinised in xylene followed by a graded series of alcohol washes. The samples were incubated with 3% hydrogen peroxide (H_2O_2) for 5 min at RT to block endogenous peroxide followed by blocking the samples using bovine serum albumin (BSA) (0.1%) in PBS for 1 hour. The primary antibody incubation was performed in a humidified chamber overnight at 4°C. Next day, biotinylated link incubation for 30 min followed by streptavidin for 1 hour was performed at RT. The DAB-substrate chromogen solution was added until sufficient color developed. The slides were immersed in a bath of hematoxylin to counterstain the nucleus followed by several washing. We used MMP13 (Abcam, 1:250), ADAMTS5 (Abcam, 1:250), and type II collagen (Millipore, 1:500) primary antibodies. For negative control, no primary antibodies were added.

Mouse primary chondrocyte cell culture

Mouse articular cartilage was carefully dissected avoiding subchondral bone. Mouse primary chondrocytes were prepared from the isolated femur head and knee joint using

enzymatic digestion as previously described (Monemdjou et al., 2012). First, dissected articular cartilage was rinsed with PBS twice, then pronase digestion for 30 min at 37°C followed by collagenase digestion (1mg/ml in DMEM/F12) for 2 hours at 37°C incubator with rotation. Supernatant was collected, spun down by centrifugation (4000RPM for 5 minutes at RT) and finally the pellet was collected. The pellet was washed with sterile PBS, re-suspended and plated into T25 flask with 10% FBS containing DMEM/F12 with antibiotics and grown at 37°C until they became confluent with 5% CO_2. Only 1-2 passaged chondrocytes were used for the experiments.

Conditioned media analysis

Conditioned media was collected from mouse primary chondrocytes. The collected conditioned media was centrifugated for 5 min at 4000 RPM at 4°C to remove debris and dead cells. We precipitated proteins from the conditioned media using a cold ethanol precipitation method. Briefly, we added 9 volumes ethanol to 1 volume of the conditioned media, inverted the samples 5 times, and left overnight at 4°C. Next day, the samples were centrifugated for 20 minutes at 14,000 RPM in a refrigerated centrifuge. Ethanolic supernatant was carefully discarded and the pellet was suspended in 5X SDS buffer with vortexing and heating in a boiling water bath for 10 minutes.

RNA isolation

Total RNA was isolated from mouse primary chondrocytes using Trizol (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini kit (QIAGEN, Toronto, Ontario, Canada) including on-column DNase digestion to eliminate DNA genomic contamination

(RNase-Free DNase Set, QIAGEN) following the manufacture's protocol.

Statistical analysis

Numerical results are represented as means of n≥4 independent experiments ± standard error of the mean (SEM). For statistical tests where only two data sets were being compared, a Student's t test (two-tailed) was used where $P<0.05$ was deemed statistically significant.

3.3 Results

Endoglin levels are increased in human OA cartilage compared to normal cartilage tissue.

First, we examined endoglin protein levels in human cartilage samples obtained from the knee of OA patients and from healthy individuals with no history of OA (three different patients each for OA and normal). Protein was extracted from the cartilage tissue as described in the Method section and analyzed by western blot using an anti-endoglin antibody. Our results demonstrate increased levels of endoglin in OA compared to normal cartilage (Fig. 3.1 A). Ponceau staining of the membrane confirms that equal amounts of protein are loaded in each lane. Similar results were obtained from immunohistochemistry of endoglin protein showing increased endoglin levels in OA cartilage tissue (Fig. 3.1 B). No staining was detected when the primary antibody was omitted in parallel experiments (data not shown).

Endoglin inhibits TGF-β1-induced Smad2 phosphorylation and enhances TGF-β1-induced Smad1/5 phosphorylation in human chondrocytes

We investigated mechanisms by which endoglin regulates TGF-β signaling pathways using a siRNA-based approach. Endoglin siRNA transfected chondrocytes display reduced levels of endoglin as compared to control siRNA transfected cells (Fig. 3.2 A, bottom panel). Blocking endoglin expression in primary articular chondrocytes displays enhanced TGF-β1-induced Smad2 phosphorylation without altering total Smad2 protein levels (Fig. 3.2 A). Furthermore, decreased endoglin levels show a decrease in TGF-β1-induced Smad1/5 phosphorylation as compared to control cells while total Smad1 levels remain unchanged (Fig. 3.2 B). An anti-STAT3 antibody was used as a loading control for the endoglin blot.

Endoglin inhibits TGF-β1-induced ECM production in human chondrocytes

Previously, our laboratory has shown that TGF-β1 stimulates type II collagen and PAI-1 protein production in human chondrocytes (Finnson et al., 2008). Here, we examined the role of endoglin in regulating TGF-β induced ECM proteins. Blocking endoglin expression displays an increase in TGF-β1-induced type II collagen expression. PAI-1 is detected as a double band and may represent non-glycosylated (lower band) and glycosylated (upper band) forms of PAI-1 (Gils et al., 2003). Endoglin siRNA transfected cells show an increase in (non-glycosylated) PAI-1 protein expression as compared to control cells (Fig. 3.3) indicating that endoglin inhibits TGF-β1-induced ECM proteins. Equal protein loading is confirmed by reprobing the membrane with an anti-actin antibody.

Endoglin levels are increased following subculture-induced dedifferentiation

We have observed that endoglin levels are increased in human OA compared to normal cartilage. Here, endoglin protein levels were measured in primary human articular chondrocytes from early passage cells (differentiated, more chondrogenic chondrocytes) and from late passage cells (dedifferentiated, more hypertrophic like chondrocytes). Fig. 3.4 A shows that endoglin levels are increased while type II collagen levels are decreased in late passage chondrocytes as compared to early passage chondrocytes. Reprobing the membrane with an anti-STAT3 antibody confirms equal amounts of protein loading in each lane (Fig. 3.4 A, bottom panel). Endoglin gene expression was also examined in early and late passage primary articular chondrocytes using semi-quantitative PCR. Endoglin mRNA levels are higher in late passage as compared to early passage chondrocytes (Fig. 3.4 B). Morphologies of the early and late passage human chondrocytes were observed under the phase-contrast microscopy (Fig. 3.4 C). The early passage chondrocytes display round shaped whereas the late passage (subculture induced) chondrocytes exhibit fibroblastic, elongated phenotype. Together, our data suggest that endoglin may represent a potential marker for chondrocyte dedifferentiation.

The schematic Figure shown in Fig. 3.4 D depicts the role of endoglin based on our current results in human chondrocytes (Fig 3.1 to 3.4). Endoglin promotes chondrocytes to exhibit OA-like phenotype by switching from TGF-β/Smad2/3 towards TGF-β/Smad1/5 signaling pathway and decreases type II collagen levels in human chondrocytes (Fig 3.4 D). Also our current data suggest that endoglin may represent a

potential marker for chondrocyte dedifferentiation.

Endoglin heterozygote ($Eng^{+/-}$) chondrocytes show increased type II collagen levels, decreased endoglin levels and decreased TGF-β1-induced Smad1 phosphorylation.

Endoglin heteterozygous mice *($Eng^{+/-}$)*, which are missing one allele of endoglin, were obtained from the laboratory of Dr. Michelle Letarte (University of Toronto). Endoglin knockout mice die due to cardiovascular malformation by embryonic day (E) 10.5 of gestation (Nomura-Kitabayashi et al., 2009) and therefore could not be used. Fig. 3.5 A shows the genotyping result of *$Eng^{+/-}$* mice where the primers amplified the recombinant product (476bp) that was inserted to disrupt exon 1 whereas this product was not detected in WT littermates control mice (Bourdeau et al., 1999).

We isolated mouse primary chondrocytes from joint tissue of both *$Eng^{+/-}$* and WT mice as described in the Materials and Method section. At confluence, cell lysates were prepared and western blot analysis was performed using anti-endoglin and anti-type II collagen antibodies. Here, we observe that endoglin levels are decreased in *$Eng^{+/-}$* compared to WT chondrocytes (Fig. 3.5 B). Both cell lysates and conditioned media from *$Eng^{+/-}$* chondrocytes show increased levels of type II collagen while β-actin levels remain the same between *$Eng^{+/-}$* and WT mouse chondrocytes.

Then, we examined whether endoglin regulates TGF-β1-induced Smad phosphorylation in mouse chondrocytes. *$Eng^{+/-}$* chondrocytes show a decrease in basal and TGF-β1-induced Smad1 phosphorylation (Fig. 3.5 C). However, *$Eng^{+/-}$* and WT chondrocytes

show similar levels of TGF-β1-induced Smad 2 and Smad3 phosphorylation. β-actin confirms the equal amount of protein loading in each well (Fig. 3.5 C).

Eng$^{+/-}$ mice do not display any structural alterations in knee joints at 14 weeks after DMM surgery when compared to WT mice

Since we observed the increased levels of type II collagen when endoglin levels were decreased in mouse chondrocytes, we examined the role of endoglin during cartilage degeneration *in vivo*. We subjected the 14-week old male *Eng*$^{+/-}$ and WT mice to DMM surgery to induce OA or were sham operated (as a control group) as described in the Experimental Design (Fig. 3.6 A). Histological analysis was performed at 14 weeks post-DMM surgery. Our data demonstrate that there is no difference in proteoglycan content (as indicated in the Safranin-O-staining, red color), roughness of the articular cartilage, and articular chondrocyte cellularity between the knee joints of WT and *Eng*$^{+/-}$ mice in the control (sham operated) group (Fig. 3.6 B). The knee joints of the WT mice from the DMM group (Fig. 3.6 B. bottom panel) show the decreased proteoglycan content, decreased chondrocyte cellularity and increased roughness of the articular cartilage compared to the WT mice from the control group indicating that the mice subjected to the DMM surgery developed OA. The graphical representation of the evaluated sections using OARSI scoring system is represented in the Figure 3.6 C. We also evaluated sections for synovial fibrosis and articular chondrocyte cellularity (cartilage fibrosis) but both *Eng*$^{+/-}$ and WT mice show similar levels of synovial and cartilage fibrosis (Fig. 3.6 D and E).

Endoglin heterozygote (Eng$^{+/-}$) mice show the similar levels of type II collagen and proteases compared to their WT counterparts

Although the safranin-O-staining data do not show any apparent difference between *Eng*$^{+/-}$ mice and WT mice knee joints after the DMM surgery, we examined whether levels of ECM proteins such as type II collagen or proteases (MMP13 and ADAMTS5) that are known to be elevated in OA chondrocytes, were altered in *Eng*$^{+/-}$ mice. 14-weeks old male mice knee joint were dissected followed by histological staining and assessment. Our data show the similar levels of MMP13 in both femoral condyle and tibial plateau from the *Eng*$^{+/-}$ mice and WT counterparts (Fig. 3.7 A). Similarly, no differences in the levels of type II collagen or ADAMTS5 were observed between *Eng*$^{+/-}$ and WT knee joints (Fig. 3.7 B and C).

3.4 Discussion

TGF-β is an important anabolic factor in cartilage homeostasis (Blaney Davidson et al., 2007b) and deregulation of TGF-β signaling is implicated in joint tissue diseases such as OA (Blaney Davidson et al., 2007b). Previously, our group has shown that endoglin forms a heteromeric complex with TβRII and TβRI (ALK5 and ALK1) in human chondrocytes (Parker et al., 2003) but the functional significance of endoglin in human chondrocytes has not been determined. Our current *in vitro* data demonstrate that endoglin enhances TGF-β1-induced Smad1/5 phosphorylation while decreasing TGF-β1-mediated Smad2 phosphorylation and ECM protein production in human chondrocytes. Moreover, we show that endoglin expression is increased in human chondrocytes

following subculture-induced dedifferentiation *in vitro* and that endoglin expression levels are increased in human OA cartilage as compared to normal cartilage *in vivo*. Collectively, our results establish endoglin as a regulator of TGF-β signaling and ECM protein production with opposing effects on the ALK1/Smad1/5 and ALK5/Smad2/3 pathways in human chondrocytes and suggest that endoglin may represent a potential marker for the loss of chondrogenic phenotype.

Our finding that endoglin enhances TGF-β1-induced Smad1/5 phosphorylation in both human and mouse chondrocytes while decreasing TGF-β1-mediated Smad2 phosphorylation in human chondrocytes but not in mouse chondrocytes suggest that role of endoglin in modulating TGF-β signaling pathways is cell type and species specific. In support of this finding, several studies show a role for endoglin in regulating TGF-β-mediated Smad1/5, Smad2 and Smad3 signaling activities in a negative or positive fashion in various cell types indicating that the mechanisms by which endoglin regulates TGF-β signaling pathways are highly cell-type specific. For example, endoglin has been shown to enhance Smad2 protein levels (not Smad3) potentiating TGF-β canonical pathway which leads to increased eNOS expression in rat myofibroblasts (Santibanez et al., 2007). On the other hand, *Velasco et al.* demonstrate that ectopic expression of endoglin has no significant effect on TGF-β-induced Smad1 and Smad2 phosphorylation in rat myofibroblasts (Velasco et al., 2008). In human bone marrow stromal cells, endoglin has been shown to promote both ALK1/Smad1 and ALK5/Smad2 signaling pathways as well as their downstream target genes such as ID-1 and PAI-1, respectively (O'Connor et al., 2007). Taken together, these studies further support the idea that the mechanisms by which endoglin regulates TGF-β signaling pathway are highly cell-type

specific and context dependent.

Our results in the current study showing that endoglin levels are increased in human OA compared to normal cartilage and in late passage chondrocytes (dedifferentiated chondrocytes) establish endoglin as a potential marker for the loss of chondrogenic phenotype. We demonstrate that primary human articular chondrocytes change their morphologies from polygonal shape to fibroblast-like shape showing reduced levels of type II collagen following serial passages in monolayer culture. Our current results are in line with other reports showing that primary human articular chondrocytes in monolayer culture initially maintain a rounded, polygonal shape but undergo a progressive phenotypic change to an elongated fibroblast-like morphology upon culturing (Goldring, 2005). These changes are characterized by a decrease in cartilage-specific markers such as type II collagen and aggrecan levels and an increase in type I collagen and type X collagen levels; and this process is known as "chondrocyte dedifferentiation' (Duan et al., 2015; Goldring, 2005). The fibroblastic morphology of the cells and decreased collagen levels are observed in hypertrophic chondrocytes and these hypertrophy-like changes are found in both human OA and experimental OA models (van der Kraan and van den Berg, 2012). We demonstrate that both endoglin protein and mRNA levels are higher in late passage as compared to early passage chondrocytes. Our results are consistent with the previous report that identified endoglin as one of upregulated cell surface proteins in primary articular chondrocytes during cell expansion in monolayer (Diaz-Romero et al., 2005). Our results indicate that endoglin may represent a novel marker for chondrocyte dedifferentiation.

Our current data showing endoglin decreasing ECM protein levels (type II collagen and PAI-1) in human chondrocytes and decreasing type II collagen levels in mouse chondrocytes suggest that endoglin promotes chondrocytes to exhibit OA-like phenotype. To determine role of endoglin during cartilage degradation *in vivo*, we used endoglin heterozygote ($Eng^{+/-}$) mice that are missing one allele of endoglin gene to examine whether missing one allele in $Eng^{+/-}$ mice delays OA progression. Our *in vivo* data showing the similar degree of cartilage degradation in the knee joints of the $Eng^{+/-}$ mice and the WT littermates that were challenged with the DMM surgery indicate that missing one allele of endoglin gene in $Eng^{+/-}$ mice is not protected from cartilage degradation. In addition, the immunohistochemistry data showing the similar levels of type II collagen, MMP13, and ADAMTS5 between $Eng^{+/-}$ and WT knee joints suggest that endoglin does not alter the levels of type II collagen and proteases in mouse cartilage. Using heterozygous mice to examine the role of endoglin *in vivo* has been a useful tool to understand role of endoglin in certain diseases including fibrosis in various tissues, chronic inflammatory diseases, heart diseases and cancers (Pericacho et al., 2013; Peter et al., 2014; Satomi et al., 2003; Scharpfenecker et al., 2009; Shen et al., 2014a). However, there are limitations using a heterozygous mouse model. It has been noted that heterozygous mice often develop compensatory mechanisms (Minamisawa et al., 1999; Wang et al., 2001). To avoid this limitation, using endoglin knockout mice would be more suitable. However, since endoglin knockout mice are embryonically lethal, developing cartilage specific endoglin knockout mice will be a better way to examine role of endoglin in cartilage function *in vivo*. Additionally, it is possible that factors released by neighboring tissues or surrounding environment modulate endoglin levels

and/or influence endoglin's role in articular cartilage. Moreover, endoglin function in synovium or in subchondral bone has not been fully elucidated. Further studies are warranted to determine the factors that regulate endoglin function in joint tissues which will provide insight into the role of endoglin in cartilage homeostasis and disease progression.

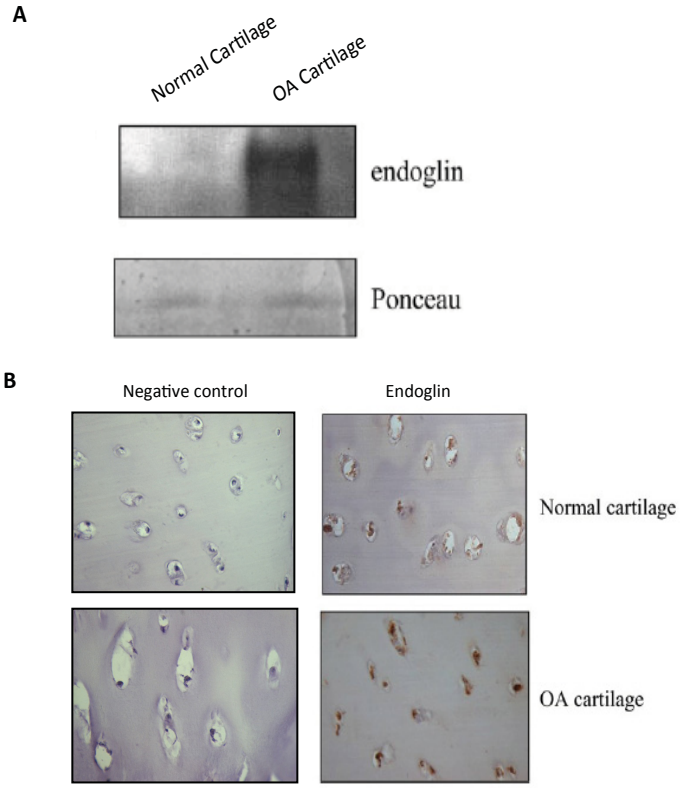
In summary, our *in vitro* data using human chondrocytes reveal that endoglin promotes ALK1/Smad1/5 signaling pathway while inhibiting ALK5/Smad2/3 pathway resulting in a decrease in ECM protein production. Our findings reveal that endoglin may represent a potential marker for chondrocyte dedifferentiation or loss of chondrogenic phenotype. Unraveling the precise molecular mechanisms governing TGF-β action in chondrocytes will help us to better understand intricate molecular mechanisms underlying cartilage homeostasis and disease progression.

3.5 Acknowledgements

We would like to thank the laboratory of Dr. Mohit Kapoor (University of Montreal) for insightful discussions and resources, Meryem Blati for helping generating histology sections and Dr. Bertrand Lussier for performing DMM surgery on mice joint knees. The study was supported by a Canadian Institutes of Health Research (CIHR) operating grant and a CIHR Proof of Principle grant to AP.

3.6 Figures

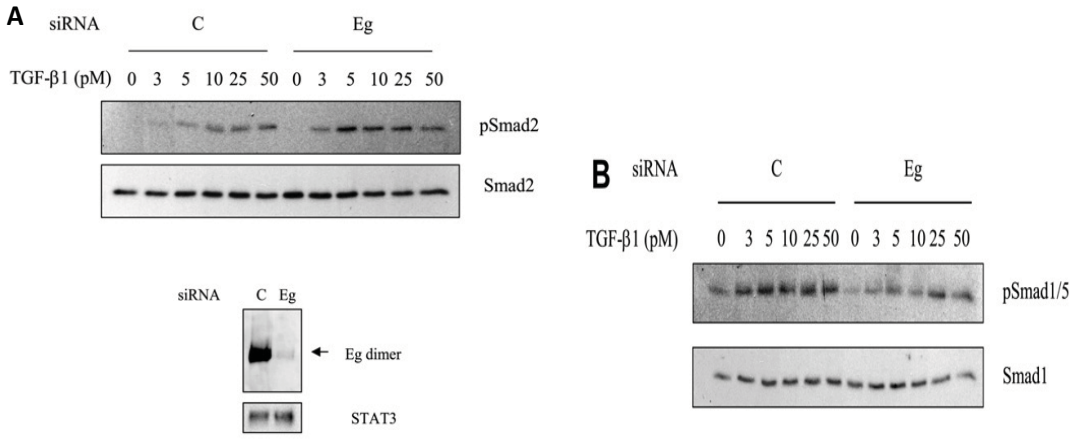
Fig. 3.1: Endoglin expression is increased in human OA cartilage as compared to normal cartilage.



(A) Western blot: Protein was extracted from OA and normal cartilage as described in Methods section. Samples were analyzed by Western blot using an anti-endoglin antibody (top panel). The membrane was stained with Ponceau S to confirm that equal amounts of protein were loaded in each lane (bottom panel). The lanes were selected from non-adjacent regions of the same gel. Results shown are representative of three independent experiments performed on cartilage of three different OA and three different normal donors. **(B)** Immunohistochemistry: Cryostat sections of OA and normal cartilage were analyzed by immunohistochemistry using an anti-endoglin (Dako, SN6h, 1:100

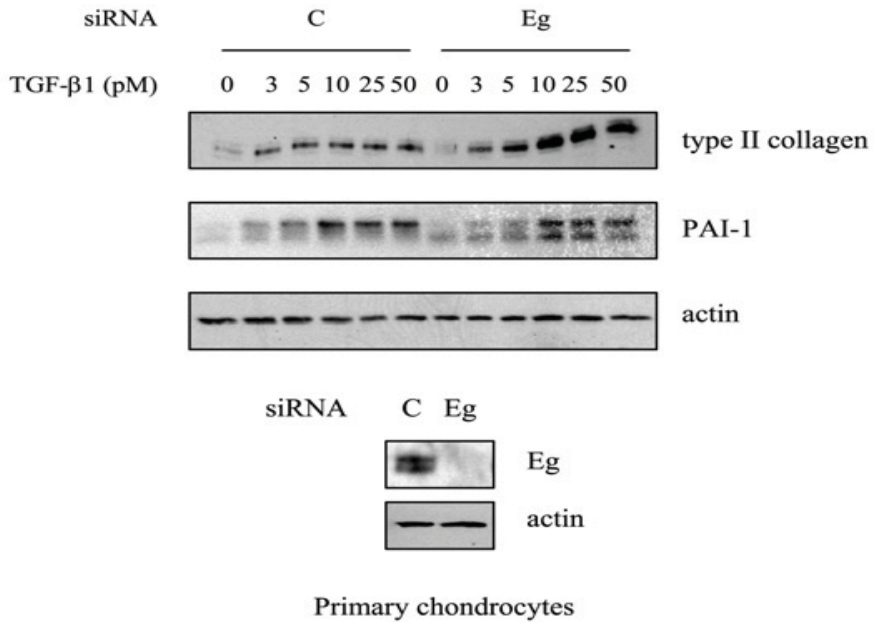
dilution) antibody. Detection was performed using Vectostain ABC immunostaining kit. Results shown are representative of two independently performed experiments.

Fig. 3.2: Endoglin inhibits TGF-β1-induced Smad2 phosphorylation while it promotes TGF-β1-induced Smad1 phosphorylation in human chondrocytes.



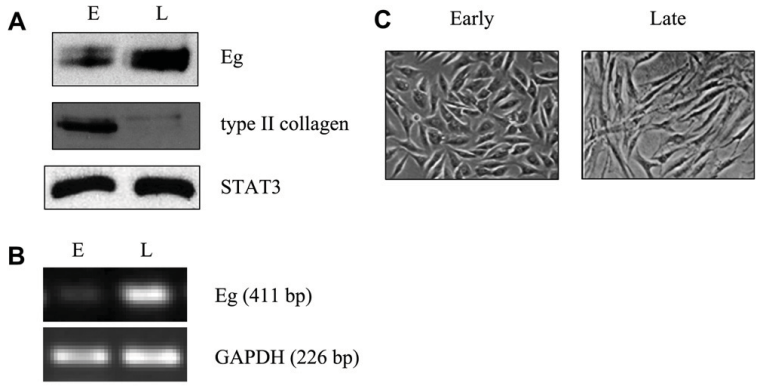
Primary human articular chondrocytes transfected with endoglin-specific (Eg) siRNA or control (C) siRNA were treated for 30 min with 0-50 pM TGF-β1 under serum-free conditions. Cell lysates were prepared and analyzed by western blot using **(A)** anti-phospho-Smad2, anti-Smad2, anti-endoglin or **(B)** anti-phospho-Smad1 and anti-Smad2 antibodies as indicated. Results shown are representative of three independent experiments.

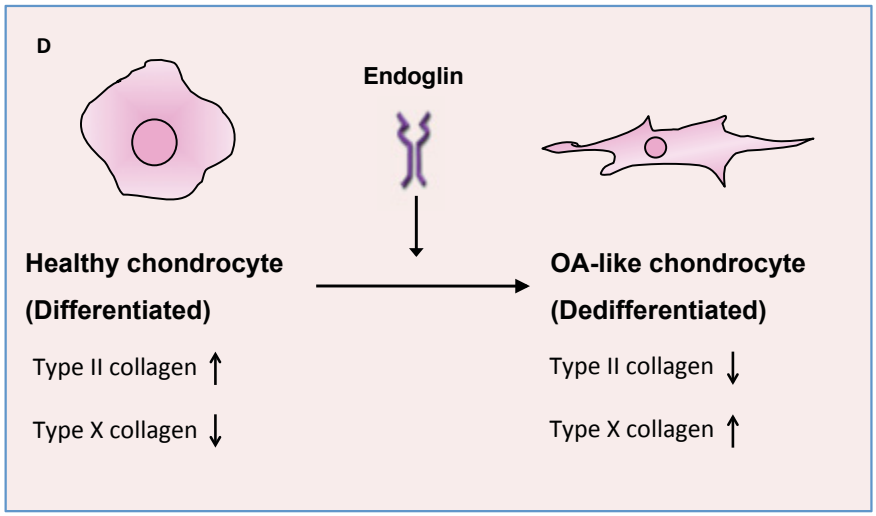
Fig. 3.3: Endoglin inhibits TGF-β1-induced type II collagen and PAI-1 protein expression in human chondrocytes.



Primary human articular chondrocytes were transfected with endoglin-specific (Eg) or control (C) siRNA. The cells were treated for 24 h with 0-100 pM TGF-β1 under serum-free conditions. Cell lysates were prepared and analyzed by western blot using anti-type II collagen, anti-PAI-1, anti-endoglin and anti-actin antibodies. Results shown are each representative of four independent experiments.

Fig. 3.4: Endoglin expressions are increased in primary human articular chondrocytes following subculture-induced dedifferentiation.

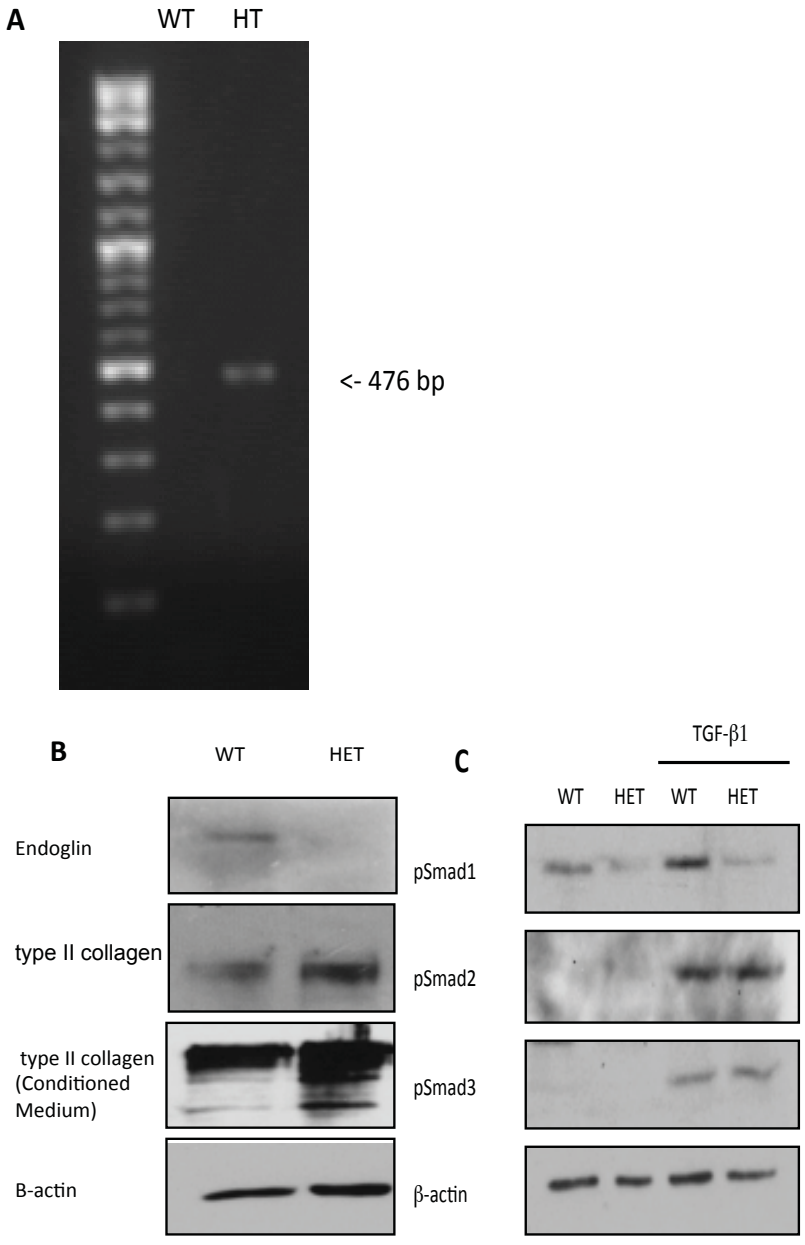




(A) Cell lysates from early and late passage primary human articular chondrocytes were analyzed by western blot using anti-endoglin (top panel) or anti-type II collagen (middle panel) antibodies. Membranes were reprobed with an anti-STAT3 antibody (bottom panel) to confirm that equal amounts of protein in each lane. **(B)** Total RNA and cell lysates were prepared from early and late passage primary human articular chondrocytes. Total RNA was analyzed by semi-quantitative RT-PCR using endoglin-(top panel) and GAPDH-specific (second panel) oligonucleotide primers. PCR product sizes for endoglin

(e.g., 411 bp) and GAPDH (226 bp) are indicated. **(C)** Cell morphology: Phase contrast microscopy of early and late passage primary human articular chondrocytes in monolayer culture. Results shown are representative of three independent experiments. **(D)** The schematic illustration describes the role of endoglin in human articular chondrocytes. Endoglin promotes chondrocytes to exhibit OA like phenotype and characteristics with decreased type II collagen levels.

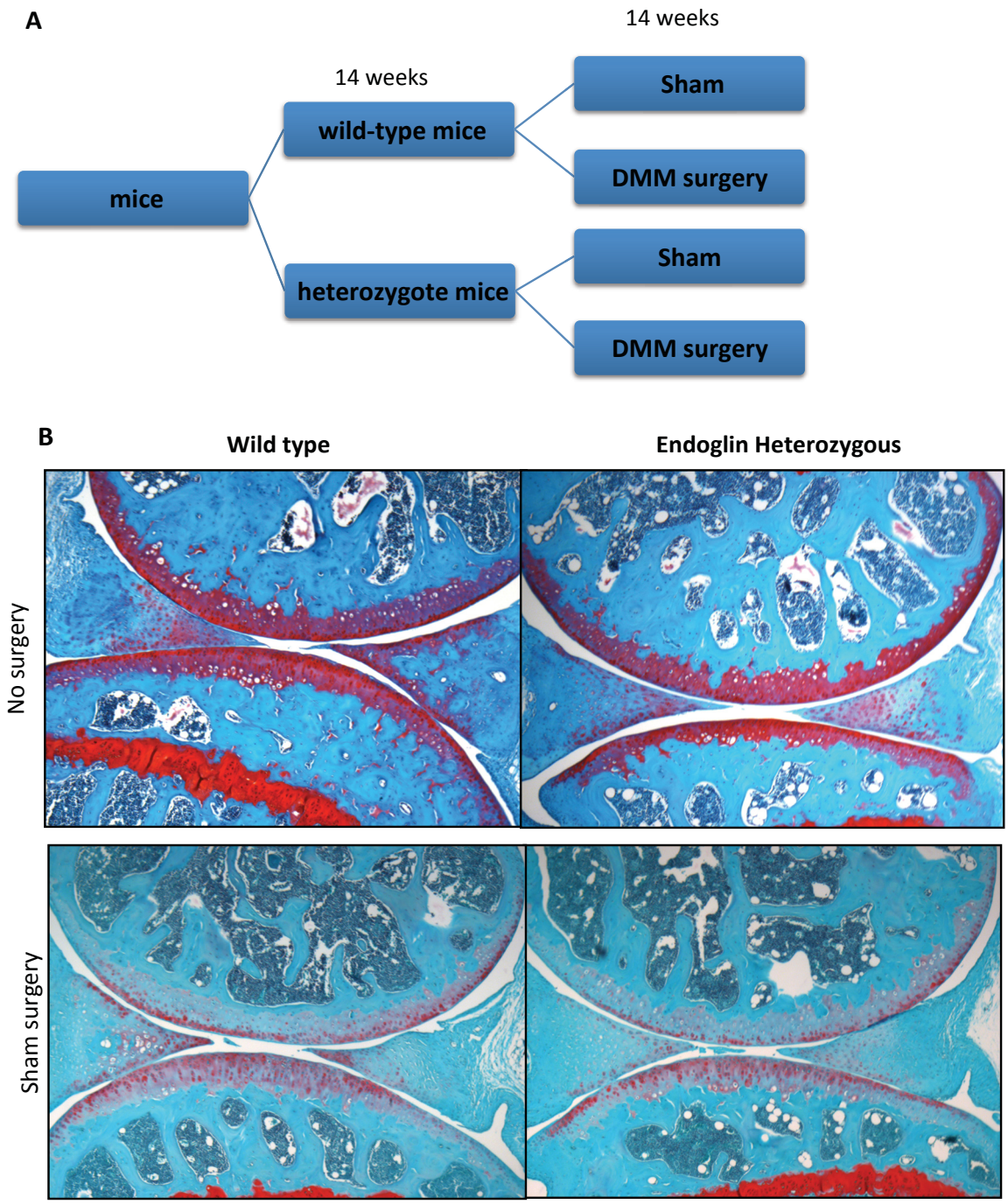
Fig. 3.5: Endoglin heterozygote ($Eng^{+/-}$) chondrocytes show increased type II collagen levels, decreased endoglin levels and decreased TGF-β1-induced Smad1 phosphorylation but show similar levels of TGF-β1 induced Smad2 and 3 phosphorylation.

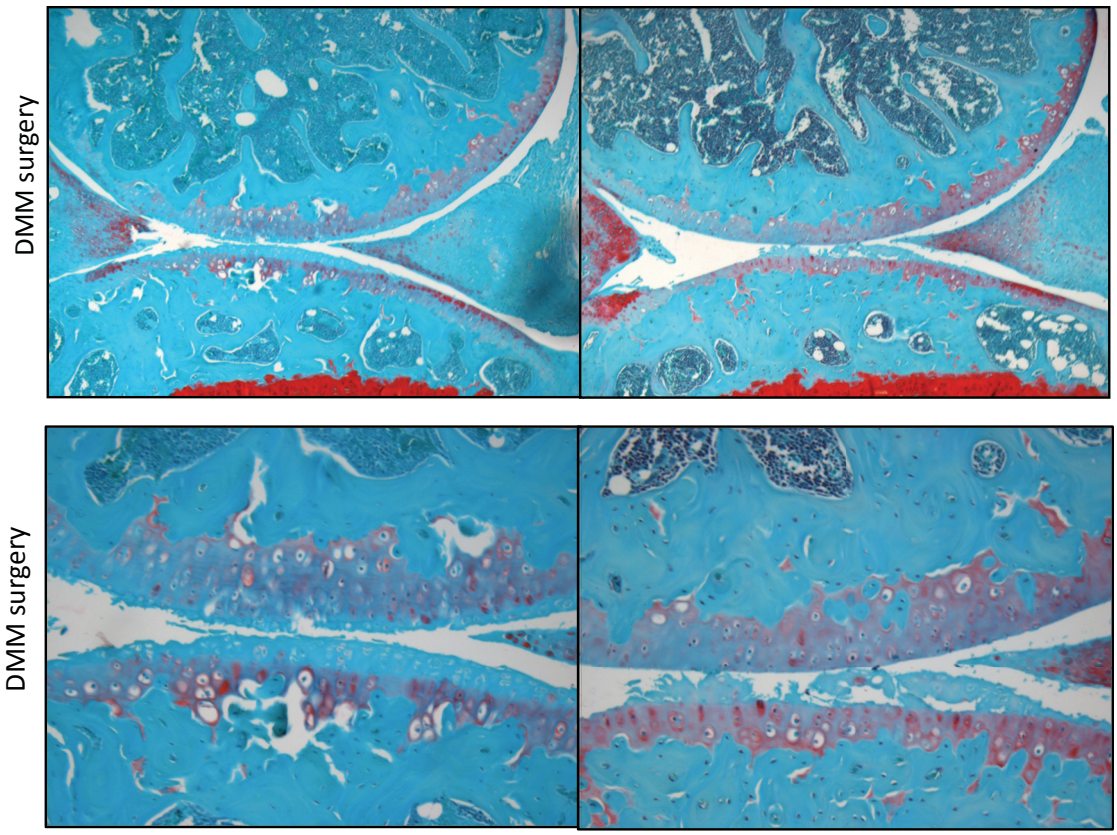


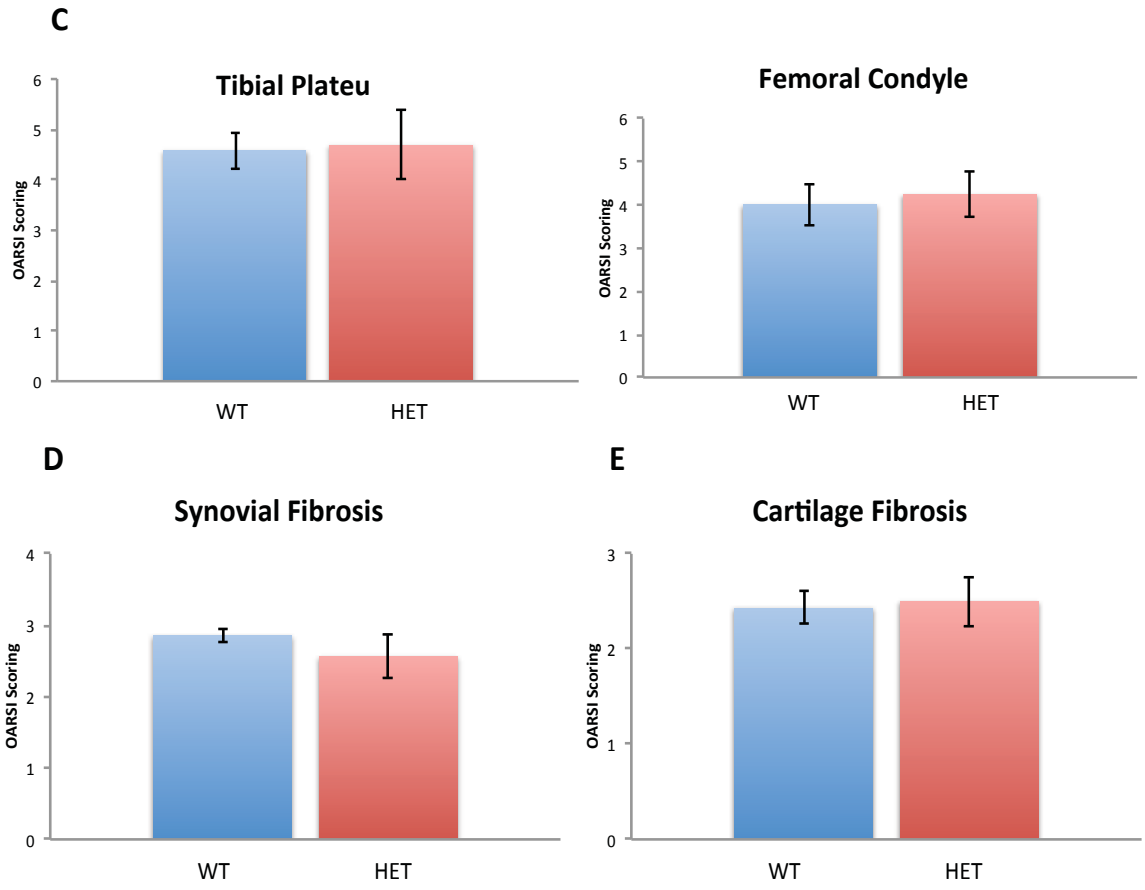
(A) DNA was extracted using a HotSHOT method (hot sodium hydroxide and tris) as previously described followed by PCR reaction. PCR products were resolved by agarose gel electrophoresis and visualized by SYBR safe DNA gel stain. Genotyping confirmed

the presence of recombinant product (476bp) in endoglin heterozygote mice and the absence in the wild type littermates. **(B)** Cell lysates were prepared from the *Eng*$^{+/-}$ and WT mouse primary chondrocytes and were subjected to western blot analysis using anti-endoglin, anti-type II collagen and anti- β-actin as indicated. Conditioned media was collected and ethanol precipitated as described in the Method section. Precipitated proteins from the conditioned media were subjected to western blot analysis for anti-type II collagen. **(C)** *Eng*$^{+/-}$ and WT mouse primary chondrocytes were treated with 100pM of TGF-β1 for 45 minutes under serum-free conditions. Cell lysates were prepared from the *Eng*$^{+/-}$ and WT mouse primary chondrocytes and were subjected to western blot analysis using anti-endoglin, anti-phosphoSmad1, anti-phosphoSmad2, anti-phosphoSmad3 and anti- β-actin as indicated. The β-actin panel demonstrates equal protein loading and transfer. Results shown are representative of two independent experiments.

Fig. 3.6: *Eng*$^{+/-}$ *mice* do not display any defective structural alterations in knee joints at 14 weeks after DMM surgery when compared to WT mice.



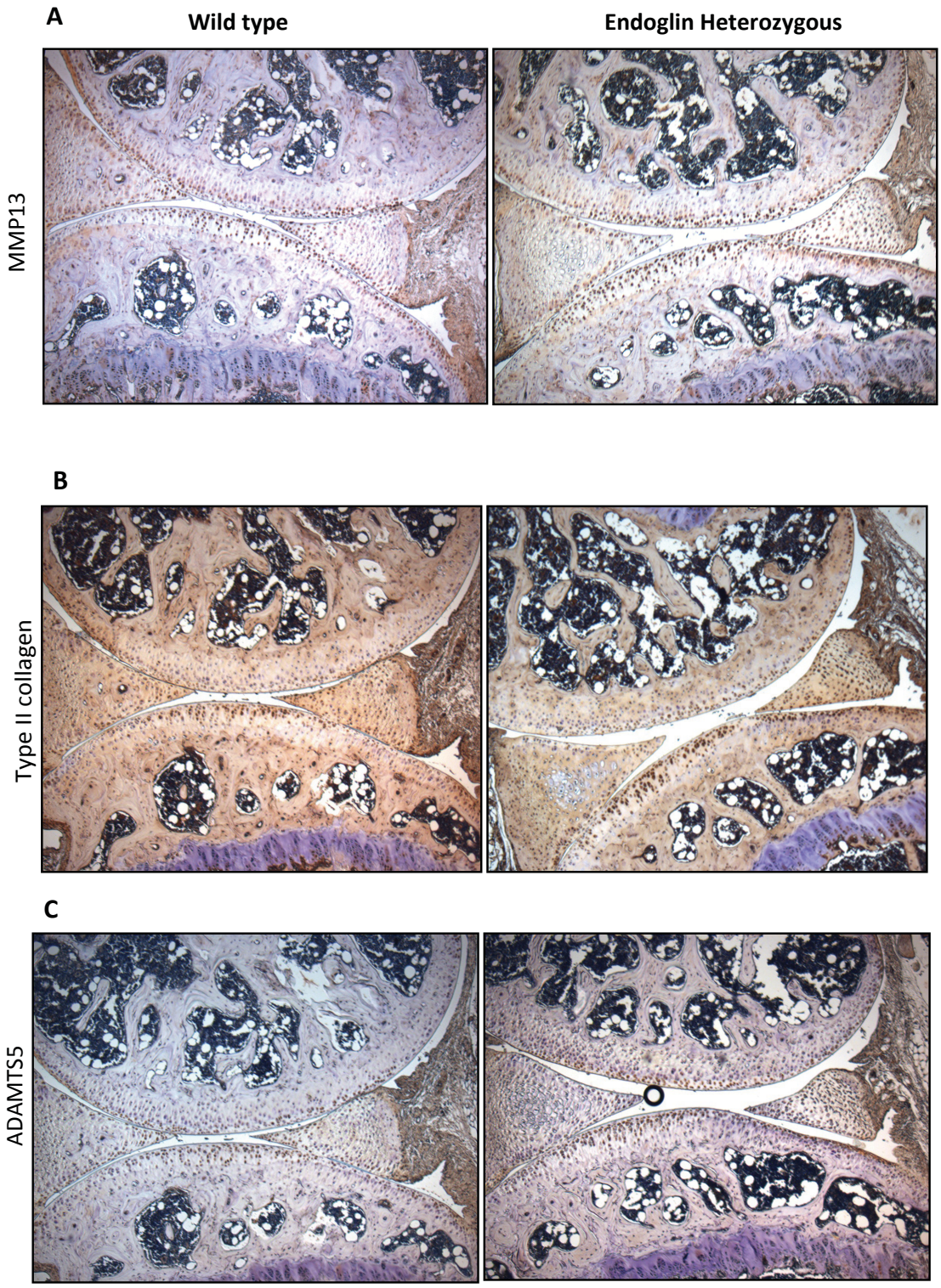

DMM surgery
DMM surgery



(A) The study consisted of 20 mice in total, comprising 10 endoglin heterozygous mice and 10 wild-type littermates. Eng (+/-) and wild-type mice were further subdivided into the 2 arms: controls group (sham operation) and DMM surgery group. All mice were sacrificed 14 weeks after the surgery **(B)** Histological analysis using Safranin O/fast green staining of 14 weeks post-OA surgery knee joint sections demonstrate that *Eng*$^{+/-}$ mice and WT mice show similar degree of cartilage degradation when the mice are surgically challenged (magnification: x 40 and x 100); **(C)** Osteoarthritis Research Society International (OARSI) scoring of medial tibial plateau and medial femoral condyle from the surgically challenged WT and Eng$^{+/-}$ mice are performed by two individuals independently. **(D)** Synovial fibrosis was evaluated and compared between

WT OA and *Eng*$^{+/-}$ OA and **(E)** Condrocyte cellularity was also evaluated and compared between WT OA and *Eng*$^{+/-}$ OA groups. Results shown are representative of minimum five mice.

Fig. 3.7: *Eng*$^{+/-}$ mice and WT mice show similar levels of MMP13, ADAMTS-5 and type II collagen expressions.



The prepared tissue sections were deparaffinised in xylene followed by a graded series of alcohol washes. The samples were incubated with 3% hydrogen peroxide (H_2O_2) for 5 minutes at RT to block endogenous peroxide followed by blocking the samples using bovine serum albumin (BSA) (0.1%) in PBS for 1 hour. The sections were then incubated with primary antibodies for **(A)** MMP13, **(B)** type II collagen and **(C)** ADAMTS5 in a humidified chamber overnight at 4°C. Next day, the slides were incubated with biotinylated link for 30 min followed by streptavidin incubation for 1 hour at RT. The DAB-substrate chromogen solution was added until sufficient color developed. Hematoxylin was used to counterstain the nucleus. Results shown are representative of minimum five mice.

Bridging statement for the next chapter

In the previous manuscript, we have shown that endoglin differentially regulates TGF-β signaling pathways and inhibits ECM protein production in human chondrocytes *in vitro*. Our findings indicate that endoglin promotes TGF-β/ALK1/Smad1/5 signaling and inhibits TGF-β/ALK5/Smad2/3 pathway, and reduces type II collagen production in human chondrocytes. We also have shown that endoglin levels are increased in human OA cartilage and in subculture-induced dedifferentiated human chondrocytes. Consistent with these findings, we have demonstrated that endoglin promotes TGF-β induced Smad1 phosphorylation and inhibits type II collagen expression in mouse chondrocytes suggesting that endoglin differentially regulates TGF-β signaling pathways and alter ECM production in mouse chondrocytes as well.

Besides endoglin, CD109 is another TGF-β co-receptor that was first identified and characterized in our laboratory. Our group has previously reported that CD109 is a potent regulator of TGF-β signaling pathway in skin cells. CD109 inhibits TGF-β canonical pathway (TGF-β/ALK5/Smad2/3) and decreases ECM protein production in skin cells both *in vitro* and *in vivo*. Whether CD109 protein is expressed in human chondrocytes or it plays a functional role in chondrocytes has not been delineated. The third part of the thesis was aimed at establishing that CD109 is expressed in chondrocytes and to determine the function of CD109 in human chondrocytes and to investigate the mechanisms by which CD109 regulates TGF-β signaling pathway.

4. CD109 inhibits TGF-Beta Receptor expression, signaling and ECM protein production in human chondrocytes.

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4.1 Abstract

Objective: Transforming growth factor-beta (TGF-β) is a pleotropic growth factor that maintains the integrity and function of cartilage and dysregulation of TGF-β action is implicated in joint tissue diseases such as osteoarthritis (OA). Our group has previously reported CD109 as a novel TGF-β co-receptor and has shown that CD109 is a potent negative regulator of TGF-β signaling and action in the skin. The objectives of the current study were: (i) to determine whether CD109 is expressed in human articular chondrocytes, and (ii) to examine whether CD109 regulates TGF-β signaling and ECM protein expression in human chondrocytes. **Methods:** Primary articular chondrocytes were isolated from normal cartilage harvested from adult patients with traumatic open joint injury who have no history of joint diseases. CD109 expression was determined by western blot and CD109 function was analyzed by blocking CD109 expression using CD109-specific siRNA. **Results:** Our results show that CD109 is expressed in human articular chondrocytes and that loss of CD109 expression markedly enhances ALK5 levels, TGF-β1-induced Smad2 and 3 phosphorylation, Smad3-driven transcriptional activity, type II collagen and plasminogen activator inhibitor-1 (PAI-1) protein levels and aggrecan gene expression. On the other hand, loss of CD109 expression decreases ALK1 levels, TGF-β1-induced Smad1 phosphorylation and inhibits MMP13 expression at both mRNA and protein levels. **Conclusion:** Our findings suggest that CD109 differentially

regulates TGF-β signaling pathways and inhibits ECM protein production while promoting proteases expression in human articular chondrocytes. We conclude that CD109 may play an important role in maintaining cartilage function and integrity.

4.2 Introduction

Osteoarthritis (OA) is a progressive joint disease characterized by cartilage degradation, synovitis, osteophyte formation, and subchondral sclerosis (Shen et al., 2013). Homeostasis of cartilage is maintained by chondrocytes which produce extracellular matrix (ECM) proteins, including collagen fibrils and aggrecans (Heinegard and Saxne, 2011). The MMPs (matrix metalloproteinases) and ADAMTS (a disintegrin and metalloproteinases with thrombospondin motifs) are families of key enzymes responsible for collagen and aggrecan breakdown, respectively. They are upregulated in cartilage and synovial tissues in patients with rheumatoid arthritis (RA) and OA (Little et al., 1999; Tetlow et al., 2001).

Among the growth factors that regulate cartilage homeostasis, transforming growth factor-beta (TGF-β) is an important cytokine for cartilage development and integrity (Serra et al., 1997). Aberrant TGF-β signaling activity is implicated in pathological conditions related to cartilage. *Serra et al*. and *Yang et al*. have demonstrated in their *in vivo* studies that mice with abnormal TGF-β signaling activity develop joint degeneration similar to human OA (Serra et al., 1997; Yang et al., 1999). TGF-β exerts its diverse actions by transducing the signal through a pair of transmembrane serine/threonine kinases namely type I (TβRI) and type II TGF-β (TβRII) signaling receptors. First, the

TGF-β ligand binds to constitutively active TβRII, which transphosphorylates the TβRI (or activin receptor-like kinase, ALK5) receptor. The activated TβRI phosphorylates receptor-regulated Smads (R-Smad, Smad2 and Smad3) which subsequently bind to common Smad (Smad4) (Shi and Massague, 2003). This complex translocates to the nucleus to regulate gene expression through interactions with transcription factors, co-activators and co-repressors (Gaarenstroom and Hill, 2014).

Besides the TGF-β canonical pathway (TGFβ/ALK5/Smad2/3), TGF-β is able to activate alternative receptor combinations in a cell-type specific manner. Previously, our group has shown that TGF-β activates ALK1 receptor which phosphorylates Smad1/5 in human chondrocytes and that activation of the ALK1 pathway elicits opposing effects on chondrocyte function to the ALK5 signaling pathway (Finnson et al., 2008). The activation of the ALK1 pathway has been shown to promote an OA-like phenotype while the activation of the ALK5 pathway promotes the anabolic activity of TGF-β by increasing ECM protein production such as type II collagen in human chondrocytes (Finnson et al., 2008). Based on this observation, it is speculated that ALK1/Smad1/5 signaling pathway promotes chondrocytes to exhibit OA like characteristics. The factors that regulate the balance between ALK5/Smad2/3 and ALK1/Smad1/5 signaling pathways may alter chondrocyte function.

Our group has discovered CD109, a GPI-anchored protein, as a novel TGF-β co-receptor and has shown that CD109 inhibits TGF-β/ALK5/Smad2/3 canonical pathway in skin cells (Bizet et al., 2011). Furthermore, the role of CD109 in skin has been extensively studied *in vivo* by our group. CD109 transgenic mice overexpressing CD109 in the epidermis exhibit reduced scarring and fibrosis during wound healing as compared to the

wild-type littermates (Vorstenbosch et al., 2013a). The functional significance of CD109 expression and its modulation of TGF-β signaling pathways in chondrocytes are unknown. In the current study, we demonstrate that CD109 increases the ALK1 to ALK5 ratio by markedly decreasing ALK5 levels and increasing ALK1 levels. We show that CD109 switches TGF-β signaling pathways from ALK5/Smad2/3 pathway to ALK1/Smad1/5 pathway and decreases ECM protein levels while increasing expression of proteases in human chondrocytes. Our current findings establish CD109 as a critical regulator of TGF-β signaling pathways and chondrocyte function.

4.3 Materials and Methods

Primary chondrocyte isolation

Articular cartilage samples were collected from medial and lateral femoral condyles obtained at hip joint surgery from adult patients diagnosed as having OA. Normal cartilage (N) was also collected from adult patients with traumatic open joint injury or from organ donors (within 12 hours of death), with no history of degenerative joint disease. Both OA and N cartilage samples were provided by Dr. John Antoniou from Jewish General Hospital. All procedures were approved by the Research Ethics Board of the McGill University Health Center and all cartilage samples were obtained with informed consent. The cartilage was rinsed with cold phosphate buffered saline (PBS), minced and then incubated for 1 hour at 37°C with 1 mg/ml pronase followed by overnight digestion at 37°C with 1 mg/ml collagenase (Sigma Aldrich, Oakville, ON). The suspension was passed through a 70 mm cell sieve and chondrocytes were collected

by 10 min centrifugation (1,500 x g), washed with PBS and plated at a density of $2.5X10^5$ cells/cm^2.

Cell culture

All cells were cultured in DMEM/F12 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin, 0.25 μg/ml amphotericin, 50 μg/ml gentamicin (Invitrogen) and maintained at 37°C in a humidified incubator under 5% CO_2/air.

Once the primary chondrocytes reach passage 3, they were put in the 3-dimensional (3-D) alginate culture system to promote chondrocyte redifferentiation. This system has been described previously (De Ceuninck et al., 2004). Briefly, chondrocytes ($4x10^6$ cells/ml) were suspended in a 1.2% alginate solution and passed drop-wise through a 25-gauge needle into a 102 mM $CaCl_2$ solution resulting in the formation of alginate beads. The beads were washed with 0.15 M NaCl and transferred to culture flasks with serum-containing media. The alginate beads were cultured for 21 days and the media changed every third day. Alginate beads were depolymerised with 55 mM sodium citrate/ 0.15 M NaCl and the recovered chondrocytes washed in PBS, resuspended in serum-containing media, plated ($2.5X10^5$ cells/cm^2) and grown to confluence.

Transient transfections and cytokine/inhibitor treatment

Human normal and OA primary articular chondrocytes were transfected with CD109-specific siRNA (cat.#129083) or a negative control siRNA (cat.#4611) (Ambion, Austin, TX, USA) using a calcium phosphate transfection method (Qureshi et al., 2008). The cells were transfected for 48 hours followed by 6 hours of serum starvation before TGF-

β1 treatment.

For inhibitor treatment, we used MG132 (Cayman Chemical, MI. USA) to block proteosomal degradation. After transfection and serum starvation, the cells were treated with MG132 for 3 hours at the final concentrations of 20uM.

Luciferase assays

Primary articular chondrocytes were transfected with CD109-specific or control siRNA. The cells were co-transfected with Smad3-responsive CAGA12-lux luciferase reporter constructs (Dennler et al., 1999) and with pCMV-β-galactosidase. After 48 hours transfection, all cells were treated for 24 hours with 0-50 pM of TGF-β1 under serum-free conditions. Cell lysates were prepared and analyzed for luciferase and β-galactosidase activity as described previously (Finnson et al., 2008). The data are presented as a fold-change in luciferase activity from control.

Preparation of cell lysates for western blot analysis

Cell lysates were prepared in RIPA buffer (radioimmunoprecipitation assay buffer)
[25 mM Tris-HCl pH 7.6, 10% glycerol, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS] containing the protease inhibitor mixture (1mM sodium fluoride, 1mM PMSF, 1mM Orthovandate and Phosphatase Inhibitor Cocktail Tablet; Roche). Cell debris was removed by centrifugation. Protein concentration was determined by Lowry protein assay (Biorad) and Prism software, and the samples were prepared in 5X sample buffer.

Western blot analysis

Cell lysates were separated by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Fisher Scientific). The membranes were blocked with Tris-buffered saline-Tween 20 (TBST) containing 5% milk for 1 hour at room temperature and incubated with primary antibodies at 4°C overnight. The membranes were washed 3 times for 10 minutes each and were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Cell Signaling) for 1 hour at room temperature. The signals on the membranes were detected using enhanced chemiluminescence (ECL) system (Millipore, Canada). The primary antibodies were diluted in 5% milk with TBST or 3% BSA with TBST and were immunoblotted with rabbit anti-human ALK5; rabbit anti-MMP13 (Santa Cruz, TX); mouse anti-human ALK1 (R&D); mouse anti-human type II collagen (Chemicon); mouse anti-PAI-1 (BD bioscience); rabbit anti-human phospho-Smad1/5, phospho-Smad2, and phospho-Smad3, mouse anti-human Smad2 (Cell Signaling); rabbit anti-human Smad1 (Zymed); and mouse anti-human Smad3 (Abnova). The membranes were stripped and then reprobed with GAPDH (Cell Signaling) or alpha-tubulin (Abcam) as a loading control.

RNA extraction, reverse transcriptase-PCR and real-time PCR reaction

Cells from six-well plates were extracted with TRIzol™ reagent (Invitrogen). Total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real time PCR was performed using oligonucleotide primers for human ALK5 (forward primer, 5-GGGGCGACGGCGTTACAGTGTTTCTGCCAC-3; reverse primer, 5-TGAGATGCAGACGAAGCACACTGGTCCAGC-3(Choi, 1999); human HPRT (forward primer, 5- TGTAATGACCAGTCAACAGGG-3; reverse primer, 5-

TGGCTTATATCCAACACTTCG-3); human aggrecan (forward primer, 5-CAACAACAATGCCCAAGACTAC-3; reverse primer, 5-AGTTCTCAAATTGCAAGGAGTG-3); human MMP13 (forward primer, 5-AAGGAGCATGGCGACTTCT-3; reverse primer, 5- TGGCCCAGGAGGAAAAGC-3); human ADAMTS5 (forward primer, 5- CATTTGGACCAGGGCTTAGA-3; reverse primer, 5- CTTCACTGTGGCTCACGAAA-3). Quantitative PCR reaction was performed using DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific) with an initial denaturation step of 15 min at 95°C, followed by 40 cycles of denaturation at 94°C (10 s), annealing at 60°C (30 s), and extension at 72°C (30 s) and analyzed in the Applied Bioscience's Stepone real-time instrument. Quantification of target mRNA was carried out by comparison of the number of cycles required in order to reach the reference and target threshold values (ΔΔ CT method).

Statistical analysis

Numerical results are represented as means of n≥3 independent experiments ± standard error of the mean (SEM). Statistical differences were calculated using Student's t-test using the Prism software where $P<0.05$ was deemed statistically significant.

4.4 Results

CD109 decreases ALK5 levels while increasing ALK1 levels in human primary articular chondrocytes.

Our group has previously reported that CD109 decreases TGF-β receptor levels in human keratinocytes (Bizet, 2011). In order to determine the effect of CD109 on TGF-β receptor levels in human chondrocytes, we inhibited CD109 expression using a siRNA approach in normal primary chondrocytes. CD109 siRNA transfected chondrocytes show significantly reduced CD109 levels as compared to control siRNA transfected cells (Fig. 4.1A). Blocking CD109 displays a significant increase in ALK5 levels ($p<0.05$) and a moderate decrease in ALK1 levels (Fig. 4.1 A and B). It has been reported that the increased ratio of ALK1 to ALK5 receptor levels alters the balance from TGF-β/ALK5/Smad2/3 canonical pathway to TGF-β/ALK1/Smad1/5 and exerts opposing effects in chondrocyte function (Bush and Beier, 2013). Therefore, we also compared the ALK1 to ALK5 levels in our study and observed that there is a significant decrease in ALK1 to ALK5 ratio in human chondrocytes ($p<0.05$, Fig. 4.1 C). Taken together, the results indicate that CD109 decreases ALK5 levels while increasing ALK1 levels and increasing ALK1 to ALK5 ratio in human articular chondrocytes.

CD109 differentially regulates TGF-β signaling pathways in human chondrocytes.

To confirm that the change in ALK1 to ALK5 ratio leads to differential activation of TGF-β signaling pathways in human chondrocytes, we further investigated the effect of CD109 on TGF-β downstream signaling. CD109's effect on Smad-3 driven signaling was examined using the Smad3-responsive CAGA12-lux luciferase reporter constructs in human normal articular chondrocytes. Blocking CD109 expression significantly increases TGF-β1-induced CAGA12-lux activity as compared to control siRNA transfected cells ($P<0.05$, Fig. 4.2 A) indicating that CD109 inhibits TGF-β1–induced Smad3

transcriptional activity in human chondrocytes. Next, we examined whether CD109 regulates TGF-β1-induced Smads phosphorylation levels in human normal chondrocytes. CD109 knockdown in these cells displays enhanced TGF-β1-induced Smad2 and Smad3 phosphorylation (Fig. 4.2 B). Furthermore, decreased CD109 levels show a significant decrease in TGF-β1-induced Smad1/5 phosphorylation as compared to control cells ($p<0.01$, Fig. 4.2 B and C). These results suggest that the increase in ALK1 to ALK5 ratio leads to a shift from TGF-β canonical pathway towards TGF-β/ALK1/Smad1/5, non-canonical pathway in human chondrocytes.

CD109 differentially regulates ECM proteins and proteases in human chondrocytes

Our group has previously shown that activation of the TGF-β canonical pathway induces ECM protein production while activation of the ALK1/Smad1/5 pathway inhibiting ECM protein production in human chondrocytes (Finnson et al., 2008). Since we observed that CD109 promotes ALK1/Smad1/5 signaling pathway, we set to determine the levels of ECM proteins and proteases in response to CD109 knockdown. Alleviation of CD109 enhances TGF-β1-induced PAI-1 levels in human chondrocytes (Fig. 4.3 A). We also analyzed the conditioned media collected from the transfected cells. The decreased levels of CD109 also increase type II collagen levels while inhibiting MMP13 levels (Fig. 4.3 A).

Moreover, knockdown of CD109 significantly enhances TGF-β1-induced *aggrecan* gene expression (Fig. 4.3 B) while significantly diminishing *MMP13* gene expression and

TGF-β1-induced *ADAMTS5* gene expressions in human normal articular chondrocytes (Fig. 4.3 C and D). Collectively, our data suggest that CD109 inhibits ECM proteins such as PAI-1, type II collagen and aggrecan while increasing levels of proteases such as MMP13 (both at mRNA and protein) and *ADAMTS5* (gene expression).

CD109 promotes ALK5 protein degradation and inhibits ALK5 gene expression in human articular chondrocytes

Our group has previously reported that CD109 promotes TGF-β receptor (ALK5) internalization and degradation in human keratinocytes (Bizet et al., 2011). We further analyzed the mechanisms by which CD109 inhibits the ALK5 protein in human chondrocytes. To determine whether CD109 modulates the ALK5 degradation process, we used MG132 (20uM), a proteasome inhibitor (Qin et al., 2003). Blocking CD109 expression shows a significant increase in ALK5 levels in both untreated and MG132 treated human chondrocytes (Fig. 4.4 A and B). This increase in ALK5 levels is more prominent in human chondrocytes treated with MG132 which indicates that CD109 promotes degradation of ALK5 receptor via the proteosomal degradation pathway.

Effect of CD109 on *ALK5* gene expression has never been studied in chondrocytes and other cell types. We thus further examined effect of CD109 on *ALK5* gene expression in human chondrocytes. Blocking CD109 expression significantly enhances *ALK5* mRNA expression in presence of TGF-β1 ($P<0.001$, Fig. 4.4 C). These results suggest that CD109 promotes degradation of ALK5 receptor and inhibits *ALK5* gene expression in human chondrocytes.

CD109 differentially regulates TGF-β signaling pathways and inhibits ECM protein levels in human OA articular chondrocytes.

So far, we have performed studies on human normal articular chondrocytes to determine the effect of CD109 on TGF-β signaling pathways and chondrocyte function. In order to determine if CD109 function is altered in diseased cells, we used human OA primary articular chondrocytes to analyze the effect of CD109 on TGF-β signaling pathways. CD109 knockdown significantly increases ALK5 receptor levels while decreasing ALK1 levels (Fig. 4.5 A and B) which led to a decrease in ALK1 to ALK5 ratio in human OA chondrocytes (Fig. 4.5 C). Blocking CD109 expression significantly increases *ALK5* mRNA expression ($P<0.05$, Fig. 4.5 D). Moreover, CD109 knockdown increases TGF-β1-induced CAGA12-lux activity as compared to control siRNA transfected cells (Fig. 4. 5 E) indicating that CD109 decreases TGF-β1-induced Smad3 transcriptional activity in human OA chondrocytes. The diminished levels of CD109 increase TGF-β1-induced PAI-1 and type II collagen levels in human OA chondrocytes. In summary, CD109 decreases ALK5 levels, TGF-β1-induced Smad3 transcriptional activity, ECM protein production while increasing ALK1 to ALK5 ratio. Therefore, the mechanisms by which CD109 acts show a similar regulation of TGF-β signaling in both human OA and in normal articular chondrocytes.

4.5 Discussion

Our laboratory has previously identified CD109 as a novel TGF-β co-receptor that negatively regulates TGF-β signaling pathway in human keratinocytes (Bizet et al., 2011;

Bizet et al., 2012; Finnson et al., 2006a). Moreover, our group has demonstrated that CD109 inhibits TGF-β signaling and responses during wound healing and skin fibrosis *in vivo*. Transgenic mice overexpressing CD109 in the epidermis show reduced scarring and fibrosis in skin (Vorstenbosch et al., 2013a; Vorstenbosch et al., 2013b), establishing CD109 as a potent anti-fibrotic and anti-scarring protein. In the current study, we have demonstrated that CD109 acts as a molecular switch from the TGF-β canonical to TGF-β/ALK1/Smad1/5 signaling pathway and regulates ECM synthesis and degradation in human chondrocytes.

Our data from the current study showing that CD109 decreases ALK5 levels, TGF-β1-induced Smad3 transcriptional activity and TGF-β1 induced Smad2 and 3 phosphorylation reveal that CD109 negatively regulates the TGF-β canonical pathway in human chondrocytes. In addition, our results demonstrating that CD109 increases ALK1 levels and TGF-β1-induced Smad1 phosphorylation indicate that CD109 promotes ALK1/Smad1/5 pathway in human chondrocytes. The present results indicate that CD109 shifts TGF-β signaling activity from the canonical ALK5/Smad2/3 pathway to ALK1/Smad1/5 signaling pathway in human chondrocytes. This observation is in agreement with the previous report from our laboratory demonstrating that CD109 acts as a similar molecular switch, shifting the TGF-β/ALK5/Smad2/3 signaling towards TGF-β/ALK1/Smad1/5 pathway in mouse skin. CD109 transgenic mice overexpressing CD109 in the epidermis show diminished ALK5 expression and Smad2/3 phosphorylation while increasing ALK1 expression and Smad1/5 phosphorylation (Vorstenbosch et al., 2016). Previously, we also have demonstrated that endoglin, another TGF-β co-receptor, promotes the TGF-β/ALK1/Smad1/5 signaling pathway while

inhibiting TGF-β/ALK5/Smad2/3 pathway in human chondrocytes (Finnson et al., 2010a). Whether there is an interplay between endoglin and CD109 to promote ALK1/Smad1/5 pathway in human chondrocytes, would be an interesting avenue to explore. It is conceivable that there are other factors involved in differentially regulating the balance between TGF-β/ALK5/Smad2/3 canonical pathway versus ALK1/Smad1/5 pathway depending on the cell-types and context of the tissue, even though skin and chondrocytes appear to follow similar fashion.

Our present data showing that CD109 promotes ALK5 proteasomal degradation in human chondrocytes is in accordance with our previous finding that CD109 promotes TGF-β receptor (ALK5) degradation in skin cells (Bizet et al., 2011). TGF-β receptors are degraded by proteasomal machineries (Kavsak et al., 2000; Kowanetz et al., 2008). However, unlike in skin cells where CD109 predominately regulates ALK5 proteasomal degradation, we have demonstrated for the first time in human chondrocytes that CD109 also inhibits *ALK5* gene expression, indicating that CD109 is a powerful inhibitor of ALK5 in human chondrocytes.

Our data demonstrating that CD109 promotes ALK1/Smad1/5 signaling pathway by significantly increasing ALK1/ALK5 ratio in human chondrocytes indicate that maintaining a fine balance between ALK1 and ALK5 levels is important in chondrocyte function. Our results support that a change in ALK1/ALK5 ratio in chondrocytes modulates TGF-β action and alters chondrocyte function. This idea is supported by another study showing increased ALK1/ALK5 ratio in an OA experimental model where ALK1 overexpression is related to MMP-13 expression, whereas ALK5 is related with aggrecan synthesis and cartilage integrity (Blaney Davidson et al., 2009). Our data

showing that CD109 inhibits ECM protein expression while expression of specific proteases in human chondrocytes suggest that CD109 promotes chondrocytes to exhibit OA-like characteristics. In view of our current findings and other previous reports, it is conceivable that activation of ALK1 pathway promotes chondrocytes to display OA-like characteristics and that CD109 mediates this process via ALK1/Smad1/5 pathway.

Although we observe that CD109's function is not altered in OA chondrocytes compared to the normal cells, it would be interesting to determine the levels of CD109 in OA compared to normal cartilage and establish the clinical relevance of CD109.

Our present data establish CD109 as one of the critical factors that regulates the balance between the ALK5/Smad2/3 and ALK1/Smad1/5 signaling pathways in human chondrocytes. However, the role of CD109 during the OA disease progression needs to be further investigated. In addition, as OA is a joint tissue disease, it will be important to investigate the role of CD109 in joint tissues other than cartilage such as synovial fluid and subchondral bone. Taken together, our present study has demonstrated for the first time that CD109 modulates TGF-β type I receptor levels leading to differential regulation of TGF-β signaling pathways and ECM biosynthesis. Identification of the factors such as CD109 that regulate TGF-β signaling pathways and action in cartilage may provide novel insights into cartilage biology and diseased state.

4.6 Role of the funding source

This study was supported by a CIHR Grants MOP 69060 (to AP) and scholarships from the Fonds de recherche du Québec en Sante and the Arthritis Society (to YC).

4.7 Contribution

Yoon Chi contributed to the conception and design, collection and assembly of the data, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content and final approval of the article.

John Antoniou contributed to the provision of study materials, collection of the data, critical revision of the article for important intellectual content and final approval of the article.

Anie Philip contributed to the conception and design of the study, obtaining funding, the assembly of the data, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content and final approval of the article.

4.8 Conflict of Interest

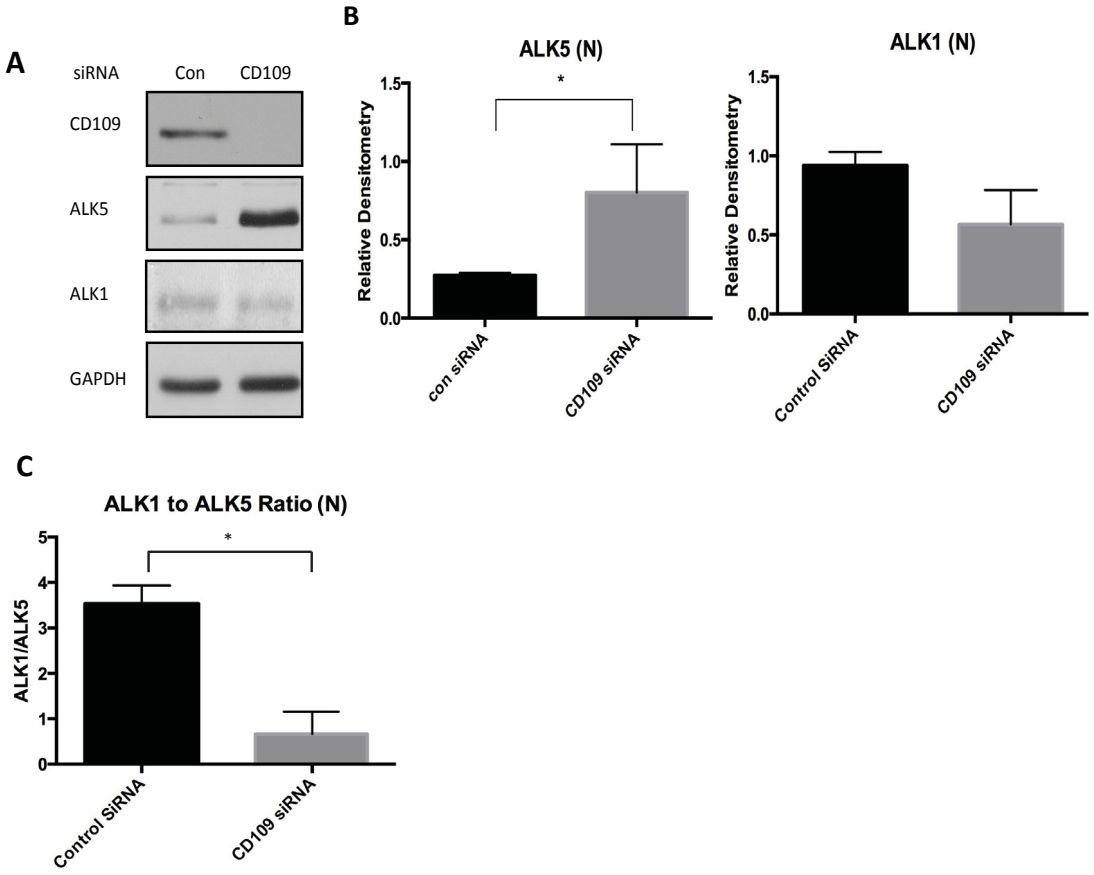
The authors state that they have no conflicts of interest.

4.9 Acknowledgement

We thank our post-doctoral fellow, Dr. Priyanka Sehgal for her constructive feedback and in-depth comments on an earlier version of the manuscript that greatly improved the manuscript.

4.10 Figures

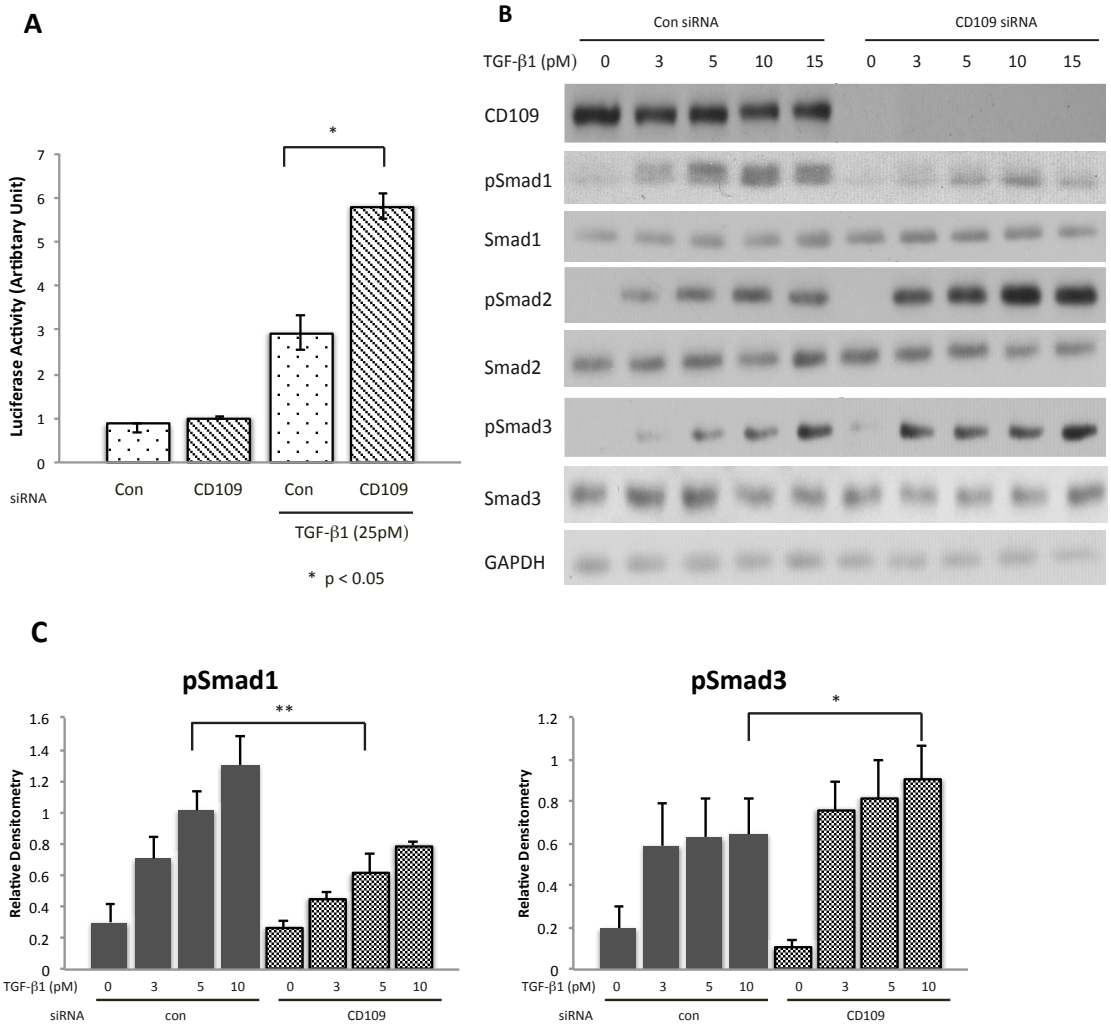
Fig. 4.1: CD109 decreases ALK5 levels and increases ALK1 levels in human primary articular chondrocyte.



Human normal primary articular chondrocytes were transfected with CD109-specific (CD109) siRNA or control (Con). Cell lysates were prepared and analyzed by western blot using **(A)** anti-CD109, anti-ALK5, and anti-ALK1 as indicated. GAPDH was used as a loading control to confirm the equal amount of protein in each lane. **(B)** Densitometry

of ALK5 and ALK1 levels from four independent experiments. ±SEM; *: P<0.05. **(C)** Ratio of ALK1 to ALK5 levels was analyzed from the same four independent experiments. ±SEM; *: P<0.05.

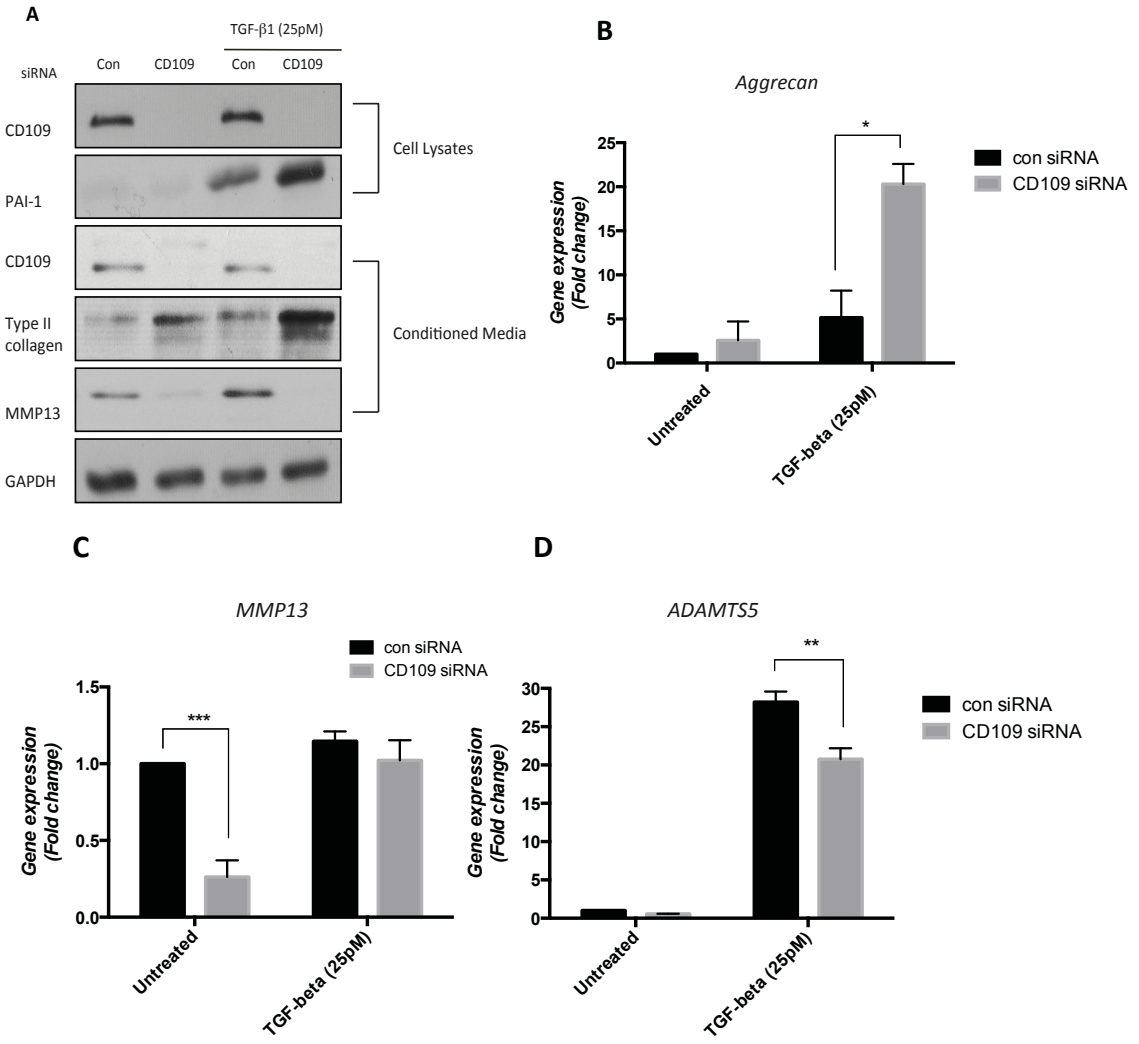
Fig. 4.2: CD109 inhibits TGF-β1-induced Smad-3 transcriptional activity, Smad2 and Smad3 phosphorylation while promoting TGF-β1-induced Smad1 phosphorylation.



(A) Human normal articular chondrocytes were co-transfected with CD109-specific siRNA or scrambled (Con) siRNA along with CAGA-lux and β-galactosidase followed by TGF-β1 treatment (25 pM) for 24 hours under serum-free conditions. Cell lysates were analyzed for luciferase activity and expressed as luciferase activity normalized to β-galactosidase activity. Results shown are representative of four independent experiments. ±SEM; *: P<0.05. **(B)** Human normal articular chondrocytes were transfected with CD109-specific (CD109) siRNA or control siRNA. Cells were treated for 30 minutes

with 0-15pM of TGF-β1 under serum-free conditions. Cell lysates were prepared and analyzed by western blot using anti-phosphoSmad1/5, anti-phosphoSmad2, anti-phosphoSmad3, anti-Smad1, anti-Smad2, anti-Smad3 and anti-CD109 antibodies. GAPDH was used as a loading control to confirm the equal amount of protein in each lane. **(C)** Densitometry of the three independent experiments of (B) at selected time points ±SEM; *: $P<0.05$ **:$P<0.01$.

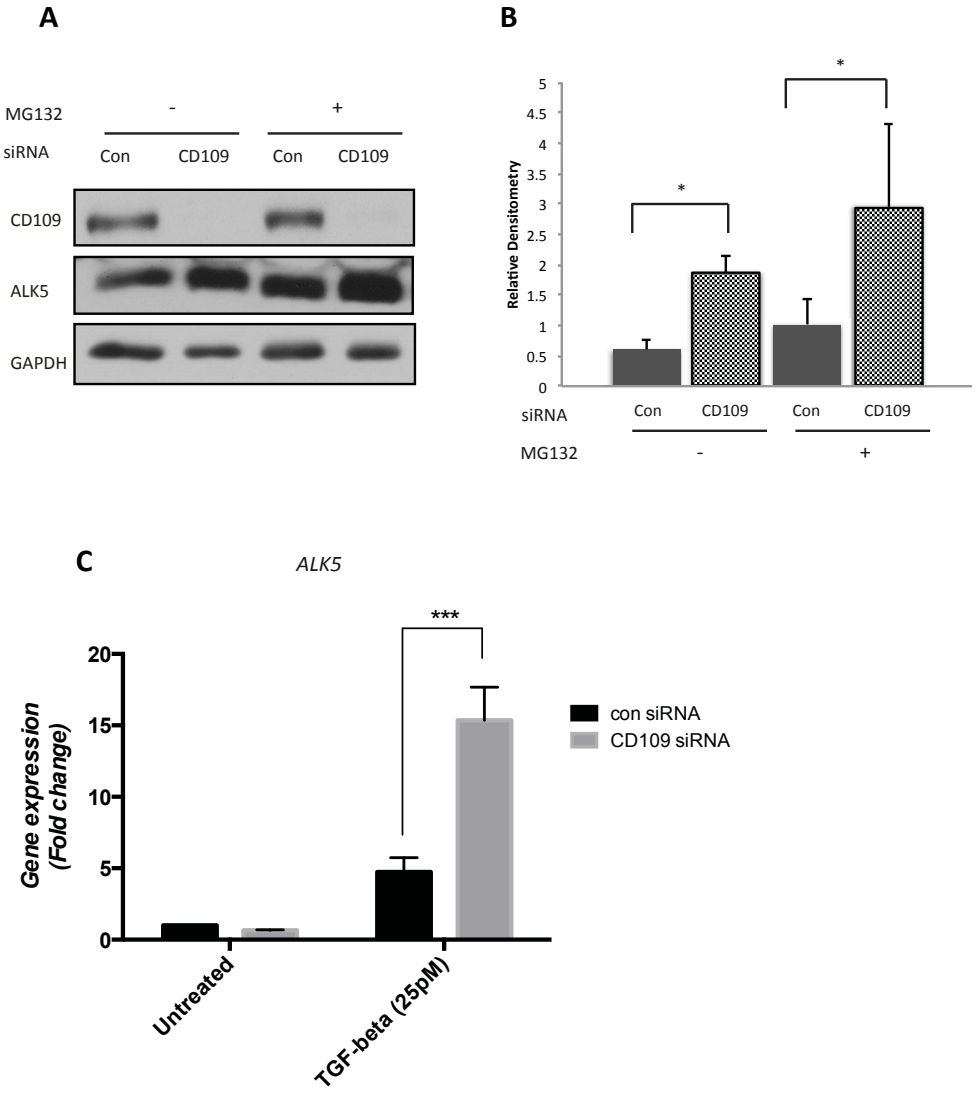
Fig.4.3: CD109 inhibits expression of chondrogenic markers while enhancing protease expression.



Human normal primary articular chondrocytes were transfected with CD109-specific (CD109) siRNA or control siRNA. The transfected cells were treated with TGF-β1 for 24 hours under serum-free conditions for western blot and real-time PCR analysis. **(A)** Cell lysates were prepared and analyzed by western blot using anti-CD109, anti-PAI-1, anti-GAPDH as a loading control to confirm the equal amount of protein in each lane. Conditioned media was collected and concentrated using Amicon Ultra-0.5ml centrifugal filters (Millipore) followed by western blot analysis using anti-CD109, anti-type II

collagen and anti-MMP13. Results shown are representative of four independent experiments. **(B-D)** Total RNA was extracted using TRIzol™ method. The collected total RNA was converted into cDNA for real-time PCR analysis for **(B)** *aggrecan*, **(C)** *MMP13* and **(D)** *ADAMTS5*. *HPRT* gene expression served as an internal control (Mean values of n=3 independent experiments ±SEM; * $P<0.05$, ** $P< 0.01$. *** $P<0.001$).

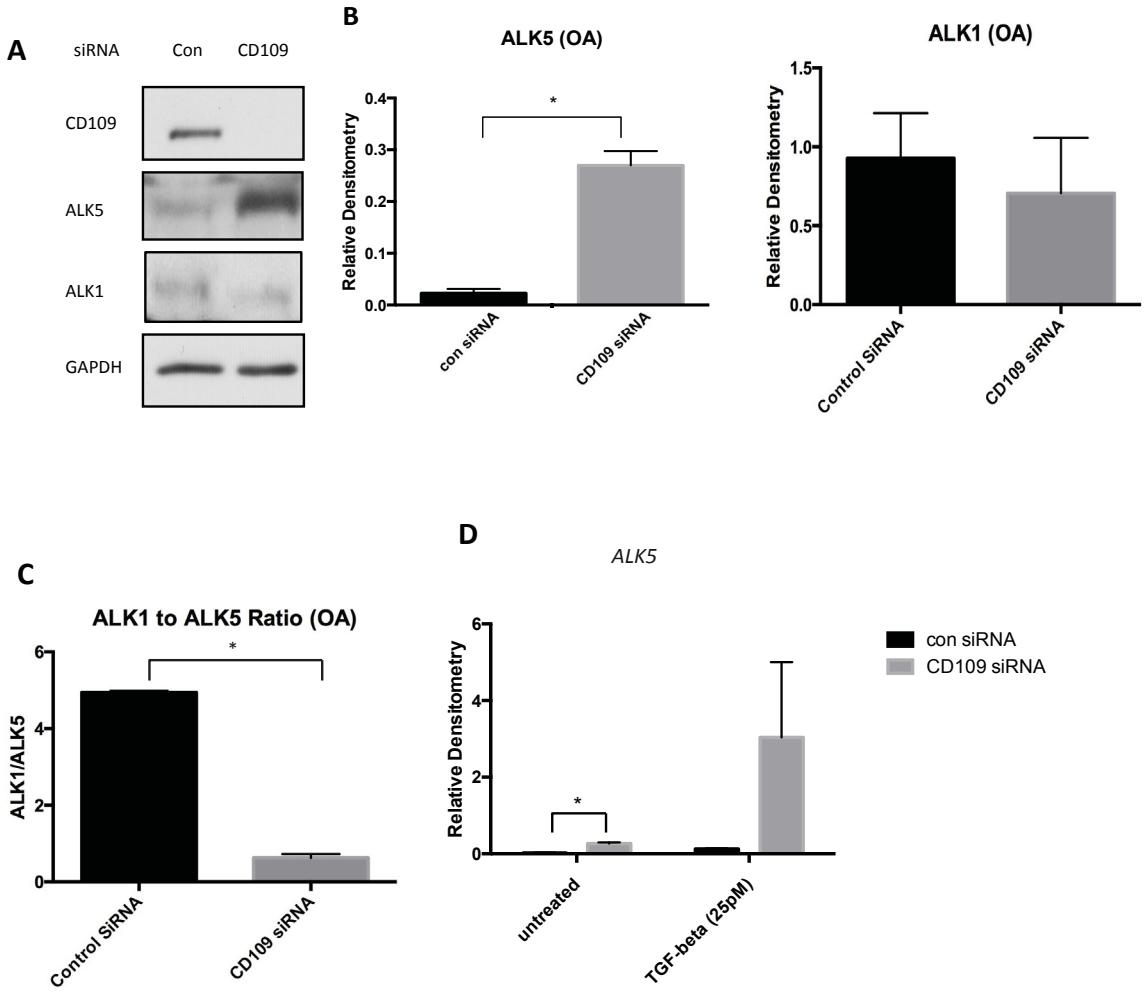
Fig.4.4: CD109 promotes ALK5 protein degradation while inhibiting *ALK5* gene expression.

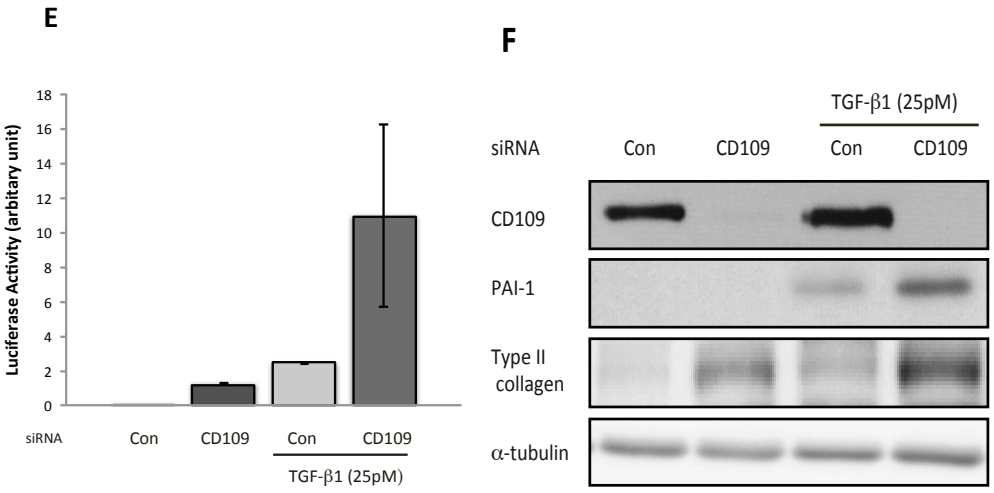


Human normal primary articular chondrocytes transfected with CD109-specific (CD109) siRNA or control (Con) siRNA. **(A)** After 48 hours of transfection, all cells were treated with 20uM for MG132 for 3 hours under serum-free conditions. Cell lysates were prepared and subjected for western blot analysis using anti-CD109, anti-ALK5, anti-GAPDH as indicated. **(B)** Densitometry of n=3 independent experiments, ±SEM; *:

P<0.05. GAPDH was used as a loading control to confirm the equal amount of protein in each lane. **(C)** After 48 hours of transfection, all cells were treated with 25pM of TGF-β1 under serum-free conditions for 24hours. Total RNA was collected and converted into cDNA for real-time PCR analysis for *ALK5* and *HPRT* gene expression, *HPRT* serving as an internal control (Mean values of n=3 independent experiments ±SEM; * P<0.05, ** P< 0.01. *** P<0.001).

Fig. 4.5: CD109 differentially regulates TGF-β signaling pathways and inhibits ECM protein levels in human OA articular chondrocytes.





Human OA primary articular chondrocytes were transfected with CD109-specific (CD109) siRNA or control (Con). Cell lysates were prepared and analyzed by western blot using **(A)** anti-CD109, anti-ALK5, anti-ALK1 and anti-GAPDH as indicated. GAPDH was used as a loading control to confirm the equal amount of protein in each lane. **(B)** Densitometry of ALK5 and ALK1 levels from four independent experiments. ±SEM; *: P<0.05. **(C)** Ratio of ALK1 to ALK5 levels was analyzed from the same four independent experiments. ±SEM; *: P<0.05. **(D)** After 48 hours of transfection, all OA cells were treated with 25pM of TGF-β1 under serum-free conditions for 24hours. Total RNA was collected and converted into cDNA for real-time PCR analysis for *ALK5* and *HPRT* gene expression, *HPRT* serving as an internal control (Mean values of n=3 independent experiments ±SEM; * P<0.05). **(E)** Human OA articular chondrocytes were co-transfected with CD109-specific siRNA or scrambled (Con) siRNA along with CAGA-lux and β-galactosidase followed by TGF-β1 treatment (25 pM) for 24 hours under serum-free conditions. Cell lysates were analyzed for luciferase activity and expressed as luciferase activity normalized to β-galactosidase activity. Results shown are representative of three independent experiments. **(F)** CD109-specific (CD109) siRNA or

control (Con) siRNA transfected cells were treated with 25pM of TGF-β1 for 24 hours under serum-free conditions. Cell lysates were collected for western blot analysis using anti-CD109, anti-PAI-1 and anti-type II collagen. α-tubulin was used as a loading control to confirm equal amount protein in each lane. Results shown are representative of three independent experiments.

5. General Discussion

Homeostasis of articular cartilage is maintained by chondrocytes that are regulated by cytokines and growth factors. Among them, TGF-β is one of the most important anabolic factors for maintaining articular cartilage integrity (Shen et al., 2014b). Aberrant TGF-β signaling pathways has been associated with a number of diseases (Massague et al., 2000), including osteoarthritis (OA) (Zhai et al., 2015).

OA is a chronic degenerative joint disease which is characterized by degradation of articular cartilage, inflammation of the synovium, and osteophyte formation (Troeberg and Nagase, 2011). It is well documented that TGF-β activity is important for cartilage homeostasis. Increased TGF-β activities lead to osteophyte formation in subchondral bone and fibrosis in synovium (Scharstuhl et al., 2003) and diminished TGF-β signaling activities in cartilage induce chondrocyte hypertrophy, one of hallmarks of OA features (Yang et al., 2001b). These studies suggest that the balance of TGF-β activity in joint tissue disease is important.

Previously, our group demonstrated that TGF-β transduces its signal through its canonical pathway, ALK5/Smad2/3 pathway, but it also activates ALK1/Smad1/5 pathway in human chondrocytes. Moreover, that these two signaling pathways exerted opposing effects on chondrocyte function (Finnson et al., 2008). This is congruent with the finding that was observed in endothelial cells where ALK5 inhibits migration whereas ALK1 stimulates migration and proliferation (Seki et al., 2006; Wu et al., 2006). Altogether, these findings suggest that the balanced TGF-β activities regulate the cellular functions. We set to determine factors that differentially regulate the balance between

ALK5/Smad2/3 and ALK1/Smad1/5 signaling pathways. In the current thesis, I have demonstrated that hypoxia, endoglin and CD109 differentially regulate the balance between TGF-β/ALK5/Smad2/3 and TGF-β/ALK1/Smad1/5 pathways and ECM protein productions in human chondrocytes.

Adult articular cartilage is avascular and cartilage's unique hypoxic environment has been well documented (Shen et al., 2014b). Hypoxia is known to play an important role in chondrocyte metabolism (Mariani et al., 2014) and the crosstalk between hypoxia and TGF-β signaling pathway is involved in certain pathological conditions.

My results showing that hypoxia (O_2 less than 2%) inhibits ALK1/Smad1/5 pathway and endoglin whereas hypoxia increases ALK5/Smad2/3 signaling pathway in chondrocytes support my hypothesis that low oxygen tension differentially regulates TGF-β signaling pathways in human chondrocytes. Furthermore, my data indicate that effect of hypoxia on TGF-β signaling pathways is highly cell type specific and context dependent. *Tian et al*. have demonstrated that hypoxia increases ALK1, Smad1/5 and endoglin levels in mouse infarcted ventricles and in human endothelial cells and promotes endothelial cell proliferation (Tian et al., 2010). I have also shown that hypoxia decreases TGF-β1 levels in human chondrocytes. In contrast, *Falanga et al.* demonstrated that hypoxia upregulates TGF-β1 expression in human dermal fibroblasts (Falanga et al., 1991). My data showing that ectopic addition of TGF-β1 decreases HIF-1α levels in human chondrocytes is contrary to the finding in endothelial cells where TGF-β1 has been shown to enhance HIF-1α levels by preventing it from degradation (McMahon et al., 2006). These discrepancies of the effect of hypoxia on TGF-β signaling pathways and TGF-β1 levels

might be attributable to cell-type specificity and context-specific differences.

My results demonstrating that hypoxia enhances ECM protein levels such as type II collagen and PAI-1 in human chondrocytes indicate the potent anabolic effect of hypoxia on chondrocyte function. Consistent with my results, other studies have demonstrated that culturing healthy and OA human chondrocytes or cartilage explants under hypoxia promotes chondrogenic matrix genes such as type II collagen and aggrecan with the increased level of HIF-1α expression (Coyle et al., 2009; Markway et al., 2013; Pfander et al., 2003; Thoms et al., 2013). Moreover, hypoxia has been shown to decrease expression of MMP-1, MMP13 and ADAMTS5 (Strobel et al., 2010; Thoms et al., 2013) in chondrocytes. In summary, multiple levels of evidence support that hypoxia provides more favorable conditions for chondrocyte homeostasis by increasing levels of chondrogenic markers while decreasing levels of catabolic markers in chondrocytes.

TGF-β co-receptors play a critical role in regulating TGF-β signaling and responses. In the current thesis, I have shown that endoglin and CD109 regulate TGF-β signaling pathways in a similar manner in human chondrocytes by inhibiting TGF-β/ALK5/Smad2/3 signaling and ECM protein production while promoting TGF-β/ALK1/Smad1/5 signaling pathway.

My data showing increased endoglin levels in human OA compared to normal cartilage and increased mRNA and protein levels in subculture-induced dedifferentiated primary human articular chondrocytes indicate that endoglin as a potential marker for OA chondrocyte or chondrocyte dedifferentiation. Our results are in line with an earlier report showing endoglin as one of increased cell surface molecules in primary articular

chondrocytes during cell expansion in monolayer (Diaz-Romero et al., 2005), further supporting that endoglin is a potential marker for chondrocyte phenotype or dedifferentiation. In addition, we also show that endoglin and ALK1 share similar function in human chondrocytes. Endoglin siRNA decreases TGF-β1-induced Smad3-driven transcriptional activity which was reversed when ALK1 was overexpressed in human chondrocytes (Finnson et al., 2010a). *Dell'Accio et al.* have found that ALK1 expression was upregulated during *in vitro* expansion of primary human articular chondrocytes (Dell'Accio et al., 2001). I showed that endoglin increases TGF-β1-induced Smad1/5 phosphorylation levels and inhibits type II collagen levels in mouse primary chondrocytes. Endoglin promotes a shift from the ALK5/Smad2/3 pathway in favour of the ALK1/Smad1/5 pathway and decreases ECM protein levels indicating that endoglin promotes chondrocytes to display OA-like characteristics in chondrocytes.

I proceeded to investigate the role of endoglin in cartilage function *in vivo* with the hypothesis that the presence of endoglin promotes cartilage degradation. I used endoglin heterozygote mice as endoglin null mice show embryonic lethality due to cardiovascular malformation (Nomura-Kitabayashi et al., 2009). Since endoglin heterozygous mice have only one allele, I predicted that mice having less endoglin might delay cartilage degradation in a surgical model of OA. However, the histomorphometric analysis of the safranin-o-staining showed no significant difference in cartilage degradation between endoglin heterozygote and WT littermates that were challenged with the DMM surgery. The discrepancy between the *in vivo* and *in vitro* data could be due to numerous factors. *In vivo* system provides more physiologically relevant conditions where different tissues in the joint area are in constant communication and there might be factors released by

different tissues which affect endoglin expression and function. Moreover, heterozygote mice often develop compensatory mechanisms for a missing allele. In order to avoid the limitations of using the heterozygote mice, cartilage-specific endoglin knock out mice generated by approaches such as the cre-lox system in combination of the DMM surgery could represent a better strategy to study the role of endoglin during OA progression *in vivo*.

Our group has shown that CD109, a TGF-β novel co-receptor, antagonizes TGF-β signaling pathway and inhibits TGF-β responses in fibroblasts (Man et al., 2012) and keratinocytes (Litvinov et al., 2011). More specifically, in keratinocytes, CD109 is able to control compartmentalization, internalization and degradation of TGF-β signaling receptors (Bizet et al., 2011). My data in the current thesis have demonstrated that CD109 promotes the ALK1/Smad1/5 signaling pathway while inhibiting the TGF-β canonical ALK5 stimulated signaling pathway in human chondrocytes. Consistent with this, our group has reported that CD109 also differentially regulates TGF-β signaling pathways by promoting TGF-β/Smad1/5 pathway while inhibiting TGF-β/Smad2/3 pathway in the skin (Vorstenbosch et al., 2016). I have demonstrated that CD109 significantly decreases ALK5 receptor by promoting its degradation and also by downregulating *ALK5* gene expression upon TGF-β stimulation. I observed that CD109 increases ALK1 levels and TGF-β1-induced Smad1 phosphorylation, thus indicating that CD109 promotes the ALK1/Smad1/5 signaling pathway in human chondrocytes. CD109 effect on decreasing ALK5 levels while increasing ALK1 levels lead to an increase in ALK1 to ALK5 ratio. It is conceivable that the increase in ratio of ALK1/ALK5 promotes TGF-β/ALK1/Smad1/5

pathway and inhibits TGF-β/Smad2/3 pathway in human chondrocytes. Moreover, our data showing that CD109 increases *MMP13* and *ADAMTS5* gene expression while decreasing type II collagen protein levels and *aggrecan* gene expression indicate that CD109 facilitates chondrocytes to exhibit OA-like characteristics. The next step would be to determine the levels of CD109 in OA compared to normal cartilage to establish the clinical relevance of CD109. Developing cartilage specific CD109 knock out mice and challenge them with the DMM surgery will potentially provide a clear answer to a role of CD109 in OA progression *in vivo*.

Previous results from our group have indicated that betaglycan associates with CD109 in keratinocytes (Finnson et al., 2006b) or endoglin in human chondrocytes (Parker et al., 2003), thus regulating TGF-β signaling pathway. The relative proportion of TGF-β co-receptors may determine the TGF-β canonical versus non-canonical pathways that will elicit different responses in a given cell. It would be interesting to take into consideration the role of all co-receptors present in chondrocytes and to study their functional interplay.

The current thesis has demonstrated that a delicate balance between TGF-β/ALK5/Smad2/3 and TGF-β/ALK1/Smad1/5 signaling pathways determines chondrocyte function. TGF-β/ALK1/Smad1/5 signaling pathway promotes OA-like phenotype in chondrocytes while TGF-β/ALK5/Smad2/3 signaling pathway maintains chondrocyte homeostasis. The imbalance in the activities between TGF-β/ALK1/Smad1/5 and TGF-β/ALK5/Smad2/3 signaling pathways may determine chondrocyte fate.

The notion that TGF-β signaling pathway in chondrocytes switches from the anabolic

ALK5/Smad2/3 pathway to the catabolic ALK1/Smad1/5/8 pathway during OA progression is further supported by other studies. *Davidson et al.* have shown that ALK1 signaling pathway correlates with the increased MMP13 expression in experimental mouse model of OA indicating that ALK1/Smad1/5 signaling pathway promotes chondrocytes to exhibit OA-like characteristics (Blaney Davidson et al., 2009). In addition, *Munoz-Felix et al*. have highlighted in their review that the ALK1/Smad1 pathway is involved in the fibrotic process that leads to chronic diseases affecting the kidney, the liver, lung, skin (scleroderma) and joints (arthritis) (Munoz-Felix et al., 2013). Altogether, it is conceivable that activation of ALK1/Smad1/5 pathway leads to a pathological condition and represents a powerful therapeutic target.

Furthermore, I have established low oxygen tension (hypoxia) and TGF-β co-receptors, endoglin and CD109, as critical factors that differentially regulate the balance between the TGF-β/ALK5/Smad2/3 canonical pathway versus the ALK1/Smad1/5 pathway (Fig. 5.1).

Identification of the factors that differentially regulate TGF-β signaling pathways will provide insights into understanding TGF-β function in chondrocytes. Understanding the mechanisms involved in the regulation of TGF-β action in cartilage homeostasis and disease conditions such as OA will provide novel therapeutic approaches for the treatment of OA.

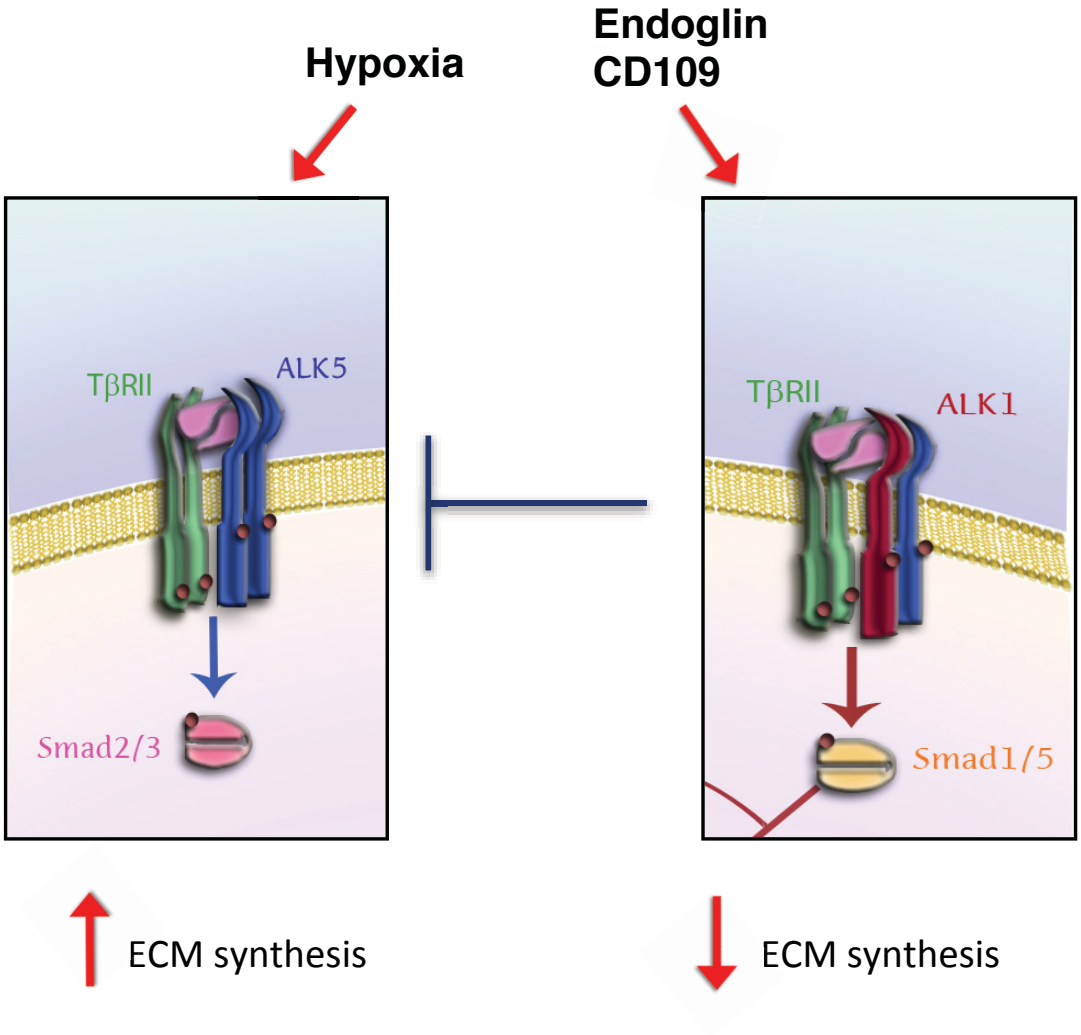


Figure 5.1: Schematic illustration of the factors that regulate the balance of TGF-β/ALK5/Smad2/3 versus TGF-β/ALK1/Smad1/5 signaling pathways in chondrocytes (Schematic diagram created by Dr. Albane Bizet and improvised by Yoon Chi)

Hypoxia, endoglin and CD109 are critical factors in the balance of TGF-β/ALK5/Smad2/3 versus TGF-β/ALK1/Smad1/5 signaling pathways in chondrocytes. We clearly showed that hypoxia promotes chondrocyte function by potentiating the TGF-β/ALK5/Smad2/3 signaling pathway while endoglin and CD109 promotes chondrocytes to exhibit an OA-like phenotype by enhancing the TGF-β/ALK1/Smad1/5 in these cells.

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