EFFECTS OF OSMOLARITY ON OSTEOARTHRITIC HUMAN ARTICULAR CARTILAGE

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Dedicated

To My Beloved

Parents & Teachers

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ABSTRACT

The cell that constitutes the articular cartilage is termed as a chondrocyte. The function of the chondrocyte is the synthesis of the extracellular matrix which comprises of proteoglycans, type II collagen and water. The proteoglycans, carry a high fixed negative charge and attract cations, (mainly sodium) into the interstitial fluid. This gives rise to an osmotic environment which is experienced by the chondrocytes. Osmolarity in the healthy human articular cartilage ranges between 350 – 450 mOsm. The osmotic environment of chondrocytes is dynamic and osmotic perturbations occur in response to the loading regimen and in pathophysiology.

The regenerative capability of articular cartilage is very poor, and consequently lead to the degeneration of the tissue, which is termed as arthritis. Extensive research work and clinical trials illustrate that neither the existing medication nor the therapy accelerates the healing process and this necessitates the repair of the cartilage defect through a surgical replacement. Certain models of the regeneration of articular cartilage has been tested using animal models. The results are promising, but, is suggestive of the fact that there is scope for betterment, which is the basic motivation to perform this research.

In this body of work, we hypothesize that the hyper-osmotic stimulus of 380 mOsm is chondro-protective, phenotype preserving and up regulates the gene expression of the critical genes necessary for the maintenance of homeostasis. To test this hypothesis we study the effect of hypo (280 mOsm) and hyper (380 mOsm) osmotic stimulus on chondrogenic markers such as COL2 and AGC1, TGF-β receptors such as ALK1 and ALK5 and members of BMP family namely; BMP2 and BMP4. Additionally, we study the effects of osmolarity on genes such as CHPF, CHST 13 and B3GAT3, which are involved in the synthesis of the GAG molecules.

Osteo-arthritic human articular cartilage is obtained from three patients undergoing TKR. The tissue is digested with collagenase to liberate to the cells. The de-differentiation experiment is performed, in which the cells are allowed to proliferate, and, parallelly they are cultured in 6 well plates for seven days. The chondrocytes are studied from passage P0 up to passage P2 RNA isolation and purification, c-DNA synthesis and PCR is performed at each passage with both the osmotic conditions. The final results are tested for significance with SPSS.

The hyper osmotic environment, proved beneficial for the chondrocytes. The phenotype was preserved to a certain extent and also the up regulation of the aforementioned genes was observed. For the first time, sensitivity of genes responsible for synthesis of GAG molecules was established and an upregulation in these specific genes was observed.

In a nutshell, chondrocytes showed good adaptation to the hyper-osmotic stimulus. It proved beneficial because the 380 mOsm condition is chondro-protective as it enhances the gene expression of the critical genes and aids in the preservation of the phenotype. This study opens up a new plethora for research with respect to osmolarity in combination with genes responsible for GAG synthesis. Targeting various critical genes, by varying osmolarity within physiological limits, would aid in engineering phenotypically stable in vitro cartilage constructs.

Keywords: Cartilage, Osteoarthritis, Hyper-osmolarity, GAG, TGF-β

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ABBREVIATIONS

A	Adenosine
ACAN	Aggrecan
ACVRL1	Activin A receptor like 1
AGC1	Aggrecan
ALK	Activin like kinase
ALK1	Activin like kinase 1
ALK5	Activin like kinase 5
B3GAT3	Beta 1,3 glucuronyltransferase 3
BMP	Bone morphogenetic protein
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
C	Cytosine
C4ST3	Chondroitin-4 Sulfotransferase 3
CDMPs	Cartilage derived morphogenic proteins
CHPF	Chondroitin polymerizing factor
CHST 13	Carbohydrate sulfotransferase 13
CLD	C-type Lectin domain
COL2	Collagen type II
COL2A1	Collagen type II, alpha 1
CSS2	Chondroitin sulfate synthase 2
CTGF	Connective tissue growth factor
DEPC	Di-Ethyl Pyro Carbonate
DMB	Dimethyl blue
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxy ribo nucleic acid
ECM	Extra cellular matrix
ERK	Extracellular regulated kinase
FBS	Fetal bovine serum
FCD	Fixed charge density
FGF	Fibroblast growth factor
G	Guanine
GAG	Glyosaminoglycan
GalNAc	N-acetyl galactosamine
GlcUA	Glucuronic acid
GOI	Gene of interest
HA	Hyaluronic acid
HPRT1	Hypoxanthine Phospho-Ribosyl Transferase 1

IGD	Inter globular domain
IGF	Insulin like growth factor
IL1	Interleukin 1
JNK	C-Jun N-terminal kinase
KD	Knockdown
ME	Mercapto ethanol
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
OA	Osteoarthritis
OAHAC	Osteoarthritic human articular chondrocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Proteoglycan
PGA	Poly glycolic acid
PI3K	Phosphatidyl inositol 3 kinase
PLA	Poly lactic acid
RNA	Ribo nucleic acid
RT-PCR	Real time polymerase chain reaction
RT-QPCR	Real time quantitative polymerase chain reaction
SD	Standard Deviation
SLRPs	Small leucine rich repeat proteoglycans
SOX9	Sex determining region-Y box 9 gene
SPSS	Statistical package for social sciences
T	Thymine
TGF	Transforming growth factor
TGFBR1	Transforming growth factor beta receptor 1
TIMP	Tissue inhibitor of metalloproteinase
TKR	Total knee replacement

NOTATIONS

Δ	Delta
∴	Therefore
⊢	Implies that
µ	Micro
µl	Micro litre
^0C	Degree centigrade
2D	Two dimensional
3D	Three dimensional
A	Angstrom
c	Complimentary
Ca^{2+}	Calcium ion
cm^2	Square centimeter
Co	Common
CORREL	Correlation function
C_T	Cycle threshold
g	Gram
g	gram
I	Inhibitory
K^+	Potassium ion
l	Litre
M	Molar
m	messenger
ml	Milli litre
mM	Milli molar
mOsm	Milli osmolar
N	Number of patients
n	Number of samples considered per patient
Na^+	Sodium ion
NaCl	Sodium Chloride
P	Passage
R	Receptor
rpm	Rotations/revolutions per minute
STDEV	Standard deviation function
β	Beta

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Chapter I

Introduction

This chapter encompasses the description of the cartilage biology, proteoglycans (PG) and glycosaminoglycans (GAG), osmolarity, Activin Like Kinase (ALK) and Bone Morphogenetic Proteins (BMPs): members of Transforming Growth Factor – beta (TGF-β) superfamily, osteoarthritis (OA) and the concepts in cartilage tissue engineering. The motivation, objective/problem statement and the organization of the report are included towards the end of this unit.

In this project, osteoarthritic cartilage is obtained from patients (three) undergoing total knee arthroplasty. The tissue is digested to obtain cells (chondrocytes), which are cultured in media with varying osmolarity (280 and 380 mOsm) for a period of seven days. The cell culture media with the chondrocytes is then subjected to Ribo Nucleic Acid (RNA) isolation and purification and complementary – Deoxy ribo Nucleic Acid (c-DNA) synthesis. With the c-DNA as the template, gene expression analysis for various genes of interest, such as; chondrogenic marker – COL2 and AGC, GAG synthesizing enzymes, ALK1 and ALK5, and BMP2 and BMP4 is performed with the polymerase chain reaction (PCR). The results thus obtained, are evaluated employing the statistical package for social sciences (SPSS) software to check for significance.

1.1 Cartilage Biology

Cartilage is a specialized connective tissue. It is found in the articular surface of synovial joints, intervertebral discs, ears, respiratory tract and nose. The cartilage present at the aforementioned locations differ in function due to the variation in their biochemical composition. Cartilage is avascular, alymphatic and aneural [1-3]. Barring the osseus junctions and at the synovial surfaces, cartilage is lined by a protective covering called the fibrous perichondrium. Three main types of cartilage exist; hyaline, fibro and elastic cartilage [4-9], as illustrated in figure 1.1. This section elucidates in detail the biology and biochemistry of the articular cartilage.

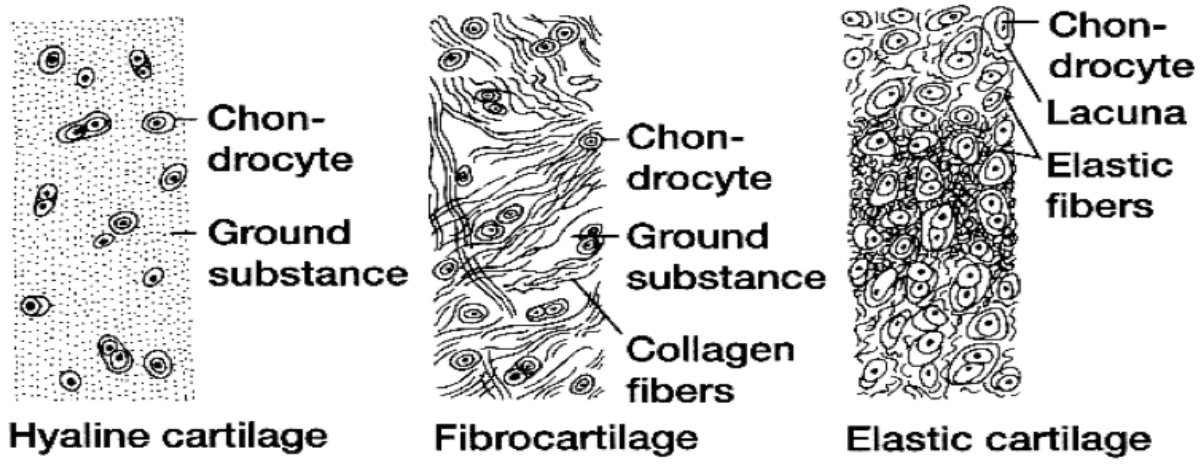

Figure 1.1 The three types of cartilage
[courtesy: http://classconnection.s3.amazonaws.com]

The articular cartilage serves two main functions: (i) to limit the load experienced by the bone extremities, (ii) To provide a smooth surface, for the distribution of the load [3,10-14]. The

energy of the mechanical stress is absorbed by the cartilage due to its viscoelasticity. Fibrous collagen and insoluble PG, constitute the prime components of the articular cartilage. These bestow the tissue with the ability of reverse deformability, which is necessary for its proper functioning [3]. The chondrocyte is the only cell type that populates the cartilage and is responsible for the synthesis and maintenance of the extra cellular matrix (ECM) [15]. Mesenchymal stem cells differentiate to form the chondrocytes [14,16-21].

Chondrocytes are placed within small lacunae in the ECM. This depicts the compartmental arrangement in which the chondrocyte is bounded by the pericellular matrix amidst the network of collagen fibers. The chondrocyte along with its pericellular matrix and the collagenous capsule is termed as a chondron, which is the functional unit of the cartilage. Interaction between the chondrocyte and the ECM is essential for the maintenance of homeostasis [9,22-24].

The components of cartilage ECM are as follows: water (65-80 %), collagen (15-25 %), PGs and non-collagenous proteins (10%). Type II collagen is found extensively in cartilage and is responsible for the tensile strength, while PGs endow compressive strength to the cartilage [9,25-27]. Water content of the cartilage tissue imparts resiliency. The movement of water inside/out of the chondrocyte aid in providing nutrition [15,28,29].

Histological studies reveal that articular cartilage comprises majorly of the ECM with a scanty population of chondrocytes. The ECM molecules and the chondrocytes are arranged in a meticulous fashion, giving rise to the zonal organization of the cartilage tissue. Four zones are identified as follows: superficial, intermediate or mid, deep and calcified zones [9,30], which is represented in figure 1.2

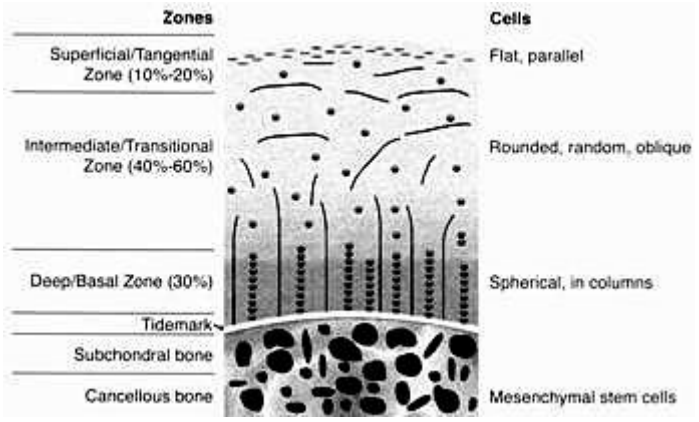

Figure 1.2 Zonal arrangement of cartilage
[courtesy: http://www.azcartilagerestoration.com/images/cart2.jpg]

The superficial zone is characterized by the arrangement of collagen fibrils tangentially. This is the thinnest layer with the parallel alignment of chondrocytes [31]. This zone is constantly bathed by the synovial fluid an accounts for the tensile strength of the cartilage. The middle zone is formed by the PGs and a radial arrangement of thick collagen fibers and accounts for 40-60 % of the total cartilage height. The deep zone is characterized by the highest aggrecan content with the collagen fibers being perpendicularly aligned to the articular surface. The tidemark separated the calcified cartilage from the other zones and aids in the anchorage of the cartilage [9,15,30].

The PGs comprise of polyanionic polysaccharide chains of the GAGs. The GAGs possess a high fixed negative charge density, which attracts water [32], will be discussed in the subsequent sub-heading.

1.2 Proteoglycans

The high aggrecan content is a characteristic of the cartilage. The PGs give rise to the concept of osmolarity, which aids to combat the applied compressive stress. The PG is a polymer, comprised of several monomers or aggregates. Each aggregate is made of a central hyaluronic acid (HA) filament, to which are attached the aggrecan molecules [15].

The core protein of aggrecan comprises three globular regions; G1, G2 and G3. The interglobular domain (IGD) separates G1from G2, while the GAG attachment region separates G2 from G3. The globular regions are stabilized by disulphide bonds. The location of the G1 region is the amino terminus of the core protein. This entails the sites for interaction between the link protein and HA. The carboxyl terminus of the core protein marks the G3 region, and its main function is post-translational aggrecan processing and secretion [33]. Three domains are identified within the GAG attachment region. The domain proximal to the G2 region is the keratin sulfate attachment domain, while CS1 and CS2 are identified as chondroitin sulfate attachment domains [34]. The structure of the PG molecule is sown in figure 1.3

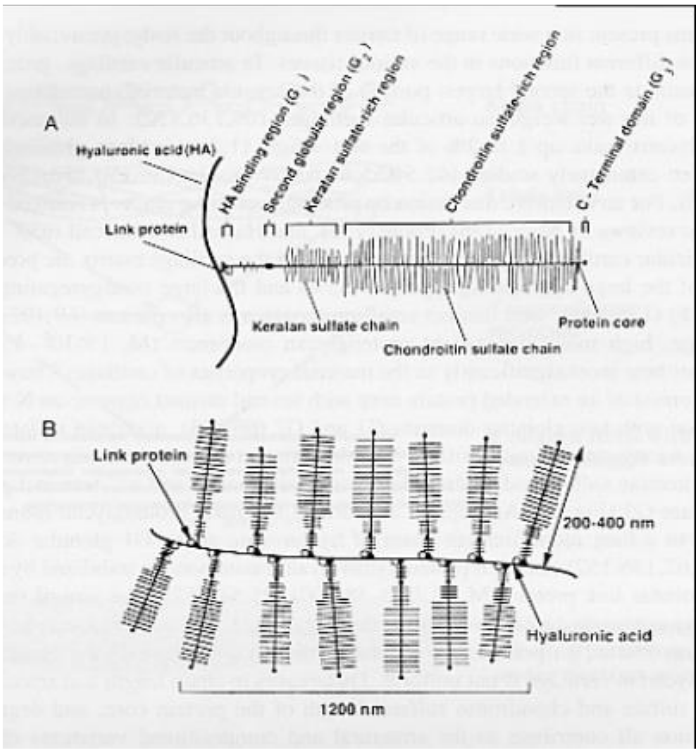

Figure 1.3 Schematic description of aggregating proteoglycan monomer
[courtesy: http://wings.buffalo.edu/academic/department/eng/mae/courses/417-208a.pdf]

The two main functions of the link protein are: (i) attachment of the central filament to the HA, (ii) stabilization of the PG polymer [35]. HA is synthesized by the activity of hyaluronan synthase and is poured into the extracellular space, where the aggrecan molecule is linked to the HA by the link protein [36].

Versican is a PG found in traces in the cartilage [37]. Perlecan which is found in the cartilage, interacts with the ECM molecules and thereby influence the metabolism of the chondrocytes [38,39]

A high concentration of aggrecan is advantageous to the cartilage tissue, as it aids in the resistance of the applied compressive load. Decline in the PG content in the ECM, leads to over-compression of the cartilage. Consequently, this promotes increase in secretion of proteases, thereby leading to degeneration [15,40].

Chondroitin-6-sulfate is a major GAG component in the articular cartilage [41,42]. Traces of keratin sulfate and chondroitin-4-sulfate are also found. The GAGs are termed as long chain hetero polysaccharides, composed of the disaccharide units which are repetitive. A hexosamine and a non-nitrogenous sugar which are linked by a glycosidic bond, make up the disaccharide [43].

The chondroitin sulfate is made of the disaccharide unit which is comprised of glucuronic acid and N-acetylgalactosamine [44,45]. When the sulfate group is attached to C4, the resultant is chondroitin-4-sulfate and when attached to C6, the resultant is chondroitin-6-sulfate. Both these GAGs are present in the articular cartilage tissue [46-50]

Keratin sulfate is composed of N-acetylglucosamine and galactose. Interestingly, it does possess the uronic acid. This is found in cartilage, inter-vertebral disc and cornea [51-54].

The content of GAGs was analyzed by monitoring the amount of radio-labelled sulfate binding to the cartilage. For the first time, in this project, the amount of GAGs will be estimated by the investigation of the response of genes coding for them, namely; Chondroitin Polymerizing Factor (CHPF) – responsible for the polymerization of the chondroitin sulfate, Chondroitin-4-Sulfotransferase-3 (C4ST3 or CHST 13) – responsible for the transfer of the sulfate group and Beta-1,3-Glucuronyltransferase-3 (B3GAT3) – responsible for the transfer of glucuronic acid during biosynthesis of GAGs, in association with various concentration of the culture medium.

The chondrocytes reside in an osmotic cellular milieu, which is controlled by the PG molecules. The PG molecules which are highly, negatively charged, give rise to fixed charge density (FCD), which attracts positively charged ions namely sodium, and water – giving rise to osmolarity [55], which is the topic of discussion in the ensuing segment.

1.3 Osmolarity

The application of stress to the articular cartilage causes a deformation in the matrix, leading to fluid flow within the tissue. This combination brings about a change in the FCD and thereby, the chondrocytes are exposed to changes in the osmolarity [56]. The characteristic feature of GAG is the high negative fixed FCD. This negative charge attracts cations, mainly sodium, and this forms the basis for the determination of the physiological osmolarity [57].

The water moves out of the tissue during loading, causing a deformation of the ECM. This leads to an increase in the concentration of PG, which consequently exposes the chondrocytes to an environment which is hyper-osmotic [58-60]. The extracellular osmolarity is in the range 350-450 mOsm in healthy cartilage [61,62]. The osmolarity varies with the depth of the tissue [62], and this is represented in the table 1.1 adapted from the study by Andrew Hall [63].

Table 1.1 Ionic and osmotic environment of articular chondrocytes

	Na^+ *(mM)*	K^+ *(mM)*	Ca^{2+} *(mM)*	*Osmolarity (mOsm/l)*
Surface zone	240 – 270	7 – 9	6 – 9	310 – 370
Deep zone	300-350	9 – 12	14 - 20	370 – 480
Tissue culture medium	140	5	1.5	280 – 300

By the addition of salts such as NaCl, the osmolarity of the culture media can be varied. The cell culture media have an osmolarity of 260-320 mOsm, while that of human plasma is 290 mOsm [64,65]. It is evident that these ranges of osmolarity are lower than that experience by the cartilage in native state. In this study, the osmolarity of the culture medium is adjusted to the in-vivo osmolarity by addition of NaCl salt solution.

Chondrocytes when exposed to hypo tonicity imbibe water and swell, while hypertonic stimulus leads to osmotic efflux of water, resulting in cell shrinkage. Studies show that at hypotonic conditions, the chondrocytes are weak due to excessive water imbibition and are susceptible to mechanical injury, and conversely the higher extracellular osmolarity showcases a chondro-protective behavior [63] as shown in the figure 1.4.

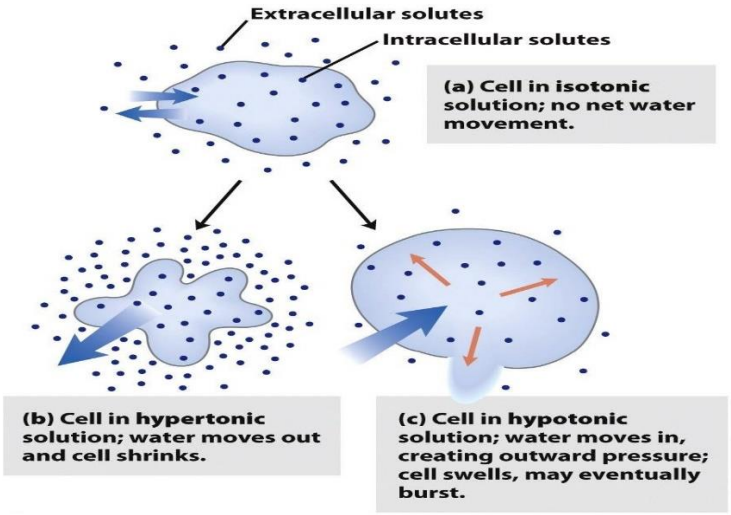

Figure 1.4 Response of chondrocytes to osmolarity
[courtesy: biology-forums.com/index.php]

During joint movement, the osmolarity of the interstitial fluid is altered as the water moves from one zone to another, without the movement of PGs or their fixed ions. As a result, increased osmolarity is observed in areas where water moves out and decreased osmolarity is noticed in regions where water moves in [66].

The articular cartilage in the native state experiences the osmotic pressure, which is dynamic in nature. The osmolarity changes under physiological (application of stress due to joint loading) and pathophysiological (in OA). Due to degeneration of the tissue in OA, the osmolarity is reduced to the range 280-350 mOsm [67-70].

Cell volume regulation in chondrocytes is essential and occurs in many physiological contexts such as (i) cellular swelling of hypertrophic chondrocytes of growth plates, (ii) decrease in chondrocyte number, but increase in cell volume due to aging and exercise, (iii) shrinkage of chondrocytes on account of apoptosis [71-73].

The results from various studies [56-58,61-63,65,67,69-71,74-77] on the effects of osmolarity on chondrocyte cell populations are enlisted in the Table 1.2. From the description, inference can be drawn, favoring the 380 mOsm condition as most suitable for culturing the chondrocyte cell population, due to the fact that the cell number and morphology are preserved along with the upregulation of chondrogenic markers.

Table 1.2 Effect of osmolarity on chondrocyte cell population

Osmolarity (mOsm)	Effects on chondrocyte cell population
280	Significant number of viable cells with normal morphology, increased AGC1 levels
320 - 380	Maximum concentration of cells which appear spherical, marked upregulation of AGC1, SOX9 and COL2 genes, sulfate incorporation and GAG retention, and, suppression of COL1
420 - 480	Cells with depleted growth and limited proliferation ability
550 - 580	Low cell number with abnormal morphology, limited proliferation ability, blebbing, and, upregulation of SOX9 and GAG synthesis

Maintenance of the cartilage homeostasis and repair mechanism is controlled by a number of growth factors. The prime growth factor affecting the cartilage is identified to be the members belonging to the TGF-β superfamily. The next section deals with the elucidation of the roles of TGF-β receptors (ALK1 & ALK5) and BMPs (BMP2 & BMP4).

1.4 TGF-β superfamily

Under this subheading the discussion would comprise of TGF- β and its isoforms, functions in cartilage, signaling pathway, ALK1 and ALK5 receptors and BMPs.

1.4.1 TGF-β: isoforms and functions in cartilage

The TGF-β superfamily consists of 40 ligands. Each of the 40 ligands have a specific function which may not be limited to tissue patterning, homeostasis, bone formation, wound healing and fibrosis [78,79]. In cartilage, the main representatives are TGF-β and BMPs. Both of them are crucial for normal joint development and homeostasis [80].

TGF-β 1,2 and 3, are the three isoforms of TGF-β.They are expressed by the cells in the perichondrium and the chondrocytes which are hypertrophic. TGF-β exerts an influence on the proliferation and differentiation of the chondrocytes [81-83].

The role of TGF-β in cartilage is regarded as controversial [84]. This is due to the fact that it modulates the homeostasis in normal and healthy state and manifests its influence in the pathophysiology of OA too [85]. On one hand, TGF-β promotes chondrogenesis, drives the synthesis of ECM molecules, increases the synthesis of tissue inhibitor of metalloproteinase (TIMP) and decreases the synthesis of MMPs and contradicting to this, increases the secretion of MMPs during OA and drives the chondrocytes towards a hypertrophic lineage [86-90].

1.4.2 TGF-β superfamily signaling pathways

Members of TGF-β superfamily exert its biological effects via canonical (Smad) pathway and also via non-canonical also referred to as non-Smad pathways. In non-canonical pathways, the signals may be transduced via MAP kinase, extracellular regulated kinase (ERK), p38 kinase,

c-Jun N-terminal kinase (JNK), Rho-like GTPase or phosphatidylinositol-3-kinase (PI3K) pathways, to regulate cell function and coordination with the Smad pathway [80]. The emphasis will be laid on the Smad pathway as shown in figure 1.5.

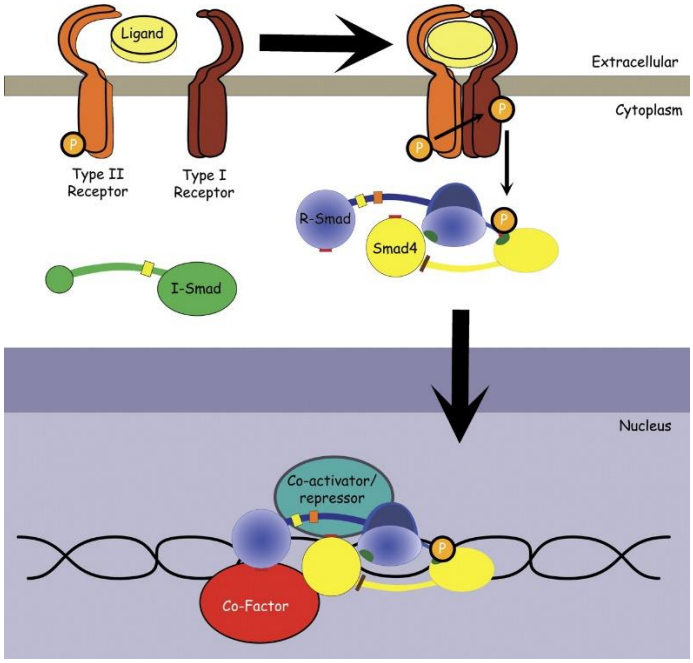

Figure 1.5 Canonical signaling pathway of TGF-β superfamily
[courtesy: http://jap.physiology.org/content/104/3/579]

The signal transduction via the canonical pathway occurs through three components: ligands, receptors (Serine/threonine kinase) and intercellular mediators (Smads). The signal transduction cascade is triggered by the attachment of the ligand to the type II receptor. The type II receptor then trans-phosphorylates the corresponding type I receptor. This leads to the formation of an activated receptor complex which phosphorylates a receptor regulated (R-Smad), which then combines with the common Smad (Co-Smad), Smad4. This Smad complex moves into the nucleus and interacts with the Smad binding partners to regulate transcription, and thereby bring about the regulation of the target genes [91].

For TGF-β the type II receptors would be ACTRII and/or TβRII, while that for BMP it would be ACVRII and/or BMPRII. The type I receptors for TGF-β would be ALK4/5/7 while that for BMP would be ALK2/3/6. TGF-β phosphorylates Smad2/3 while BMP phosphorylates Smad 1/5/8 [80].

In this project the discussion would be focused on ALK1 and ALK5, type I receptors of TGF-β, which will be the highlight in the following subheading.

1.4.3 Alkali Like Kinase (ALK)

ALK1 and ALK5 are type I receptors for TGF-β signaling pathway [80]. ALK5 phosphorylates Smad 2/3 while ALK1 phosphorylates Smad 1/5/8 [92-94].

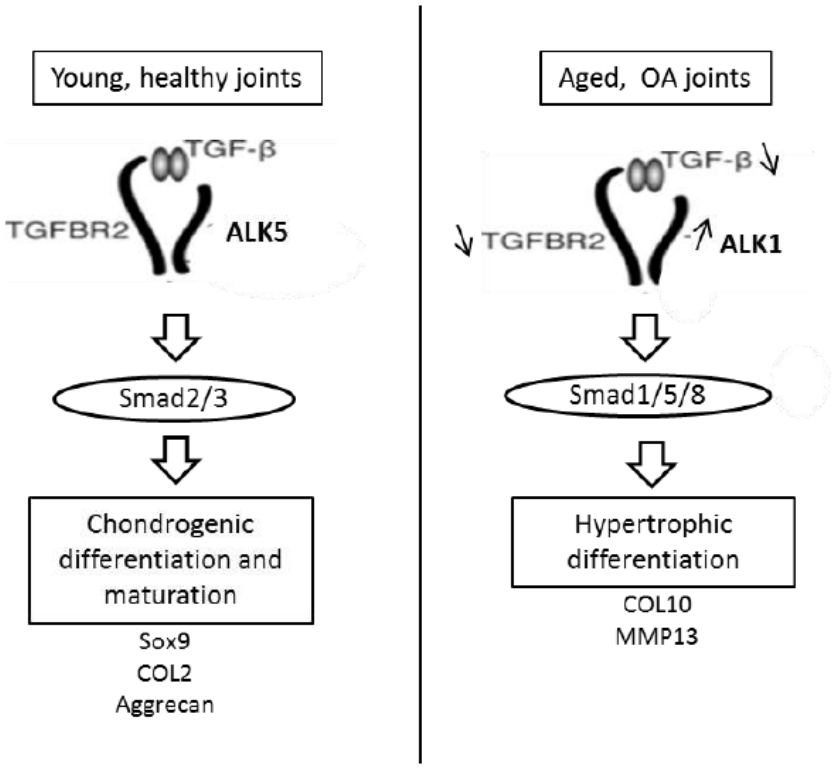

Figure 1.6 Role of TGF-β in healthy and OA cartilage
[courtesy: Catherine Bauge et al, 2014]

The ALK5/ALK1 ratio is of importance in chondrocyte differentiation. Age related reduction in ALK1 is more pronounced than that of ALK5. Studies have shown that the reduction of this ratio could shift chondrocyte differentiation towards more hypertrophic phenotype [92-94]. Figure 1.6, depicts the role of TGF-β in healthy and OA cartilage, throwing light on the concentration levels of ALK1 and ALK5. In this project, investigation to the response of ALK1/5 to varying osmolarity will be studied.

1.4.4 Bone Morphogenetic Proteins (BMP)

Bone morphogenetic proteins (BMPs) are a sub-group of growth factors which belong to the TGF-β superfamily [95]. They represent almost one-third of the TGF-β superfamily and are involved in bone and cartilage formation [96]. Only traces of BMPs are found in healthy normal cartilage [97].

The BMPs regulate chondrogenesis, lineage commitment, promote cell-cell interaction, upregulate SOX9 gene expression and drive the synthesis of ECM molecules [98-102]. If the BMP signaling is blocked in healthy cartilage, it inhibits the chondrocytes from terminally differentiating. This leads to an elevated BMP signaling, which would accelerate the degeneration of the tissue [80]. In this study, focus will be laid on the effect of hypo- and hyper-osmotic stimulus on BMP2 and BMP4.

The strength and function of the cartilage tissue depends on the structural parameters of the chondrocyte and the composition of the ECM. When there is an equilibrium between the synthesis and breakdown, the homeostasis will be maintained, else would lead to arthritis, which is the topic for discussion in the following subheading.

1.5 Osteoarthritis

Healthy and normal cartilage exists in a steady state; which means there us equilibrium between the synthesis and degradation. The loss of this equilibrium leads to the onset of osteoarthritis (OA) [103-108]. OA is a musculoskeletal disorder and is characterized by the destruction of the articular cartilage which affects the entire joint, leading to pain, stiffness and impairment in joint motion [109-112]. OA can happen in the hand, knee, hip and foot. The pathological changes which are noticeable on the radiograph are joint space narrowing, subchondral bone sclerosis and osteophyte formation [15,32,113,114], as shown in figure 1.7

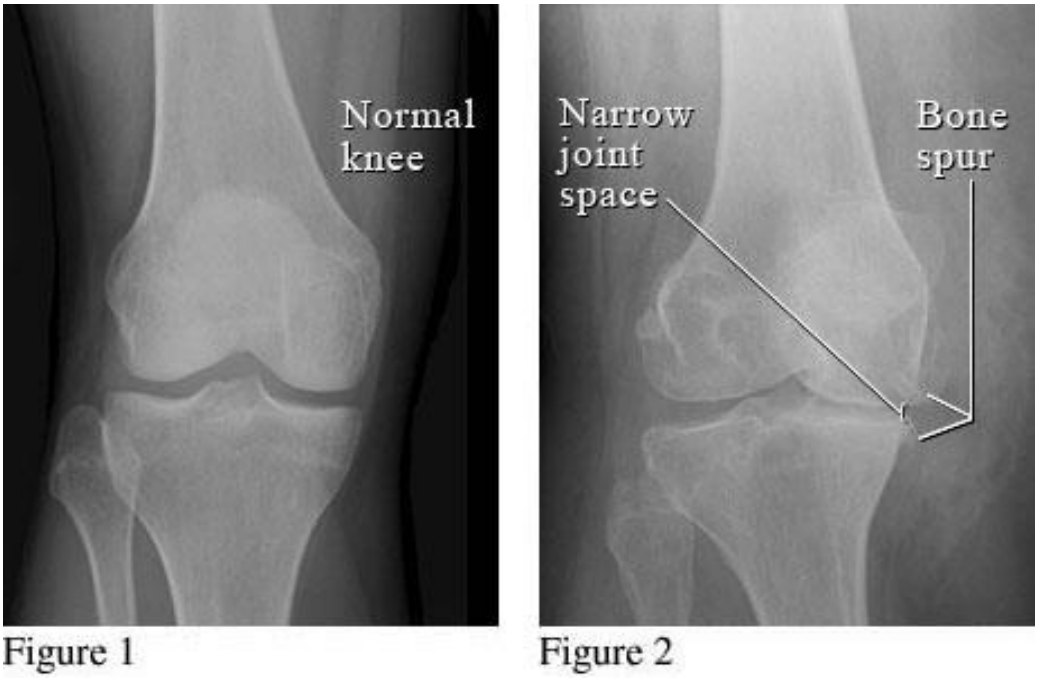

Figure 1.7 Radiological assessment of OA
[courtesy: www.myhealth.alberta.ca]

The biochemical investigation of OA suffers the drawback of; (i) the definition of the disease in biochemical terms is vague, leading to indecisiveness of the severity of the pathology, (ii) tissue sampling [3].

Many types of injury and diverse pathogenic mechanisms can produce in the articular cartilage of all vertebrates a reaction pattern termed OA [116]. The earliest morphological changes characterizing the disease include loss of cartilage elasticity and loss of cartilage flakes from the surface, due to cleavage of the tissue along the horizontally aligned fibrillary planes. Eventually, when the more randomly disposed collagen fibers in the tissue are exposed, deep vertical fissures arise. This process is termed as fibrillation. In severe cases, total erosion of the cartilage with exposure of the underlying bone occurs [117]. The amount of abnormal physical stress, generated by both weight bearing and muscle pull, appears to be an important factor in the induction of cartilage breakdown [118]. Progression of OA is illustrated in the figure 1.8

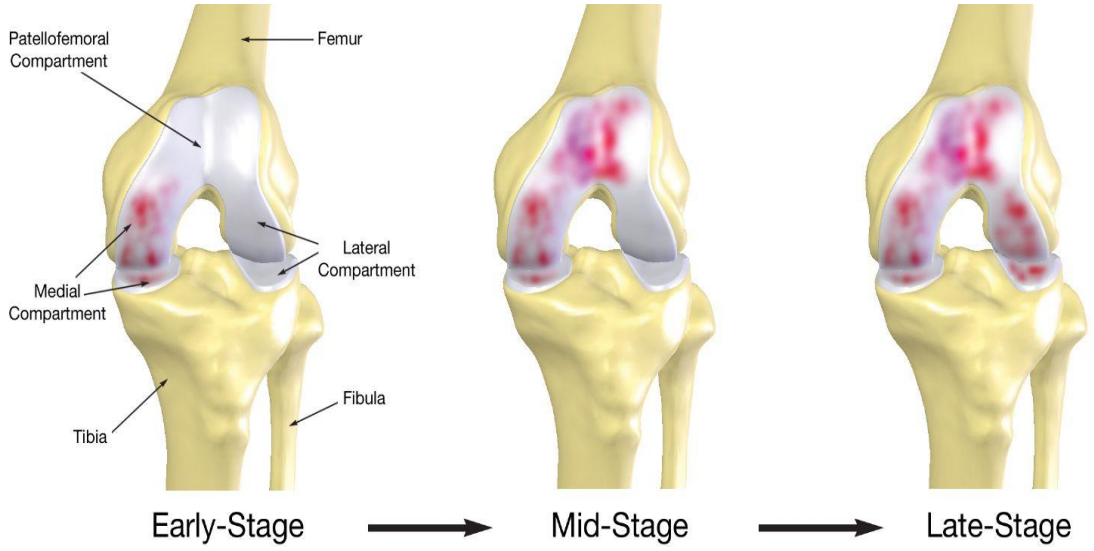

Figure 1.8 Stages of OA
[courtesy: http://www.jupitermed.com/osteoarthritis]

Regenerative capacity of the articular cartilage is very poor and limited [119,120]. The frequently used option for treatment of OA is that of joint replacement [121]. Due to advancement in tissue engineering, the therapies based on the principles of regenerative medicine, surely be an alternate option for joint replacement, and this is the next topic which will be discussed in the succeeding segment.

1.6 Strategies for cartilage tissue engineering

Repair mechanism in articular cartilage is limited. The two problems in cartilage repair are; (i) filling of the defect void with a tissue with the same mechanical properties of the native cartilage, (ii) enhance the integration among the repair tissue and the native cartilage [122]. This can be achieved with the use of triads (cells, scaffolds and culture medium) of tissue engineering [123], which is depicted in figure 1.9

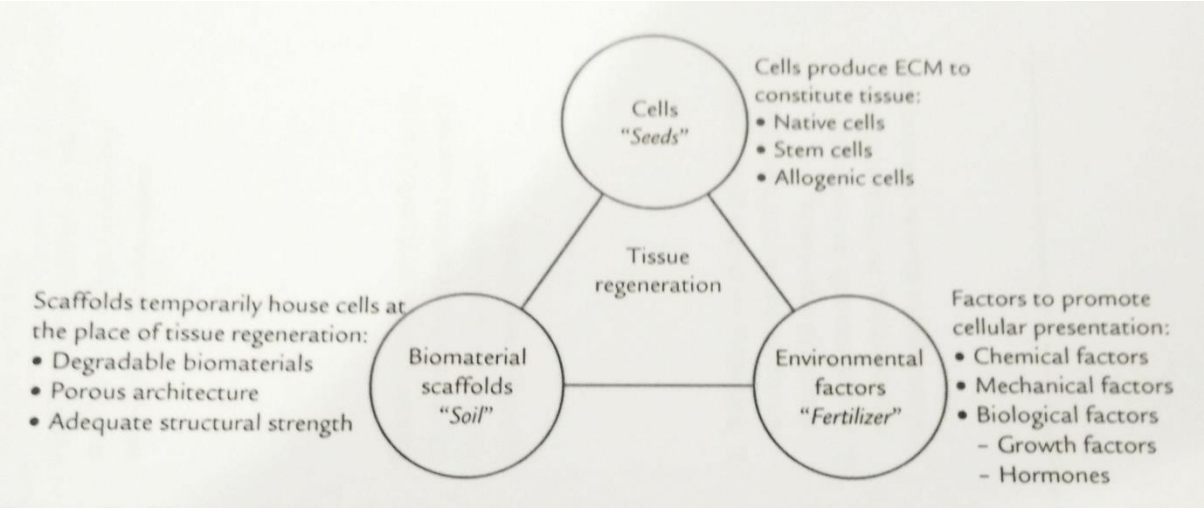

Figure 1.9 Tissue engineering triad
[courtesy: Hongsen Chiang et al, 2009]

Cells, The choice of the stem cell based therapy is in wide use, because of the ease of extraction of the stem cells [124]. The cell population(s) considered for therapy include bone marrow derived MSCs [125], adipose tissue derived MSCs [126,127], synovial membrane derived MSCs [128,129], periosteum progenitor MSCs [130,131], umbilical cord blood MSCs [132], MSCs harvested from trabecular bone, amniotic fluid and Wharton's jelly [133-135], and induced pluripotent stem cells (iPSCs) [136-138]. The inherent problems faced during the culture of the chondrocytes obtained from the aforementioned cell sources are; (i) de-differentiation into fibroblast like cells [139,140], (ii) slow rate of proliferation [141], (iii) loss of phenotype [142].

Scaffolds, The culturing of chondrocytes in scaffolds was employed to solve the aforementioned shortcomings during chondrocyte cell culture. The adoption of bioactive molecules during the culture of chondrocytes in scaffolds enhances the ability of the cells to differentiate and mature, without the loss of phenotypical stability [143]. Poly-glycolic acid (PGA) and poly-lactic acid (PLA) based scaffolds have a positive effect on the chondrocytes by driving the PG synthesis, improving chondrocyte proliferation and maturation [144].

Culture Conditions, The chondrocytes in culture, will possess the ability to synthesize the ECM molecules. Externally growth factors may be incorporated into the culture medium and be administered. This would drive the synthesis of ECM molecules. The chondrocytes in culture, derive their nutrition from the culture medium [145-147]. Additionally, bioreactors may be used to control a multitude of factors like pH and oxygen concentration, which would enhance chondrogenesis as this would mimic the in-vivo environment [148-152].

1.7 Motivation

During the literature survey, the following scientific articles generated an interest to explore deeper, which forms the rationale of this thesis.

- The study by Palmer G et al [56], demonstrated for the first time that aggrecan is affected by osmolarity. From the experiments is it evident that the aggrecan gene expression is best at optimal osmolarity of 280 mOsm, and reduction in gene expression is observed as there is an increase in osmolarity.

- The scientific work by Catherine Bauge et al [80], showed the duality of TGF-β: normal activity due to presence of ALK5 in young, healthy joints and promoting OA due to increased ALK1 in aged, OA joints

- The investigation by Simon R Tew et al [153], established systematically for the first time a set of glycosyl- and sulfotransferase enzymes needed for the synthesis of GAG chains and paved way by opening new vistas of research

1.8 Objectives

In this scientific exploratory work, we propose to evaluate the effects of osmolarity on chondrocytes.

For this purpose, the chondrocytes are obtained from three osteoarthritic patients undergoing total knee replacement surgery. Chondrocyte cell culture experiment is performed, wherein the

chondrocytes are cultured for a period of seven days in six well plates at 280 and 380 mOsm conditions for 3 passages – P0, P1 and P2.

We would like to hypothesize that hyperosmolarity enhances chondrogenic marker expression and is thereby chondro-protective in nature.

The specific goals of this body of work is enlisted as follows:

- To examine and verify the effect of osmolarity on COL2 and AGC, which are widely accepted chondrogenic markers.

- We hypothesize that genes involved in GAG synthesis, namely CHPF, CHST 13 and B3GAT3 are sensitive towards osmolarity and are affected by it. In this context, we wish to determine the response of the aforementioned genes to hypo (280 mOsm) and hyper (380 mOsm) osmolarity conditions.

- We hypothesize that elevated osmolarity increases the activity of ALK5, thereby preventing the progression of OA. In connection to this, we desire to scrutinize the effects of hypo- and hyper-osmolarity on type I receptors (ALK1 and ALK5) of the TGF-β family.

- We hypothesize that osmolarity impacts the activity of BMP2 and BMP4. In connection with this, we examine, if they are up- or down-regulated when exposed to hypo- and hyper-osmotic stimulus.

1.9 Organization of the report

The report is segregated into four chapters. The first chapter is the Introduction, which deals with presenting the basic biology and biochemistry of normal and OA cartilage along with the motivation and objectives. The second chapter is the Research Methodology, which includes the methods and procedures followed to accomplish the objectives. The third chapter is the Results and Discussion, which reveals the results and outcome of the project. The fourth chapter is the Conclusion & Future Scope, which gives the brief summary of the work, conclusions and discusses about the future scope of work.

Chapter II

Research Methodology

This chapter elucidates the procedures involved, accompanied with the block diagram of the entire process. Subsequent sections of the unit provide description of the individual steps mentioned in the block diagram.

2.1 Procedure

The project is divided into various tasks as illustrated in the figure 2.1 which are performed in the same mandate as mentioned.

- Culture medium preparation and osmolarity induction
- Cartilage extraction from the patient undergoing arthroplasty, and consequently, isolation of the chondrocytes
- Isolated chondrocytes are then subjected to chondrocyte cell culture experiments, which involve; expansion of chondrocytes for passaging from P0 to P2, culturing of these cells in monolayers for seven days, and storing the cells
- RNA is to be isolated from the cells in monolayer culture and purified
- c-DNA is synthesized with the RNA as the template, obtained in the previous step
- Gene expression analysis using the Real –Time Quantitative Polymerase Chain Reaction (RT-QPCR) technique with the c-DNA as the starting material, obtained in the preceding stage
- Processing of data obtained from the RT-QPCR, using MS Excel
- Statistical analysis is performed employing SPSS software on the results of the PCR to check the significance of the results

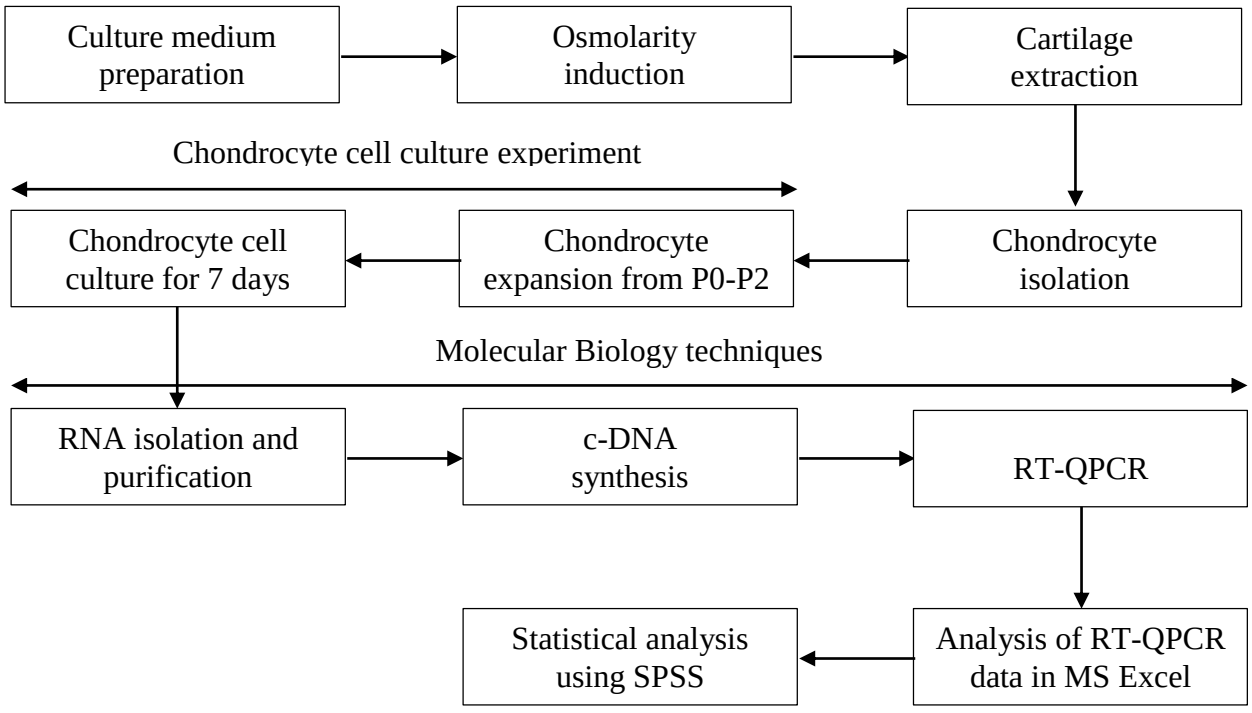

Figure 2.1 Block Diagram of the procedure

2.2 Culture medium preparation and osmolarity induction

The culture medium used for this experiment is Dulbecco's Modified Eagle's Medium (DMEM). It is available in a bottle of 500ml as shown in figure 2.2. Two bottles are categorically used, as the cells need to be exposed to hypo- (280 mOsm) and hyper- (380 mOsm) osmolarity conditions. To each bottle add 10% by volume i.e. 50 ml Fetal Bovine Serum (FBS), 1% i.e. 5ml gentamycin and 1ml fungizone.

Figure 2.2 500ml flask of DMEM
[courtesy: www.thermofisher.com]

In this research work, we use 5M NaCl to induce osmolarity. A 50 ml stock solution of 5M NaCl is prepared (for calculation refer appendix). Add 5.8ml of the 5M NaCl solution, to one of the flasks of DMEM, and label this is as 380 mOsm to avoid confusion. The other flask without 5M NaCl is at the osmolarity of 280 mOsm.

Hence the culture medium is prepared and the osmolarity is induced.

2.3 Cartilage extraction and chondrocyte isolation

After informed consent was obtained, human articular cartilage was harvested from the osteoarthritic knees of three patients undergoing total knee replacement surgery. The harvested cartilage is then chopped into small pieces to increase surface area during tissue digestion. This step is carried out in the flow chamber to maintain sterility. The small chunks of cartilage are weighed, and divided equally into two portions. Each portion is then loaded onto 50 ml tubes: of which one contains 30 ml of 280 mOsm culture medium DMEM, while the other has 30 ml of 380 mOsm culture medium DMEM. For digestion process to be effective, 1.5 mg/ml of collagenase type II enzyme is used. The two 50 ml tubes containing the mixture of cartilage chunks, collagenase type II enzyme and the culture medium is allowed to rest in an incubator for a period of 24 hours at 37^0C.

Consequently, due to the action of the collagenase type II enzyme, the tissue matrix is dissolved and the chondrocytes are now free and floating in the medium provided for their nourishment. The tube with the floating chondrocytes is placed in the flow chamber to proceed further with the isolation of chondrocytes. The culture medium is aspirated into another 50 ml tube through a sieve. The sieve allows the cells and the culture medium to pass through while separating the insoluble debris. The debris on the sieve is washed with the culture medium to free any cells bound to it and direct them into the tube. The insoluble debris and the sieve are now disposed.

2.4 Chondrocyte cell culture experiment

After digestion, the osteoarthritic chondrocytes are obtained. With this as the starting material chondrocyte cell culture experiment is planned. The cells will undergo chondrocyte expansion from passage P0 to passage P2. Similarly, the chondrocytes will be cultured in six well plates for a time period of seven days to perform the RNA isolation and purification, c-DNA synthesis and PCR of the Gene Of Interest (GOI).

The various stages in the cell culture experiment are as follows:

- Cell counting
- Cell seeding and passaging, in culture flasks, starting with passage P0 up to passage P2
- Cell culture in monolayers in six well plates for seven days
- Refreshing cells

2.4.1 Cell counting

Post isolation of chondrocytes, the number of cells need to be counted, to make an effective plan to use the cells maximally for expansion and downstream analyses. The cell count is determined for hypo- (280 mOsm) and hyper- (380 mOsm) osmolarity conditions, by following the procedure mentioned below.

The 50 ml tube containing the floating chondrocytes in the culture medium is subjected to centrifugation at 1700 rpm for 8 minutes, twice. At completion, the supernatant is discarded and the cell pellet is re-suspended in a small volume (2-3 ml) of the culture medium. For the cell counting, 15µl of the cell suspension and an equal volume of the dye dimethyl blue (DMB) is used. This mixture is then loaded onto a counting chamber as shown in figure 2.3

Figure 2.3 Cell counting chamber
[courtesy: http://www.marienfeld-superior.com]

The cell number can be determined using the formula:

$$Cell\ count = \frac{No\ of\ cells\ counted}{16} \times 25 \times 2 \times 10000$$

Where 16 – number of squares counted
02 – dilution factor
25 – correction factor
10000 – 1/volume of suspension

The value thus obtained reflects the number of cells/ml. If this value is multiplied with the re-suspension volume the total cell number is achieved. With the aforementioned protocol, the cell count is hence determined.

For this experiment, the chondrocytes will be cultured for a period of 7 days in six well plates for downstream analyses. Additionally, the cells will be passaged from P0 upto P2 and cultured in medium with varying osmolarity of 280 and 380 mOsm, which is elaborated in the ensuing section.

2.4.2 Cell seeding and passaging

The primary human articular osteoarthritic chondrocytes are cultured for expansion in monolayers (in T175ml flask) at a seeding density of 7500 cells/cm^2 for further passaging. The culturing of cells in the culture flasks is performed till the cells are 85-90% confluent, as shown in figure 2.4. This is performed for 2 passages. Separate flasks are employed for expansion of chondrocytes for the 280 mOsm and the 380 mOsm conditions.

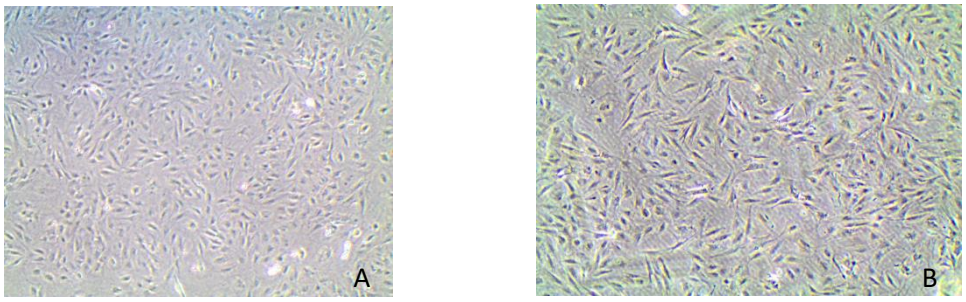

Figure 2.4 Chondrocyte expansion in T175 flask: (A)280 mOsm (B)380 mOsm

After chondrocytes have achieved the desired confluency, cells are removed via trypsin treatment. The culture medium is aspirated completely and discarded. The cells are then washed with Phosphate Buffered Saline (PBS), to extract any left over medium. This is an important step, because, the medium contains trypsin inhibitors, and, if not completely removed the action of trypsin would be ineffective. At this juncture, 2.5-3 ml of trypsin is added into the culture flask and is observed under the microscope to see the rate of detachment and time point (usually within 5 minutes), at which the action of trypsin can be suppressed by the addition of culture medium into the flask. Once the cells have detached, which can be visually inspected under the microscope, the protocol for cell counting is followed, post which, the cells can be seeded in 6 well plate and/or culture flask for further passaging. This process is started with P0 cells and repeated with P1 cells to reach P2.

2.4.3 Cell culture in six well plate

P0, P1 and P2 cells are seeded at 20,000 cells/cm^2 and are cultured for 7 days in 6 well plates, as shown in figure 2.5.

Figure 2.5 Six well plate
[courtesy: biomaterialsusa.com]

Two six well plates are used – one for 280 mOsm condition and the other for 380 mOsm condition. At the end of the 7 day period, the culture medium is discarded. The cells adhering to the wall of the 6 well plate will be used for the downstream analyses.

2.4.4 Refreshing cells

Once the seeding of cells is performed, the culture flasks, with the lids partially unscrewed (to ensure exchange of gases), and the 6 well plates are left in the incubator. The medium is refreshed once in two days with fresh medium to replenish the growing cells with the necessary nutrients. Table 2.1 lists the quantity of medium provided.

Table 2.1 Quantity of medium used

Cell culture vessel	Quantity of culture medium in ml
T175 flask	25
6 well plate	2

2.5 RNA isolation and purification

This section explains the principle and the procedure involved in the RNA isolation and purification. For this purpose, RNeasy Mini Kit, manufactured by Qiagen is employed.

2.5.1 Principle

The RNeasy procedure is an effective technology for the purification of RNA. The basis of this technology is the property of selective binding property of a silica membrane and the speed of the microspin technology. "Biological samples are first lysed and homogenized in the presence of a denaturing guanidine-thiocyanate containing buffer". This immediately deactivates the RNAses to ensure the purification of the intact RNA. The addition of ethanol provides the appropriate binding conditions. The sample is loaded onto an RNeasy mini spin column. Through this, the RNA will bind to the membrane and the impurities will be eliminated. The high quality RNA is thus obtained, which is eluted in 30-100 µl of water [154]. The steps involved in RNA purification is depicted in the figure 2.6

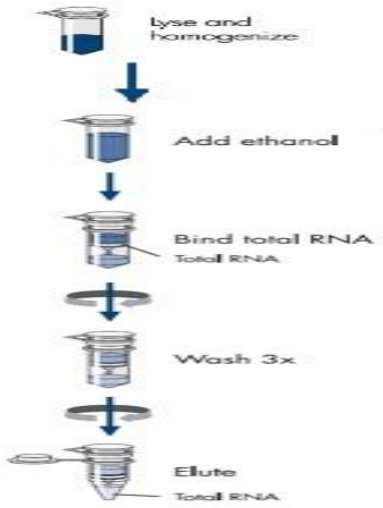

Figure 2.6 Steps in RNA purification
[courtesy: RNeasy Mini Handbook, 2010]

2.5.2 Procedure for RNA isolation

The isolation of RNA is performed according to the protocol provided by the manufacturer [154]. The steps involved in the RNA isolation are enlisted as follows:

1. A mix of 120 µl β-mercaptoethanol (β-ME) and 1.98 ml of RLT buffer is prepared and homogenized
2. The medium in the six well plate is discarded post aspiration
3. Wash the wells with PBS to remove any left over medium
4. To each well, add 350 µl of the mix prepared in step 1
5. Allow to rest for 5 minutes
6. The resultant solution (lysate), containing RLT buffer, β-ME and the disrupted cells are stored in cuvettes

2.5.3 Procedure for RNA purification

The purification of the isolated RNA is performed according to the protocol provided by the manufacturer [154]. The steps involved in the RNA purification are stated as below:

1. The lysate is homogenised for 30s using the rotor-stator homogenizer
2. 50 ml of 70% ethanol solution is prepared
3. An equal volume of 70% ethanol solution prepared in step 2, is added to the lysate
4. Vortex this mixture to ensure homogenization
5. Transfer the entire volume of solution obtained in step 4, to an RNeasy spin column which is placed in a 2ml collection tube
6. Centrifuge for 15s at 10000 rpm and discard the flow through
7. 350 µl of RW1 buffer is added, centrifuged for 15s at 10000 rpm and the flow through is discarded
8. DNAse I solution is prepared by dissolving 10µg of lyophilized DNAse I in 550 µl of RNAse free water and mix gently by inverting the vial
9. DNAse I mixture is prepared by adding 10 µl DNAse I solution to 70 µl of RDD buffer and centrifuge gently
10. 80 µl of DNAse I mixture prepared in step 9, is added directly to the RNeasy spin column membrane and allow to rest for 15 minutes
11. Repeat step 6
12. Add 500 µl of RPE buffer and centrifuge for 15s at 10000 rpm and discard the flow through
13. Repeat step 12 and centrifuge for 2 minutes
14. The RNeasy spin column is placed in a new 2 ml collection tube and centrifuge for 1 minute at 24000 rpm
15. The RNeasy spin column is placed in a new 1.5 ml collection tube and 50 µl of RNAse free water, is added directly to the spin column membrane
16. Centrifuge for 1 minute at 10000 rpm
17. The concentration of RNA is determined by measuring the absorbance at 260 nm
18. The ratio of A_{260}/A_{280} is determined, which is used to estimate the purity of RNA

By following the aforementioned procedure the RNA is isolated and purified. Additionally, the concentration and purity of RNA is calculated and estimated respectively. This forms the starting material for the synthesis of c-DNA.

2.6 c-DNA synthesis

This unit enlists the principle and the procedure involved in the c-DNA synthesis. For this purpose the RevertAid first strand c-DNA synthesis kit, manufactured by MBI Fermentas is employed.

2.6.1 Principle

c-DNA is synthesized from the mRNA as the template. This reaction is catalyzed by the reverse-transcriptase and DNA polymerase enzymes. This is adopted once there is a need, to express a specific protein in a cell, which otherwise is not expressed, the c-DNA will code for the specific protein, which will be transferred to the target (recipient cell) [155].

2.6.2 Procedure

The c-DNA synthesis is performed as per the protocol provided by the manufacturer [156]. The procedure is elucidated as follows:

1. The reaction mixture comprising of RNA and the primer is prepared.
2. To the mixture in step 1 add DEPC treated water to attain a volume of 12 µl
3. Homogenize the reaction mixture by mixing gently and centrifuge for 5s in a micro-centrifuge
4. Add 4 µl of 5X reaction buffer, 1 µl of ribo-nuclease inhibitor and 2 µl of dNTP mix to the solution obtained after homogenization
5. Repeat step 3
6. Incubate at 37^0C for 5 minutes
7. Add 1 µl of reverse transcriptase and incubate at 42^0C for 60 minutes
8. The reaction is stopped by heating at 70^0C for 10 minutes and then chill on ice

By following the aforementioned procedure the c-DNA is synthesized. This will be utilized in the RT-QPCR explained in the next segment.

2.7 Real-Time Quantitative Polymerase Chain Reaction

This segment describes the principle and the procedure involved in the PCR. For this purpose the PCR kit, equipment and software manufactured by Bio-Rad is employed.

2.7.1 Principle

Real-Time Quantitative Polymerase Chain Reaction (RT-QPCR), measures the PCR product at the end of every cycle. This enables the precise determination of the initial amount of the target which will be required [157].

PCR amplifies the DNA exponentially. The number of molecules are doubled, with every amplification cycle. In RT-QPCR, the amount of DNA is measured by the use of fluorescent markers. The strength of the fluorescence signal is directly proportional to the number of PCR product molecules or amplicons that are generated [157].

2.7.2 Procedure

The RT-QPCR is performed as per the protocol devised at the Department of Orthopedics, Erasmus University Medical Center. The steps are listed as below:

1. Prepare a premix comprising of 20X primer (PCR primer sequence shown in table 2.2), 2X SYBR Green master mix and RNAse free water
2. Prepare the premix on ice and centrifuge gently for homogenization
3. Prepare a plate layout of the PCR to be performed, using MS Excel
4. Add 2 µl of the c-DNA sample and 8 µl of the premix to each well of the plate
5. Seal the plate and centrifuge gently
6. Place the sealed PCR plate onto the CFX 96 Touch^{TM} Real Time PCR Detection System, manufactured by BioRad Laboratories
7. Adjust the settings to SYBR Green option and start the PCR

HPRT1 is used as a housekeeping gene in this experiment. The data analysis is explained in the subsequent unit.

Table 2.2 PCR Primer Sequence

Gene of Interest	Oligonucleotide Sequence (5'-3')	Reference
HPRT1	Forward – TGCAGACTTTGCTTTCCTTGGTCAGG Reverse - CCAACACTTCGTGGGGTCCTTTTCA	158
COL2	Forward – GGCAATAGCAGGTTCACGTACA Reverse - CGATAACAGTCTTGCCCCACTT	159
AGC	Forward – TCGAGGACAGCGAGGCC Reverse - TCGAGGGTGTAGCGTGTAGAGA	159
B3GAT3	Forward - TAGGGGAGTGCGAGGAAGG Reverse - GACACCAGGAAGTAGGCGAG	-
CHPF	Forward - AGTCCGAGACTTTCTCTGGG Reverse - AGCCGGTTTAGGACACAGTC	-
CHST 13	Forward - TTTTCAACTACTCCGCCCCC Reverse - GGTCTTGTCGGAAAGGCACT	-
ALK1	Forward – TTAAAAGGCGCAACCAAGAAC Reverse - GTGGTGATGAGCCCTTCGAT	160
ALK5	Forward – GACATCAATCTGAAGCATGAGAACA Reverse - GGCGGTGATCAGCCAGTATT	160
BMP2	Forward - AACACTGTGCGCAGCTTCC Reverse - CTCCGGGTTGTTTTCCCAC	-
BMP4	Forward - GGATGTTCTCCAGATGTTCTTCGT Reverse - TCCACAGCACTGGTCTTGAGTATC	-

2.8 PCR data analysis in MS Excel

The C_T value of the Gene of Interest (GOI) and the housekeeping gene is obtained. The housekeeping gene used is HPRT1 and the control is P0-280 mOsm condition. The data is processed using the $2^{-\Delta C}{}_T$ method [161]. The steps in the data analysis is as follows:

1. C_T value of the GOI is obtained in duplicates for each condition and is tabulated
2. C_T value of the housekeeping gene is obtained and is tabulated
3. The difference in the C_T value is computed as $\Delta C_T = C_T\ (GOI) - C_T\ (HPRT1)$
4. Compute $2^{-\Delta C_T}$ for each sample in every condition
5. Calculate the average of the sample for each condition using the AVERAGE function
6. Normalize all samples in each condition to the control which is P0-280 mOsm condition
7. Repeat step 5
8. Compute the standard deviation for each condition using the STDEV function
9. Repeat step 1-8 for the cartilage sample obtained 3 patients
10. For pooling data, calculate the average value of each condition, obtained from 3 patients
11. Compute the mean SD of each condition, obtained from 3 patients, using the formula $SD_{mean} = \sqrt[2]{(SD_1^2 + SD_2^2 + SD_3^2)/3}$
12. Using the values obtained in step 10 & 11, plot a bar graph of normalized ΔC_T expression versus osmolarity, using Graph-Pad software

2.9 Statistical analysis

Statistical verification was performed using the SPSS tool. The samples obtained from three donors (N=3) were tested for all the genes of interest (GOI) mentioned above. Each condition was performed in duplicate (N=3, n=2, N'=6).

The effect of the experimental conditions was tested using the mixed effect linear regression model. By adopting this method, the correlation in the data, that exists within each donor was accounted. In the software, donor was incorporated as a random effect to correct for basal differences in the specific expression between patients. Experimental conditions were introduced as a fixed effect. P values ≤ 0.05 were considered significant, which are enlisted in the table 2.3

Table 2.3 Statistical significance with p-values

p-values	Symbolic representation
≤ 0.05	*
≤ 0.005	**
≤ 0.001	***

Chapter III

Results and Discussion

This chapter lists the results obtained due to sequentially following the protocols. This is then followed by the discussion, where the obtained results will be validated.

3.1 Results

This section presents the outcome of the chondrocyte cell culture experimental study performed on the human osteoarthritic cartilage obtained from three patients. P0-280 mOsm condition is chosen as the control and the results are normalized maintaining this as a reference point.

3.1.1 Chondrogenic markers

The osteoarthritic chondrocytes cultured in varying osmolarity showed good adaptation to osmolarity. The chondrogenic markers tested are COL2 and AGC1 as shown in figure 3.1. It can be noted that both the chondrogenic markers are upregulated in the 380 mOsm condition.

In the case of COL2, there is a decrease in the gene expression as the passage number increases, which is due to the change in phenotype. But the increased osmolarity has preserved the gene expression of COL2 to some extent.

AGC1 gene shows upregulation in the 380 mOsm condition.

Hence, it can be deduced that hyper-osmolarity is enhancing the expression of chondrogenic markers, which are essential for synthesis of the ECM proteins and, in the maintenance and preservation of the chondrogenic phenotype.

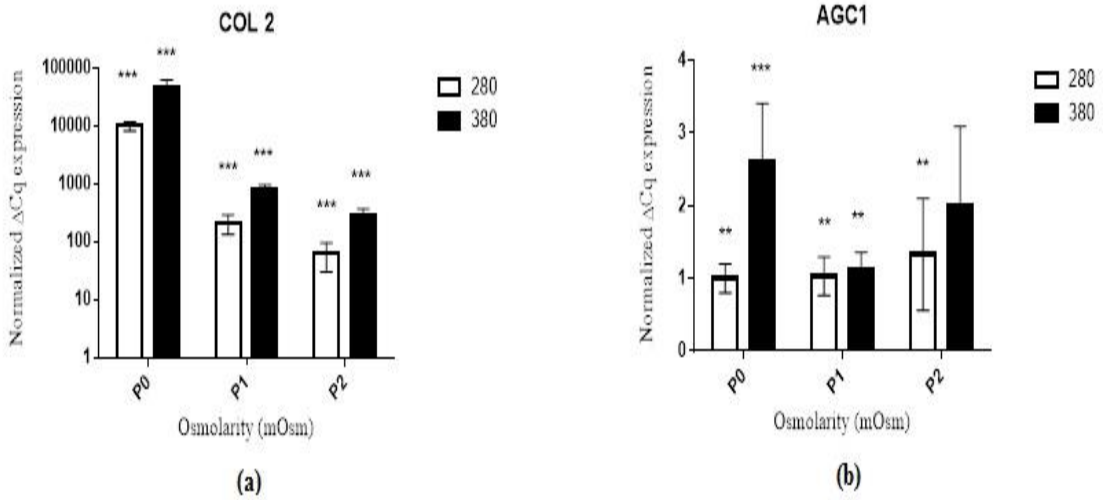

Figure 3.1 Chondrogenic markers **(a)**COL2 **(b)**AGC1

3.1.2 GAG synthesizing enzymes

Previously, the cartilage samples tested for AGC1, have reported to be sensitive towards osmolarity. This section deals with the outcome of the experiment with respect to the genes

controlling the synthesis of GAGs. The genes tested are CHPF, CHST13 and B3GAT3 as shown in figure 3.2

All the genes are upregulated in the hyper-osmotic stimulus i.e. the 380 mOsm condition. Another important revelation is that, initially the gene expression is at a minimum, and, as the passage number increases, there is an increase in the quantity of the gene expression.

Once again, it can be inferred that hyper-osmotic stimulus is favorable for the maintenance of the chondrogenic phenotype, as it promotes the production of GAGs, by increasing the expression of the GAG synthesizing genes.

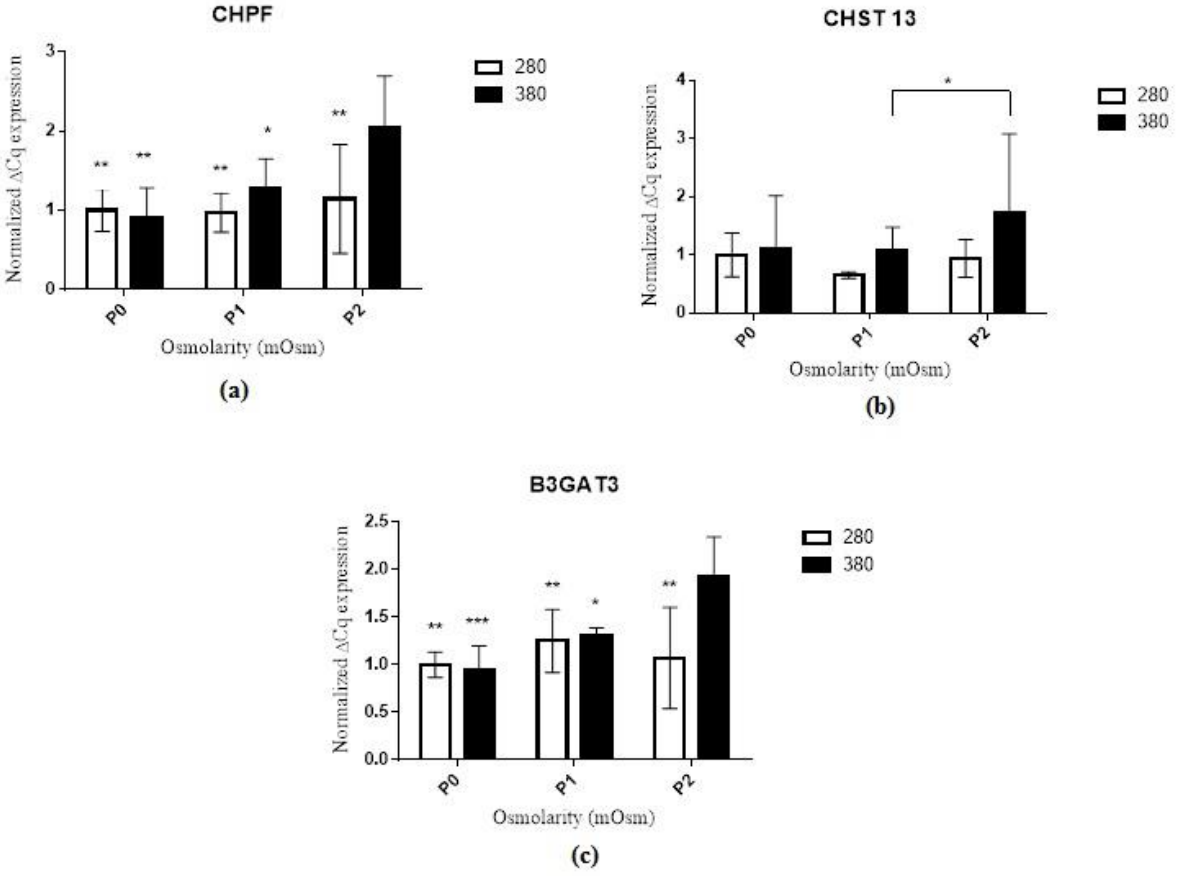

Figure 3.2 Genes for GAG synthesis **(a)**CHPF **(b)**CHST13 **(c)**B3GAT3

3.1.3 TGF-β type I receptors

This segment deals with the testing of ALK1 and ALK5 with varying levels of osmolarity, as shown in figure 3.3

ALK1, the receptor of TGF-β, is said to be increased in OA. But when tested with varying osmolarity, it is seen that passaging of the OAHAC lead to an increase in the level of gene expression. However the gene expression of ALK1 is downregulated in the 380 mOsm condition.

ALK5 is the receptor of TGF-β, which is found in normal and healthy joints. It is upregulated in the 380 mOsm condition. Inference can be drawn that, the hyper-osmotic stimulus accentuates the activity of the ALK5 receptor to help maintaining homeostasis.

In a nutshell, the 380 mOsm condition suppresses the ALK1 and upregulates the activity of ALK5 to prevent progression of OA and help in preserving steady and constant internal environment respectively.

The ALK5:ALK1 ratio decreases as the passage number increases, and this can be corroborated with the fact that there would be a loss of chondrocyte phenotype and the chondrocytes would have turned hypertrophic. This ratio is upregulated in the 380 mOsm condition, which is a pointer to the fact that the hyper-osmolarity is favorable for the culture of chondrocytes in vitro.

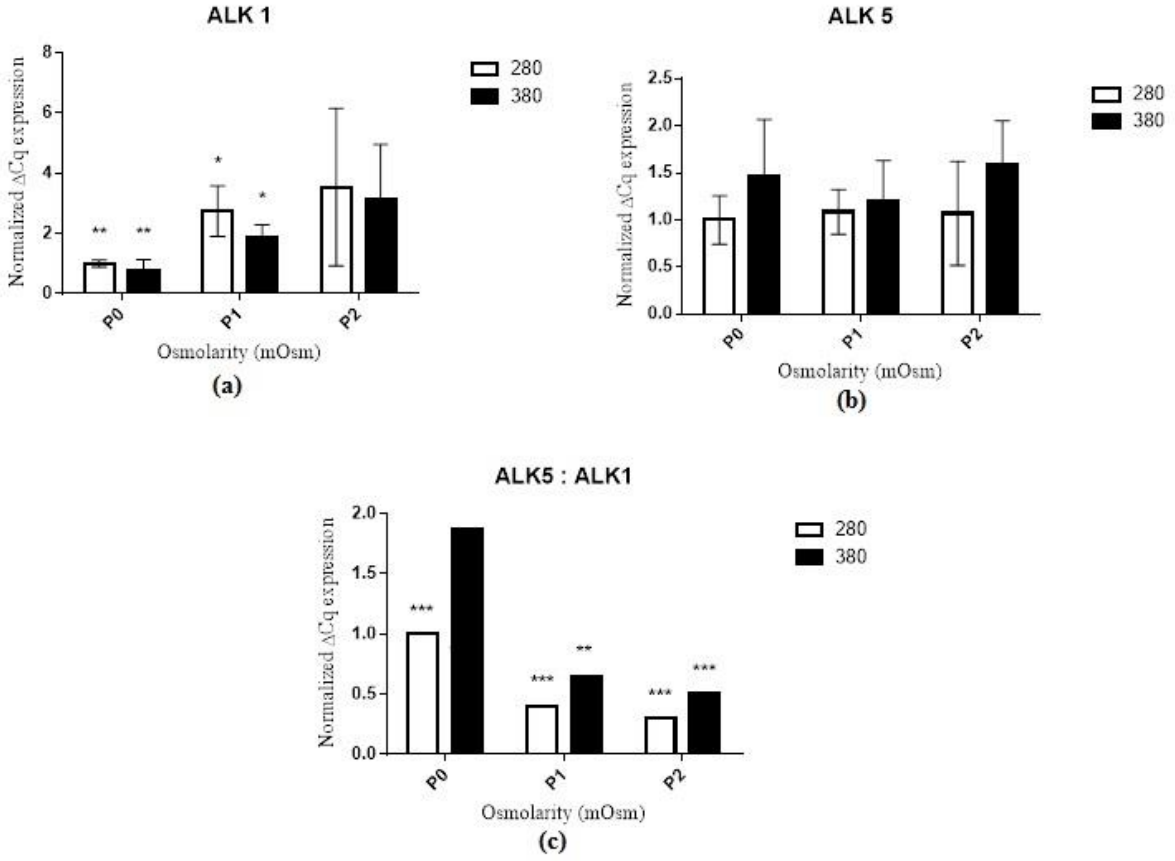

Figure 3.3 TGF-β type I receptors **(a)**ALK1 **(b)**ALK5 **(c)**ALK5/ALK1 ratio

3.1.4 Bone Morphogenetic Proteins

This scientific work involved the testing of bone morphogenetic proteins – BMP2 and BMP4 with varying osmolarity as shown in figure 3.4

BMP2 is upregulated in the 380 mOsm condition in passage P2. Although a clear pattern is not established in the behavior of the gene in response to hyperosmotic stimulus, the gene depicts a pattern of decline of the gene expression profile of BMP2, in the case of 280 mOsm condition.

On the other hand, BMP4 is significantly upregulated in 380 mOsm condition in all passages.

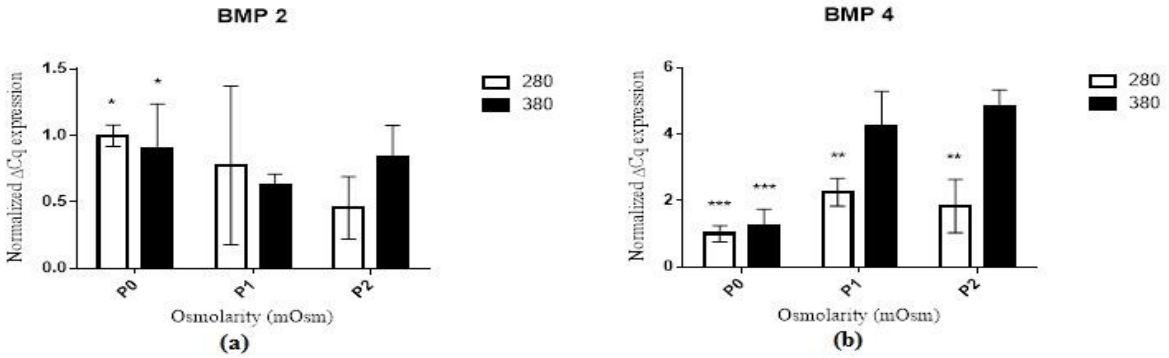

Figure 3.4 Bone Morphogenetic proteins **(a)**BMP2 **(b)**BMP4

3.2 Discussion

This systematic study was performed to elucidate the effect(s) of osmolarity on OAHACs. As part of the investigation, the chondrogenic markers were first analyzed, to understand the impact of osmotic stimulus. This is then followed by the evaluation of the outcome of osmolarity on genes responsible for GAG synthesis, TGF-β receptor type I and BMPs.

The most prominently used biomarkers of chondrocytes are COL2, AGC and SOX9. In this study, we test the effect of hypo- (280 mOsm) and hyper- (380 mOsm) osmolarity on COL2 and AGC.

COL2 gene expression is upregulated in the 380 mOsm condition, as compared to the P0-280 mOsm control condition. It is observed that there is a decline in the gene expression in both the hypo- and hyper-osmotic stimuli, as the cells are passaged from P0 to P2. This may be attributed to the change in phenotype due to multiple passaging and effect of monolayer culture. The decreased gene expression of COL2 gene, would mean reduced synthesis of COL2 protein, resulting in the chondrocytes becoming fibroblastic in nature. Nonetheless, our results are in agreement with the findings of van der Windt et al [162]. Since the cells turn towards a fibroblastic phenotype, it can be inferred that COL1 is being excessively produced. Certain studies state that chondrocytes can be cultured in COL1 scaffolds to prevent the elevated production of COL1, and, this thereby prevents the change in phenotype.

AGC gene expression is also upregulated in the 380 mOsm condition. There is noticeably a significant increase in the AGC gene expression in P0-380 mOsm, which further reduces in P1 and is better expressed in P2. There is an observable increment pattern in the AGC gene expression levels in 280 mOsm from passage P0 to P2. The decrease in AGC gene expression, is due to the change in phenotype, which is already observed in the case of COL2. The results obtained are contradicting with the study by Clark et al [163], the reason being the choice of a different control condition. On the other hand, the outcome of the AGC gene expression profile versus osmolarity is in full agreement with the research by Palmer et al [56].

Hence the effect of osmolarity on gene expression profile of COL2 and AGC are thus studied and we conclude that hyper-osmolarity increases the gene expression of both the chondrogenic markers.

This is the first study to evaluate the effect of osmolarity on the genes involved in the GAG synthesis. The genes tested are CHPF – which is involved in the transfer of a glucuronic acid, CHST 13 – responsible for transfer of sulfate to position 4 of the chondroitin sulfate and

B3GAT3 – catalyzes the transfer of glucuronyl group during the synthesis of linkage region of the PG.

Gene expression profiles of CHPF, CHST 13 and B3GAT3, are all upregulated in the 380 mOsm condition with reference to the control, which is P0-280 mOsm. An observable pattern of increase in the gene expression of CHPF and B3GAT3 are noticed in the 380 mOsm condition. The gene expression of CHST 13 at 380 mOsm condition is expressed similarly in passage P0 and P1, and there is a significant increase in gene expression in P2. In a nutshell, we may infer a similar behavior of the three GAG synthesizing genes. This holds true, because all the three genes work in a systematic manner, due to the fact that there is a transfer of sulfate, followed by the glucuronic acid and finally the linkage to the PG.

There is an increase in the gene expression profiles of the aforementioned GAG synthesizing genes as the passage number increases. This can be corroborated with the fact that in osteoarthritis, initially the GAGs are lost at a rapid rate in comparison to their synthesis, and, as time progresses, GAGs are produced in high quantity, but of the lower quality.

Our hypothesis of the genes involved in the GAG synthesis being sensitive to osmolarity is found to be true. Additionally, all the genes are upregulated in the 380 mOsm condition

ALK1 and ALK5 are the type I receptors of the TGF-β family. They are involved in the TGF-β signaling through the signaling pathway. ALK1 is found abundantly in OA cartilage, whereas ALK5 is present in healthy and young cartilage [80].

The gene expression of ALK1 is downregulated in the 380 mOsm condition as compared to the control which is P0-280 mOsm. Although there is noticeable increase in the gene expression profile at 380 mOsm from P0 to P2, this may be attributed to the effect of passaging and monolayer culture. The hyper-osmotic stimulus (380 mOsm), reduces the gene expression profile of ALK1. The consequence of this would be a decrease in the formation of ALK1 protein, implies less or no signal transduction through this receptor protein, thereby leading to arrest the progression of OA. Hence, in this situation, we may refer to the hyper-osmotic stimulus of 380 mOsm to be chondro-protective.

The gene expression of ALK5 is upregulated in the 380 mOsm condition in all the passages with respect to P0-280 mOsm which is the control. Increased expression of ALK5 gene, would lead to synthesis of ALK5 protein in abundance. This leads to the signal transduction of the TGF-β family to occur via ALK5. This could be a pointer to the fact that increased osmolarity of 380 mOsm, is functioning as a counter to the pathophysiology of OA, by increasing the signal transduction through the receptor protein, ALK5, which is reflective of the healthy state of the cartilage. In this scenario, the hyper-osmotic stimulus of 380 mOsm, presents itself as combatting and a healing agent against osteoarthritis.

The ratio of ALK5:ALK1 can be used as a metric to predict the nature of the chondrocytes. The decrease in this ratio, refers to the loss of chondrogenic phenotype and turning towards a hypertrophic nature [80]. There is a rapid decrease in the ratio, in the 380 mOsm condition, as the cells are passaged from P0 to P1, and a negligible decrease when the cells are passaged from P1 to P2. In our experiment, the ratio is upregulated when compared to the 280 mOsm condition within each passage. Hence, the hyper-osmotic stimulus of 380 mOsm preserves the chondrogenic phenotype.

With respect to the study of ALK1 and ALK5 versus osmolarity, we can conclude that our hypothesis of increased activity of ALK5 in response to hyper-osmotic stimulus is found to be true. Additionally, the hyper-osmolarity condition, prevents the progression of OA and

preserves the chondrogenic phenotype. The results of our study are in complete agreement with the theory postulated by Catherine Bauge et al [80].

The gene expression profile of BMP2 is down-regulated in 380 mOsm condition with respect to the P0-280 mOsm which is the control. The gene expression of BMP2 in the 380 mOsm condition reduces when passaged from P0 to P1, and slightly increases in P2.

BMP2 is found abundantly in OA tissue, associated with proliferation of OA cells and involved in the endochondral bone ossification [164]. The elevated osmolarity of 380 mOsm, again acts as a chondro-protective agent by reducing the gene expression profile of BMP2, which otherwise could accelerate the rate of progression of OA and osteophyte formation.

The gene expression profile of BMP4 is up-regulated in 380 mOsm in all passages when compared with the control P0-280 mOsm condition. There is a noticeable increase in the gene expression level of BMP4, at 380 mOsm condition, when passaged from P0 to P1, and a negligible increase when passaged from P1 to P2.

BMP4 is responsible for chondrogenesis, promotes aggrecan synthesis and sulfated matrix accumulation and is associated with chondrogenic lineage commitment [165]. The elevated osmolarity, increases the gene expression profile of BMP4, which proves beneficial to the cartilage, due to the aforementioned reasons. The elevated BMP4 gene expression in higher passages, is a pointer to the fact that, it is synthesizing BMP4 protein abundantly, to aid in chondrogenic lineage commitment of the chondrocytes which have turned hypertrophic or fibroblastic in nature. Hence, we may term the role of hyper-osmolarity to be phenotype preserving in this case.

Our hypothesis that elevated osmolarity will impact the activity if BMP2 and BMP4 is found to be true. The hyper-osmotic stimulus of 380 mOsm suppresses the activity of BMP2 and enhances that of BMP4, thereby, prevention in the progression of OA, lineage commitment and preservation of the phenotype.

Table 3.1 provides the correlation co-efficient, for each GOI studied in this experiment. The correlation co-efficient is computed using the CORREL function of MS-Excel software. The computation is based on the assumption that the variables (here, gene of interest) have a linear relationship.

Table 3.1 Correlation co-efficient for the genes studied in this research work

	COL2	**AGC**	**B3GAT3**	**CHPF**	**CHST13**	**ALK1**	**ALK5**	**BMP2**	**BMP4**
COL2	1	0.75	-0.5	-0.42	0.01	-0.73	0.37	0.47	-0.51
AGC	0.75	1	0.11	0.2	0.53	-0.23	0.84	0.27	0.02
B3GAT3	-0.5	0.11	1	0.94	0.74	0.54	0.6	-0.004	0.89
CHPF	-0.42	0.2	0.94	1	0.88	0.51	0.63	-0.05	0.85
CHST13	0.01	0.53	0.74	0.88	1	0.11	0.8	0.21	0.63
ALK1	-0.73	-0.23	0.54	0.51	0.11	1	-0.0001	-0.68	0.45
ALK5	0.39	0.84	0.6	0.63	0.80	-0.0001	1	0.24	0.52
BMP2	0.47	0.27	-0.004	-0.05	0.21	-0.68	0.24	1	-0.23
BMP4	-0.51	0.02	0.89	0.85	0.63	0.45	0.52	-0.23	1

The following inferences can be drawn from the correlation table:

- COL2 and AGC are ECM proteins of cartilage synthesized by their respective genes. The gene expression profile of COL2 and AGC are positively and strongly correlated.

This means that, if there is an increase in gene expression of COL2, there would be an increase in the gene expression of AGC too. The correlation co-efficient corroborate well with the results presented in figure 3.1.

- The gene expression profiles of CHPF, CHST 13 and B3GAT3 are all positively and strongly correlated with each other. This finding perfectly matches with the results presented in figure 3.2.

- The gene expression profiles of AGC and the genes involved in GAG synthesis also show a positive correlation co-efficient. This should be the case because, GAGs are side chains of the AGC molecule, and hence their synthesis is inter-related.

- The gene expression profile of ALK1 is negatively correlated with that of COL2 and AGC. Theoretically, the cartilage tissue lowers ALK1, as it is associated with OA and averts the tissue from entering into a degenerative mode. This is the desired state because, there is reduction in the agents initiating the pathophysiology of OA and parallelly promotes COL2 and AGC, which are responsible for synthesis and maintenance of ECM and homeostasis of cartilage.

- The gene expression of ALK5 is positively correlated with that of COL2 and AGC. This matches the theory and also our findings: TGF-β drives the synthesis of COL2 and AGC via the Smad signaling pathway through the ALK5 receptor. The opposite behavior must be noted in case of correlation of gene expression profiles of ALK1 with that of COL2 and AGC. Interestingly, this is the scenario represented by our results and fits the theory postulated by Catherine Bauge et al [80].

- Surprisingly, the gene expression profiles of both ALK1 and ALK5 are positively correlated with that of the genes involved in the GAGs synthesis. This fits into the theory postulated by Matsunobu et al [81], about the controversial role of ALK1 in the TGF-β signaling pathway.

- The gene expression profile of BMP2 and that of COL2 and AGC are positively correlated, while the correlation co-efficient for gene expression profile of BMP2 and that of GAG synthesizing genes is negatively correlated. This can be explained based on the theory by Mei Wan et al [95], wherein, the role of BMP2 in cartilage environment is termed controversial. The positive correlation co-efficient of BMP2 v/s AGC and negative correlation co-efficient of BMP2 v/s GAG synthesizing genes is contradicting.

- The gene expression profile of BMP4 is positively correlated with that of AGC and is positively and strongly correlated with that of the GAG synthesizing enzymes. This finding is in accordance with the theory postulated by Yuji Hatakeyam et al [165] and, is in agreement with the results obtained in our study.

- The gene expression profiles of BMP2 and BMP4 are negatively correlated. This finding is in accordance with the results obtained in our study.

The deviation in the results may be attributed to the following factors:

- Harsh enzymatic treatment which compromises on the signaling pathway of the chondrocytes.
- The difference in the progression of OA among the three patients could lead to a difference in the chondrocyte cell population obtained after digestion of the cartilage tissue.
- Exposure of chondrocytes to static osmolarity condition, elicit a response that is different from the chondrocytes in-vivo which experience an intermittent osmotic loading.

In conclusion, we observed better gene expression of chondrogenic markers, such as COL2 and AGC1 in hyper-osmolarity. We may say that our hypothesis, that GAGs are affected by osmolarity is true, and the expression of genes tested, was up-regulated in the hyper-osmotic stimulus. Further, the elevated osmolarity, suppressed the activity of ALK1, promoted ALK5 expression and enhanced the ALK5/ALK1 ratio and preserved the chondrogenic phenotype. Finally, increased osmolarity downregulated the activity of BMP2 gene, and upregulated the BMP4 gene expression. When closely observed correlation can be found in the expression of chondrogenic markers, genes involved in GAG synthesis, ALK5, ALK5/ALK1 ratio and BMP4 activity. This suggest that in OA, the elevated osmolarity is chondro-protective in nature and enhances the expression of genes involved in the synthesis of ECM and maintenance of homeostasis.

Chapter IV

Conclusion & Future Scope

This chapter re-iterates the problem statement. This is followed by recapitulation of the results and the conclusion. This would be accompanied with the future scope, which navigates the attention towards the possibilities of probing deeper into the work to gain sufficient insights about the behavior of osmolarity with the genes responsible for maintenance of chondrogenic phenotype.

4.1 Project Summary

This section presents in brief the objectives of the project as follows:

- To examine the effect of osmolarity on
    - Chondrogenic markers – COL2, AGC1
    - Genes for GAG synthesis – CHPF, CHST13, B3GAT3
    - TGF-β receptors – ALK1, ALK5
    - Bone Morphogenetic Proteins – BMP2, BMP4

4.2 Conclusion

This subunit acquaints the reader with the final results and the conclusion of this body of work. Table 4.1 summarizes the results obtained.

Table 4.1 Summary of the results

GOI	P0		P1		P2	
	280	380	280	380	280	380
COL2		✓		✓		✓
AGC1		✓		✓		✓
CHPF	✓			✓		✓
CHST13		✓		✓		✓
B3GAT3		✓		✓		✓
ALK1		✓		✓		✓
ALK5		✓		✓		✓
ALK5:ALK1		✓		✓		✓
BMP2	✓		✓			✓
BMP4		✓		✓		✓

The tabulated results indicate a good adaptation of OAHAC to increased osmolarity (380 mOsm) of the culture medium. This might suggest that implementing the hyper-osmotic stimulus to in vitro cell culture would mimic the native environment of cartilage and aid in synthesis of ECM and maintenance of homeostasis.

The experimental findings reveal the following:

- Upregulation of chondrogenic markers: COL2 and AGC1 in the hyper-osmotic condition
- Increase in gene expression of GAG synthesizing enzymes to hyper-osmotic stimulus
- The increased osmolarity, suppresses the ALK1 and enhances the ALK5 activity, thereby preserve the phenotype of the chondrocytes
- Increased activity of BMP4, as a consequence of the hyper-osmotic environment

In a nutshell, the study reveals, the functions of the hyper-osmotic stimulus to be that of chondro-protective and phenotype preserving. The hyper-osmotic stimulus is thus beneficial for the culture of chondrocytes in vitro.

Therefore, the integration of hyper-osmolarity, within the physiological limits, in the cell culture regimen of chondrocytes, would yield cells which are more chondrogenic, rather than fibroblastic. Consequently, this can be utilized as a suitable therapeutic strategy in the treatment of OA.

4.3 Future Scope

This section deals with the realistic and achievable ideas, which could be addressed to gain insight and provide better understanding about the osmolarity and culture conditions, which are the pre-requisites for successfully engineering an in vitro cartilage.

There is a pressing need to quantify the osmolarity condition, with respect to chondrocyte cell culture, which could be used as a golden standard, and there could prevail a unique control condition.

The osmotic properties can either be passive (due to osmotic gradients without use of energy) and/or active (with the use of energy). The present day experimental setup measures both of them, without probing the individual contributions. Hence, it might be necessary to employ chemicals which would block either of the properties, enabling to throw better light on the un-suppressed osmotic property.

The effects of raised saline osmolarity on adjacent soft tissues such as synovium, menisci and ligament need to be studied in order to understand their response to changing osmotic stimuli.

Research can be focused to investigate the effects of cell isolation and digestion under hyperosmotic condition, which could mimic the in-vivo native osmolarity of the cartilage, consequently lead to increased cell number, minimal cell death and prevent transformation into fibroblasts.

Extensive and systematic studies are necessary to evaluate the best agent to increase culture medium osmolarity among NaCl, sucrose and PEG taking into consideration various factors such as in-vivo cell environment, ionic equilibrium and native osmolarity which are just a few to name.

Experiments should be aimed at identifying specific osmolarity targets, which could help gain deeper insights on the effects of varying the osmolarity in the culture medium.

Sequential research work is necessary to study and understand the dynamics of the osmolarity in the osteoarthritic cartilage. This then can be applied to the culture regimen, which would

yield better results, rather than applying the osmolarity of normal cartilage on the OAHAC in culture.

There is a need to establish scientific findings on various other genes involved in the GAG synthesis and their effects on osmolarity conditions.

Although cartilage is described as a stratified tissue, with clearly distinct populations of cells within each zone, which experience a different history of osmolarity and tissue environment. Hence there is a need to utilize the zonal tissue engineering concept, to quantify the cells from each zone, qualitatively, which thereby enable us to better understand the expected response or the deviation of results.

Co-culture may be employed to study the behavior of chondrocytes when in a group of similar cells such as osteoblasts, bone marrow derived stem cells and embryonic stem cells; to better understand the cell signaling between these multitudes of cells.

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Appendix

Part A – Details of GOI

This section presents brief information about the genes of interest probed in this project work.

COL2 The official name of the gene is *Collagen type II, alpha 1*. The official symbol is COL2A1. For simplicity purpose, it is symbolized as COL2.

The COL2A1 gene is responsible for the synthesis of collagen type II fibers. Type II collagen forms the ECM and imparts tensile strength to the cartilage.

AGC1 The official name of the gene is *Aggrecan*. The official symbol is *ACAN*. For simplicity purpose, it is symbolized as AGC1.

The ACAN gene is responsible for synthesis of aggrecan protein. Aggrecan is the major proteoglycan found in the ECM of cartilage and imparts compressive stability by attracting water molecules.

B3GAT3 The official name of the gene is *Beta-1,3-glucuronyltransferase 3*. The official symbol is *B3GAT3*.

This gene acts as a catalyst in the process of glycosaminoglycan-protein linkage by the transfer of a glucuronyl group in the final step of the biosynthesis of the linkage region of proteoglycans.

CHST13 The official name of the gene is *Carbohydrate Sulfotranferase 13*. The official symbol is *CHST 13*. Alternatively the gene is also referred by *Chondroitin 4-sulfotransferase 3* and abbreviated as *C4ST3*.

This gene is responsible for the transfer of sulfate to position 4 of the N-acetylgalactosamine (GalNAc) residue of chondroitin.

CHPF The official name of the gene is *Chondroitin Polymerizing Factor*. The official symbol is *CHPF*. Alternatively the gene is also referred by *Chondroitin sulfate synthase 2* and abbreviated as *CSS2*.

This gene is responsible for the transfer of glucuronic acid (GlcUA) from UDP-GlcUA and GalNAc to the non-reducing end of the elongating chondroitin polymer.

ALK1 The official name of this gene is *Activin A receptor type-II like 1*. The official symbol is *ACVRL1*. Alternatively the gene is referred by *Activin like kinase* and abbreviated as *ALK1*.

The *ACVRL1* gene is responsible for the formation of a protein called activin receptor-like kinase 1, which is found on the surface of cells. This can be used to study the TGF-β activity via the Smad signaling pathway.

ALK5 The official name of the gene is *Transforming growth factor, beta receptor 1*. The official symbol is *TGFBR1*. Alternatively the gene is referred by *Activin like kinase* and abbreviated as *ALK5*.

This gene is responsible for the synthesis of a protein called activin like kinase 5, which is found on surface of cells. . This can be used to study the TGF-β activity via the Smad signaling pathway.

BMP2 The official name of the gene is *Bone Morphogenetic Protein 2*. The official symbol is *BMP2*.

This gene is responsible for the synthesis of BMP2 protein which induces bone and cartilage formation.

BMP4 The official name of the gene is *Bone Morphogenetic Protein 4*. The official symbol is *BMP4*.

This gene is responsible for the synthesis of BMP4 protein which aids in chondrocyte terminal differentiation, lineage commitment and promotes synthesis of ECM molecules.

Part B – Osmolarity Calculation

This section presents the calculation for preparing 50 ml 5M NaCl solution.

Atomic weight of Sodium (Na) = 23 g
Atomic weight of Chlorine (Cl) = 35.5 g

∴ Molecular Weight of NaCl = (Atomic weight of Na) + (Atomic weight of Cl)
= 23 + 35.5
= 58.5 g

∴ 1 mole of NaCl = Molecular weight of NaCl = 58.5 g
⊢ 5 moles of NaCl = 5 * molecular weight of NaCl
= 5 * 58.5
= 292.5 g

By dissolving 292.5 g NaCl in 1000 ml distilled water, we attain 5M Nacl solution.
To prepare 5M NaCl solution in 50 ml distilled water we would require:
X = (50*292.5)/1000
= 1462.5 *10(^-2)
= 14.625 g

∴ We can prepare 5M NaCl solution by dissolving 14.625 g of NaCl in 50 ml distilled water

It is postulated that 11.6 µl of the 5M NaCl solution is required per ml of the culture medium to reach an osmolarity of 380 mOsm.

Since we use 500 ml culture medium flasks, we would need:
Y = 11.6 * 10^(-6) *500
= 5800 * 10^(-6)
= 5.8 * 10^(-3)
= 5.8 ml

∴ 5.8 ml of 5M NaCl should be added to a 500 ml flask containing the culture medium to raise the osmolarity to 380 mOsm

Part C – Pilot Experiments

This section presents the results of the pilot experiments and the underlying motive behind choosing the specific GOI.

The figure C.1 and C.2 illustrates the results of the final PCR and the statistical analysis without and with TGF-β knockdown. These cells are cultured in 2D for a period of 5 hours with varying osmolarities like 280 and 380 mOsm respectively. HPRT-1 is used as the housekeeping gene and 280 mOsm condition is adopted as the control.

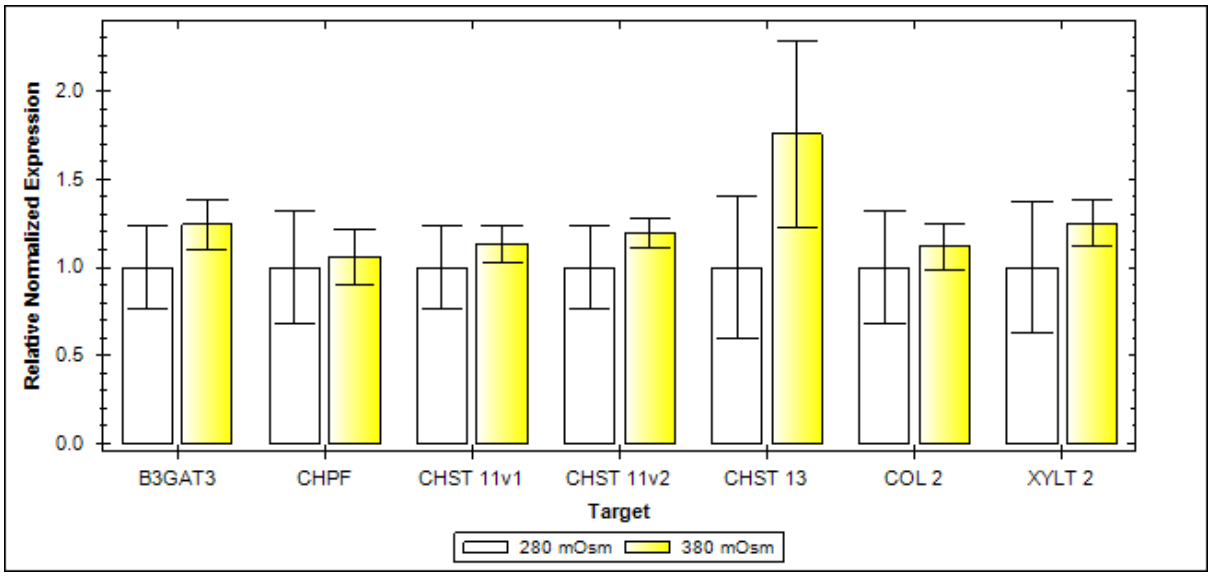

Figure C.1 Results without knockdown

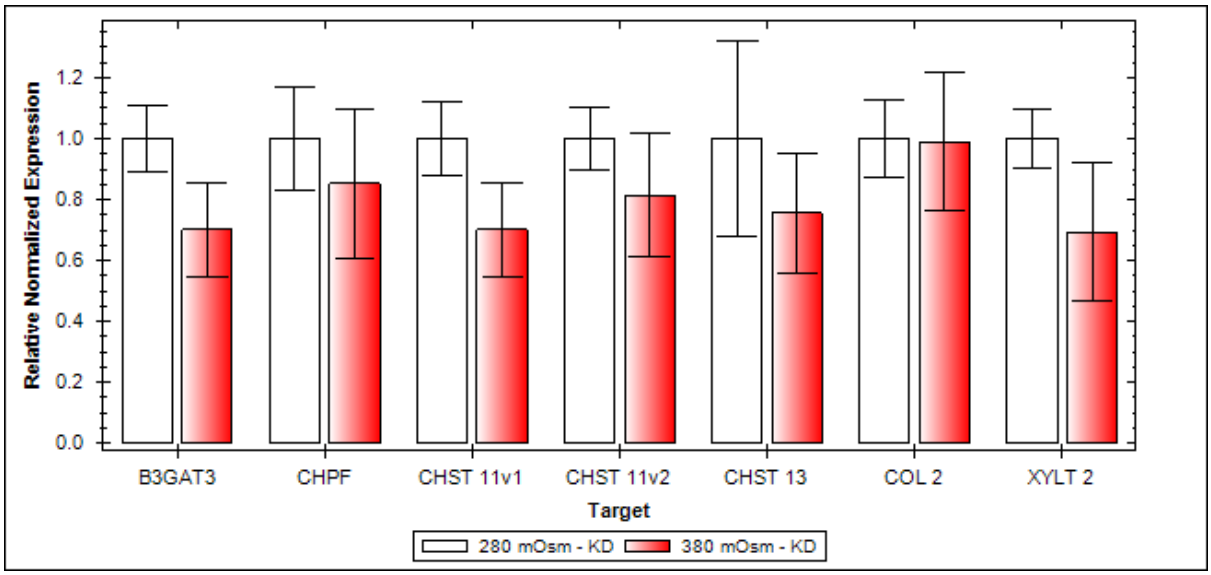

Figure C.2 Results with knockdown

From these results it is evident that the GAG synthesizing molecules are sensitive to osmolarity and osmolarity + TGF-β knockdown. CHPF, CHST 11v2 and COL2 show negligible upregulation due to variation in osmolarity, without the knockdown. The same behavior of COL2 is observed in the knockdown.

The figure C.3 depicts the outcome of the final PCR and the statistical analysis of the experiment. The conditions are: cells are cultured in 2D for a period of 6 hours with varying osmolarities like 280 and 380 mOsm respectively. HPRT-1 is used as the housekeeping gene and 280 mOsm condition is adopted as the control.

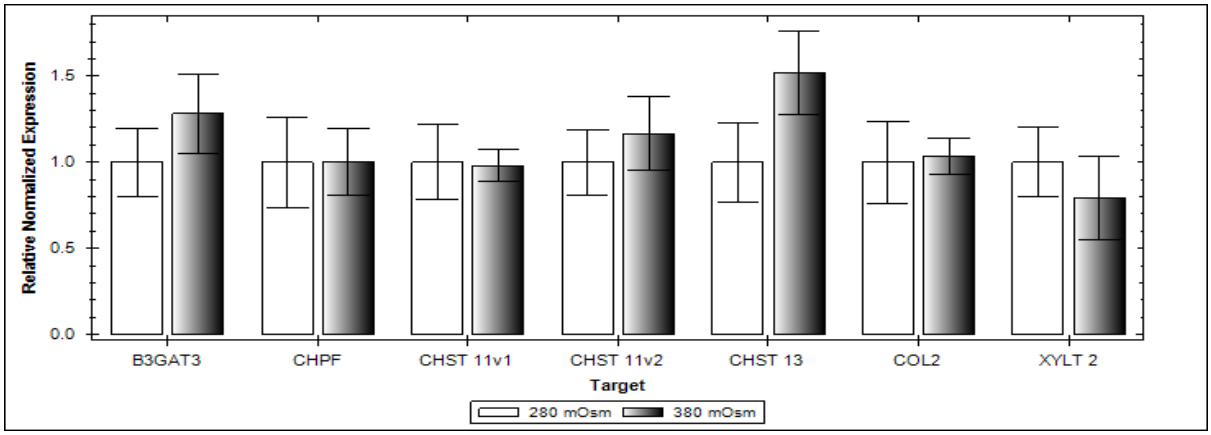

Figure C.3 Results at 6 hour time point

From the results pictorially represented it is evident that COL2 has a very negligible upregulation, which confirms the previous findings. B3GAT3 and CHST13 are considerably upregulated while XYLT-2 is downregulated in the hyper osmotic condition.

The figure C.4 depicts the outcome of the final PCR and the statistical analysis of the experiment. The conditions are: cells are cultured in 3D for a period of 48 hours with varying osmolarities like 280 and 380 mOsm respectively. HPRT-1 is used as the housekeeping gene and 280 mOsm condition is adopted as the control.

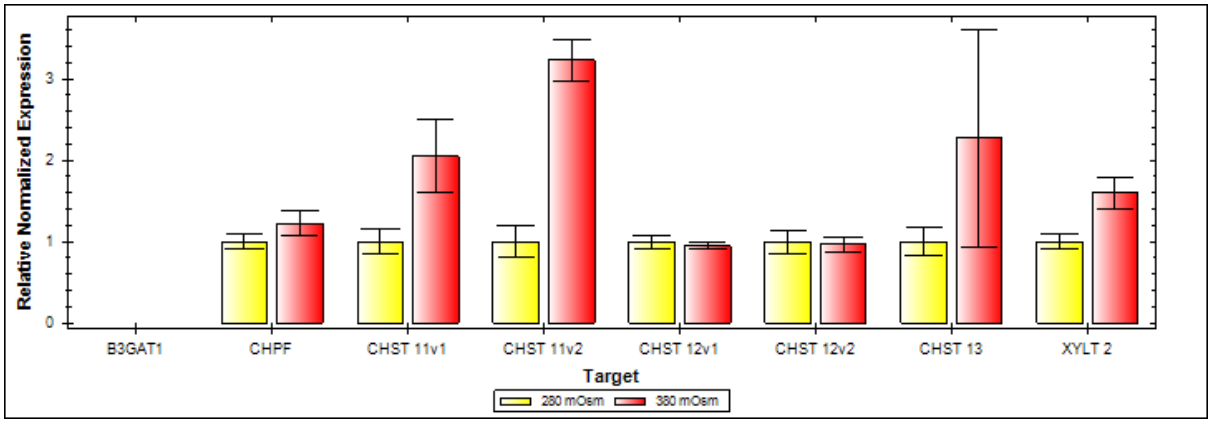

Figure C.4 Results at 48 hour time point

From this scientific work, it can be concluded that CHPF shows negligible changes, while CHST 11v1, CHST 11v2, CHST13 and XYLT2 show sufficient upregulation in the 380 mOsm condition.

From these pilot studies the following conclusions can be drawn.

- The genes responsible for synthesizing GAGs are sensitive to changes in osmolarity
- At early time point B3GAT3 and CHST13 show considerable upregulation and the reversal occurs in the knockdown experiment
- CHST11 v1 and v2 show marked response at a later time point and in 3D culture condition

Since this project is restricted to 2D cell culture and taking into account the results of the pilot study, CHST13, CHPF and B3GAT3 genes are selected to analyze their response to variations in osmolarity.