TGF-β3 mediated chondrogenic differentiation of synovial mesenchymal stem cells in gene transferred co-culture systems

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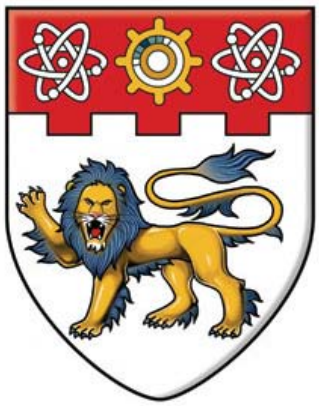

NANYANG TECHNOLOGICAL UNIVERSITY

TGF-β3 MEDIATED CHONDROGENIC DIFFERENTIATION OF SYNOVIAL MESENCHYMAL STEM CELLS IN GENE TRANSFERRED CO-CULTURE SYSTEMS

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ABSTRACT

Articular degenerative diseases like osteoarthritis significantly affect the life quality of worldwide populations including Singaporean communities. Traditional approaches, typically transplantation of autologous tissue grafts, exhibit many limitations in clinic because of implants resorption and poor shaping with articular defects, as well as lack of sufficient materials donors. In the recent decade, novel strategies with tissue engineering and gene therapy have been developed from laboratory-based investigation to clinical applications. With the development of gene delivery to chondrocytic/progenitor therapeutic cells such as mesenchymal stem cells, treating disorders of articular cartilage by tissue engineering has borne much promise.

Mesenchymal stem cells (MSCs) have been proven to be efficacious in articular cartilage repair and have even been used in a few in vivo clinical trials. Recently the focus has extended to synovium-derived MSCs (SMSCs) owing to their excellent chondrogenic potential. These cells are highly proliferative and can be expanded to sufficiently large numbers. Research has been carried out to induce chondrogenesis and engineer cartilaginous constructs from SMSCs using gene therapy and tissue engineering, with some degree of success. This study focuses on SMSCs and transgenic chondrocytes based co-culture system for engineered chondrogenesis.

The general strategy in this project relies on adenovirus mediated transfection of the growth factor (GF) transforming growth factor-beta 3 (TGF-β3) in partially de-

differentiated chondrocytes in order for these chondrocytes (The Transfected Companion Cells, TCCs) to provide exogenous GF supply to SMSCs (The Therapeutic progenitor cells, TPCs) in a co-culture system. This co-culture system proves to be an effective methodology for cartilage tissue engineering. It maintains the viability of TPCs – as TPCs do not undergo invasive gene treatment therapy themselves; TCCs provide sustained, localized and over-expressed supply of GF to TPCs; transient gene expression from adenovirally transduced TCCs leads to timely termination of GF supply to the TPCs.

Accordingly, an adenoviral vector containing the TGF-β3 gene was constructed. SMSCs and TGF-β3 transfected early passage rabbit articular chondrocytes were separately encapsulated in alginate beads and co-cultured in the same pool of chondrogenic medium. The adenovirally transfected early passage chondrocytes successfully re-differentiate in the presence of self-expressed TGF-β3. The TCC sourced transgenic TGF-β3 also successfully induces chondrogenesis and leads to the formation of a cartilaginous tissue in the TPCs.

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List of Abbreviations

3D: Three Dimensional

Ad.TGF-β3: Adenoviral Vector carrying Human TGF-β3 and fluorescent marker Zs-Green Genes

BMP: Bone Morphogenetic Protein

BMSC: Bone Marrow Derived Mesenchymal Stem Cells

BSA: Bovine Serum Albumin

CDS: Coding Sequence

COMP: Cartilage Oligomeric Matrix Protein

CPE: Cytopathic Effects

DF: human Dermal Fibroblasts

DMEM: Dulbecco's Modified Eagle's Medium

ECM: Extracellular Matrix

ELISA: Enzyme-linked Immunosorbant Assay

ESC: Embryonic Stem Cells

FBS: Fetal Bovine Serum

FGF: Fibroblast Growth Factor

GAG: Glycosaminoglycans

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

GF: Growth Factor

HEK293: Human Embryonic Kidney 293 Cells

hTGF-β3: Human Transforming Growth Factor - Beta 3

IGF: Insulin-like Growth Factor

IL: Interleukin

ITR: Inverted Terminal Repeat

MCS: Multiple Cloning Site

MOI: Multiplicity Of Infection

MSC: Mesenchymal Stem Cells

PBS: Phophate Buffered Saline

PI: Propidium Iodide

rAAV: recombinant Adeno-Associated Viral Vectors

RCA: Replication Competent Adenovirus

SMSC: Synovial Mesenchymal Stem Cells

TCC: Transfected Companion Cells

TEM: Transmission Electron Microscopy

TGF-β3: Transforming Growth Factor - Beta 3

TPC: Therapeutic Progenitor Cells

Chapter 1

Introduction

1.1 Overview

Articular cartilage – cartilage lining the bones in joints – heals poorly, and cartilage defects continue to pose a challenging clinical problem [2-3]. Osteoarthritis (OA) and cartilage traumas/injuries lead to progressive degeneration and defects in the cartilage respectively. Osteoarthritis is a debilitating, painful and chronic condition that affects people worldwide. It is the most common disorder associated with bones [4].

Adult hyaline articular cartilage is avascular [5]. Due to this lack of vascularization, access of progenitor cells to the site of injury or degradation is limited. Articular cartilage repair is defined as leading to a tissue that shares some structural similarities with hyaline articular cartilage. This kind of repair can be seen taking place in injuries that penetrate deep into the cartilage. Articular cartilage regeneration is defined as the re-establishment of cartilage in the area of the defect, indistinguishable from the neighboring, uninjured articular cartilage. Cartilage regeneration, however, does not occur in adults. No conservative or operative treatment procedure has convincingly proven to promote cartilage regeneration.

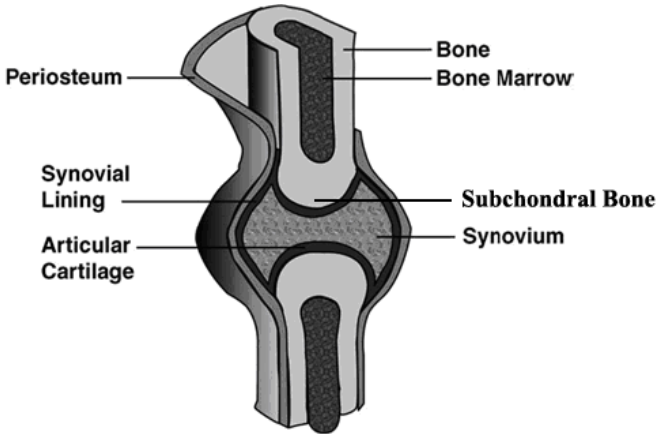


Figure 2.1: Schematic representation of a knee joint [6]

Treatment modalities employed by Orthopaedic surgeons and tissue engineers range from conservative treatment, continuous passive motion of the joint [7], to various surgical interventions. Many surgical procedures aim at recruiting mesenchymal stem cells by artificially creating connections between the bone marrow and the defect. Other strategies focus on the transplantation of autologous tissue grafts taken from the periosteum [8] or osteochondral transplants from uninjured, lesser weight-bearing areas of the joint [9]. More recently, the transplantation of isolated and expanded autologous chondrocytes/MSCs [10-11] in the absence [12] or presence [13] of supportive biodegradable matrices and in presence of therapeutic agents capable of inducing chondrogenesis and stimulating cell proliferation, maturation, and matrix synthesis, such as growth factors [14] to sites of articular cartilage damage has been investigated.

Cells derived from a variety of tissues including articular cartilage, bone marrow, periosteum, perichondrium or the synovial membrane can be modified by gene transfer ex vivo. Subsequently, they may be injected into the joint cavity or transplanted directly

into cartilage defects. Intra-articular release of the therapeutic proteins could also be achieved by application of a biochamber loaded with protein-secreting cells. Alternatively, gene vectors may be directly injected into the joint space or administered via surgical incision into a focal cartilage defect. Preformed cartilage or osteochondral constructs prepared using gene therapy and tissue engineering techniques can also be implanted into the lesions.

However, even such highly sophisticated procedures do not predictably lead to the formation of articular cartilage that is identical in its structure to the normal cartilage and capable of withstanding mechanical stresses over time [3, 15]. The regeneration of hyaline articular cartilage therefore remains one of the greatest challenges for orthopaedic surgeons and tissue engineers alike [3].

1.2 Hyaline articular cartilage and its defects

Cartilage is a connective tissue, broadly categorized into three types – hyaline cartilage, fibro-cartilage and elastic cartilage. Hyaline cartilage is the predominant cartilage type in the body. Articular cartilage is a specialized form of hyaline cartilage and is typically found along gliding surfaces of joints. Hyaline articular cartilage is formed by chondrocytes that are surrounded by a network of extracellular matrix. This cartilaginous matrix is rich in proteoglycans, primary and most abundant of which is aggrecan; and collagen fibrils composed of type-II collagen. It also contains type- IX, XI, VI and XIV collagens and a number of additional macromolecules including cartilage oligomeric matrix protein (COMP), link protein, decorin, fibromodulin, fibronectin and tenascin [16].

Normal hyaline articular cartilage contains about 70–80% water, which is mainly bound to proteoglycans. The extracellular matrix proteins are synthesised and degraded by the articular chondrocytes. Chondrocytes regulate the structural and functional properties of the cartilage. Adult hyaline articular cartilage is avascular and aneural[5].

Articular cartilage consists of multiple layers or zones. The thinnest and most superficial zone is the articular surface, where flattened ellipsoidal chondrocytes are covered by a film of synovial fluid. Cells here are aligned parallel to the surface. This surface is important in maintaining low friction for gliding. The middle/transitional zone has a low cell count and high concentration of proteoglycans and provides the first damping area for compressive forces. The deep zone contains a large amount of perpendicularly arranged collagen fibrils, and is the main damping zone for compressive forces. The calcified zone contains hypertrophic chondrocytes that produce Collagen X. This area acts as a shock absorber and maintains structural integrity [17-18]. Composition-wise (wet-weight), articular cartilage consists of 65-80% water, 10-20% collagen (about 95% of collagen is type-II, the next common types are type-IX and type-XI collagen), 10-20% proteoglycans and 1-5% chondrocytes [18].

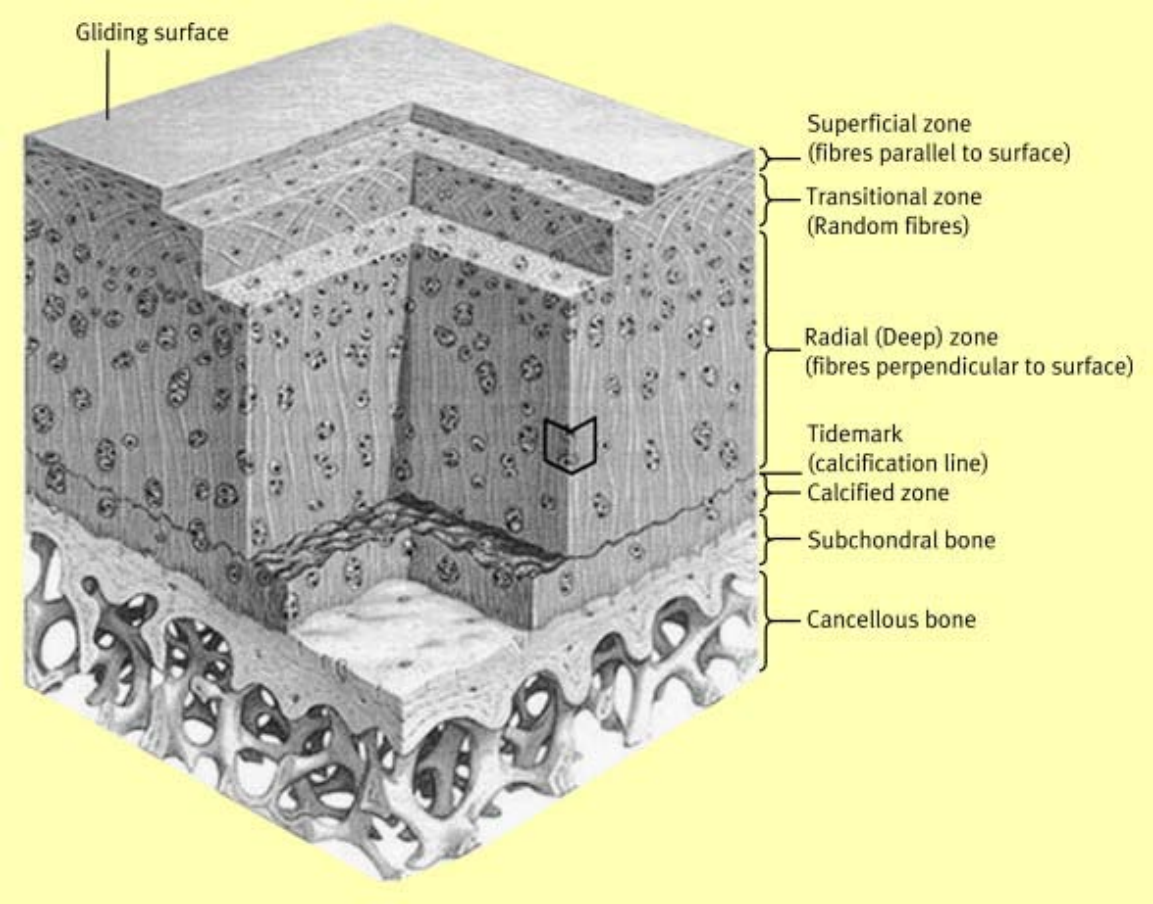


Figure 1.2: Schematic 3D representation of hyaline articular cartilage[19]

1.2.1 Chondrocytes

Chondrocytes are predominantly round cells located in cavities in the ECM called lacunae, with the exception of those on the articular surface. Chondrocytes synthesize and maintain the cartilage ECM. Chondrocytes constitute about 1-5% of the articular cartilage, the rest being the ECM.

Osteo/Chondrogenesis in native tissue

Chondrogenic cells arise from pluripotent MSCs via a series of differentiation pathways regulated by numerous cytokines and GFs like transforming growth factor-beta (TGF-β)

family, bone morphogenetic proteins (BMPs), cartilage-derived morphogenetic proteins, fibroblast growth factors (FGFs), and IGF-1 [20-22]. Upregulation of gene expression associated with chondrogenesis includes the transcription factor Sox-9 followed by Col2A, which encodes Collagen type II [23-24]. After the upregulation of transcription factors, the differentiation of chondroprogenitor cells progresses through various steps to finally result in mature chondrocytes [25]. During this phase of differentiation, the chondroblasts differentiate into chondrocytes and start synthesizing Collagen II and highly sulfated cartilage proteoglycans.

Chondrocytes may further undergo de-differentiation, turn hypertrophic and start expressing hyperthrophic chondrocyte marker – Collagen X [25]. Hypertrophic chondrocytes are involved in endochondral bone development. As the chondrocytes turn hypertrophic, their intracellular volume greatly increases and they start expressing matrix components which lead to the calcification of the matrix. Formation of blood vessels takes place next, and the cartilage is replaced by bone.

The differentiation steps involved in the process of osteo/chondrogenesis are: Chondrogenic MSCs —> Prechondrocytes —> Early Chondroblasts —> Columnar Chondroblasts —> Prehypertrophic Chondrocytes —> Hypertrophic Chondrocytes —> Terminal Chondrocytes.

1.2.2 ECM components

Collagens

A large portion of connective tissues in the body is made up of collagens. Collagens have diverse functions such as bearing tensile and/or compressive loads as well as allowing varying degrees of freedom to the moving parts of the body. In articular cartilage, collagens make up for about two thirds of dry weight. Collagen fibers within the 4 zones of articular cartilage vary mostly in their fiber orientation. The superficial layer contains long thin fibers parallel to the surface plane. The middle zone has seemingly random orientations with varying thickness of fibers while the deep zone has orthogonally orientated fibers. Cartilage is mainly made up of Collagen II, Collagen IX and Collagen XI fibers in all zones (about 90%, 1% and 3% respectively). Young growing cartilage is mainly composed of the same 3 collagen types with percentages <80%, >10% and >10% respectively. The calcified zone contains Collagen X as well, produced by hypertrophic chondrocytes. TEM studies show fibers becoming coarser and more banded as one moves further from the chondrocytes. Collagen II, Collagen IX and Collagen XI exist as cross-linked polymers in articular cartilage. Collagen IX forms covalent bonds with Collagen II and Collagen XI molecules and they are usually found on the surfaces of fibrils. It is thought that they act as mechanical restraints. Collagen XI is most concentrated in the superficial layers [26-27]. Collagen I is not normally present in healthy, well functioning articular hyaline cartilage. The presence of Collagen I in articular cartilage indicates the formation of fibrocartilage and/or calcification of the cartilage. This compromises the

mechanical integrity and functioning of normal cartilage and hinders the damping of compressive forces.

Proteoglycans

In vitro studies have shown that human and canine articular cartilage contain a collagen-glycosaminoglycan matrix [28]. Studies have been conducted to make use of proteoglycans for the maintenance of cartilage. The synthesis and catabolism of Aggrecans (or aggregating proteoglycans) trapped within Collagen II matrix has also been studied. Aggrecans, and proteoglycans in general, play a role in the damping of compressive forces. Glucosamine sulphate, a basic component of glycosaminoglycans (GAG) in articular cartilage stimulates proteoglycan and GAG production [29]. Insulin-like Growth Factors (IGFs) have also been known to induce proteoglycan production and reduce catabolism in bovine cartilage.

COMP

The pentameric glycoprotein - COMP, is a short length protein commonly found in cartilage and cartilage-derived bones. COMP is localized mainly near chondrocytes in the ECM. COMP does not get degraded after calcification of cartilage tissue [30]. COMP is primarily a component of cartilage but at least one study has detected the presence of COMP in other tissues such as tendons, meniscus and ligaments [31]. COMP is an integral part of cartilage tissue and an indicator of chondrogenic differentiation. It is involved in human limb development and ameliorates matrix degradation in osteoarthritis.

Other components

Various other macromolecules like leucine-rich protein condroadherin, link protein, leucine-rich proteoglycans biglycan and decorin, fibromodulin, fibronectin, tenascin and core protein versican play important roles in controlling the structure and organization of the matrix [16, 32]. Another molecule that is of importance is hyaluronic acid which is essential in regulating the structural organization, function, cell adhesion and migration, and differentiation in cartilage [33-34]. The ECM is thus composed and organized so as to enable the cartilage to withstand biomechanical stresses and loads.

1.2.3 Defects

Two kinds of cartilage defects can occur as a result of trauma: chondral – limited to the cartilaginous zones and osteochondral – reaching further down into the subchondral bone. As there is no vascularization in articular cartilage, there is very limited supply of progenitor cells at the site of chondral injury. A chondral defect is partly repopulated by cells migrating from the synovial membrane [35-36]. These cells are insufficient in filling the defect and the surrounding cartilage starts degenerating. An osteochondral defect is filled with a blood clot if bone marrow manages to reach the defect from the injured subchondral bone. The MSCs present in the blood clot differentiate into chondrocytes and osteoblasts that later form a cartilaginous repair tissue and the new subchondral bone. The repair tissue however does not integrate with the existing ECM and the neighboring tissue, and the repair tissue itself starts degenerating over time due to mechanical load.

1.3 Cartilage tissue engineering

There are two approaches to cartilage tissue engineering as described by Hunziker in his review of articular cartilage repair[36]. In the first, repair tissue is engineered completely *in-vitro*, the fully-differentiated construct being then implanted within the defect void. Its advantage is that cell metabolism and differentiation are subject to better control *in-vitro* than *in-vivo*. Its drawbacks are that problems associated with repair tissue integration and mechanical fixation cannot be readily anticipated and solved. Mechanical loading conditions are difficult to simulate *in-vitro*; hence, the composition of the extracellular matrix may not be ideally suited to the specific needs of the prospective repair site. Another challenge is the press-fit implantation of such engineered implants.

The second approach, which is more frequently adopted, aims to engineer only the basic building block, namely, a matrix scaffold containing a homogeneous population of cells and signaling molecules entrapped within an appropriate delivery system, to ensure that the desired differentiation process takes place in-vivo and to assure the long-term maintenance of chondrocytic activity within the repair tissue formed. According to this approach, the differentiation and remodeling of repair tissue occurs in-vivo under physiological conditions of mechanical loading. Repair tissue formed in situ is more likely to adhere to, and integrate with, native articular cartilage than that produced in-vitro. One problem associated with this approach is difficulty of control of cell activity on a long-term basis. In addition, appropriate measures must be taken to prevent interference from cells and signaling substances involved in the spontaneous healing which would compromise the quality and mechanical competence of the final repair composite.

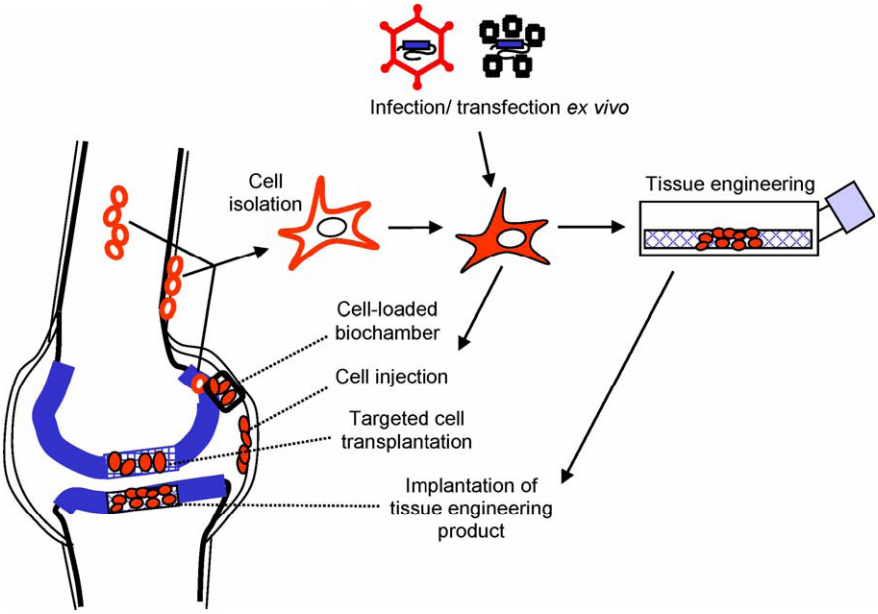


Figure 1.3: Schematic showing various approaches to cartilage tissue engineering[37].

Articular chondrocytes taken from non-load bearing cartilage area of the knee joint have been transplanted into cartilage defects since the late 1980s [12, 38]. In a first experiment of its kind, genetically modified cells were transplanted into a cartilage defect and the transgene was observed to be expressed for up to a month [10]. A GF gene was applied to a focal cartilage defect with no observed side effects in another first of its kind study [39]. Other *in vivo* experiments showing prolonged transgene expression [40-41] and trials in a large animal model [14] convincingly show the potential of transplantation of *in vitro* genetically modified cells into cartilage defects.

In 1994, Brittberg et al. [12] reported successfully treating cartilage defects by means of autologous chondrocyte implantation. Autologus chondrocytes expanded in monolayer were transplanted to a cartilage defect as a suspension. Although short and medium term

results were reported to be successful, this procedure still had some disadvantages. Monolayer culture of chondrocytes has been employed widely but chondrocytic phenotype fails to be maintained during long-term monolayer culture [42-43]. The chondrocytes tend to assume a dedifferentiated fibroblastic morphology, lose their ability to form ECM and start synthesizing Collagen type I [44]. Therefore, it is doubtful whether the fibroblastic cells re-express chondrocytic phenotypes after transplantation [45]. Another concern is the risk that some of the chondrocytes may leak from the site of the graft during load bearing because they have been injected in suspension. Also, when cartilage is damaged to the subchondral bone-tissue spaces, a spontaneous repair is initiated involving various cell types and signaling molecules which can have undesirable or unpredictable effects on the transplanted chondrocytes.

In order to address the concerns mentioned above, tissue engineering strategies for cartilage regeneration are now directed at producing models which incorporate 1. a reliable source of chondrogenic cells, 2. a three dimensional (3D) environment for chondrogenesis such as appropriate scaffolds or matrices, 3. favourable culture conditions using bioreactors to enhance mass transfer rates, nutrient supply and apply dynamic loading [46] and 4. nutrients, GFs and up-regulation of genes to direct chondrogenesis or maintain chondrocytic behavior.

1.3.1 Cell sources

Although chondrocytes have been primarily used for cartilage repair and regeneration, the source of autologous chondrocytes is limited. *In vitro* expansion of these

chondrocytes results in their de-differentiation and loss of phenotype. Due to these limitations and challenges in the use of chondrocytes, focus has shifted to the use of progenitor cells for cartilage tissue engineering. MSCs, and synovial MSCs (SMSCs) in particular, have been increasingly used as a cell source for cartilage regeneration.

1.3.1.1 MSCs

The differentiated and committed cell lineages in adult organisms arise from and are sustained by highly regulated processes involving cell proliferation, migration, differentiation and maturation. Stem cells are the first rung in this chain of processes. The most commonly used progenitor cells in cartilage regeneration are MSCs isolated from bone marrow [47] or skeletal muscle [48]. Other cell types being used include stem cells from adipose tissue [49], progenitor cells from periosteum [50], perichondrial cells [51] and muscle-derived cells [52]. These cells can be easily expanded *ex vivo* and induced, either *in vivo* or *in vitro*, to terminally differentiate into chondrocytes, osteoblasts, adipocytes, myotubes, tenocytes, neural cells, and hematopoietic-supporting stroma [25, 53-54]. MSCs form a homogenous population of fibroblast-like cells [55]. The antigenic phenotype of MSCs has some features in common with mesenchymal, endothelial, epithelial and muscle cells [56-57]. MSCs show extensive self-renewal potential, a defining characteristic of stem cells. Also, their easy expansion and multilineage potential make them an attractive therapeutic tool [58].

The source of origin plays an important role in MSC's differentiation potential for a given cell lineage. For example, adipose tissue derived MSCs have higher adipogenic

potential while periosteum derived MSCs have better osteogenic and chondrogenic potential [59]. MSCs can be used in various ways for clinical therapies: incorporation into a 3D scaffold to regenerate tissues; cell replacement therapy whereby genetically pathologic cells are replaced by normal autologous MSCs; and as cytokine/GF producers to simulate regeneration and inhibit degeneration [60].

Studies that compare SMSCs with other adult stem cells in terms of their proliferative and differentiation potential for chondrogenesis have found SMSCs to be superior in these aspects [61-62]. SMSCs are extracted from the synovium tissue in knee joints. They are multipotent and capable of differentiating into different mesenchymal lineages. Moreover, their multipotent ability is not affected by the age of the donor, the number of passages undergone in culture or by cryopreservation. They are highly proliferative and can be expanded to large numbers *in vitro*[63]. SMSCs are particularly efficient in undergoing chondrogenesis[63-65]. SMSCs have been shown to produce more ECM during *in vitro* pellet culture compared to other MSCs[66]. SMSCs have also been shown to undergo better chondrogenesis than bone marrow derived MSCs (BMSCs), periosteum derived MSCs or MSCs derived from adipose or skeletal muscle tissues[64]. These studies highlight the particular effectiveness of SMSCs in undergoing chondrogenic differentiation.

1.3.1.2 Clinical trials utilizing MSCs for articular cartilage repair and regeneration

Clinical trials making use of MSCs are in the preliminary stages, but they have already shown the safety and feasibility of using MSC therapy. Autologus MSCs have been used

in clinical trials for the treatment of articular cartilage defects with a certain degree of success. In one such study, BMSCs were isolated from osteoarthritic patients, expanded *in vitro*, encapsulated in collagen gel and then implanted back into cartilage defects. About 10 months later a hyaline cartilage like tissue was seen to be formed at the defects [67]. In another study, *in vitro* cultured MSCs encapsulated in collagen gel were transplanted into patellar defects. After two years, there were improvements in pain and walking ability, but formation of a fibrocartilage was seen in the repair tissue.[68] More recently, percutaneous injection of BMSCs in a patient with degenerative joint disease resulted in an increase in cartilage volume and decreased pain after six months. [69] Transplantation of BMSCs encapsulated in a collagen gel to a cartilage defect resulted in formation of a hyaline-like cartilage tissue and significantly improved clinical symptoms after a year [70].

An important factor concerning MSC therapy for cartilage repair that needs to be addressed is whether the transplanted MSCs are primarily involved in repopulating the defect or if they influence regeneration indirectly by paracrine signalling with cytokines, GFs and other bioactive agents. Another important factor that needs to be addressed is the formation of fibrocartilaginous tissue and the need to arrest *in vivo* formation of fibrous scar tissue. Besides, MSCs were also found to be involved in tumour development *in vivo*, owing to their immunosuppressive properties [71]. Spontaneous transformation of MSCs after long time culture and calcification and vascularisation of cartilaginous tissue derived from MSCs are some other concerns in using MSCs in the clinic. All these

factors need to be addressed in further studies before MSCs can be successfully used in clinical trials.

1.3.2 3D environment for chondrogenesis

3D cell culture closely mimics native microenvironment for cell growth, differentiation and tissue formation. Chondrocytes are anchorage independent cells and their differentiation and commitment best takes place in a 3D non-adhesive environment [72]. Chondrocytes start de-differentiating, lose their phenotype and turn fibroblastic in monolayer cultures. Quite a few studies have shown that MSC differentiation into chondrocytes requires a 3D semisolid environment that does not promote cell adhesion [73]. Cells transfected by viral or non-viral vectors show a gradual decrease in transgene expression in monolayer culture. 3D culture also helps in prolonging transgene expression [74-77]. Various synthetic (poly (lactic acid), poly (glycolic acid), poly (ethylene glycol)) and natural (alginate, collagen, fibrin, hyaluronate, chitosan) polymers have been used for chondrogenesis and cartilage tissue engineering in 3D environments.

Chondrocytic differentiation increases with scaffold hydrophilicity [78] and as articular cartilage has high water content, hydrogels are particularly suited for chondrogenesis. Hydrogels are cross-linked networks of polymers that can absorb large quantities of aqueous solutions. Hydrogels such as alginate have proven to be particularly effective for chondrogenesis [78-81]

1.3.2.1 Alginate

Alginate promotes chondrocyte viability and differentiation and many studies on chondrogenesis have been done using alginate matrices. Alginate is mainly derived from

seaweed, but is not constrained to a certain species. Differing alginate compositions from different type of seaweed may affect cell growth and differentiation in different ways. One study has shown that alginate with high mannuronic acid content and long polymer chain lengths results in better GAG synthesis and chondrocytic density compared to alginate with high guluronic acid content or with short polymer chains [82]. Alginate has emerged as particularly suitable for easy and efficient transfection of chondrocytes [80], prolonged transgene expression [74], increase in cell numbers [81] and transplantation of cells genetically modified *ex vivo* [41, 77, 81, 83-84]. It has already been applied in human studies [85]. Alginate hydrogels have also been widely used in chondrogenic studies involving SMSCs[86-87]. In order to improve structural and biocompatible properties of 3D culture, some studies have used composites of alginate/chitosan and alginate/fibrin. However, the effects of these biomaterials still need to be further researched. A recent study even suggests chondrocytes cultured in alginate and alginate-composite hydrogels exhibit lower Sox9 triggered Collagen II expression in comparison to pellet cultured chondrocytes [88]. Notwithstanding these results, overall, alginate proves to be highly effective in promoting chondrogenesis and maintaining chondrogenic phenotype in a 3D environment for cartilage repair and regeneration and its benefits in cartilage tissue engineering far outweigh the few minor drawbacks it might have.

1.3.3 Therapeutic GFs

GFs help in promoting chondrogenesis, cell proliferation, maturation, and matrix synthesis [89]. GFs that induce chondrogenesis include members of the TGF-β superfamily

(which includes BMPs) [90-94], members of the FGF family [95] and the parathyroid hormone-related protein (PTHrP) [96]. FGF-2 [97] and IGF-I [98] promote cell proliferation. IGF-I [99], members of TGF-β superfamily BMP-2 and BMP-7, and the cartilage-derived morphogenetic proteins [100] help stimulate ECM synthesis. Apart from GFs, genes for transcription factors [23], matrix proteins [101] and inhibitors of articular cartilage degeneration [102] have also been used in cartilage regeneration.

1.3.3.1 Transforming growth factor-beta isoforms

TGF- β superfamily of cytokines is a group of polypeptides that are further divided into TGF- β *sensu stricto*, BMPs and activins, all of which operate via similar downstream signaling mechanisms, through heteromeric complexes consisting of type I and type II serine/threonine kinase receptors. Also, these GFs are all structurally similar, forming dimers as mature proteins and they share similar mechanism of synthesis - via cleavage of propeptide [103]. Extracellular influence of TGF- β is determined by its active TGF- β concentration. While a large amount of TGF- β may exist within a tissue, only a small amount is usually active. This can happen due to several reasons. The TGF- β propeptide and cytokine may still be tightly bound even after cleavage, rendering the cytokine latent. Also, the TGF- β large latent complex may be bound to the ECM and may have to be proteolytically released. Latent TGF- β can be activated by several means – via proteolytic activation, thrombospondin-1, integrins, reactive oxygen species and mild acidic pH levels of about 4.5 [104].

Currently, three types of TGF- β have been identified - TGF- β1, TGF- β2 and TGF- β3 [105]. TGF-β1, TGF-β2 and TGF-β3 are expressed in most types of cells. TGF-β1 gets expressed in the hypertrophic zone of cartilage. TGF-β2 is present in all zones of cartilage. TGF-β2 and TGF-β3 are expressed most in hypertrophic and calcifying zone of cartilage [106]. TGF-β1 initiates cellular interaction in progenitor cells [107]. TGF-β1 is also an anti-inflammatory molecule and induces chondrocytes to synthesize ECM [2, 108]. The hypertrophic differentiation of chondrocytes is regulated by TGF-β2 [109]. TGF-β1,2 and 3 prevent vascularization in cartilage during development [110].

TGF- β signal is transmitted into the nucleus via smad pathway [111] which leads to the activation of sox 9. Sox 9 stimulates the expression of Collagen II and Aggrecan [112]. TGF-β is also able to prevent the effects of Interleukin-1 (IL-1) induced genes involved in cartilage injury [113]. Furthermore molecules that block endogenous TGF-β such as murine latency-associated peptide 1 (mLAP-1), increase the loss of proteoglycans and cartilage damage [114-115]. All three isoforms of TGF-β have been shown to be able to induce chondrogenic differentiation of human adult BMSCs [32]. TGF-β1 has been shown to induce chondrogenic differentiation in mesenchymal cell lines [116]. TGF-β3 induces chondrogenesis in progenitor cells under varied strategies [117-119]. It has also been shown to differentiate human MSCs to chondrocytes in micromass pellet culture [120]. TGF-β3 has also been reported to induce differentiation and cartilage-specific ECM production in chondrocytes in hydrogel constructs *in vivo* [121].

1.4 Gene therapy for cartilage tissue engineering

Various gene delivery vectors, GFs and cell types have been experimented with for chondrogenesis and cartilage regeneration. Viral and non-viral vectors have been used to transfer a myriad of genes into progenitor cells as well as chondrocytes.

The most commonly used MSCs for gene therapy are those derived from bone marrow or skeletal muscle tissue. MSCs obtained from adipose tissue and periosteum, as well as perichondrial cells have also been utilized for gene transfer. Transduction efficiencies in stem cells can reach up to 50% with adenoviral vectors [122]. Enhanced efficiencies have been reported in MSCs and attached stromal cells using retroviral vectors [123]. Positive results were obtained with recombinant adeno-associated viral (rAAV) vectors in stem cells [124]. When transduced retrovirally with BMP-2, murine embryonic MSCs exhibited a chondrocytic phenotype *in vitro* [125]. Adenoviral transduction with BMP-13 also induced chondrogenic differentiation in murine MSCs [126]. Chondrogenesis of progenitor cells has also been achieved by retroviral transduction with TGF-β1 [127].

Articular chondrocytes have been transduced with Adenovirus [128] with greater than 90% efficiency. Transfection of articular chondrocytes with retrovirus has led to efficiencies of up to 100% [129]. Baculovirus [130], lentivirus [131], rAAV [132] have also been used to tranduce chondrocytes with high efficiencies. Articular chondrocytes transduced with BMP-2 carrying adenoviral vector exhibited increased matrix synthesis [2]. Transduction of chondrocytes by an adenoviral vector carrying TGF-β1 was reported to induce the

synthesis of ECM [2]. Better synthesis of ECM and formation of cartilaginous tisssue was observed in articular chondrocytes transfected with BMP-7 using adenovirus [133].

Cells from the synovial cavity or synovial cells have also been transduced using lentiviruses [134] and other viral and non-viral vectors [135-136] and with a variety of genes - IL-1 receptor antagonist IL-1 RA [137], IGF-I [122], TGF-β [138].

The above results show that various kinds of cells, vectors and genes have been used to achieve chondrogenesis or cartilage synthesis with some degree of success. Transfection of chondrocytes with viral vectors carrying genes from TGF-β superfamily has consistently resulted in high transfection efficiencies, increased matrix synthesis, and formation of cartilage-like tissues.

There are mainly two approaches to gene delivery for therapeutic purposes. The first comprises direct insertion of genes *in vivo.* The second involves harvesting of cells, amplification of harvested cells and then introduction of gene of interest into the target cells *in vitro,* followed by subsequent transplantation. Choosing a method and vector requires consideration of the pathology, target tissue, vector titer and gene product activity, among others. For example, *in vivo* gene delivery is more suitable for synovium compared to cartilage due to the low level of chondrocytes within cartilage itself [139].

Autologous chondrocyte transplantation has been clinically used for repairing damaged articular cartilage since 1987 [140]. As such, gene delivery into chondrocytes has been

explored to be used in tandem with autologous chondrocyte transplantation. Chondrocytes seem to be resistant to transfection by plasmids, but can still be transfected with lipid-based vectors and virus-based vectors. *In vitro* cultured autologous chondrocytes are preferably used for transfection as chondrocytes *in vivo* have a rich matrix around them which makes them inaccessible to gene delivery vectors.

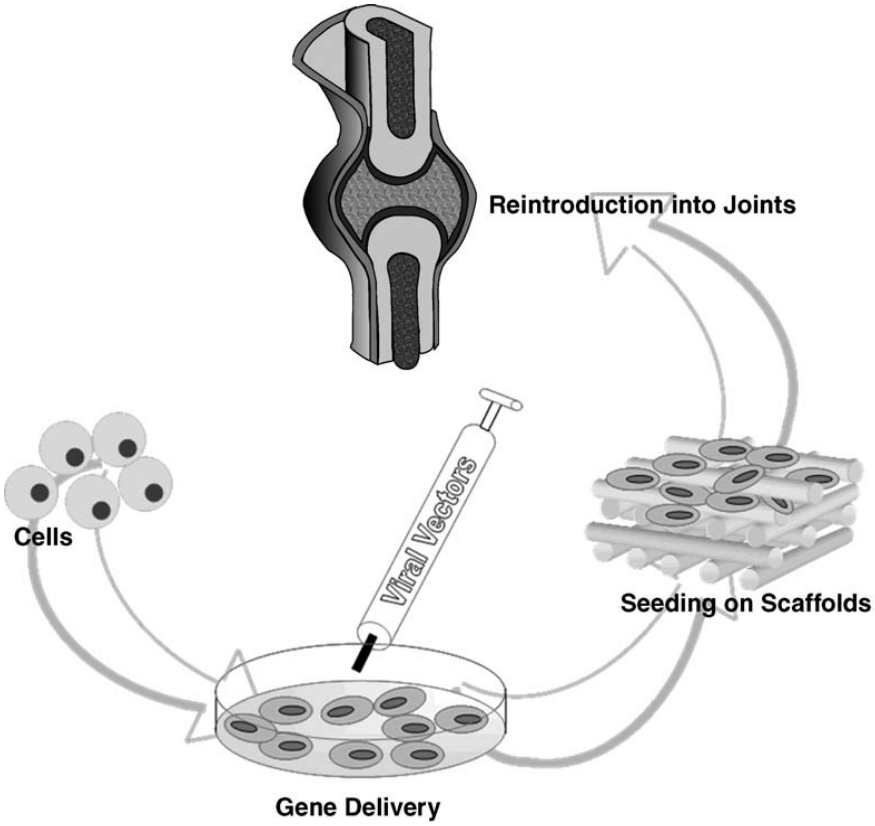


Figure 1.4: Ex vivo gene delivery for cartilage tissue engineering[6]

1.4.1 Gene delivery vectors

Various viral and non-viral vectors have been used for gene delivery in cartilage tissue engineering. One example of a non-viral vector is plasmid DNAs. Gene activated matrices (matrices of biodegradable materials with plasmid DNA encapsulated within)

have been designed as scaffolds for cartilage tissue engineering. This prevents degradation of the plasmid DNA and allows transfection of the cells seeded on the scaffold, consequently allowing the cells to secrete relatively high and sustained levels of the protein encoded by the plasmid DNA. Studies have shown the effectiveness of these scaffolds in synthesis of new cartilage *in vitro* [141].

Viral vectors have been preferred over non-viral vectors in gene delivery for cartilage repair and regeneration. Viral vectors are a lot more efficient compared to non-viral vectors. They can have transfection efficiencies as high as 90%. In studies, articular chondrocytes as well as MSCs have been modified via viral vectors to secret large amounts of protein before they are transplanted within the damaged cartilage [142]. Viral vectors, however, may induce some undesirable responses, such as inflammatory response and changes in cell surface markers. The full extent of these responses is still being explored in studies using viral transfections.

1.4.1.1 Adenoviral vectors

Despite the extensive development of non-viral gene delivery systems as a result of concerns over immune responses from viral vectors, viral gene delivery remains the most efficient way of transferring genes to therapeutic cells[128-129]. It has been rather difficult to transfect chondrocytes with cationic liposomes, such as Lipofectin [143] or LipofectAmine [144], whereas improved transduction has been observed with the lipid based reagent FuGENE 6 [145].

Adenoviral transfection results in highly efficient gene transfer to dividing as well as non-dividing cells [146]. The Adenoviral genetic material does not integrate with the host genome [147]. This eliminates the risk of insertional mutagenesis. Alginate derived chondrocytes that are transfected with adenovirus exhibit extremely high transduction efficiencies of nearly 99% [80]. Though adenoviral gene transfer is transient, gene expression in transfected chondrocytes cultured in alginate could be seen up to six weeks in more than half of the cells [74]. Gene expression in rat chondrocytes transfected *in vitro* and subsequently transplanted *in vivo* lasted for up to eight weeks *in vivo* [148].

Despite their high transfection efficiencies in most cell types, certain cell types are refractory to the most commonly used adenoviruses. This may be due to lack of specific cell surface receptors that facilitate adenoviral entry into the cell. Various methods have been used to improve adenoviral transfection efficiencies in refractory cells. Changing adenoviral cell tropism by creating chimeric vectors such as ad5/35 [149] or fiber modified vectors [150] improves their transfection efficiencies in the target cells. Polycations or cationic lipids[151-152] have also been used to improve adenoviral transfection efficiencies in refractory cells *in vitro*. The polycation-adenovirus complexes exhibit better adsorption into cells than adenoviral particles alone.

The most commonly used recombinant adenoviruses are generated from human adenoviruses of serotypes 2 and 5 of subgroup C [153]. They are non-enveloped particles of 60-90 nm in diameter, possessing icosahedral symmetry. Their capsids are made of 252 capsomers comprising 240 hexons and 12 pentons [154]. A thin trimeric glycoprotein fiber

protrudes from the center of each penton, terminating in a bulbous knob. Initial interactions of adenoviruses with the cells occurs through the binding of this knob to a host cell surface molecule, Coxsackie and adenovirus receptor (CAR), and this mediates internalization of the adenovirus [155]. Also, the adenoviral penton base interacts with the α_v integrin family of cell-surface heterodimers via the RGD (Arg-Gly-Asp) motif [156]. The α_v integrins act as co-receptors and assist in viral endocytosis into clathrin-coated vesicles [157]. On entry into the cells, the virus core uncoats from endocytotic vesicles and migrates to the nucleus, where DNA replication and transcription occurs to produce progeny virions that ultimately come out by lysing the host cells [158]. The genome of adenovirus serotype 5 is a double-stranded linear DNA molecule with an inverted terminal repeat (ITR) sequence of 102 bp on each end. The genome is functionally divided into two major overlapping regions, early (E) and late (L). The proteins encoded in the early regions E1A and E1B mediate viral replication. Most recombinant adenoviral vectors used for *in vitro* and *in vivo* gene transfer are usually deleted for the E1 and/or E3 region. As a result, adenoviruses are unable to replicate and spread in transfected cells and tissues. This deletion also generates space for introduction of foreign DNA/target gene for gene delivery. In order the assemble a complete E1 deleted adenoviral vector, a shuttle plasmid containing the left adenoviral ITR, packaging domain and gene of interest has to be combined with a template consisting of most of the adenoviral genome, and the right ITR. The final vector is generated by transfecting E1 complementing cell lines with the combined shuttle plasmid and adenoviral template.

1.4.1.2 Safety considerations in using adenovirus for transfection

Adenoviruses as vectors for gene therapy have been extensively studied and researched [159]. In fact, about 25% of all the clinical gene therapy trials as of 2005 made use of adenoviruses, a large portion of which were recombinant adenoviruses [9]. These trials are grouped in phases I, I/II, II, II/III and III. Phase I trials are primarily for determining safety and sometimes, maximum tolerable doses. The subsequent phases are primarily used to assess efficacy and have higher statistical significance to detect possible side effects. Numerous Phase I, I/II [160] and II [161] trials have been conducted where patients are reported to have tolerated the treatment with no significant adverse effects. Adenoviral p53 gene therapy, widely used for numerous types of cancers, has been reported to be a well tolerated and efficacious treatment [162].

Two concerns need to be addressed before using adenovirus *in vivo*. The first is the presence of replication competent adenovirus (RCA). RCA can lead to formation of infectious adenoviruses. Almost all of the *in vivo* applications of adenovirus involve using a replication incompetent adenovirus to prevent a viral infection in the subject. RCA occurs because production stocks of adenovirus vectors are made using 293 cells, which express the E1-encoded trans-complementing factors. To ensure that the presence of RCA for *in vivo* clinical use is within a certain limit, various biological RCA Assays are used including detection of cytopathic effects (CPE), immunostaining and PCR – using A549 as the indicator cell line.

The other concern is the immune responses in the subject and viral toxicity. These have been addressed by a series of deletions in the adenoviral genome to remove some, most or all of the viral genes except the ITRs and packaging genes. First generation adenoviruses were the E1 and E3 deleted viruses which were replication deficient. To further alleviate their immunogenicity, further deletions in E2 and E4 regions were made to reduce toxic protein synthesis as well as decrease the probability of RCAs. These were called the second generation viruses. However to introduce heightened safety and better deal with immune responses triggered by residual viral gene expression, all the viral genes except the ITRs and packaging sequences were deleted. These third generation (high capacity) adenoviruses have proven to be vastly better in regards to safety and prolonged expression of the gene of interest[163]. Furthermore, when adenovirally transfected cells are cultured *in vitro* - before being transplanted back to the host body - the viral capsid (the immunogen responsible for cytotoxic response from T lymphocytes) undergoes disintegration [164] and clearance from the cells (after nuclear translocation of the viral (vector's) genome), leaving the cells free of any immunogenic viral residues upon further transplantation.

While these advances in the use of adenoviruses are highly encouraging, *in vivo* immune responses to the vectors need to be thoroughly researched and possibly eliminated before any treatments using adenoviral vectors can be clinically implemented.

1.5 Co-culture of stem cells with autologus chondrocytes

In the recent decade, various co-culture strategies have been practiced, wherein progenitor cells – stem cells of varied origins – have been co-cultured with target cells or other companion cells, that lead to desired induction by paracrine signaling, with or without the aid of transgenic GFs. When mice embryonic stem cells (ESCs) were co-cultured with progenitor cells from the limb buds of a developing embryo, about 60-80% of the ESCs exhibited chondrocytic phenotype [165]. In other studies, chondrogenesis was induced in porcine BMSCs *in vitro* and *in vivo* by co-culturing them with articular chondrocytes [166-167]. Co-culturing sheep BMSCs with synovial fluid or synovial cells resulted in the expression of chondrocytic markers in the MSCs [168], while chondrogenesis was induced in human ESCs by co-culturing them with primary chondrocytes using well inserts [169]. Chondrogenesis was also investigated in rat BMSCs by co-culturing them with allogenic cartilage explants, with or without the addition of TGF-β3 [170]. More recently, MSCs [171-172], human adipose derived stem cells [173] and ESCs [174] have been reported to undergo chondrogenesis by co-culture with mature articular chondrocytes. ESCs have also been made to undergo chondrogenesis by co-culturing them with hepatic cells, which reportedly secrete factors/signals that induce chondrogenesis [175]. As a slight variation of this basic co-culture strategy, co-culture of human dermal fibroblasts (DF), which are not stem cells but committed cells that possess chondrogenic potential, with porcine articular chondrocytes resulted in chondrogenic induction in the DF [176]. In one study, passaged chondrocytes were co-cultured with primary chondrocytes using filter inserts and this resulted in their re-differentiation [177]. These co-culture studies represent a

promising methodology for chondrogenesis in particular and tissue regeneration in general, and provide a background for the current research.

1.6 Significance of current work

Researchers have conceived various strategies to direct the chondrogenesis of stem cells. Some of these involve direct/indirect co-culture of progenitor cells with either conditioned media obtained from cartilage explants [178] and synovial fluids [168], or with other companion cells like chondrocytes themselves [169], hepatic cells [175] and synovial cells [168]. GFs are essential to potentiate and accelerate chondrogenic differentiation of stem cells. Direct *in vitro* administration of GFs to stem cells requires prolonged *in vitro* culture periods. This mode of GF application is not translatable to clinical use. Direct in situ application of GFs to transplanted stem cell constructs *in vivo* requires repeated administration at the site of defect and is also clinically impractical. This calls for efficient gene transfer techniques that can lead to constitutive, endogenous and in situ GF production. Most of the gene transfer strategies up to this point have focused on transduction and transplantation of the same population of cells, be it chondrocytes or progenitor cells like MSCs or ESCs. It is known that besides cellular damage induced by invasive transfection – particularly significant in the fragile and vulnerable stem cells - long term transgenic GF over-expression may also accelerate hypertrophy and apoptosis of the transfected cells [179-181]. Studies have shown that the main role of these transduced cell populations is to produce the GF rather than to repopulate the defect [14, 41, 182]. Transfection of stem cells with GF genes has been proposed and practiced. However, the outcomes are not completely desirable not only due to the damage caused by the invasive

procedure of transfection but also because long term over-expression of endogenous GF may result in undesirable changes in the transfected cells.

To overcome these challenges, a co-culture strategy was devised in this study wherein chondrogenesis is induced in stem cells, the therapeutic progenitor cells (TPCs), by co-culturing them with autologous chondrocytes transfected with GF genes, the transfected companion cells (TCCs). The introduction of the co-culture system avoids directly transfecting the TPCs, but instead transfers GF genes to their co-cultured TCCs. By doing so, we can generate and modulate endogenous therapeutic GF supply for the progenitor cells from their co-cultured transfected cells so as to trigger and guide the chondrogenic differentiation in situ. The advantages of such an approach are: (1) the viability and chondrogenic specificity of TPCs can be maximally maintained as they are prevented from undergoing invasive gene transfection treatment; (2) during the early period of cell transplantation, TCCs can provide sustained, localized and over-expressed GF supply to stimulate the chondrogenic differentiation of TPCs; (3) when the chondrogenic differentiation of TPCs proceeds to completion, a timely termination of the TCC-sourced GF supply is programmed by TCC apoptosis induced by long-term presentation of self-generated overexpressed GFs. This turn-off mechanism functions to maintain the newly transformed (TPC-derived) chondrocytic cells from the fate of apoptosis equivalently catalyzed by the overshot GFs.

1.7 Rationale of current work

Human articular hyaline cartilage is avascular in nature [2-3, 5] Due to this lack of vascularization, access of progenitor cells to the site of injury or degradation is limited. In case of defects beyond a critical size, substantial self-regeneration does not occur in the adult articular cartilage. Thus we need clinical regeneration techniques to solve the problem of cartilage lesions. Using autologous chondrocytes as therapeutic cells for cartilage regeneration is not suitable due to the inevitable phenotype loss (de-differentiation) during cell expansion and the source of usable cell lines being too limited to fulfill the requirement of clinical applications [183]. Autologus progenitor cells, in particular multipotent stem cells, can be expanded to clinically relevant numbers with minimal loss in viability and plasticity.

The most commonly used progenitor cells for cartilage regeneration/repair are MSCs isolated from bone marrow [47] or skeletal muscle [48]. Other cell types being used include ESCs [174], adult stem cells derived from adipose tissue [49], progenitor cells from periosteum [50], perichondrial cells [51] and muscle-derived cells [52]. SMSCs are being increasingly used nowadays, owing to their excellent and well documented chondrogenic potential [184-185]. This led to the selection of SMSCs as TPCs in the current study. Early passage chondrocytes were used as TCCs in this study as they bring minimal non-cartilagenous impurities into the system and are robust enough to withstand transfection and still maintain functionality. Given the limited number of primary chondrocytes, by expanding the cells for a few times, sufficient numbers of early passage chondrocytes can be obtained to suit the requirements for co-culture use.

As described above, desired differentiation of stem cells relies on proper GFs. Various GFs have been used to promote chondrogenesis, cell proliferation, maturation, and matrix synthesis [89]. Members of TGF-β superfamily, in particular, are widely used and have been proven to be effective in promoting chondrogenesis to various degrees [90-94]. TGF-β3 has been known to induce chondrogenesis in numerous progenitor cells under varied strategies [117-119, 186]. TGF-β3 has also been reported to induce neo-cartilage formation *in vivo* in hyaluronic acid blended hydrogel constructs encapsulating rabbit chondrocytes [121]. Therefore, TGF-β3 was selected as the therapeutic GF in this study and TGF-β3 gene was adenovirally transfected in the TCCs that were co-cultured with TPCs in pursuit of chondrogenesis.

The TPCs and TCCs were separately encapsulated in alginate beads and cultured in the same pool of chondrogenic medium. The chondrogenic differentiation of TPCs was investigated under different initial TGF-β3 concentrations and exposure profiles while maintaining the same duration of co-culture.

Chapter 2

Construction of Adenoviral Vector Carrying Human TGF-β3 Gene

2.1 Introduction

This chapter is broadly separated into sections dealing with synthesis of Adenovirus encoding TGF-β3, transfection of Human Embryonic Kidney (HEK293) cells and subsequent characterization.

The adenoviral vector used in this research - pLP-Adeno-X-CMV-E3-ZsGreen1 (Clontech) – has been derived from replication deficient type 5 adenovirus (Ad5) with the deletion of E1 and E3 regions of the genome and can be used *in vivo* in animal models. pLP-Adeno-X-CMV and pAdeno-X adenoviral vectors (Clontech), which have essentially the same adenoviral backbone, have already been used *in vivo* [187-190]. All the present work with adenovirus has been carried out in a Biosafety Level 2 facility as required for working with adenoviruses.

An overview of the experimental procedure for the work done in chapter 2 is shown below in **Fig. 2.1**

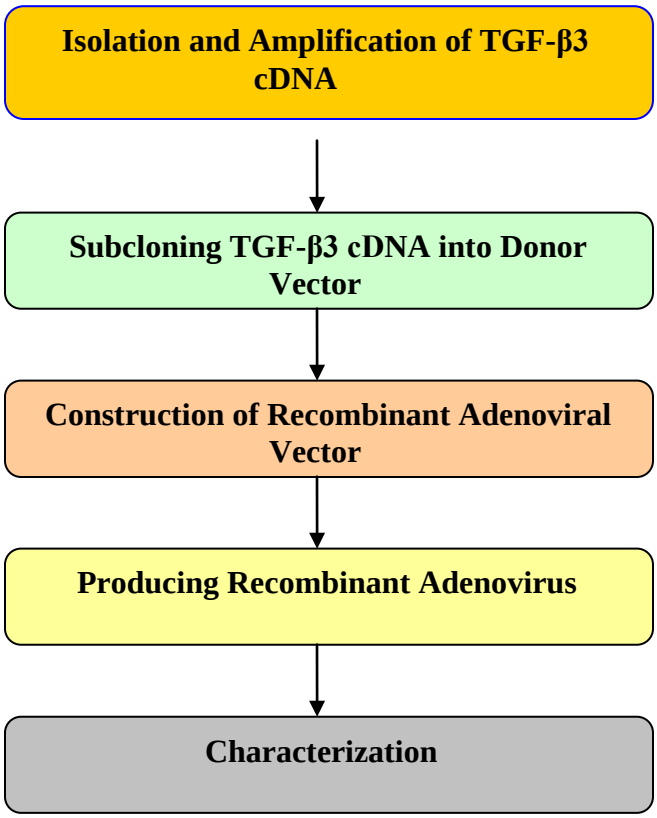


Figure 2.1: Overview of the workflow in constructing an Adenoviral Vector encoding TGF-β3 gene

2.2 Materials and Methods

2.2.1 Amplifying plasmid DNA encoding TGF-β3

2.2.1.1 Transformation of high-efficiency competent cells with plasmid DNA

(TGF-β3)-pCMV6-XL5 plasmid DNA (Origene Technologies, Rockville, MD, USA.) was used to transform MAX Efficiency DH5α Competent Cells (Invitrogen). One-

hundredth of the transformation solution (10ng plasmid DNA + 100µL competent cells + 900µL S.O.C. medium) was plated on LB-agar/ampicillin (100µg/mL) plates.

2.2.1.2 Plasmid extraction and purification

The bacterial colonies were inoculated into a starter culture of 3 ml LB-broth/Ampicillin (100µg/mL). The starter culture was incubated for 8 h at 37°C with vigorous shaking (at 300 rpm). The starter culture was then diluted 600 times into 25 ml LB-broth/Ampicillin (100µl/mL) and grown at 37°C for 12–16 h with vigorous shaking (300 rpm). Amplified plasmid was extracted/purified using Plasmid Midi Kit (QIAGEN). The plasmid DNA was stored at -20°C for future use. The bacterial strain was stored at -80°C.

2.2.2 Isolation and amplification of target gene by PCR

TGF-β3 cDNA was isolated from (TGF-β3)-pCMV6-XL5 plasmid DNA and amplified using the following primers: Forward -5' ACGC GTCGAC ATGAAGATGCACTT 3', Reverse - 5' TGCA CTGCAG TCAGCTACATTTAC 3'. The forward primer was used to add *Sal1* recognition site and the reverse primer to add *Pst1* recognition site at the two ends of the human TGF-β3 (hTGF-β3) cDNA Coding Sequence (CDS). The PCR conditions were: Initial denaturation - 94°C for 5 minutes; 30 cycles of: denaturation - 94°C 30 seconds, annealing - 55°C 30 seconds, extension - 72°C 30 seconds; Final extension - 72°C for 10 minutes. The product size was 1259 bp. After confirmation of PCR product with gel electrophoresis, gel electrophoresis was done again and TGF-β3 cDNA CDS was extracted/purified using QIAquick Gel Extraction Kit (Qiagen). The product was named Sal1-CDS-Pst1 (SCP) and stored at -20°C for future use.

2.2.3 Construction of shuttle vector

2.2.3.1 Digestion of donor vector and TGF- β3 cDNA with Sal I and Pst I

pDNR-CMV donor vector (**Fig. 2.2**) – part of Adeno-X™ Expression Systems 2 (Clontech) – was digested with *Sal1* (New England Biolabs) and *Pst1* (New England Biolabs). TGF-β3 cDNA CDS – Sal1-CDS-Pst1 (SCP) – was also digested with *Sal1* and *Pst1*. The digestion products were extracted/purified using QIAquick Gel Extraction Kit (Qiagen). Gel electrophoresis was done to confirm the presence of Plasmid DNA after extraction. The digested and purified donor vector and SCP were stored at -20°C.

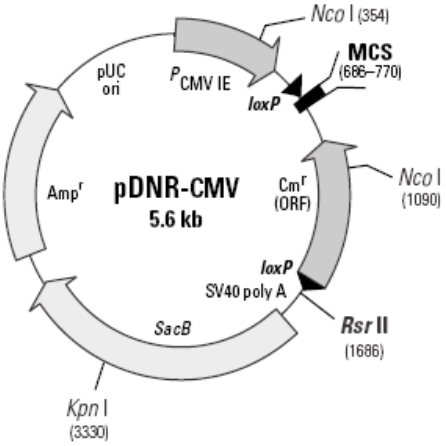


Figure 2.2: pDNR-CMV Donor Vector used to clone the gene of interest (**Clontech**)

Fig. 2.3 shows the restriction map and Multiple Cloning Site (MCS) of the Donor Vector.

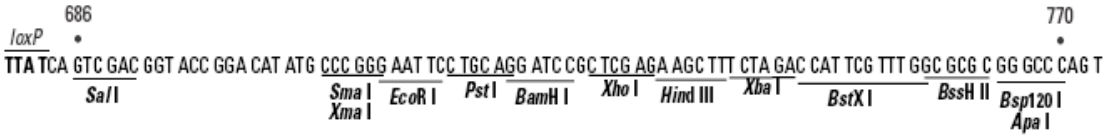


Figure 2.3: Restriction Map and Multiple Cloning Site of pDNR-CMV Donor Vector (**Clontech**)

2.2.3.2 Subcloning TGF-β3 cDNA into donor vector

The *Sal1* and *Pst1* digested TGF-β3 cDNA CDS and pDNR-CMV donor vector were ligated using T4 DNA Ligase (Invitrogen) in the ratio of 1:6::pDNR-CMV:TGF-β3 cDNA CDS. The ligation product was amplified by transformation in MAX Efficiency DH5α Competent Cells (Invitrogen). Colonies were picked and used for PCR colony screening with primers for TGF-β3 cDNA CDS: Forward -5' ACGC GTCGAC ATGAAGATGCACTT 3', Reverse - 5' TGCA CTGCAG TCAGCTACATTTAC 3'. More bacterial colonies were picked and all the colonies were propagated in LB-broth/Ampicillin (100µl/mL) and used for plasmid extraction (QIAfilter Plasmid Midi Kit, QIAGEN). To confirm the presence of ligated shuttle vector (pDNR-CMV + TGF-β3 cDNA CDS) (**Fig. 2.4**), the extracted plasmids were digested with *Sal1* and *Pst1* and used for gel electrophoresis. The shuttle vector from a colony which showed positive results from gel electrophoresis was sent to 1st BASE (Singapore) for DNA sequencing. The extracted plasmids were stored at -20°C and the bacterial strains at -80°C.

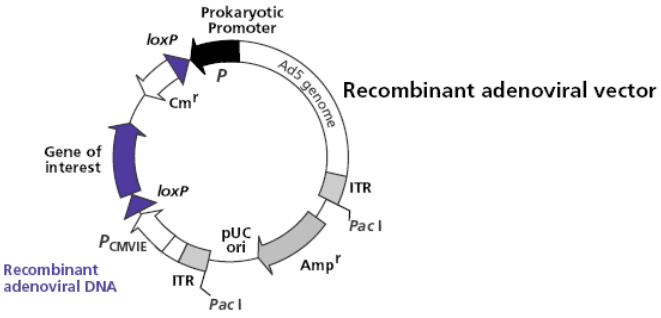


Figure 2.4: A Schematic Diagram of Recombinant Adeno-X *Shuttle* vector containing the gene of interest (Clontech)

2.2.4 Construction of recombinant adenoviral vector

TGF-β3 cDNA CDS from Shuttle Vector (pDNR-CMV + TGF-β3 cDNA CDS) was cloned into Acceptor Vector (pLP-Adeno-X-CMV) (**Fig. 2.5**) using Adeno-X™ Expression Systems 2 (Clontech). This process makes use of *Cre-loxP* site specific recombination (**Fig. 2.6**).

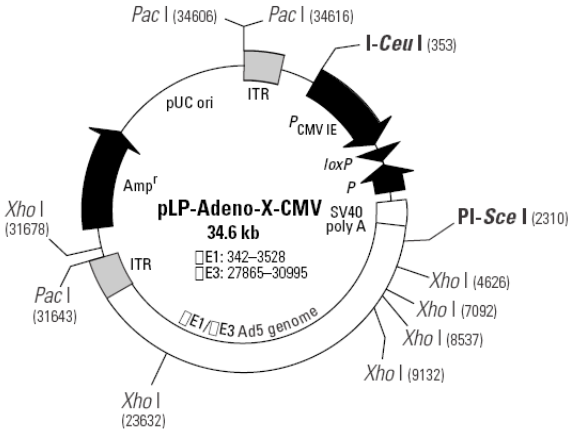


Figure 2.5: pLP-Adeno-X-CMV Viral DNA, Acceptor Vector (Clontech)

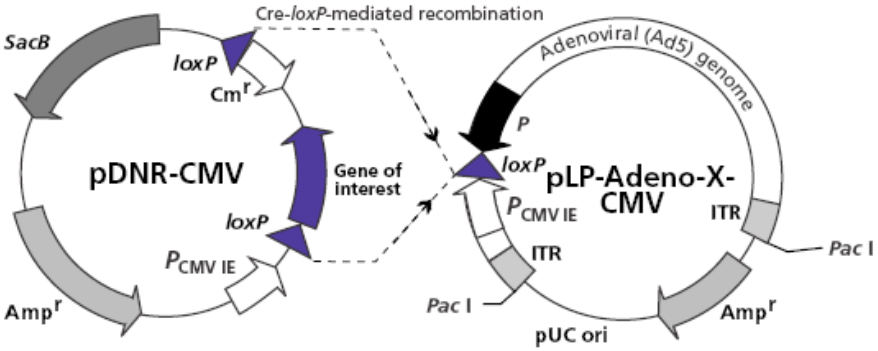


Figure 2.6: Cre-*loxP*-mediated recombination for transferring gene of interest from shuttle vector to the viral vector (Clontech)

The products of recombination were electroporated into ElectroMAX™ DH10B™ Cells (Invitrogen). The cells were electroporated once at 2kV, 200Ω, 25μF. After electroporation, cells were plated onto LB-agar/chloramphenicol (30μg/mL)/sucrose (7% w/v) plates to screen against Shuttle Vector and the non recombinant Acceptor Vector. *SacB* in the Shuttle Vector prevents colonies from forming on sucrose rich medium, while non recombinant acceptor vectors lack the chloramphenicol resistant gene – which gets transferred from the Shuttle Vector along with the Gene Of Interest (GOI) – and hence cannot grow on chloramphenicol containing medium.

The resultant colonies were grown/amplified in LB broth/Ampicillin (100μg/mL)/Chloramphenicol (30μg/mL). The recombinant Adenoviral Vector (pLP-Adeno-X-CMV + TGF-β3 cDNA CDS) was extracted/purified using QIAfilter Plasmid Midi Kit (QIAGEN) from the colonies and its concentration was measured using a UV-Visible spectrophotometer. To confirm the presence of recombinant adenoviral vector, *Xho1* restriction analysis was performed. The plasmid DNA was digested with *Xho1* and run on agarose gel. The recombinant adenoviral vector was sent to 1st BASE (Singapore) for DNA sequencing. Extracted adenoviral vector was stored at -20°C and the bacterial strains were stored at -80°C for future use.

2.2.5 Producing recombinant adenovirus

Infectious adenovirus was produced by transfecting HEK 293 cells with Pac I-digested pLP-Adeno-X DNA (pLP-Adeno-X-CMV + TGF-β3 cDNA CDS) as suggested in Adeno-X™ Expression System 1 (Clontech).

2.2.5.1 Linearization of adenoviral vector

The adenoviral vector plasmid was linearized by digestion with Pac1 to expose the ITRs located at either end (**Fig. 2.7**). The ITRs help in assembling the replication complex and should be situated at either ends of the linearized DNA to aid in DNA replication. The linearized DNA was purified by phenol – chloroform – isoamyl alcohol extraction. Concentration of purified DNA was measured using spectrophotometer.

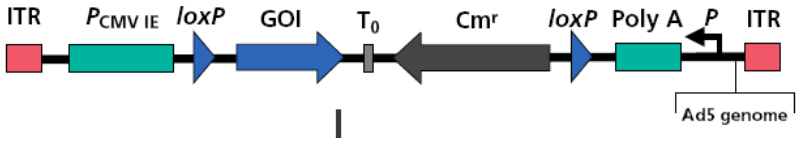


Figure 2.7: Linearized Recombinant Adenoviral DNA (Clontech)

2.2.5.2 Transfection of HEK293 cells with adenoviral vector using Lipofectamine

HEK 293 (ATCC) cells were plated at a density of 1–2 x 10^6 cells per 60-mm culture plate. When the cells were 50-70% confluent, they were transfected with Lipofectamine – Adenoviral Vector complex using Lipofectamine™ 2000 (Invitogen). The DNA (µg): Lipofectamine™ 2000 (µl) ratio used was 1:2.5 (DNA 8µg: Lipofectamine™ 2000 20µl). Four days later when sufficient CPE was observed, 500µl of Viral Particle suspension was collected by lysing the cells by repeated freeze and thaw cycles and stored at -20°C for future use.

2.2.5.3 Transfection of HEK293 cells with viral particles and subsequent characterization

HEK 293 cells were plated at a density of 1–2 x 10^6 cells per 60-mm culture plate in two culture plates. When the cells were 50-70% confluent, they were transfected with 250µl of Viral Particles per 60-mm culture plate. CPE was observed within four days.

The cells from one of the plates were suspended in the medium by repeatedly pipetting up and down and the suspension was collected in two different 15ml centrifuge tubes. The two tubes were centrifuged and the supernatant from both was collected in a separate tube and used for protein extraction using ethanol precipitation. One of the centrifuged tubes was used for RNA, DNA and Protein extraction/isolation using TRIZOL® Reagent (Invitrogen). The other centrifuged tube was used to collect protein by lysing cells using cell lysis buffer (1mol/l Tris.HCl pH 8.0 2.5ml + NaCl 0.438g + Triton X-100 0.5ml topped up to 50ml with deionized water).

RNA from TRIZOL® Reagent (Invitrogen) was used for Reverse Transcription using SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen). The single stranded cDNA thus obtained and DNA isolated from Trizol Reagent were then run on agarose gel to confirm the presence of target gene - TGF-β3 using the following primers: Forward -5’ ACGC GTCGACATGAAGATGCACTT3’, Reverse - 5’ TGCACTGCAG TCAGCTACATTTAC3’ for PCR. β-Actin Primers (Forward: 5’GTGGGGCGCCCCAGGCACCA3’,Reverse:5’CTCCTTAATGTCACGCACGATTT C3’) were also used to obtain dsDNA from the cDNA samples and run on gel.

Protein samples collected from the supernatant, from Trizol reagent and from cell lysis were characterized by Western Blot using Rabbit anti Human Polyclonal TGF-beta 3 (Abcam) and Rabbit anti Human Polyclonal GAPDH (IMGENEX) as Primary antibodies and Goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) as Secondary antibody.

2.2.5.4 Obtaining a primary amplification of viral particles

500µl of Viral Particle suspension was collected by lysing the cells form the other transfected plate by repeated freeze and thaw cycles. The suspension was labeled as Primary Amplification and stored at -80°C for future use.

2.2.6 Producing recombinant adenovirus expressing a green fluorescent protein

The Shuttle Vector constructed as described in **section 2.2.3** was used to construct a recombinant adenovirus containing a gene for a green fluorescent protein using Adeno-X™ ViraTrak™ Expression System 2 (Clontech) by following the same procedure as described in **sections 2.2.4 and 2.2.5**.

2.2.6.1 Amplification, purification and titer of adenovirus expressing a green fluorescent protein and TGF-β3

The titer of virus obtained from primary amplification **(section 2.2.5)** was determined using Adeno-X™ Rapid Titer Kit (Clontech). To obtain a high titer virus stock, this primary amplification of virus was further ampilified by transfecting multiple T150 flasks of HEK 293 cells at a multiplicity of 5 pfu/cell or greater, collecting the virus by

repeatedly freeze thawing the transfected cells and using the collected virus to further transfect another batch of HEK 293 cells. After several such amplifications (2-3), the virus was purified using Adeno-X™ Maxi purification Kit (Clontech) and titered again. In order to minimize the probability of producing RCA, several aliquots of early amplifications were stored at -80°C, for use whenever high titer stocks were required in the future.

2.3 Results and Discussion

2.3.1 Isolation and amplification of target gene by PCR

Human TGF- β3 CDS is 1239 bp long. With the addition of Sal1 and Pst1 recognition sites on 5' and 3' ends of the CDS respectively, its size changes to 1259 bp. TGF- β3 was isolated and amplified from (TGF-β3)-pCMV6-XL5 plasmid DNA using the following primers by PCR: Forward -5' ACGC GTCGAC ATGAAGATGCACTT 3', Reverse - 5' TGCA CTGCAG TCAGCTACATTTAC 3'. The resultant DNA was run on 0.8% agarose gel (120V, 30min) **(Fig. 2.8)**. All the three samples show bands between 1000 and 1500 bp indicating presence of TGF- β3 at 1259 bp.

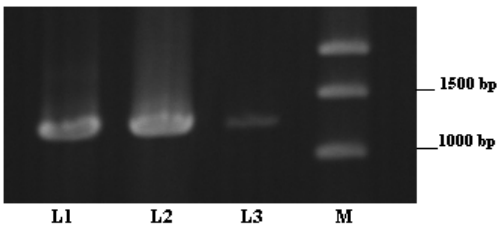


Figure 2.8: Gel Electrophoresis for PCR amplified TGF- β3 CDS cDNA. L1, L2 and L3 are samples from PCR and M is a 1000 bp Ladder (New England Biolabs). TGF-β3 CDS is 1239 bp long and corresponding bands can be seen between 1000bp and 1500bp bands of the marker.

2.3.2 Digestion of donor vector and TGF-β3 cDNA with Sal I and Pst I

pDNR-CMV and TGF-β3 cDNA CDS were both digested with *Sal1* and *Pst1* and purified by gel extraction in preparation for the next step - Ligation. To confirm that sufficient DNA was obtained after gel extraction, it was run on agarose gel again.

The size of pDNR-CMV is 5.6 kb. On digestion with *Sal1* and *Pst1*, the region between these two enzymes in the pDNR-CMV MCS (**Fig. 2.3**) gets excised leaving 5574 bp. The size of TGF-β3 cDNA CDS is 1259 bp which on digestion with *Sal1* and *Pst1* is reduced to 1249 bp. The success of digestion cannot be confirmed from gel electrophoresis as there is very little difference between the size of plasmid DNA before and after digestion. Successful digestion can only be confirmed by analysis of colonies transformed with the ligated plasmid DNA. **Fig. 2.9** does confirm that sufficient DNA was obtained from gel extraction so as to make feasible the next step of ligation (Band in Lane 1 between 5000bp and 6000 bp and in Lane 2 between 1000 bp and 1500 bp).

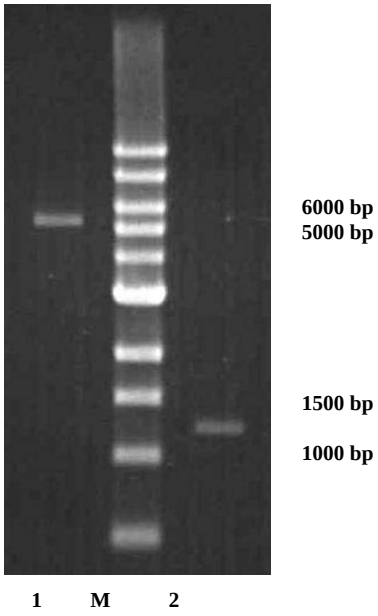


Figure 2.9: pDNR-CMV donor vector (Lane 1, 5574bp) and TGF-β3 cDNA CDS (Lane 2, 1249bp) after digestion with S*al1* and *Pst1* and gel extraction confirm that sufficient DNA was obtained from gel extraction to proceed with ligation.

2.3.3 Subcloning TGF-β3 cDNA CDS into donor vector

Fig. 2.10 shows colonies of DH5 competent cells transformed with the ligated shuttle vector pDNR-CMV + TGF-β3 cDNA CDS.

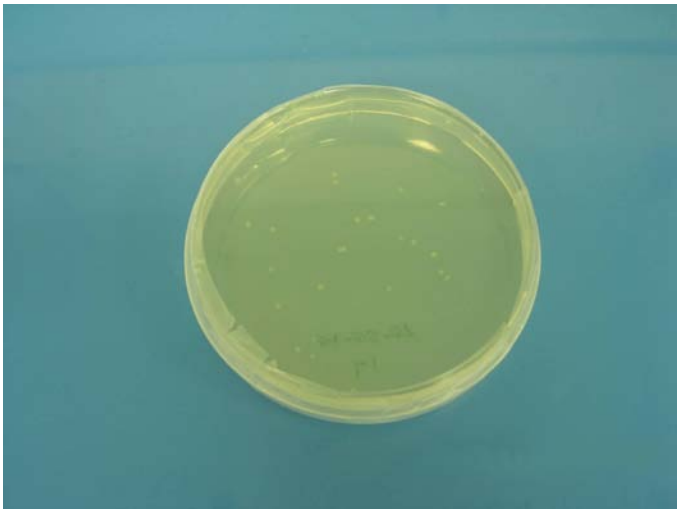


Figure 2.10: MAX Efficiency DH5α Competent Cells, successfully transformed with the ligated shuttle vector pDNR-CMV containing the TGF-β3 cDNA CDS, form Ampicillin resistant colonies.

2.3.3.1 PCR colony screening for presence of TGF-β3 cDNA CDS:

Four colonies were picked and used for PCR colony screening in duplicate PCR tubes with primers for TGF-β3 **(Fig. 2.11)**. Lane 1-1 and 1-2 are samples from colony 1. Lane 1-1 could be a false negative in PCR. Lane 2-1 and 2-2 from Colony 2 both show a positive result for TGF-β3. Lanes 3-1, 3-2, 4-1, 4-2 were negative for TGF-β3. Colonies 3 and 4 could have Donor Vector pDNR-CMV and not the shuttle vector pDNR-CMV + TGF-β3 cDNA CDS. Another alternative could be false negative results from PCR.

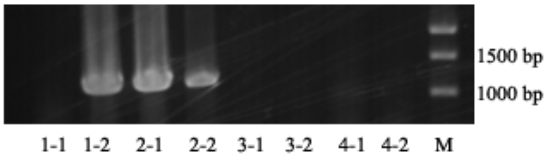


Figure 2.11: Colonies screened for presence of TGF-β3 cDNA (1259 bp) indicating successful transformation with ligated shuttle vector pDNR-CMV + TGF-β3. Lanes 1-1 and 1-2 are samples from colony 1. (Lane 1-1 could be false negative). Lanes 2-1 and 2-2 from colony 2 are positive for TGF-β3 cDNA. No bands in lanes 3-1 and 3-2 and 4-1 and 4-2 indicate that the colonies 3 and 4 could contain Donor Vector pDNR-CMV only or they could be false negative.

2.3.3.2 Confirmation of shuttle plasmids digested with *Sal1* and *Pst1*, by agarose gel electrophoresis

More colonies were picked out and used for plasmid extraction. The extracted plasmids were digested with *Sal1* and *Pst1* and run on agarose gel (**Fig. 2.12**).

A colony successfully transformed with the shuttle vector pDNR-CMV + TGF-β3 cDNA CDS should show two bands at about 5574 bp (pDNR-CMV – 5600 bp minus 26 bp between *sal1* and *pst1* cloning sites in MCS (**Fig. 2.3**)) and 1249 bp (TGF-β3).

All the colonies except colony 4 had bands between 1000 bp & 1500 bp and 5000 bp & 6000 bp indicating presence of both TGF-β3 cDNA CDS and pDNR-CMV. The extracted plasmids from all these colonies were stored at -20°C as stock for future use.

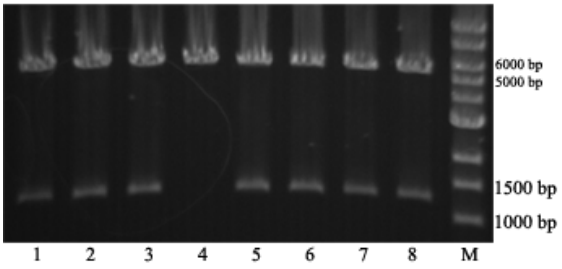


Figure 2.12: Plasmids extracted from transformed colonies and digested with *Sal1* and *Pst1*. Colonies successfully transformed with the shuttle vector pDNR-CMV + TGF-β3 cDNA CDS (all colonies except colony in lane 4) show two bands at about 5574 bp (pDNR-CMV – 5600 bp minus 26 bp between *sal1* and *pst1* cloning sites in MCS (**Fig. 2.3**)) and 1249 bp (TGF-β3).

All the colonies except colony 4 had bands between 1000 bp & 1500 bp and 5000 bp & 6000 bp indicating presence of both TGF-β3 cDNA CDS and pDNR-CMV.

2.3.3.3 Results of DNA sequencing of shuttle vector

Plasmid DNA from colony 2 was sent for DNA sequencing to 1st Base Asia (Singapore). Following is part of the BLAST (NCBI) of the sequence provided by 1st Base:

> ref|NM_003239.1| Homo sapiens transforming growth factor, beta 3 (TGFB3), mRNA

emb|X14149.1|HSTGFB3M Human mRNA for transforming growth factor-beta 3 (TGF-beta 3) Length=2574

Score = 2283 bits (1236), Expect = 0.0

Identities = 1238/1239 (99%), Gaps = 0/1239 (0%)

The one identity (Base Pair) that does not match is base number 369 in TGF-β3 CDS mRNA sequence. A point mutation – T in place of C – has occurred. Base 369 is part of the Codon UGC (367 368 369). This mutation has no effect on the expression of TGF-β3 protein as the Codon UGC codes for Cysteine which is also coded by UGU or TGT (in terms of DNA sequence). Thus the mutation replaces TGC with TGT both of which are codes for the same amino acid Cysteine. This is confirmed by analysis of protein expression later discussed in **2.3.5.2**.

2.3.4 Construction of recombinant adenoviral vector

Plasmid DNA was extracted from three colonies, digested with *Xho1* and run on agarose gel. **Fig. 2.13** shows the expected band sizes after *Xho 1* digestion of the pLP-Adeno-X-CMV Acceptor Vector

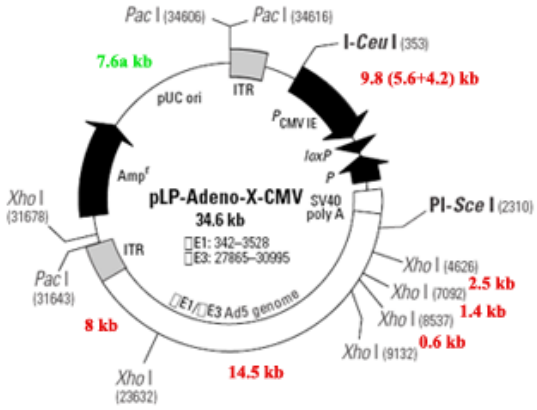


Figure 2.13: pLP-Adeno-X-CMV acceptor vector map showing expected band sizes between xho1 restriction sites

2.3.4.1 *Xho1* restriction analysis

As shown in **Fig. 2.14**, plasmid DNA from colony 1 (Lane 1) and colony 3 (Lane 3) showed positive results on *Xho1* digestion. The expected results of *Xho1* digestion of pLP-Adeno-X-CMV *Acceptor* Vector are 14.5kb, 8.0kb, 7.6akb, 2.5kb, 1.4kb & 0.6kb. The size of TGF-β3 insert (containing chloramphenicol resistance gene) is about 2200 bp and there is a Xho1 restriction site in MCS (**Fig. 2.3**) just downstream of the site of gene (TGF-β3) insertion. Thus the total size of the 7.6a kb band should be around 9.8 kb (7.6+2.2 kb) consisting of two separate bands of sizes 5.6 and 4.2 kb generated by Xho1 restriction site in MCS. Therefore the expected results of Xho1 digestion of pLP-Adeno-X-CMV *Recombinant Adenoviral* Vector are 14.5kb, 8.0kb, 5.6kb, 4.2kb, 2.5kb, 1.4kb & 0.6kb. All of these bands can be seen in colony 3 except for the 0.6 kb band. Thus both colony 1 and colony 3 can be said to possess the Recombinant Adenoviral Vector.

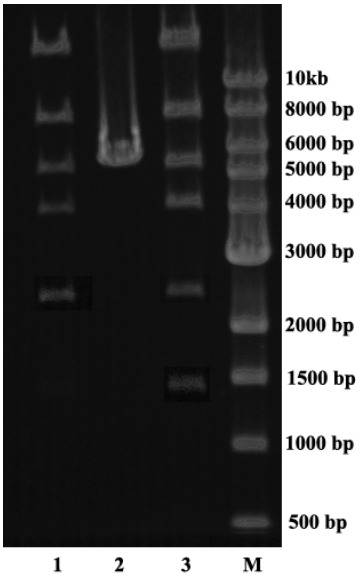


Figure 2.14: *Xho1* restriction analysis of pLP-Adeno-X-CMV *Recombinant Adenoviral* Vector plasmid DNA obtained from colony 1 (lane 1), 2 (lane 2) and 3 (lane 3). The expected band sizes resulting from Xho1 digestion are 14.5kb, 8.0kb, 5.6kb, 4.2kb, 2.5kb, 1.4kb & 0.6kb. These bands, particularly 5.6kb and 4.2kb bands indicate the presence of the gene of interest and successful recombination. Both colony 1 and colony 3 can be said to possess the Recombinant Adenoviral Vector as they give rise to the expected bands.

2.3.4.2 Results of DNA sequencing of recombinant adenoviral vector

Plasmid DNA from colony 3 was sent for DNA sequencing to 1st Base Asia (Singapore). Following is part of the BLAST (NCBI) of the sequence provided by 1st Base:

> ref|NM_003239.1| Homo sapiens transforming growth factor, beta 3 (TGFB3), mRNA

emb|X14149.1|HSTGFB3M Human mRNA for transforming growth factor-beta 3 (TGF-beta 3) Length=2574

Score = 2279 bits (1234), Expect = 0.0

Identities = 1237/1239 (99%), Gaps = 0/1239 (0%)

There were two mismatches. One of them was the same as discussed in **2.3.3.3.** The other was due to an unidentified base in the viral plasmid DNA sequence provided by 1st Base.

2.3.5 Transfection of HEK293 cells with viral particles and subsequent characterization

Fig. 2.15 shows the appearance of CPE in HEK293 cells four days after transfection with the recombinant adenoviral vector.

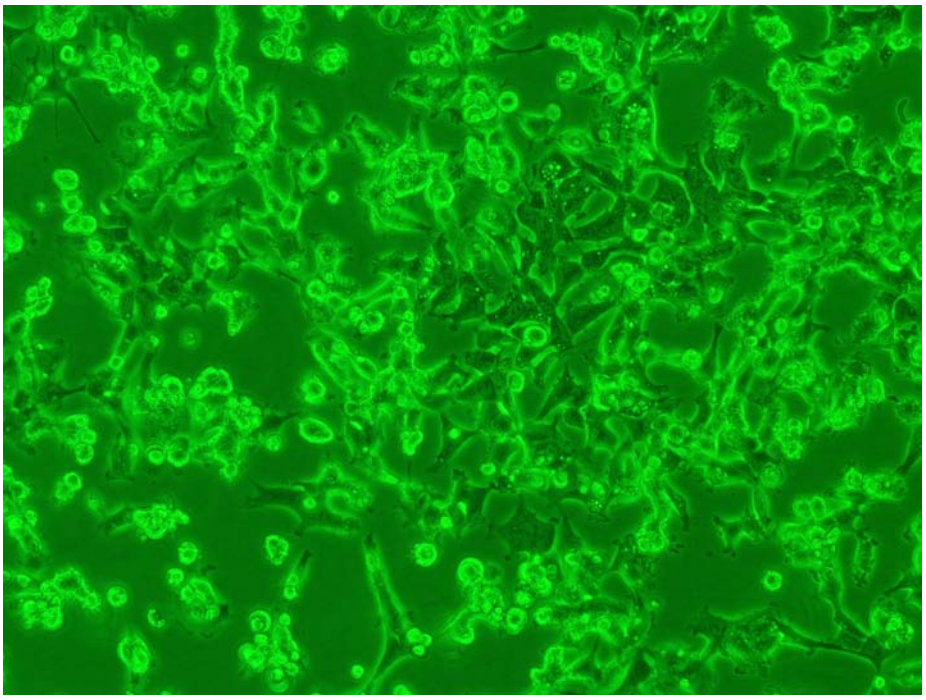


Figure 2.15: Appearance of CPE in HEK293 after 4 days of transfection with Recombinant pLP-Adeno-X-CMV Acceptor Vector. Clear morphological differences can be seen between transfected (round shaped) cells and non-transfected (stretched, with epithelial morphology) cells.

2.3.5.1 RT-PCR analysis

As shown in **Fig. 2.16**, T1 and T2 are DNA samples extracted from HEK 293 by Trizol. C1 and C2 are cDNA samples obtained by Reverse Transcription PCR of RNA extracted from HEK 293 by Trizol. P1 and P2 are TGF-β3 cDNA samples obtained in **section 2.2.2**

and used as a positive control. All of the aforementioned samples were obtained by using TGF-β3 primers for PCR. A1 and A2 are samples of cDNA obtained by RT-PCR of RNA extracted from HEK 293 by using the primers for β-Actin, used as a housekeeping gene (Size of expected product: 540 bp). Samples T1, T2, C1 and C2 are all positive for TGF-β3 (1261 bp) indicating that not only the gene is present in the cells (T1, T2) but is also being transcribed (C1, C2).

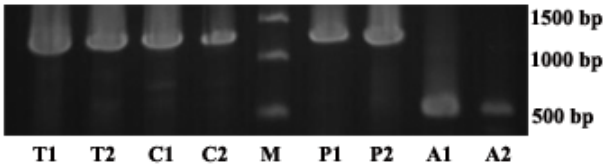


Figure 2.16: RT-PCR Analysis of HEK293 cells transfected with recombinant adenoviral vector carrying TGF-β3. T1 and T2 are DNA samples extracted from HEK 293 by Trizol while C1 and C2 are cDNA samples obtained by Reverse Transcription PCR of RNA extracted from HEK 293 by Trizol using primers for TGF-β3 (product size: 1261bp). P1 and P2 are recombinant TGF-β3 cDNA samples used as positive control. A1 and A2 are samples of cDNA obtained by RT-PCR of RNA extracted from HEK 293 by using the primers for β-Actin (product size 540bp).

2.3.5.2 Western blot analysis

Protein samples collected from the supernatant (Pro1), from Trizol reagent (Pro3) and from cell lysis (Pro2) were characterized by Western Blot using Rabbit anti Human Polyclonal TGF-beta 3 (Abcam) and Rabbit anti Human Polyclonal GAPDH (IMGENEX) as Primary antibodies and Goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) as Secondary antibody **(Fig. 2.17)**.

The molecular weight of TGF- β3 and GAPDH Proteins is 47kD and 37kD respectively. All the three samples are positive for both the proteins. It can be concluded that TGF- β3 is being sufficiently expressed by the transfected HEK 293 cells.

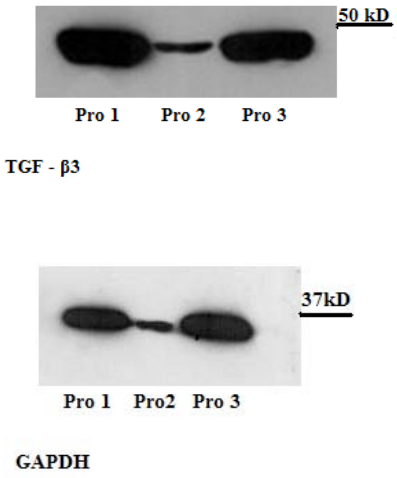


Figure 2.17: Western Blot of TGF- β3 (47kD) and GAPDH (37kD) proteins from transfected HEK 293 cells using Rabbit anti Human Polyclonal TGF-beta 3 (Abcam) and Rabbit anti Human Polyclonal GAPDH (IMGENEX) as Primary antibodies and Goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) as Secondary antibody. Protein samples collected from the supernatant – Pro1, from Trizol reagent - Pro3 and from cell lysis - Pro2.

2.3.6 Producing recombinant adenovirus expressing a green fluorescent protein

2.3.6.1 Observation of fluorescence under a fluorescent microscope.

Successful transfection was confirmed by the expression of green fluorescent protein, which was observed by fluorescence microscopy (**Fig. 2.18**).

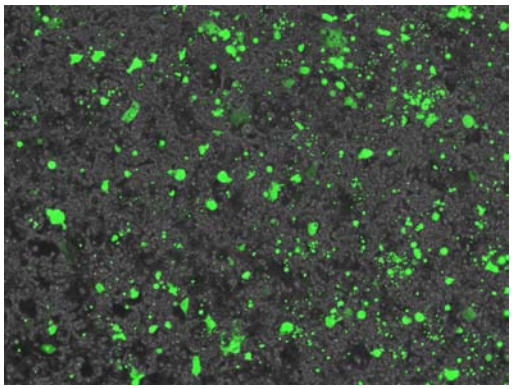


Figure 2.18: Expression of green fluorescent protein in HEK293 after 7 days of transfection with recombinant pLP-Adeno-X-CMV-E3-ZsGreen1 Acceptor Vector

2.3.6.2 *Xho1* restriction analysis of recombinant adenoviral vector

Lanes 1, 2, and 3 in **Fig 2.19** represent the three transfected colonies that were used for plasmid extraction. The extracted plasmids were digested with *Xho1*. Expected bands from the *Recombinant* pLP-Adeno-X-CMV-E3-ZsGreen1 Acceptor Vector are: 14.6 kb, 9.7 kb, 5.6 kb, 4.2 kb, 2.5 kb, 1.4 kb and 0.6 kb. All the three colonies show all of the above bands indicating successful recombination and transfection.

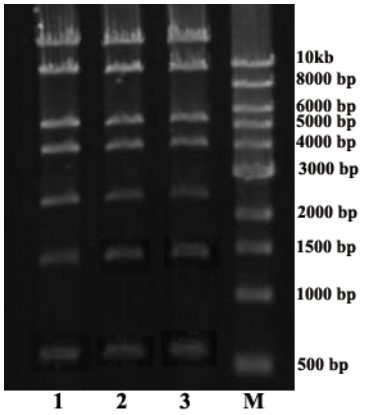


Figure 2.19: *Xho1* Restriction analysis of Recombinant pLP-Adeno-X-CMV-E3-ZsGreen1 Acceptor Vector. Expected bands from *Xho 1* digestion are: 14.6 kb, 9.7 kb, 5.6 kb, 4.2 kb, 2.5 kb, 1.4 kb and 0.6 kb. All the three transformed colonies (Lanes 1, 2 and 3) show all of the above bands indicating successful recombination and transformation. Lane M represents a 10kb DNA ladder.

2.3.6.3 RT-PCR analysis of transfected HEK293

RNA extracted from transfected HEK 293 cells was used for RT and then for PCR using primers for TGF-β3 and β-Actin **(Fig. 2.20)**. C1 and C2 represent the cDNA samples screened for TGF- β3 (1261 bp) and B1 and B2 are cDNA samples screened for β-Actin (540 bp). P1 and P2 are TGF-β3 cDNA samples obtained in **section 2.2.2** and used as a positive control. C1 and C2 bands are positive indicating that the transfected gene is being sufficiently expressed.

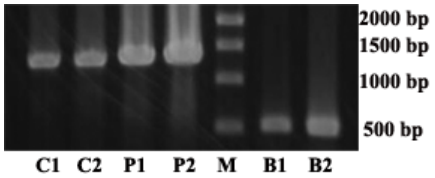


Figure 2.20: RT-PCR Analysis of HEK293 cells transfected with recombinant adenoviral vector carrying TGF-β3. C1 and C2 represent the cDNA samples screened for TGF- β3 (1261 bp) and B1 and B2 are cDNA samples screened for β-Actin (540 bp). P1 and P2 are TGF-β3 cDNA positive control. M represents a 10kb DNA ladder. C1 and C2 are positive for TGF-β3 indicating that the transfected gene is being sufficiently expressed in HEK293 cells.

2.3.6.4 Titer of primary amplification and purified virus stock

The titers of Primary Amplification and the purified virus stock obtained in **section 2.2.6.1** were $9x10^9$ and $2.8x10^9$ respectively.

2.4 Summary

A recombinant adenoviral vector carrying hTGF-β3 gene was successfully constructed. A recombinant adenoviral vector (Ad.TGF-β3) carrying hTGF-β3 gene and a gene for green fluorescent protein – ZsGreen was also successfully constructed. When HEK 293 were transfected with Ad.TGF-β3, green fluorescence could be seen under a fluorescent microscope indicating successful construction, transfection and functioning of the adenoviral vector.

Chapter 3

Re-differentiation of Chondrocytes Adenovirally Transduced with TGF-β3 Gene

3.1 Introduction

Following the successful construction of adenoviral vector carrying hTGF-β3 gene and a ZsGreen gene, we went on to characterize and optimize various factors involved in transfection of chondrocytes. We then investigated the re-differentiation of Ad.TGF-β3 transfected chondrocytes in the presence of endogenous, constitutively expressed GF.

The source of autologous chondrocytes – normal, healthy non-load bearing cartilage tissue – is limited. As a result, only a limited number of primary chondrocytes are available for any treatment procedure that involves re-implantation of these cells back into cartilage defect. On the other hand, *in vitro* expansion of chondrocytes in monolayer culture results in their de-differentiation and loss of chondrocytic phenotype. After just a few passages, the cells start turning hypertrophic and stop multiplying. Thus for any *in vitro* study that aims to utilize chondrocytes for cartilage regeneration – either as primary cell source responsible for cartilage tissue formation or as companion cells producing necessary GFs for chondrogenesis of another cell type like SMSCs – it is essential to ensure the re-differentiation of *in vitro* expanded chondrocytes. Toward this goal, the determination of an ideal passage for the re-differentiation of the chondrocytes was carried out in the studies described in this chapter. Re-differentiation studies of Ad.TGF-β3 transfected chondrocytes were also carried out in alginate hydrogels where chondrocytes were seeded at various densities.

3.2 Materials and methods

3.2.1 Isolation of chondrocytes

Six week old male Wistar rats were acclimatized for two weeks at the NTU Animal Facility. At the end of the two weeks, the rats were euthanized and sterilized in 75% ethanol for five minutes. Articular cartilage pieces were excised from the eight week old male Wistar Rats, submerged in PBS with 1x Pen/Strep and cut into small pieces the size of half a grain of rice. The cartilage pieces were then incubated for 12-14 hours with gentle rotation at 60rpm in cartilage digestion medium – Dulbecco's modified Eagle's medium (DMEM, GIBCO) + 10% Fetal Bovine Serum (FBS, GIBCO) + 1mg/ml Collagenase (type II, 250 U/mg, Worthington). Next day, the medium was collected and centrifuged for 5 minutes at 1100rpm. The cells were resuspended and plated onto a T75 culture flask in 4 parts DMEM/10%FBS and 1 part DMEM/20% FBS (CCP medium). The medium was changed every three days.

Cartilage tissue was also harvested from the tibia and femur of 3-5 months old New Zealand white rabbits. The cartilage was cut into small pieces and digested overnight with 1mg/ml Collagenase type II (250 units/mg; Worthington, MN) with gentle rotation at 60 rpm at 37°C in 5% CO2. Isolated chondrocytes were cultured at 5% CO_2, 37^{o}C in chondrocyte culture medium (CC) - 0.01 M 4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid (HEPES), 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline, 0.05 mg/mL Vitamin C, 100 U/mL penicillin, 100 U/mL streptomycin and 20% FBS in High Glucose DMEM.

3.2.2 Determination of an ideal passage for the redifferentiation of chondrocytes.

Rat condrocytes were passaged every time they reached near 100% confluence. For each passage as well as for primary chondrocytes, some cells were used for RNA extraction with TRIZOL® Reagent (Invitrogen) and RT-PCR with SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). Primers for the cDNA of the following cartilage related proteins were used for PCR **(Table 3.1)**:

Table 3.1: PCR primers for de-differentiation analysis

Gene	Accession	Forward primer	Reverse Primer
GAPDH	XM574168	5′-CCTCCTCATTGACCTCAACTAC-3′	5′-CATGGTGGTGAAGACGCCAG-3′
Collagen Type II	NM012929	5′-AGTGGAAGAGCGGAGACTA-3′	5′-GACAGGCCCTATGTCCACAC-3′
Collagen Type II (IIA/IIB)	NM012929	5′-GTGAGCCATGATCCGC-3′	5′-GACCAGGATTTCCAGG-3′
Aggrecan	NM022190	5′-TCAGGAACTGAACTCAGTGG-3′	5′-GCCACTGAGTTCCACAGA-3′
COMP	NM012834	5′-CAACTCCGATAAAGACAAGT-3′	5′-ATCTTGTCTATAACCTTGTC-3′
Collagen Type IX	NM001100842	5′-GTAGACTTCAGGATTCCA-3′	5′-CCGGAACTCCAGGAGGC-3′
Collagen Type I	NM053304	5′-AACGATGGTGCCAAGGGTGAT-3′	5′-ATTCTTGCCAGCAGGACCAAC-3′

cDNA obtained from PCR was run on agarose gel to monitor the expression of cartilage related proteins.

Real Time PCR was performed with cDNA from Primary through Passage 5 chondrocytes using iQ™ SYBR® Green Supermix (Bio-Rad). The primers used for Real Time PCR are listed in **Table 3.2** below.

Table 3.2: Real-time PCR primers for de-differentiation analysis

Gene	Accession	Forward primer	Reverse Primer
GAPDH	NM017008	5′-ATGATTCTACCCACGGCAAG-3′	5′-CTGGAAGATGGTGATGGGTT-3′
Collagen Type II	L48440	5′-CCGATCCCCTGCAGTACATG-3′	5′-TGCTCTCGATCTGGTTGTTCA-3′
Aggrecan	J03485	5′-CAGAACCTTCGCTCCAATGAC-3′	5′-CCTCAATGCCATGCATCACTT-3′
COMP	NM012834	5′-AACGCTCACCCCTGTTTCC-3′	5′-GGACAGGCTTCGCAGTGAA-3′
Collagen Type IX	S67620	5′-CTGATGGATTAACAGGACCTGATG-3′	5′-CAGGCACACCAGGTTCTCCTT-3′
Collagen Type I	NM053304	5′-CAACCTCAAGAAGTCCCTGC-3′	5′-AGGTGAATCGACTGTTGCCT-3′
Collagen Type X	RNO131848	5′-CCCAGGGTTACCAGGACCAA-3′	5′-GTTCACCTCTTGGACCTGCC-3′

WST analysis was done for Passage 2 through Passage 5 chondrocytes using Cell Proliferation Reagent WST-1 (Roche) to measure the proliferation/viability of cells. Equal number of cells – 5000 – were seeded in a 96 well plate with five replicates each for Passages 2 through 5. After the cells reached confluence, they were used for WST analysis. After measuring absorbance through a spectrophotometer, the cells were trypsinized and counted. Absorbance was then normalized with the number of cells.

3.2.3 Determination of optimum multiplicity of infection (MOI) for adenoviral transduction of chondrocytes.

Optimum MOI for transfection with Ad.TGF-β3 was determined using flow cytometry for rabbit chondrocytes. Passage 2 rabbit chondrocytes were plated in each well of six well plates at a seeding density of 2×10^5. The chondrocytes in the wells of the aforementioned 6-well plates were transfected with MOI of 10, 50, 100, 300, 500 and 1000. The cells were cultured in 1 ml of serum free DMEM containing Ad.TGF-β3 for 2 hours at 5% CO_2, 37^{o}C, after which the culture fluids were replaced with 2 ml of

complete culture medium. Flow cytometry was used to determine transfection efficiency and viability of chondrocytes 2 days after transfection following the protocol described below.

Trypsinized chondrocytes (transfected and non-transfected) were centrifuged in 15 ml centrifuge tubes at 1100 rpm for 5 minutes. Cell pellets were washed once with PBS and spun down. The cell pellets were resuspended in 1 ml of PBS and transferred evenly into two 5 ml round-bottom tubes. These tubes were labeled as transfected (TF) with PI (BD Pharmingen, 556463), TF without PI, negative (N) with PI and N without PI. 5 µl of PI was added to the tubes labeled with PI. All four 5 ml round-bottom tubes were covered with aluminum foil to minimize exposure of suspensions to light. The suspensions were vortexed and incubated for at least 20 minutes at 37°C before proceeding with flow cytometry using BD FACSCalibur™.

PI is used to indicate dead cells while green fluorescent protein expressed from transfected cells indicates live transfected cells. Non-fluorescent or unstained cells indicate live non-transfected cells.

3.2.4 Re-differentiation of chondrocytes adenovirally transduced with TGF-β3

The study consists of 4 groups **(Table 3.3).**

Table 3.3: Groups for Chondrocytes Study

Group	Conditions
N	Non-transfected chondrocytes with seeding density of 3 million/ml
T	Transfected chondrocytes with seeding density of 6 million/ml
T'	Chondrocytes transfected with excess media and with seeding density of 3 million/ml
T''	Chondrocytes transfected with seeding density of 23 million/ml

Passage 2 rabbit chondrocytes were thawed and cultured in monolayer till adequate numbers were reached. Half were transfected with MOI of 100, but with excess medium, 20 ml in T150 flasks, during the period of transfection. These were encapsulated at a seeding density of 3 million/ml in alginate beads of approximately 40 μl. The non-transfected chondrocytes were encapsulated at a seeding density of 3 million/ml, also in 40 μl alginate beads. Another batch of Passage 2 rabbit chondrocytes were transfected 1 day after and encapsulated at seeding densities of 6 million/ml and 23 million/ml.

This study spanned 6 weeks with analysis with flow cytometry, ELISA and Immuno/Histochemical staining in order to assess re-differentiation of the passaged chondrocytes in alginate beads **(Table 3.4)**.

Table 3.4: Summary of time points for data collection for chondrocytes study

Day/ Analysis	10	20	30	40
Flow Cytometry		Every 3 days		
Immuno/histochemical Staining	✓	✓	✓	✓
ELISA		Every 3 Days		

3.2.4.1 Viability of transfected chondrocytes

The viability of Ad.TGF-β3 transfected rabbit chondrocytes encapsulated in alginate hydrogels was determined using flow cytometry.

3.2.4.1.1 Optimization of flow cytometry protocol for chondrocytes encapsulated in alginate beads

When Ad.TGF-β3 transfected chondrocytes are cultured in alginate for a period of time, they start expressing abundant ECM and start forming a cartilage-like tissue. In order to extract single cells from such engineered constructs for flow cytometry, a refined and optimized protocol was established as described below.

The basic flow cytometry protocol to determine the viability or transfection efficiency for Ad.TGF-β3 transfected chondrocytes encapsulated in alginate beads is as described in the **section 3.2.3** above. This protocol was supplemented with an additional step of initial

dissolution with 55 mM sodium citrate, to dissolve alginate beads and release the encapsulated cells/construct.

Several flow cytometry assays were then carried out to determine the effect of various steps in the flow cytometry protocol. Factors investigated were incubation time with sodium citrate, incubation time with PI, effect of trypsin and effect of Collagenase II.

Non-transfected chondrocytes alginate beads were dissolved by incubating in sodium citrate for 5 minutes and 20 minutes. These two samples were then prepared as per the above described flow cytometry protocol and the results analyzed using flow cytometry to determine any effects of the different incubation time.

To determine the effects of incubation time with PI, three samples were incubated at 37°C for 5, 10 and 20 minutes with PI and analyzed with flow cytometry. All other steps of the protocol were as described above.

It was observed that the chondrocytes cultured in alginate began to aggregate over time, possibly due to secretion of ECM components. This could potentially affect the accuracy of flow cytometry as an aggregation of many cells may be counted as a singular cell. To counter this problem, we proposed that after washing the cell pellet with PBS and centrifuge, the cell pellet should be resuspended in 0.5 ml PBS and 1 ml trypsin or 1 ml Collagenase II for 5 minutes. The rationale is that trypsin has potential to cause cell detachment and could be used to dissolve the aggregates. Also, Collagen II is one of the

major ECM components present in these aggregates and hence digestion with Collagenase II would result in the separation of cells and dissolution of the aggregates. Here, we tested with non-transfected and transfected chondrocytes alginate beads using both trypsin and Collaganese II.

3.2.4.1.2 Determination of viability of transfected rabbit chondrocytes

As a result of the study done above, a refined flow cytometry protocol was developed and used to determine the viability of Ad.TGF-β3 transfected rabbit chondrocytes encapsulated and culture in alginate hydrogels as described below.

Every three days, alginate beads of non-transfected and Ad.TGF-β3 transfected chondrocytes were dissolved in 1 ml 55 mM sodium citrate in a 15 ml centrifuge tube each for 5 minutes and then centrifuged at 1100 rpm for 5 minutes. The cell pellets were resuspended in 1 ml PBS and 2µl of Ad.TGF-β3 transfected chondrocytes was used for cell counting using a haemocytometer. The cell suspensions were then centrifuged again and the pellets were resuspended in 0.5 ml PBS and 1 ml Collagenase II (100µg/ml) and incubated at 37°C for 5 minutes. After centrifuge, the pellets were resuspended in 1 ml PBS and redistributed into two 5ml round bottom tubes each, wrapped with aluminum foil and labeled as transfected (TF) with PI, TF without PI, negative (N) with PI and N without PI. 10 µl of PI (BD Pharmingen, 556463) was added to tubes labeled “with PI”. The tubes were incubated for 5 minutes at 37°C before proceeding with flow cytometry with BD FACSCalibur™.

3.2.4.2 Expression profile of TGF-β3 in Ad.TGF-β3 transfected chondrocytes

Medium was collected every three days from the group T and stored at -80°C for future use. ELISA (R&D Systems, Minneapolis, MN) was performed using the stored media according to the manufacturer's instructions in order to obtain the expression profile of TGF-β3 from Ad.TGF-β3 transfected chondrocytes.

ELISA was also done for medium collected over a period of 72 hours from manually added recombinant TGF-β3 protein in culture medium without any hydrogels or cells.

3.2.4.3 Immuno/histochemical Staining

Alginate beads from all four groups were collected and stored in 4% Paraformaldehyde for immunohistochemical staining. The alginate beads were progressively dehydrated by immersing in 30%, 50%, 70%, 80%, 90%, 95% ethanol, 3 times in 100% ethanol and finally in xylene. These were then sent to be embedded in paraffin and sectioning with a microtome. Sectioned samples were mounted on slides. Prior to staining, the mounted slides were immersed in recycled xylene for 10 minutes. This was followed by three washes with fresh xylene, 1 minute immersion in 100% ethanol, 1 minute immersion in 70% ethanol and 1 minute in deionised water. The slides were left to air dry.

For immunohistochemical staining of Collagen I and II, the slides were incubated for 30 minutes with 1% bovine blocking serum (1% BSA, Bovine Serum Albumin) to inhibit nonspecific binding of IgG. After washing thrice with PBS and drying, the respective primary monoclonal antibodies (Collagen type II - 2 ng/ml in PBS, MAB8887, Millipore;

Collagen type I - 2 ng/ml in PBS, SC-59772, Santa Cruz Biotech) were added and left overnight in the dark at 4°C. Following the incubation, the slides were washed with PBS and dried. The slides were then incubated with Anti-IgG binding immunofluorescent protein (5 ng/mL in PBS, Invitrogen Alexa Fluor®, 488) for 1 hour in the dark at 37°C. Finally, they are washed three times with PBS and mounted with a mounting solution containing DAPI (4'-6-Diamidino-2-phenylindole) and observed under a fluorescent microscope.

In addition to immunohistochemical staining of Collagen I and II, histochemical staining of GAG was also carried out. For histochemical staining, slides were immersed in Safranin-O for 5 minutes before immersing in deionised water, 70% ethanol and 100% ethanol for 1 minute each. After a final wash with xylene, a cover slip was placed over the sample with one drop of xylene and mounting solution and the slides were observed under an optical microscope.

3.3 Results and Discussion

3.3.1 Determination of an ideal passage for the redifferentiation of chondrocytes

An ideal passage for redifferentiation would be where the cells start showing decline in production of or stop producing proteins expressed by differentiated cells - Type II Collagen, Type II Collagen (IIA/IIB), Type IX Collagen, COMP, Aggrecan and start showing significant production of proteins expressed by dedifferentiated cells – Type I

Collagen. The cells should also be sufficiently viable so as to withstand transfection with adenovirus.

In **Fig. 3.1**, it can be seen that the production of Collagen IX stops after Passage 2. Also Aggrecan, COMP and Collagen IIA/IIB are produced in significantly lesser amounts in Passages 4 and 5. The production of Collagen I steadily increases from passage1 through passage 5 chondrocytes. From these results it can be inferred that Passage 4 and Passage 5 can both be used as starting points in re-differentiation of chondrocytes.

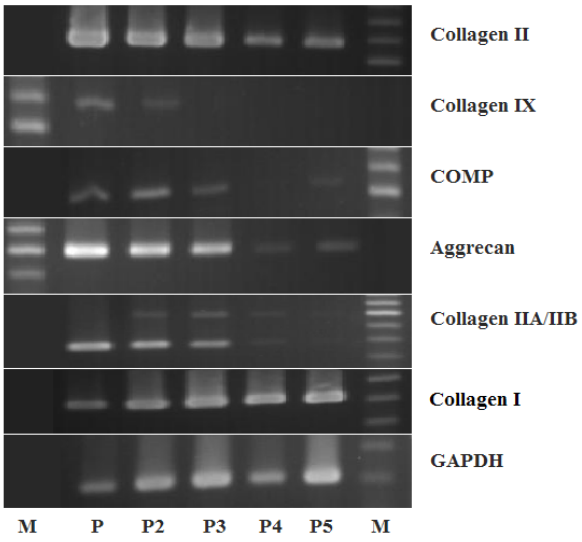


Figure 3.1: Products of RT-PCR for RNA extracted from *in vitro* cultured rat chondrocytes using primers for Collagen II, Collagen IIA/IIB, Collagen IX, COMP, Aggrecan and Collagen I. M stands for DNA Ladder and P, P2, P3, P4, P5 stand for primary chondrocytes, passage 2, passage 3, passage 4 and passage 5 chondrocytes respectively.

Real Time PCR results **(Fig. 3.2)** correlate with PCR and Electrophoresis results. It can be seen that the expression of cartilage/chondrocytic markers decreases significantly by Passage 3 compared to that in Passage 2 or primary chondrocytes.

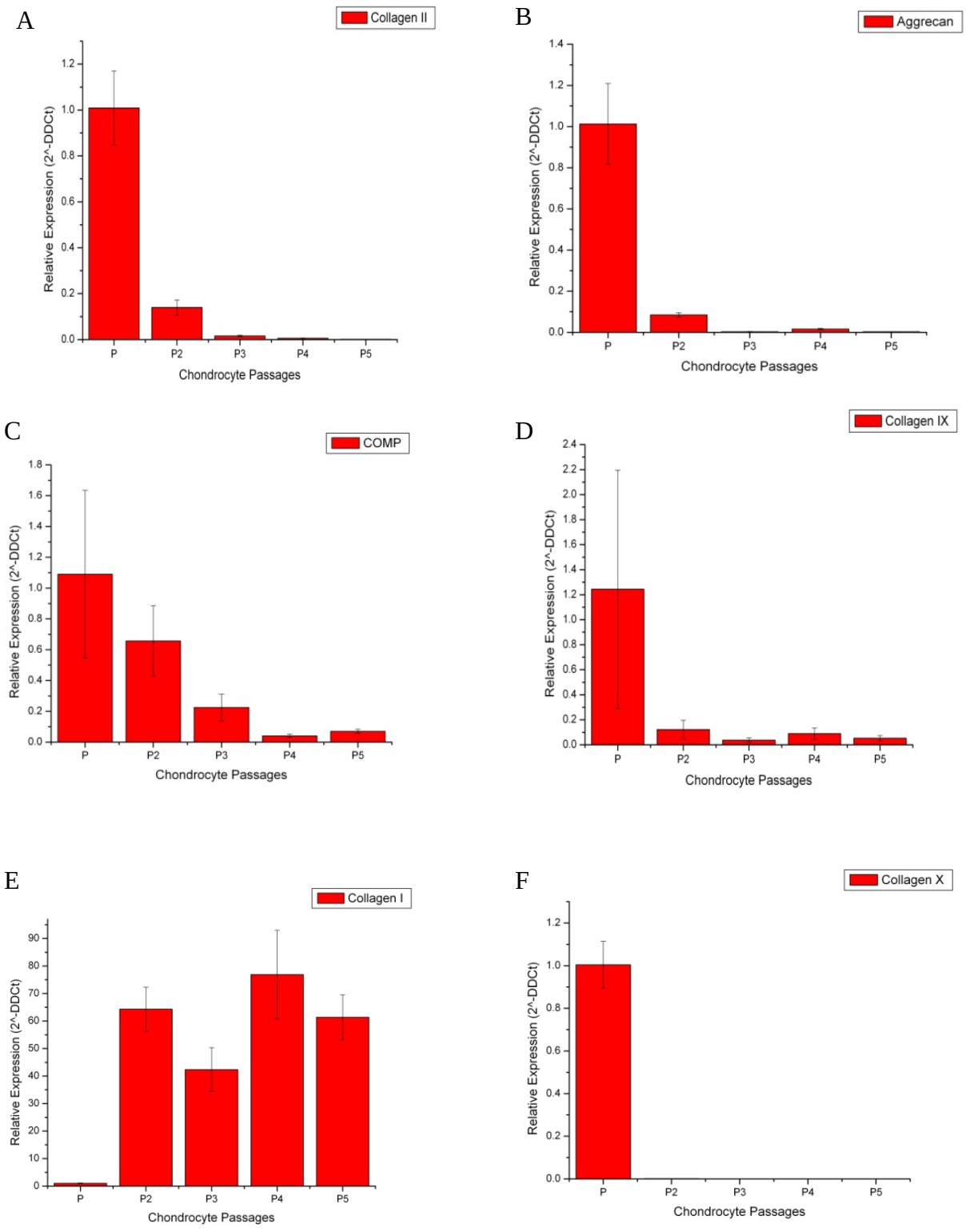


Figure 3.2: Real-time PCR results for different primers and passages of rat chondrocytes. A)Collagen II, B)Aggrecan, C)COMP, D)Collagen IX, E)Collagen I and F)Collagen X.

Passages assayed are Primary (P), passage 2 (P2), 3 (P3), 4 (P4) and 5 (P5) respectively. Expression of cartilage/chondrocytic markers significantly decreases by Passage 3 compared to Passage 2 or primary chondrocytes

WST analysis (**Fig. 3.3**) of chondrocytes from Passages 2 through 5 revealed that all the Passages, 2 through 5, showed similar proliferation. It can be said the there is very little difference between the viability/proliferation of the cells from the aforementioned passages.

The Passage 5 chondrocytes exhibit a slower growth compared to the growth rates of Passages 2, 3 and 4. The Passage 5 cells reached confluence two days later than Passage 2, 3 and 4 chondrocytes. The Passage 5 chondrocytes also start showing a slight change in their phenotype.

It would be suitable to select a higher passage so as to obtain sufficient population of cells. But the cells should also be viable/proliferative enough to withstand adenoviral transfection and the dedifferentiation should not be so further progressed that the cells start showing significant expression of proteins that are the markers of fibroblasts or chondro-progenitor cells. Based on these criteria, it would be advantageous to select Passage 4 or earlier for re-differentiation and co-culture.

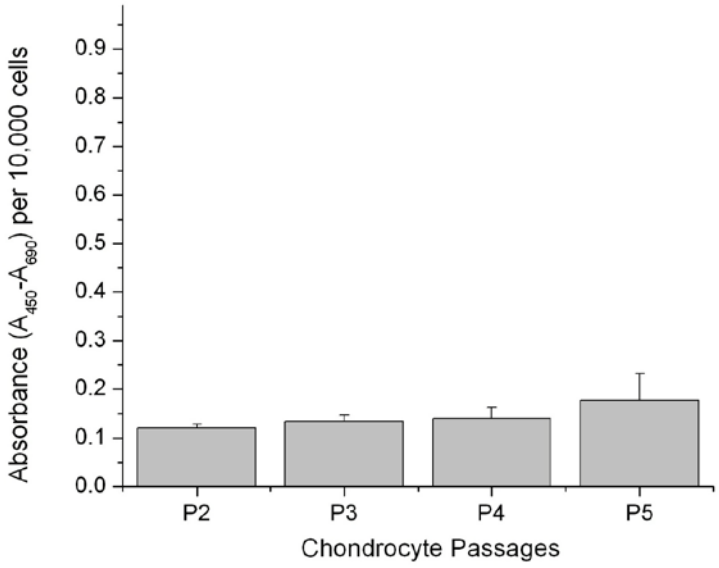


Figure 3.3: WST results of cell proliferation/viability in various passages of rat chondrocytes. Passages 2 through 5 have similar proliferation rates.

3.3.2 Determination of optimum MOI for adenoviral transduction of chondrocytes.

As shown in **Fig. 3.4,** successful transfection was determined after 2 days by the expression of green fluorescence observed under ultraviolet light by the Ad.TGF-β3 transfected chondrocytes (**Fig. 3.4a**). Nil-transfected and non-transfected chondrocytes do not express green fluorescence (**Fig. 3.4b**).

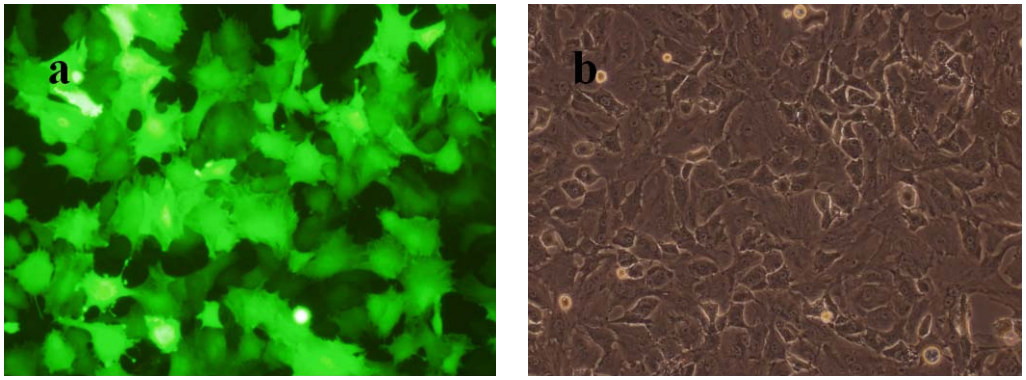
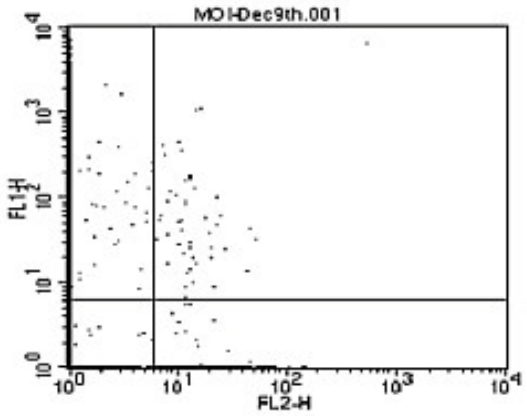


Figure 3.4: Micrographs of **(a)** Ad. TGF-β3 transfected rabbit chondrocytes under ultra-violet (2 days after transfection) and **(b)** normal rabbit chondrocytes. GFP expressed from recombinant adenoviral vector illuminated under ultra-violet light.

Results from flow cytometry analysis indicate an MOI of 300-500 gives rise to maximum number of viable transfected rabbit chondrocytes two days post-transfection (**Fig. 3.5**). MOI 300 and 500 have the highest percentage of transfection with the least percentage of dead cells: 85.51% cells were alive and transfected and 8.23% were dead for MOI of 300; 88.99% cells were alive and transfected and 7.85% were dead for MOI of 500. This is significant compared to MOI of 50, which had only achieved 60.49% transfection of the population and 17.43% deaths in the population.

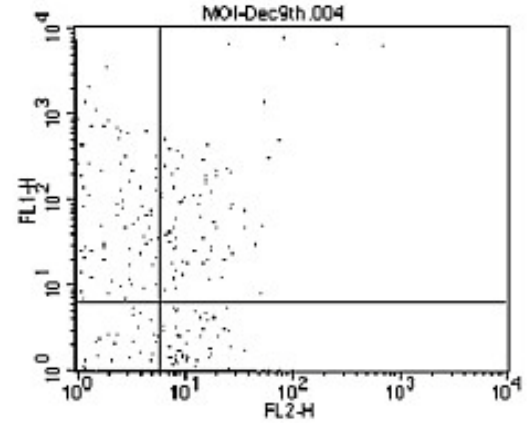
A



Quadrant Statistics

File: MOI-Dec9th.001
Sample ID: 10
Acquisition Date: 09-Dec-08
Gate: No Gate
Gated Events: 10000
Total Events: 10000

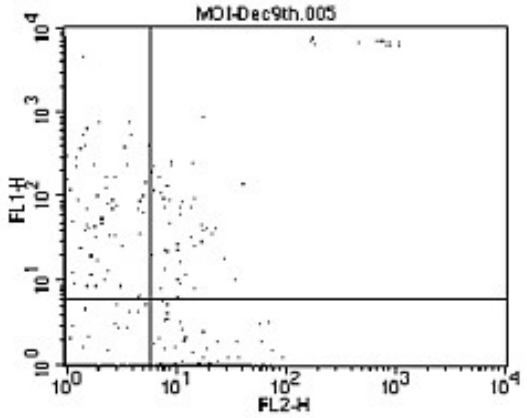
Quad	Events	% Gated	% Total
UL	2679	26.79	26.79
UR	47	0.47	0.47
LL	6673	66.73	66.73
LR	601	6.01	6.01



Quadrant Statistics

File: MOI-Dec9th.004
Sample ID: 50
Acquisition Date: 09-Dec-08
Gate: No Gate
Gated Events: 10000
Total Events: 10000

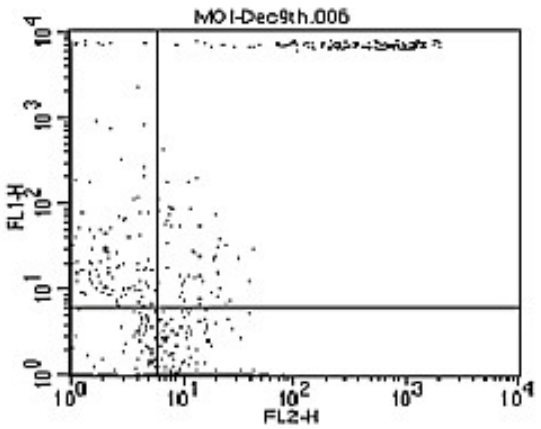
Quad	Events	% Gated	% Total
UL	6049	60.49	60.49
UR	77	0.77	0.77
LL	2208	22.08	22.08
LR	1666	16.66	16.66



Quadrant Statistics

File: MOI-Dec9th.005
Sample ID: 100
Acquisition Date: 09-Dec-08
Gate: No Gate
Gated Events: 10000
Total Events: 10000

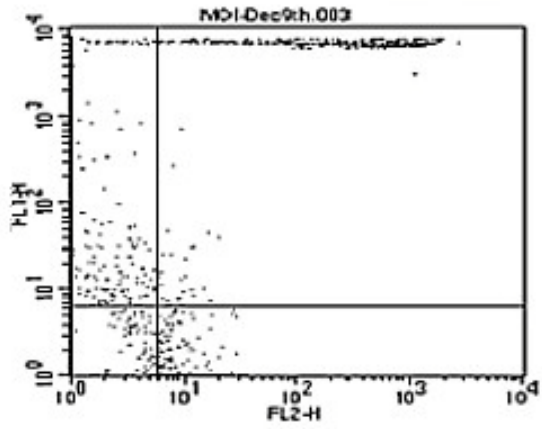
Quad	Events	% Gated	% Total
UL	7034	70.34	70.34
UR	59	0.59	0.59
LL	2146	21.46	21.46
LR	761	7.61	7.61



Quadrant Statistics

File: MOI-Dec9th.006
Sample ID: 300
Acquisition Date: 09-Dec-08
Gate: No Gate
Gated Events: 10000
Total Events: 10000

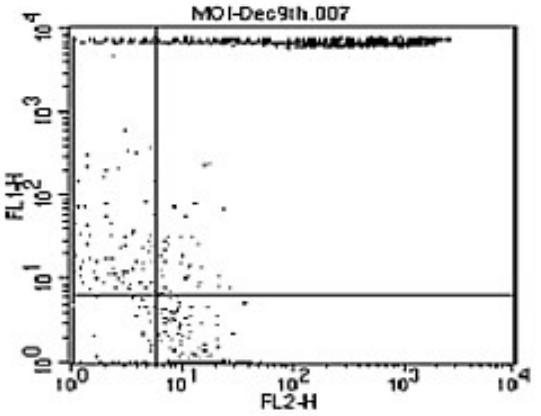
Quad	Events	% Gated	% Total
UL	8551	85.51	85.51
UR	207	2.07	2.07
LL	626	6.26	6.26
LR	616	6.16	6.16



Quadrant Statistics

File: MOI-Dec9th.003
Sample ID: 500
Acquisition Date: 09-Dec-08
Gate: No Gate
Gated Events: 10000
Total Events: 10000

Quad	Events	% Gated	% Total
UL	8899	88.99	88.99
UR	429	4.29	4.29
LL	316	3.16	3.16
LR	356	3.56	3.56



Quadrant Statistics

File: MOI-Dec9th.007
Sample ID: 1000
Acquisition Date: 09-Dec-08
Gate: No Gate
Gated Events: 10000
Total Events: 10000

Quad	Events	% Gated	% Total
UL	8346	83.46	83.46
UR	1200	12.00	12.00
LL	226	2.26	2.26
LR	228	2.28	2.28

B

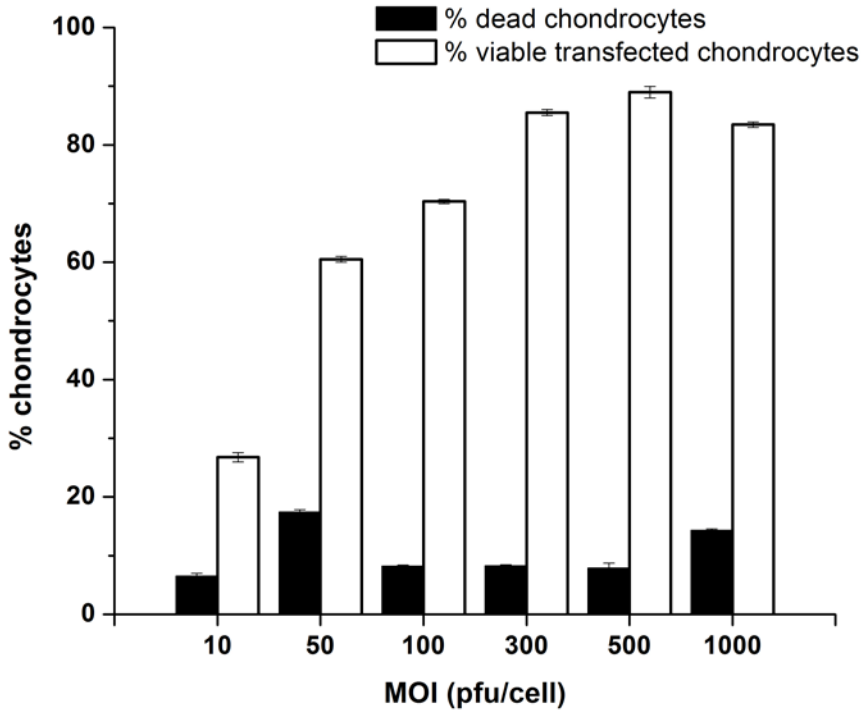


Figure 3.5: Flow cytometry analysis. **(A)** Representative flow cytometry dot plots of rabbit chondrocytes transfected at 10, 50, 100, 300, 500 and 1000 MOI, 48 hours post-transfection indicating cell population distribution based on green fluorescent protein expression and propidium iodide staining. Dots in upper left quadrant represent viable transfected cells. **(B)** Transduction efficiencies and cell viabilities of transfected rabbit chondrocytes at various MOIs. Results are statistically significant when $p<0.01$ (one-way AVONA).

3.3.3 Optimization of flow cytometry protocol for chondrocytes encapsulated in alginate beads

Flow cytometry analysis shows that incubation time with sodium citrate had little effect on the flow cytometry results. Hence, 5 minutes incubation time was used for the chondrocytes study.

Different lengths of incubation time with PI also had little or no effect on the outcome of the flow cytometry data (**Fig. 3.6**).

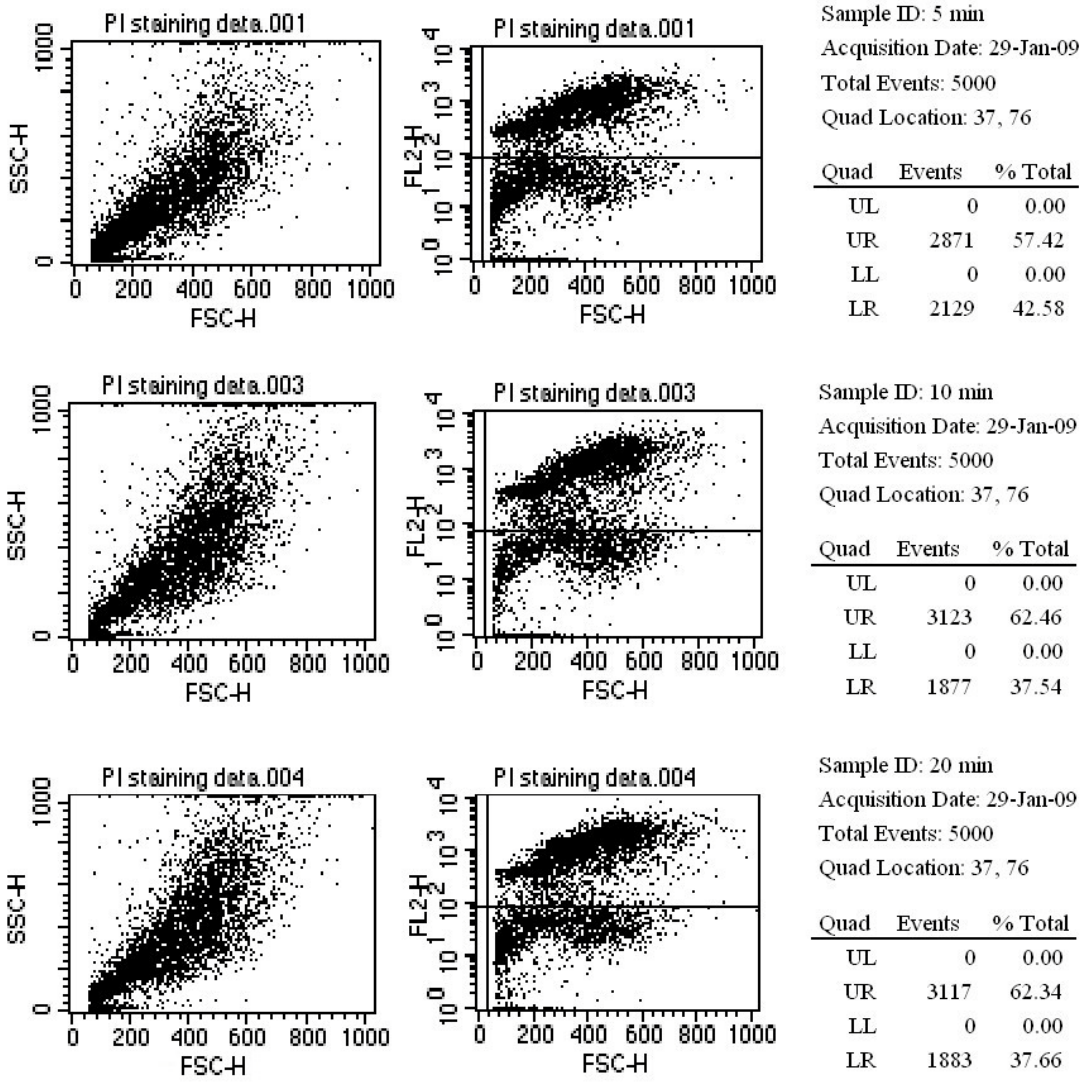


Figure 3.6: Flow cytometry analysis showing the effect of different lengths of incubation time with PI – 5, 10 and 20 minutes, for rabbit chondrocytes encapsulated in alginate beads. Upper right quadrant represents PI stained dead cells while lower right quadrant represents live unstained cells.

It was observed that percentage of dead cells increased with incubation time, 57.12% for incubation of 5 minutes and 62.31% for incubation of 20 minutes. This could be a small percentage of cells dying during the prolonged incubation time. Thus, an incubation time of 5 minutes with PI was used for chondrocytes study.

Finally, it was visually, using a microscope, ascertained that trypsin had no effect on separating the cell aggregates. Collagenase II, had an effect in separating the aggregates. Further analysis with flow cytometry also showed a significant decrease in percentage of dead cells for both non-transfected and transfected chondrocytes alginate beads when treated with Collagenase II (**Fig. 3.7**).

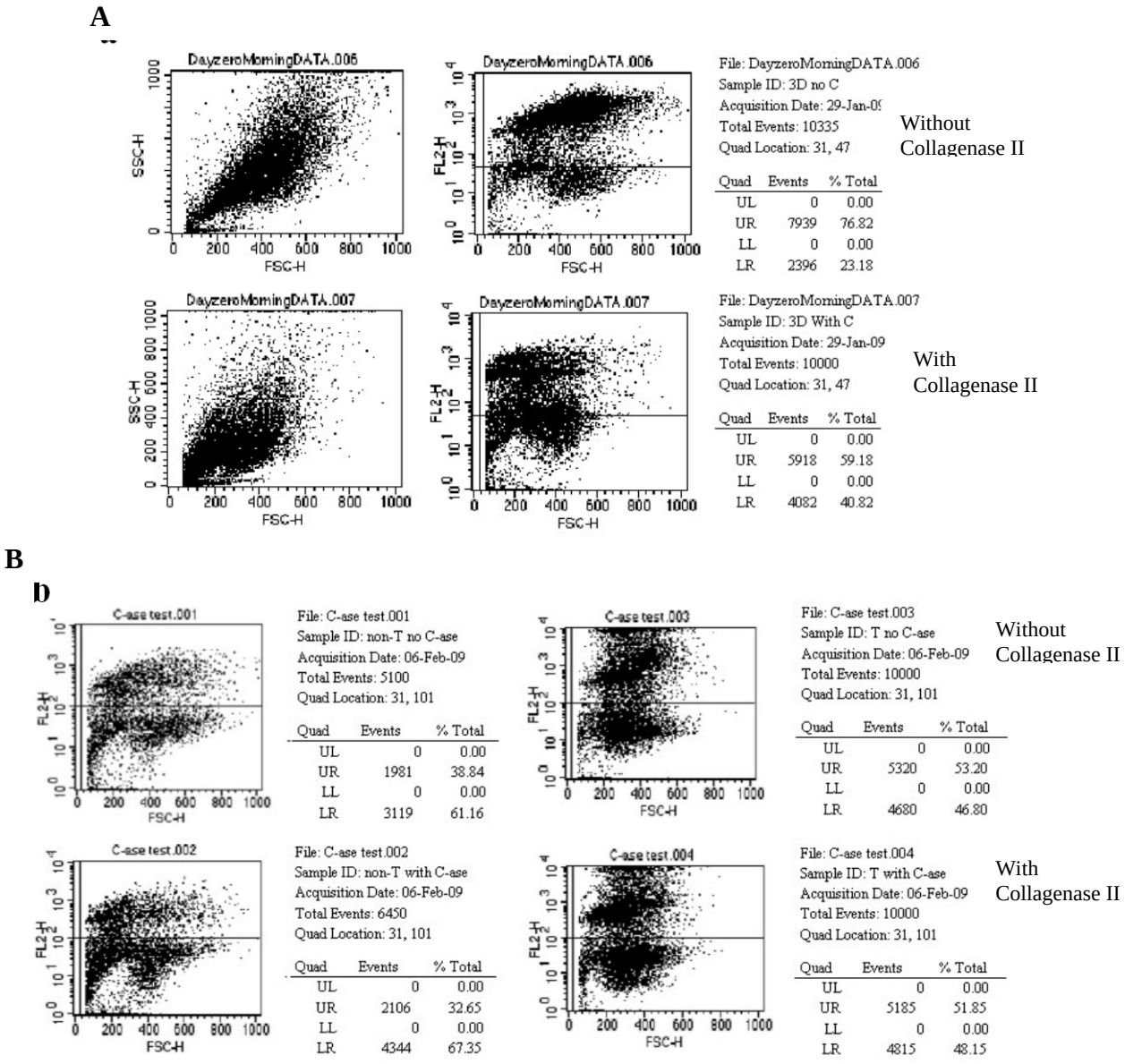


Figure 3.7: Flow cytometry data from **(A)** transfected rabbit chondrocytes and **(B)** non-transfected rabbit chondrocytes in alginate beads, showing a decrease in percentage of dead cells for non-transfected and transfected samples treated with Collagenase II during the flow cyctometry preparation. Upper right quadrant represents PI stained dead cells while lower right quadrant represents live unstained

cells. A significant decrease in percentage of dead cells for both non-transfected and transfected chondrocytes can be seen on treatment with Collagenase II

It is likely that the aggregates, which may contain some live and also dead cells are categorized as a singular dead cell by the flow cytometer due to the PI staining. Therefore, with Collagenase II, the live cells in the aggregates will be freed and counted as live cells. Based on this result, we decided to use Collagenase II for the flow cytometry protocol for chondrocytes study.

3.3.4 Determination of viability of transfected rabbit chondrocytes

The total cell number of Ad.TGF-β3 transfected chondrocytes over a period of 42 days (counted using a haemocytometer) remains more or less constant **(Fig. 3.8)**. Flow cytometry data shows that there is a steady decrease in the percentage of transfected living cells and an increase in the percentage of total dead cells, while the percentage of non-transfected living cells remains constant during these 42 days **(Fig. 3.9)**. The decrease in TGF- β3 expression seen in data from ELISA **(Fig. 3.10)** can be attributed to this decrease in number of living transfected chondrocytes, and to a smaller extent, to the transient nature of gene expression with adenoviruses.

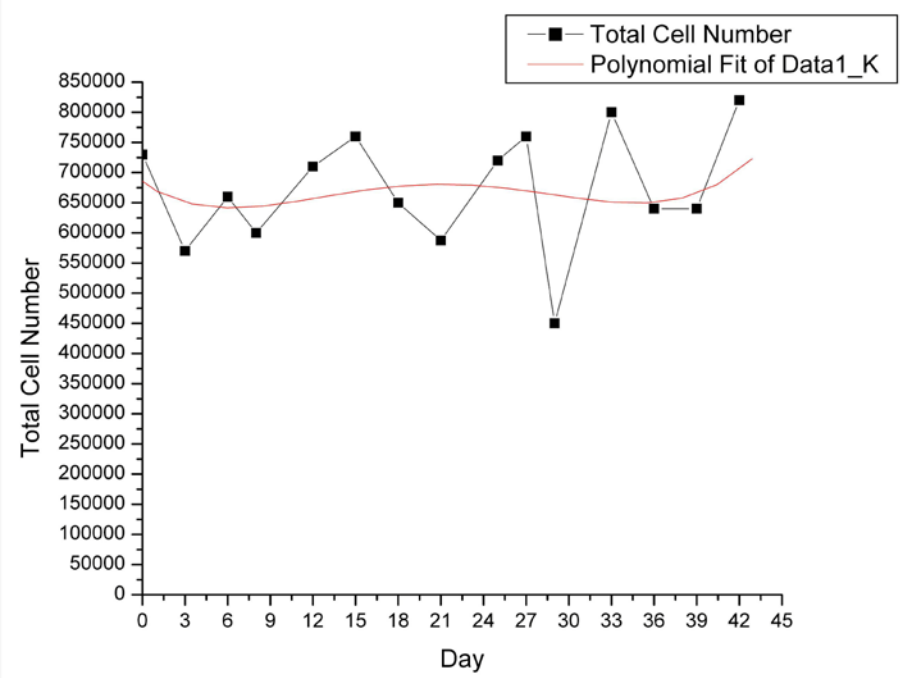


Figure 3.8: Total cell number from alginate beads encapsulating rabbit chondrocytes (6 million/ml seeding density) from one well (six 40µl alginate beads/well) counted before each flow cytometry analysis using a hemocytometer for a period of 42 days. The cells in alginate beads were counted every three days.

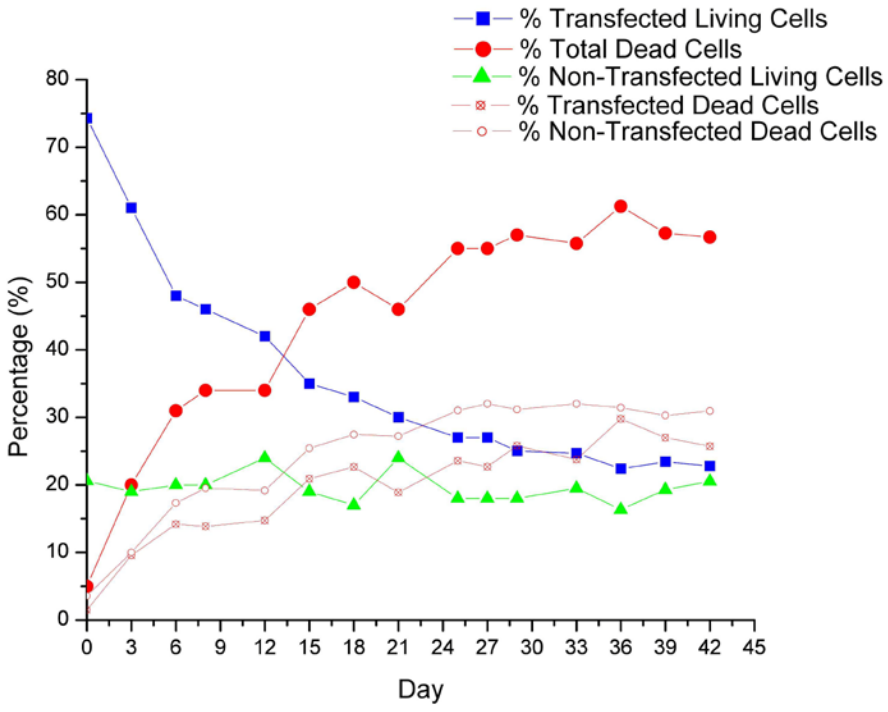


Figure 3.9: Flow cytometry data depicting the percentage of transfected living cells, total dead cells, non-transfected living cells, transfected dead cells and non-transfected dead cells. Non-transfected living cells remain constant in numbers while transfected living cells are seen to decrease over time along with the total number of dead cells. Chondrocytes for flow cytometry obtained from six alginate beads (6 million/ml seeding density) every three days. Live transfected cells are GFP positive. Transfected dead cells are GFP and PI positive. Non-transfected dead cells are PI positive and non-transfected living cells are GFP and PI negative.

The increasing percentage of dead cells can be mainly attributed to the decreasing percentage of living transfected cells. Given the constant total number of cells, this indicates that the number of live transfected cells is decreasing. The decrease in TGF- β3 expression seen in data from ELISA can be attributed to this decrease in number of living transfected chondrocytes. Cell death would be the main reason for the decrease in number of living transfected cells. To a smaller extent, another cause would be the transient nature of gene expression from adenoviruses.

3.3.5 Expression profile of TGF-β3 in Ad.TGF-β3 transfected chondrocytes

ELISA data from Ad.TGF-β3 transfected chondrocytes shows a general decreasing trend in expression of TGF- β3 (**Fig. 3.10**). Expression of TGF- β3 was highest during the first few days with expression levels greater than 100 ng/ml. TGF- β3 expression stabilized at around 4 ng/ml after Day 25. A small spike of 3 ng/ml in expression was also observed from Day 12 to 21.

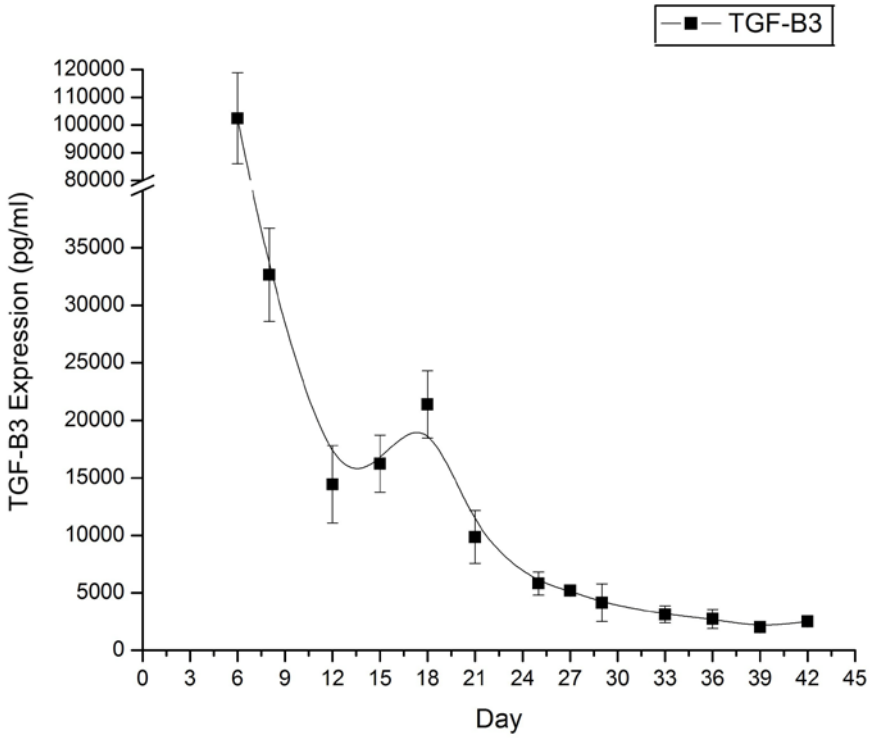


Figure 3.10: Expression of TGF-β3 (pg/ml) by Ad.TGF-β3 transfected rabbit chondrocytes over a period of 42 days as measured by ELISA for medium collected every three days from each well of a 12 well plate containing 6 alginate beads. The expression is too high (10-100 ng/ml) and beyond measurable range for the first 5 days. It gradually decreases and settles at 4 ng/ml after Day 25 for the rest of the culture period. Medium collected from 3 wells for each time point. Data represented as mean ± SD.

Unlike repeated manual dosing of TGF-β3, which has extremely short pharmacological half-life, the release of TGF-β3 by transfected cells can be maintained for weeks, and thus sustained, localized supply of TGF-β3 can be achieved. The degradation of manually added TGF-β3 over a period of 72 hours in culture medium under normal culture conditions is shown in **Fig. 3.11.** "

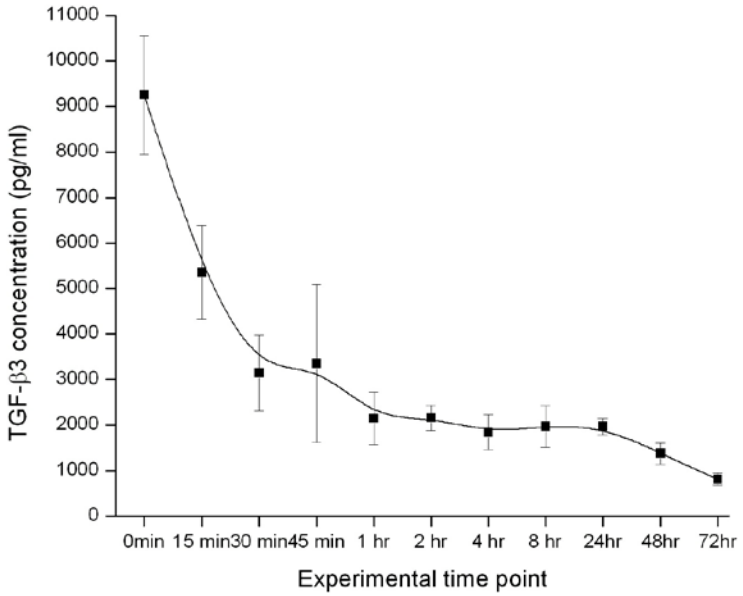


Figure 3.11: Dynamic changes in TGF-b3 concentration in medium containing 10 ng/mL recombinant hTGF-β3 over a period of 72 hours as measured by ELISA. Data represented as mean ± SD. [1]

3.3.6 Expression of ECM proteins

The expression of ECM proteins was evaluated using immuno/histochemistry. Results of the histochemical staining of GAG showed an increase in amount of GAG expression with time for all Groups T, T' and T'' (**Fig. 3.12**).

Negative control Group N showed no positive staining for GAG throughout the experiment period and is represented by the staining on Day 21 in **Fig. 3.12**. It was also observed that GAG expression varied very little between the different groups. In addition to the presence of GAG, chondrocytes can be seen clearly residing in lacunae; similar to their behavior in native tissue. From these data, we can conclude that GAG expression

increased with time and is unaffected by seeding densities as well as differences in the production of TGF- β3.

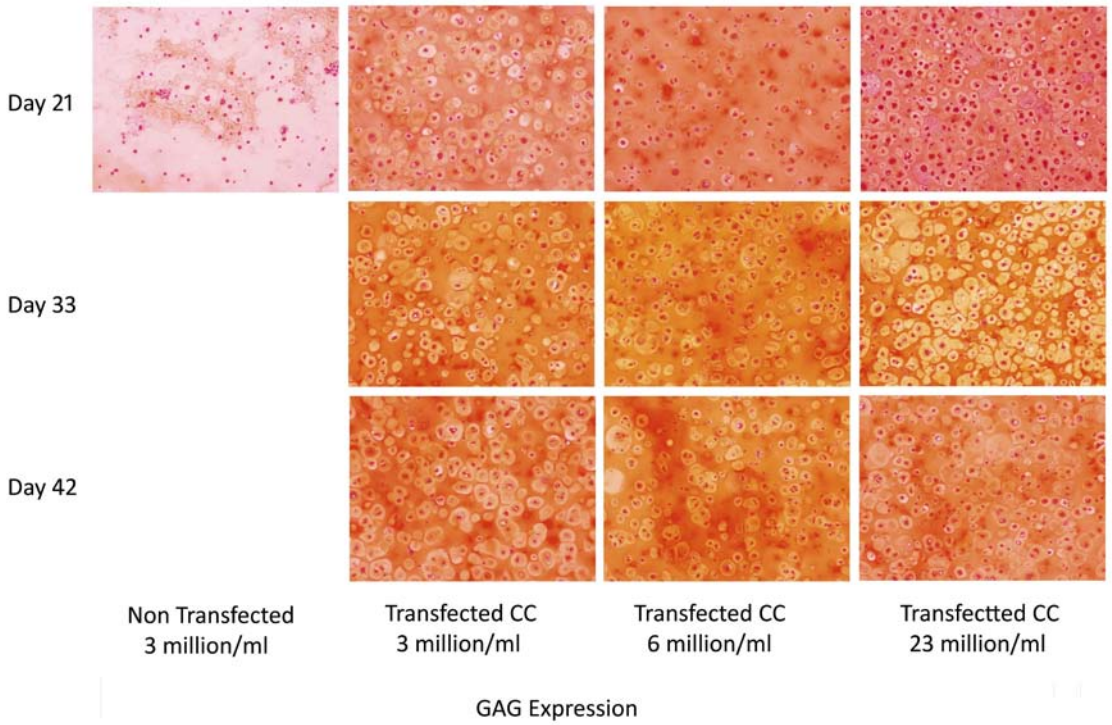


Figure 3.12: Histochemical staining of GAG using Safranin-O in alginate beads at Day 21, 33 and 42 for Group N non-transfected chondrocytes, Group T' transfected chondrocytes with seeding density of 3 million/ml, Group T transfected chondrocytes with seeding density of 6 million/ml and Group T'' transfected chondrocytes with seeding density of 23 million/ml. Red/Orange staining indicates glycosaminoglycans.

Collagen II expression also increased with time for Groups T, T' and T'' with little differences between the groups (**Fig. 3.13**). Coupled with a decrease in TGF- β3, this may indicate that the chondrocytes continue to thrive well and are unaffected by the

transfection. There is more Collagen II production in groups with higher seeding density. This difference in Collagen II production between different groups keeps decreasing as time progresses and by Day 42 is almost unnoticeable.

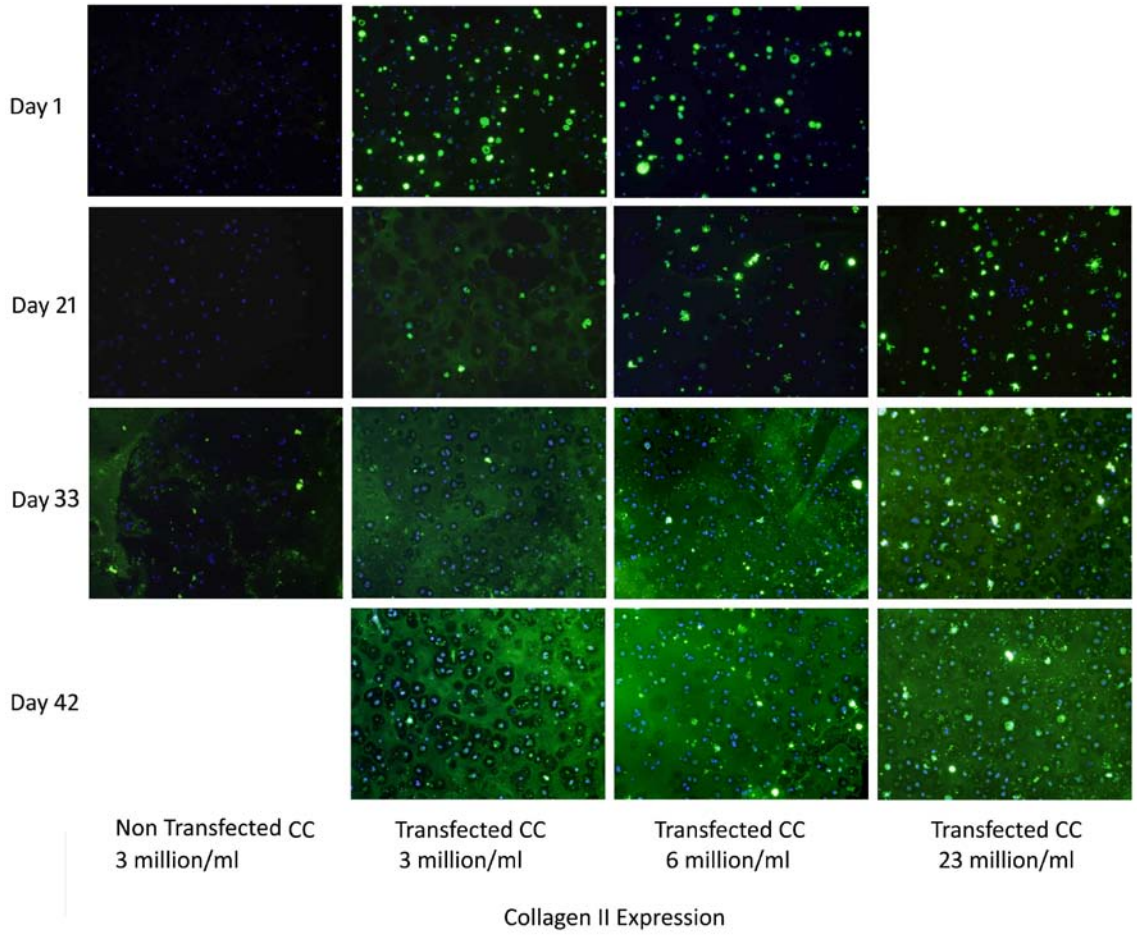


Figure 3.13: Immunohistochemical staining (at Day 1, 21, 33 and 42) for Collagen II shows an increase in Collagen II with increasing time for all transfected groups: Group T' transfected chondrocytes with seeding density of 3 million/ml, Group T transfected chondrocytes with seeding density of 6 million/ml and Group T'' transfected chondrocytes with seeding density of 23 million/ml. Group N non-transfected

chondrocytes show little or no Collagen II expression. Green fluorescence indicates Collagen II expression.

Immunohistochemical staining for Collagen I shows that there is not much difference in Collagen I expression between the various groups at different time points **(Fig. 3.14)**. As predicted, Collagen I expression is less than that of Collagen II. This is in accordance with the characteristics of ECM synthesized by chondrocytes in native tissue.

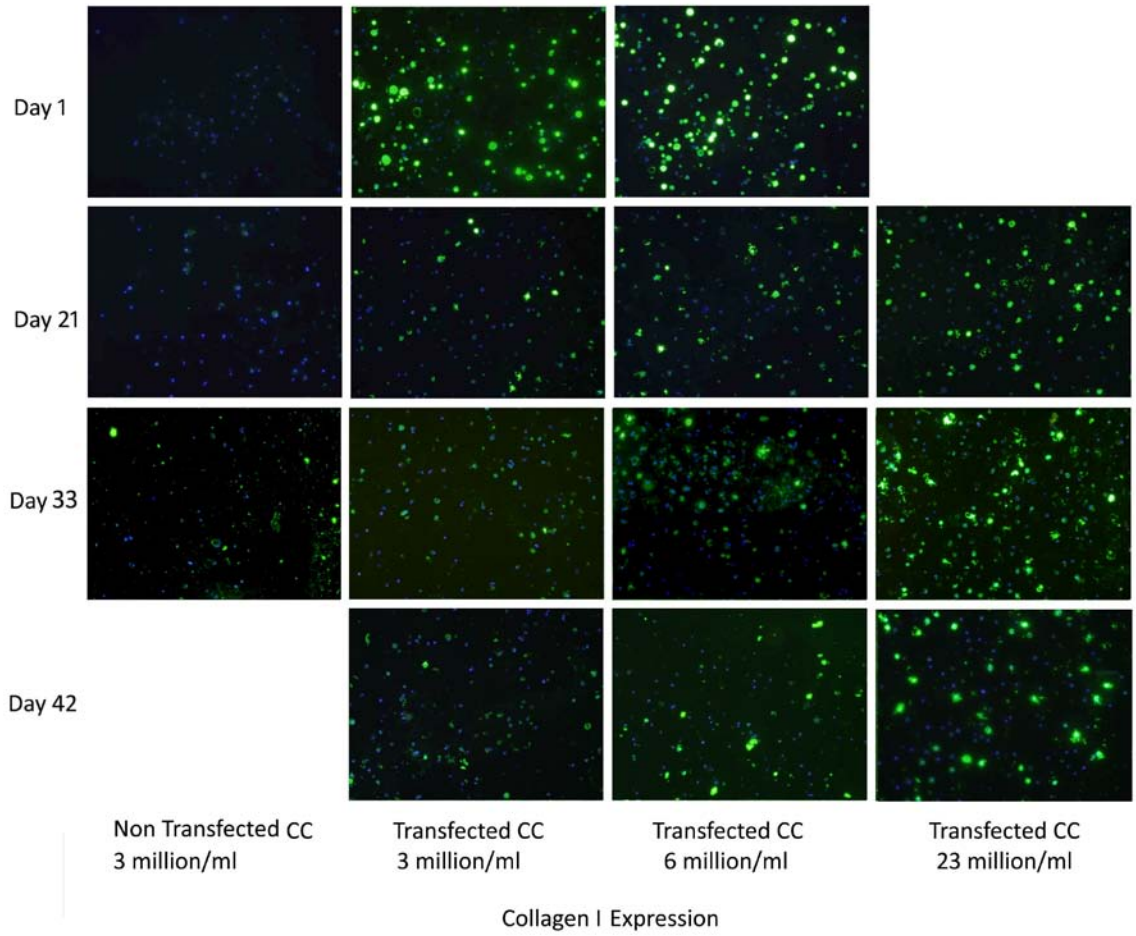


Figure 3.14: Immunohistochemical staining of Collagen I is minimal for all four time points (day 1, 21, 33 and 42) in Group N non-transfected chondrocytes, Group T'

transfected chondrocytes with seeding density of 3 million/ml, Group T transfected chondrocytes with seeding density of 6 million/ml and Group T'' transfected chondrocytes with seeding density of 23 million/ml. Green fluorescence indicates Collagen I expression.

3.4 Summary

Ad.TGF-β3 transfection helps in the re-differentiation of the chondrocytes as seen by the abundant Collagen II and GAG expression in the transfected chondrocytes. Ad.TGF-β3 transfection does lead to a slight increase in Collagen I expression. It can also be seen that the transfected cells thrive well in alginate hydrogels as seen by the presence of characteristic lacunae in the matrix. Thus transfection with Ad.TGF-β3 and the resultant constitutive expression of endogenous TGF-β3 helps the early passage chondrocytes regain their original differentiated state when cultured in 3D alginate hydrogels. Higher seeding densities give rise to better re-differentiation when the chondrocytes are transfected at optimal MOI.

Chapter 4

Chondrogenesis of Rabbit SMSCs in Co-culture with Ad.TGF-β3 Transfected Autologus Rabbit Chondrovcytes

4.1 Introduction

The source of autologous chondrocytes is limited and only a limited amount of chondrocytes can be obtained by *in vitro* expansion as the chondrocytes start turning hypertrophic, lose their phenotype and start de-differentiating into fibroblasts. Studies have also shown that when *ex vivo* genetically modified cells producing endogenous GFs are transplanted back into cartilage defects, their main role is to provide the GFs rather than repopulate the defect[14, 41, 182]. Keeping in mind these considerations, we devised a co-culture strategy wherein the chondrocytes (TCCs) provide the GFs for the differentiation of SMSCs (TPCs). SMSCs are stem cells isolated from synovial tissue surrounding the cartilage in knee joints and have excellent chondrogenic potential. They are also highly proliferative and can be expanded to large numbers *in vitro*.

In this chapter, rabbit SMSCs (TPCs) were co-cultured with Ad.TGF-β3 transfected rabbit chondrocytes (TCCs) in alginate hydrogels. The TPCs and TCCs were separately encapsulated in alginate beads and cultured in the same pool of chondrogenic medium. The chondrogenic differentiation of TPCs was investigated under different initial TGF-β3 concentrations and exposure profiles – the co-culture was started at Day 0, Day10 and Day 20 – while maintaining the same duration of co-culture (30 Days). The TCCs provide the necessary GF for the differentiation of the TPCs. This prevents the TPCs

from undergoing the invasive procedure. It also prevents unpredictable changes and cell death in TPCs caused by long term constitutive over-expression of endogenous GF.

4.2 Materials and Methods

4.2.1 Construction of adenoviral vector

Adenoviral vectors (Ad.TGF-β3), containing an E1, E3-deleted, replication-deficient, type 5 adenovirus genome (Ad5), were constructed as described previously in Chapter 2 to express human TGF-β3 [1]. This vector also carried a green fluorescent marker gene – ZsGreen.

4.2.2 Extraction and culture of rabbit chondrocytes and rabbit SMSCs

SMSC isolation and expansion was carried out as reported elsewhere by De Bari et al [63]. Briefly, random biopsies of synovial membrane tissue were aseptically harvested from the knee joint of New Zealand white rabbits aged 3-5 months. The small fragments of synovial tissue were rinsed in phosphate buffered saline (PBS) solution containing antibiotics. SMSCs were isolated by digestion with 0.1% Collagenase I in high-glucose DMEM with 10% FBS and antibiotics. Isolated SMSCs were resuspended in high-glucose DMEM with 10% FBS and antibiotics and replated in culture flasks with a suitable seeding density.

Cartilage tissue was harvested from the tibia and femur of 3-5 months old New Zealand white rabbits. The cartilage was cut into small pieces and digested overnight with 1mg/ml

Collagenase type II (250 units/mg; Worthington, MN) with gentle rotation at 60 rpm at 37°C in 5% CO2. Isolated chondrocytes were cultured at 5% CO_2, 37^oC in chondrocyte culture medium (CC) - 0.01 M 4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid (HEPES), 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline, 0.05 mg/mL Vitamin C, 100 U/mL penicillin, 100 U/mL streptomycin and 20% FBS in High Glucose DMEM.

All animal experiments were carried out in accordance with the guidelines approved by The NUS Institutional Animal Care and Use Committee (IACUC), Singapore.

4.2.3 Transfection of rabbit chondrocytes with Ad.TGF-β3

When chondrocytes reached passage 3 in monolayer culture, they were transfected – one day after seeding – with Ad.TGF-β3 with a MOI between 200-500. Briefly, the cells in T150 culture flasks were cultured in 7 ml of serum free DMEM containing Ad.TGF-β3 for 2 hours at 5% CO_2, 37^oC, after which the culture fluids were replaced with 25 ml of CC medium.

4.2.4 Encapsulation of transfected rabbit chondrocytes and SMSCs in alginate hydrogels

48 hours after transfection, the chondrocytes were trypsinized, collected and washed with 0.15M NaCl. The cells were then resuspended in sterile 1.2% alginate (Sigma) at a concentration of $5x10^6$ or $10x10^6$ cells/ml. 40 μL of cell suspension was added into 102 mM $CaCl_2$ (Sigma-Aldrich). To allow for complete polymerization, the alginate beads

were kept for 15 minutes in $CaCl_2$ and then rinsed twice with washing buffer. 6 beads were transferred into each well of a 12 well plate on one side of the silicon rubber partition in 2 ml of Chondrogenic Medium (CM) containing 100 nM dexamethasone (Sigma), ascorbic acid 2-phosphate (50 mg/ml; Sigma), sodium pyruvate (100 mg/ml; Gibco), proline (40 mg/mL; Sigma), penicillin (100 U/ml), streptomycin (100 mg/ml) (Gibco), and 5 ml of BD ITS premix (BD Biosciences, Bedford, MA) in 500 ml of high-glucose DMEM.

Passage 3 non-transfected chondrocytes and chondrocytes transfected with null-adenovirus carrying no GF gene were also collected and encapsulated in alginate beads (5 or 10 million cells/ml) following the above mentioned procedure, to be used in control groups. 6 beads were placed into select wells in a 12 well plate.

Passage 2 SMSCs were trypsinised, collected and encapsulated into alginate beads (8 million cells/ml) following the same procedure as described above. 6 beads were placed into each well of the 12 well plates on the other side of the silicon rubber partition in 2ml of CM **(Fig. 4.1)**. New SMSC beads were formed at Day 10 for group 2 and Day 20 for groups 3 and 4 from the same batch of cells, to start the co-culture at Day 10 and Day 20 respectively.

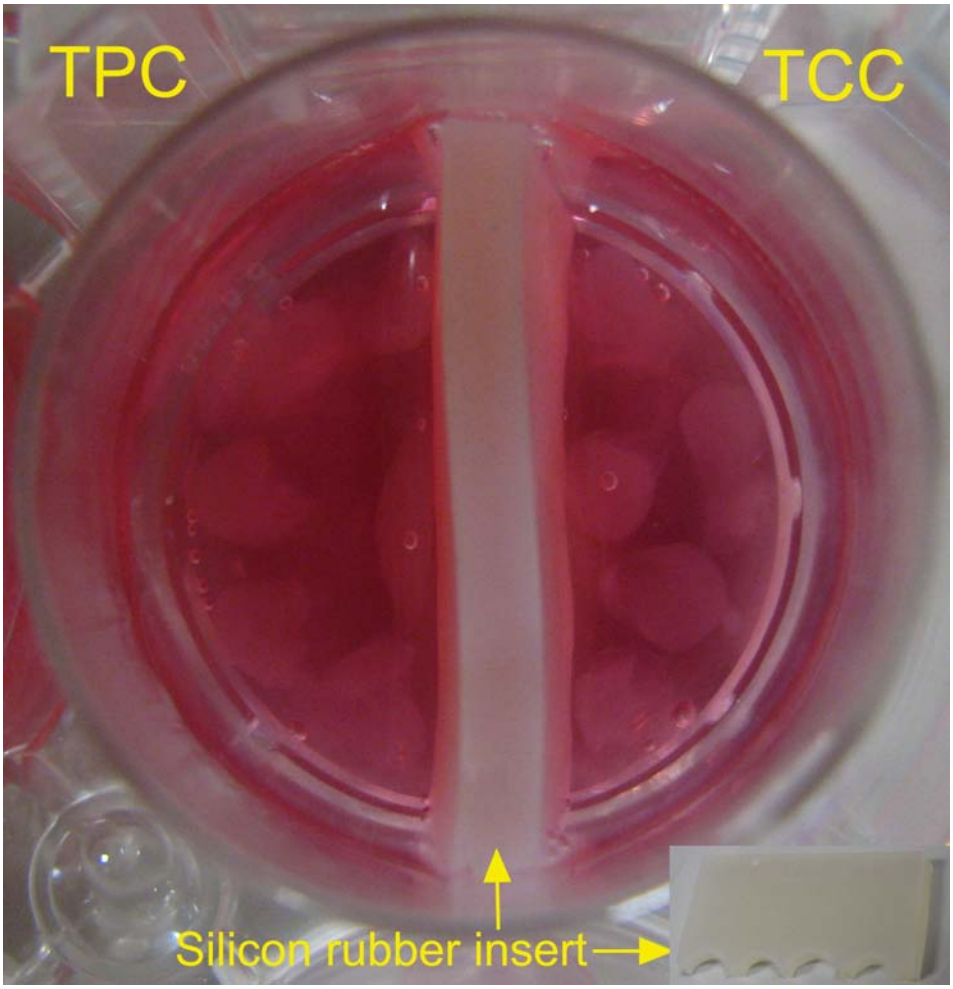


Figure 4.1: Co-culture well with silicon rubber partition and alginate beads

4.2.5 Co-Culture of SMSCs with transfected chondrocytes: setup and nomenclature

Experiments were conducted with two different seeding densities of TCCs: 5 million cells/ml (**V**) and 10 million cells/ml (**X**).

In all experiments, TPC beads were formed at a seeding density of 8 million cells/ml. Co-Culture was started either at Day 0 (**V00** and **X00**), or Day 10 (**V10** and **X10**) or Day 20 (**V20** and **X20**).

For each group, six 40µl alginate beads of TPCs were co-cultured with six 40µl alginate beads of Ad.TGF-β3 transfected TCCs in the same well in 2ml of CM.

For the group of experiments where co-culture was started at Day 20, twice the number of TCC beads (twelve 40µl alginate beads) were also used for co-culture. These groups were named **VD20** and **XD20** respectively.

Four control groups were also formed in addition to the above mentioned groups:

- TPCs in co-culture with null-adenovirus transfected chondrocytes (5 or 10 million cells/ml), **Co-Nill (V or X)**
- TPCs in co-culture with non-transfected chondrocytes (5 or 10 million cells/ml), **Co-Non (V or X)**
- TPCs cultured alone in CM with no chondrocytes, **SMSC**
- TPCs cultured alone in CM and supplemented with 10ng/ml of TGF-β3 active protein every three days, **Manual**

Six 40µl alginate beads of TPCs were co-cultured with six 40µl alginate beads of null-adenovirus transfected (Co-Nill) or non-transfected (Co-Non) chondrocytes in the same well in 2ml of CM.

The medium was changed every three days in all the groups.

In all the co-culture groups, TCC beads were formed at Day 0 while TPC beads were formed and added at Day0 or Day 10 or Day 20 so as to start the co-culture at Day0, Day10 or Day20.

Two time-points were selected for the characterization (RNA extraction and real-time PCR; Immuno/histological staining) of TPC beads: 15 days after the start of co-culture and 30 days after the start of co-culture.

Thus, **X20+15** for instance would denote the group where TCC seeding density was 10 million cells/ml, 6 TCC beads were used, and the co-culture was started at Day 20; while the characterization time-point for TPCs was 15 days after the start of co-culture, that is Day 35 in the whole scheme of the experiment.

The various groups and their nomenclature are listed in detail in **Table 4.1** and **Table 4.2**.

Table 4.1: Co-Culture groups for TCC seeding density of 5 million cells/ml

	V00	V10	V20	VD20	Manual	SMSC	Co-Non(V)	Co-Nill(V)
Start of Co-Culture	Day 0	Day 10	Day 20	Day 20	Day 0	Day 0	Day 0	Day 0
End of Co-Culture	Day 30	Day 40	Day 50	Day 50	Day 30	Day 30	Day 30	Day 30
SMSCs	6 beads	6 beads	6 beads	6 beads	6 beads	6 beads	6 beads	6 beads
Chondrocytes	6 beads; Ad.TGF-β3 transfected	6 beads; Ad.TGF-β3 transfected	6 beads; Ad.TGF-β3 transfected	12 beads; Ad.TGF-β3 transfected	Manual addition of TGF-β3 every 3 days	N.A.	6 beads; non-transfected	6 beads; null-adenovirus transfected
Time Points for characterization	V00+15 (Day 15);	V10+15 (Day 25);	V20+15 (Day 35);	VD20+15 (Day 35);	Day 15; Day 30	Day 15; Day 30	Day 15; Day 30	Day 15; Day 30

(Days after starting co-culture)	V00+30 (Day 30)	V10+30 (Day 40)	V20+30 (Day 50)	VD20+30 (Day 50)				
Change of Medium	Every three days							

Table 4.2: Co-Culture groups for TCC seeding density of 10 million cells/ml

	X00	X10	X20	XD20	Manual	SMSC	Co-Non(X)	Co-Nill(X)
Start of Co-Culture	Day 0	Day 10	Day 20	Day 20	Day 0	Day 0	Day 0	Day 0
End of Co-Culture	Day 30	Day 40	Day 50	Day 50	Day 30	Day 30	Day 30	Day 30
SMSCs	6 beads	6 beads	6 beads	6 beads	6 beads	6 beads	6 beads	6 beads
Chondrocytes	6 beads; Ad.TGF-β3 transfected	6 beads; Ad.TGF-β3 transfected	6 beads; Ad.TGF-β3 transfected	12 beads; Ad.TGF-β3 transfected	Manual addition of TGF-β3 every 3 days	N.A.	6 beads; non-transfected	6 beads; null-adenovirus transfected
Time Points for characterization (Days after starting co-culture)	X00+15 (Day 15); X00+30 (Day 30)	X10+15 (Day 25); X10+30 (Day 40)	X20+15 (Day 35); X20+30 (Day 50)	XD20+15 (Day 35); XD20+30 (Day 50)	Day 15; Day 30	Day 15; Day 30	Day 15; Day 30	Day 15; Day 30
Change of Medium	Every three days							

4.2.6 Characterization of TGF-β3 expression by western blot

To confirm the presence of both active and inactive forms of TGF-β3 in the conditioned media from Ad.TGF-β3 transfected chondrocytes, western blot was performed using rabbit polyclonal primary antibody to TGF-β3 (ab15537, Abcam) and goat anti-rabbit IgG-HRP (ab6721, Abcam) as secondary antibody.

4.2.7 Quantitative analysis of TGF-β3 expression by specific ELISA

Conditioned medium from the co-culture wells containing Ad.TGF-β3 transfected chondrocytes was collected every three days and stored at -80 °C for further analysis by ELISA (R&D Systems, Minneapolis, MN). Elisa was performed as per the manufacturer's instructions, wherein HCl is used to activate latent TGF-β3 (in acidic pH, the pro-peptide gets cleaved to release the active form). Absorbance was measured at 450 nm using a microplate reader (Thermo Electron, USA) and the concentration of TGF-β3 from each group was recorded as triplicates.

ELISA was also done without HCl activation for the same groups to measure the concentration of already active TGF-β3 present in the media.

4.2.8 RNA extraction, reverse transcription and real-time PCR

At 15 and 30 days after the start of co-culture for the corresponding groups, alginate beads from the TPCs were placed in 15ml centrifuge tubes and dissolved in 1ml 55mM sodium citrate for 10 minutes and then centrifuged at 1100 rpm for 5 minutes. 1ml of Trizol (Invitrogen, Singapore) was added to the cell pellet in each centrifuge tube. The cell pellet was resuspended in Trizol, transferred to 1.75 ml Eppendorf Tubes and total RNA was isolated according to the protocol for Trizol reagent. RNA was also extracted from passage 3 monolayer rabbit chondrocytes (CC Monolayer), to be used as a positive control, from TPC beads before the start of co-culture (Day 0), to be used as negative control and from TCC beads of group X00 to characterize the status of TCC re-differentiation in co-Culture.

Concentration of RNA was evaluated using UV spectrophotometer (Multiskan®spectrum, Thermo). The total RNA collected was transferred to -80°C refrigerator for future use. 500 ng RNA prepared from each sample was transcribed into cDNA using reverse transcriptase and oligo DT (Promega, USA). Quantification of mRNA was performed on a Real-Time PCR system (iQ5, Bio-Rad) using a 20 µL reaction volume for SYBR Green reactions [1µL cDNA amplified in 20 µL final volume with 0.5 mM of each primer suspended in iQ SYBR Green Supermix (Bio-Rad)]. The level of expression of each gene was analyzed using ΔC_T method, normalizing the expression to rabbit HPRT gene, while fold difference of each gene in each sample was analyzed using $2^{-\Delta\Delta CT}$ method. The real-time PCR primers used are detailed in **Table 4.3**.

Table 4.3: Real-Time PCR Primers

Gene	Accession	Forward primer	Reverse Primer
HPRT	NM001105671	5′-GACCTTGCTTTCCTTGGTCA-3′	5′-TCCAACAAAGTCTGGCCTGT-3′
Collagen Type II	D83228	5′-TATCCAGTAGTCACCGCTCTTCC-3′	5′-ACGCTCAAGTCCCTCAACAAC-3′
Aggrecan	L38480	5′-TGGAGGTCGTGGTGAAAGG-3′	5′-CAATGATGGCGCTGTTCTGT-3′
COMP	NM012834	5′-CTGGACCCTGAGGGCGAT-3′	5′-CCGTGTACCCCACAGCCAG-3′
Collagen Type IX	XM601325	5′-CAAGAGGCCAAATTGATGTCG-3′	5′-AGTTTCTCTCCTGGGCCGC-3′
Collagen Type I	NM001195668	5′-GGCAACAGCAGGTTCACTTACA-3′	5′-ATCAAGGAAGGGCAAACGAG-3′
Collagen Type X	D49399	5′-CAAGGTATTCCTGGACCGCC-3′	5′-AACCCTGGCTCTCCTTGCAG-3′

4.2.9 Immuno/histochemical staining

SMSC-laden hydrogel samples at 15 and 30 days after the start of co-culture were fixed with 4% (w/v) neutral buffered paraformaldehyde at room temperature for a day. The fixed hydrogels were embedded in paraffin and sectioned in two different thicknesses – 5µm and 10 µm – using a microtome. The 5µm sections were subsequently stained with Safranin-O solution and kept aside for 5 minutes before observation under optical microscope for sulfated GAG expression. For Collagen type II and Collagen type I immunostaining, the 10µm sections were incubated for 30 minutes in 10%(w/v) goat blocking serum to inhibit non-specific binding of IgG and then incubated with Collagen type II (2 ng/ml in PBS, MAB8887, Millipore) and Collagen type I (2 ng/ml in PBS, SC-59772, Santa Cruz Biotech) primary monoclonal antibodies overnight at 4^{o}C, before being washed 3 times by PBS solution. The sections were then incubated with anti-IgG binding immunofluorescent (5 ng/mL in PBS, Invitrogen Alexa Fluor®, 488) secondary antibody at 37^{o}c in dark for an hour, washed 3 times with PBS and observed under fluorescent microscope.

Statistical analysis

Statistical analysis was carried out using Student's t-test. *P* values < 0.05 were considered statistically significant. Results are expressed as mean ± SD unless otherwise specified.

4.3 Results

4.3.1 Presence of active and inactive TGF-β3 in the conditioned medium

As seen from the western blot result in **Fig. 4.2,** conditioned medium from Ad.TGF-β3 transfected chondrocytes contains both active and inactive forms of TGF-β3. Lanes 1 and 4 represent recombinant human TGF-β3 (Biosource, PHG9305) used as control and lanes 2, 3, 5 and 6 represent TGF-β3 in the conditioned medium of transfected chondrocytes. The active monomer (~12.5 kDa, mature form) is the functional form which acts as the effective GF and can be seen in both the control and in the conditioned medium. In addition to the active monomer, we can also see the presence of active dimer (20-25 kDa) in both the control and the conditioned medium; while the inactive monomer (47-50 kDa, pro form) and inactive dimer (100 kDa) can be seen in the conditioned medium only. Bands seen at 70 kDa in lanes 1, 2 and 3 are nonspecific and their identity has not been established yet.

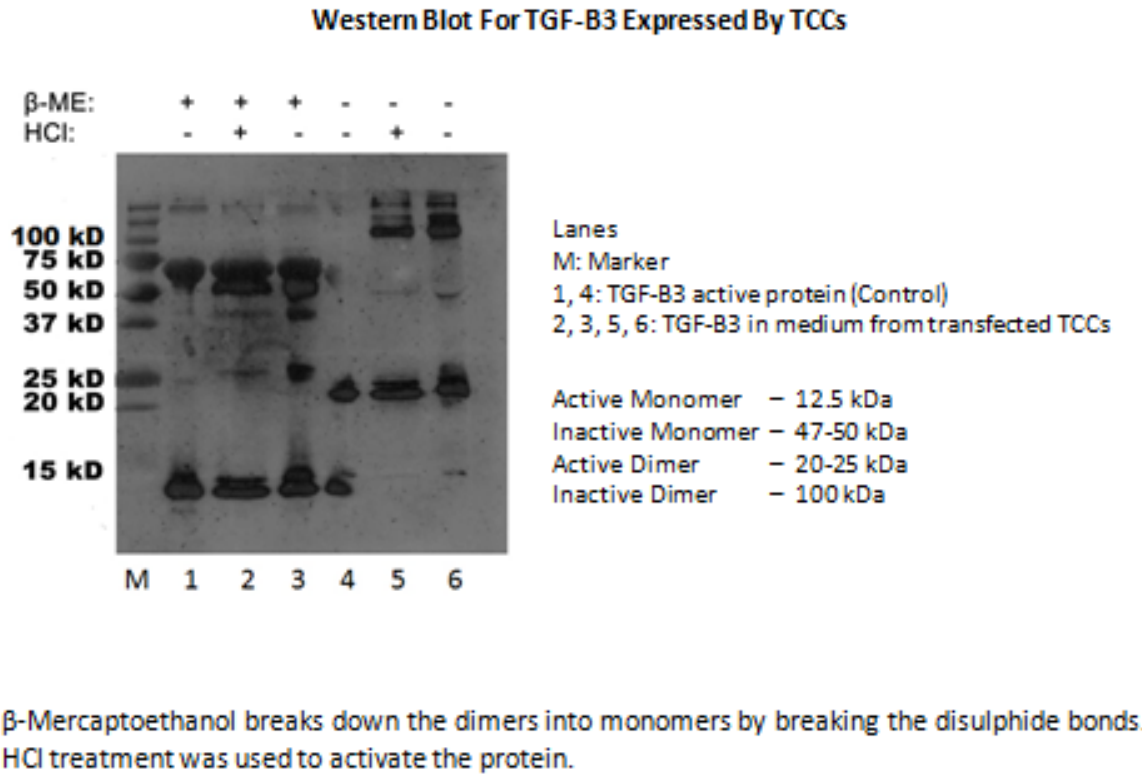


Figure 4.2: Western Blot for TGF-β3 expressed by transfected rabbit chondrocytes, showing active and inactive monomers and dimers. Lane M: Protein Ladder, Lanes 1, 4: TGF-β3 active protein (positive control), Lanes 2, 3, 5, 6: TGF-β3 in medium from transfected chondrocytes. Lanes 1, 2 and 3 are β-mercaptoethanol treated, and lanes 2 and 5 are HCL treated. Active and inactive monomers of TGF-β3 are seen in β-mercaptoethanol treated samples. Active and inactive dimmers are seen in samples not treated with β-mercaptoethanol.

4.3.2 hTGF-β3 expression by specific ELISA

TGF-β3 expression from groups V00, V10 and V20 as well as from groups X00, X10 and X20 was averaged as the three groups contained the same number of TCCs. It can be

seen from **Fig. 4.3A** and **Fig. 4.3B** that total TGF-β3 expression from groups V00, V10, V20 as well as group VD20 was greater than 200ng/ml at Day 3, between 50 and 200 ng/ml at Day 6 and kept on decreasing progressively up till Day 24-36, after which it stabilized to a value between 1-10ng/ml. Total TGF-β3 expression in group VD20 at Day 3 was beyond measurable range of the ELISA setup, and was about 200ng/ml at Day 6, the value being more than twice that in other groups at all time points. This correlates with twice the number of chondrocyte beads in each well in group VD20. The same trend can be seen in groups X00, X10, X20 and XD20 (**Fig. 4.3C** and **Fig. 4.3D**) except that the expression is, as expected, higher than in the groups with 5 million cells/ml seeding density and in case of XD20 as high as 3μg/ml at Day 3. Thus by controlling the seeding density of TCCs, we can modulate the TGF-β3 expression levels in this system.

The amount of active TGF-β3 present in the conditioned medium from groups with both the seeding densities was between 10-70ng/ml at Day 3 and rapidly decreased till Day 9, after which the expression remained between 1-10ng/ml **(Fig. 4.3 A,B,C,D)**.

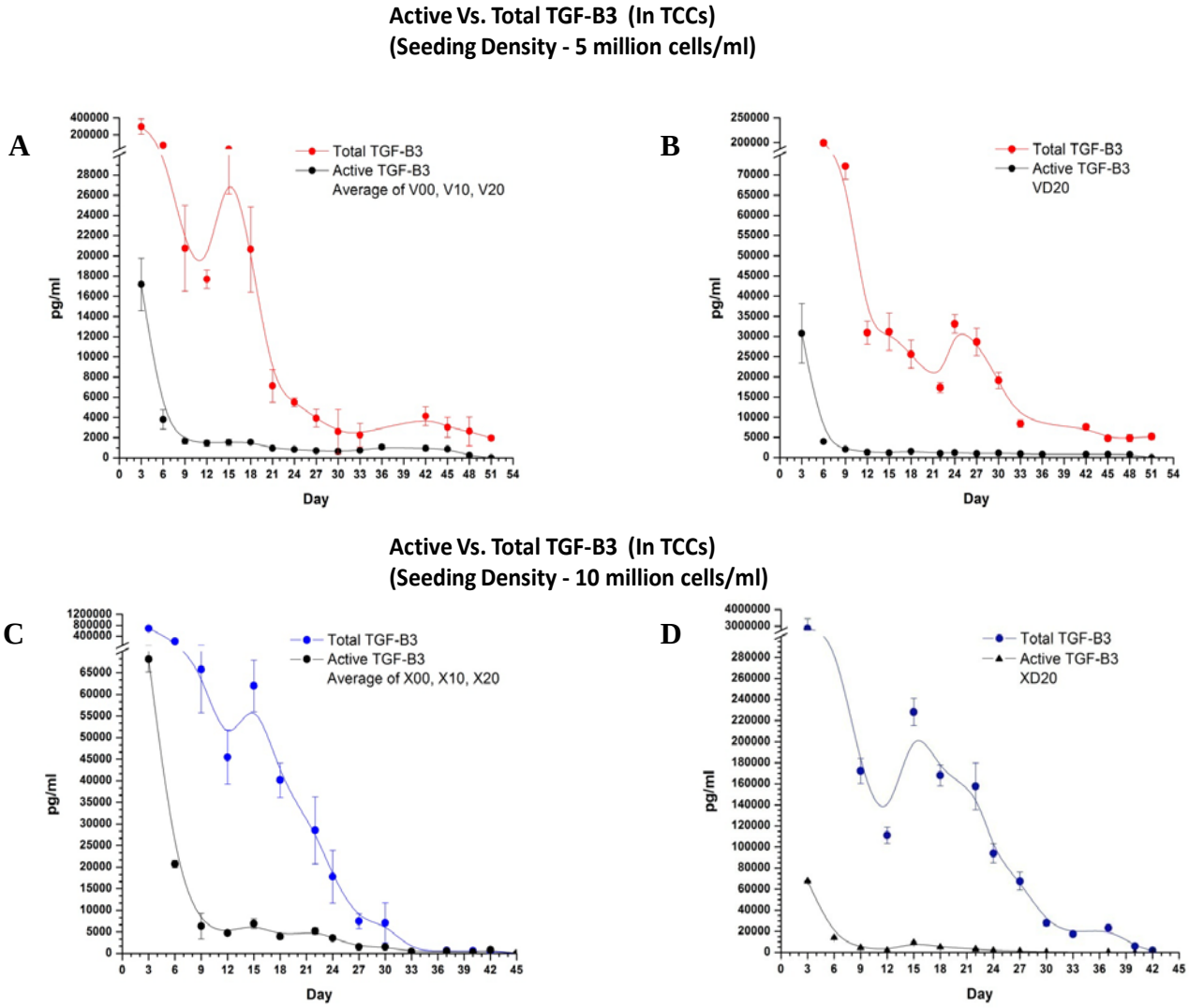


Figure 4.3: Total and active TGF-β3 (pg/ml) expressed by transfected rabbit chondrocytes seeded in alginate beads (A and B seeding density of 5 million cells/ml; C and D seeding density of 10 million cells/ml), six beads in one well, measured by ELISA for medium collected from one well. Average TGF-β3 expression from groups V00, V10 and V20 **(A)**. TGF-β3 expression from group VD20 **(B)**. Average TGF-β3 expression from groups X00, X10 and X20 **(C)**. TGF-β3 expression from group XD20 **(D)**.

4.3.3 Gene expression in transfected chondrocytes

Real time PCR was carried out to determine the status of re-differentiation in Ad.TGF-β3 transfected chondrocytes (TCCs) at transcriptional level. As can be seen from **Fig**. **4.4,** the expression of cartilage markers Collagen II, COMP, Aggrecan and Collagen IX is either comparable to or more than that in monolayer passage 3 chondrocytes (CC Monolayer) at Day 15. The expression of these cartilage markers is significantly higher than that in CC Monolayer at Day 30 (2, 2.5, 28 and 29 folds respectively for Collagen II, COMP, Aggrecan and Collagen IX). The expression of Collagen I – a marker of de-differentiation in chondrocytes – though a bit more than that in CC Monolayer at Day 15, decreases significantly by Day 30. The hypertrophic marker Collagen X is extremely high at Day 15 (5000 fold) and drastically reduces by Day 30 (100 fold). The expression of the hypertrophic marker can be correlated to TGF-β3 expression in the TCCs **(Fig. 4.3C)**, leading one to suspect that transfection with and exposure to high amounts of endogenously produced TGF-β3 initiates hypertrophy in chondrocytes.

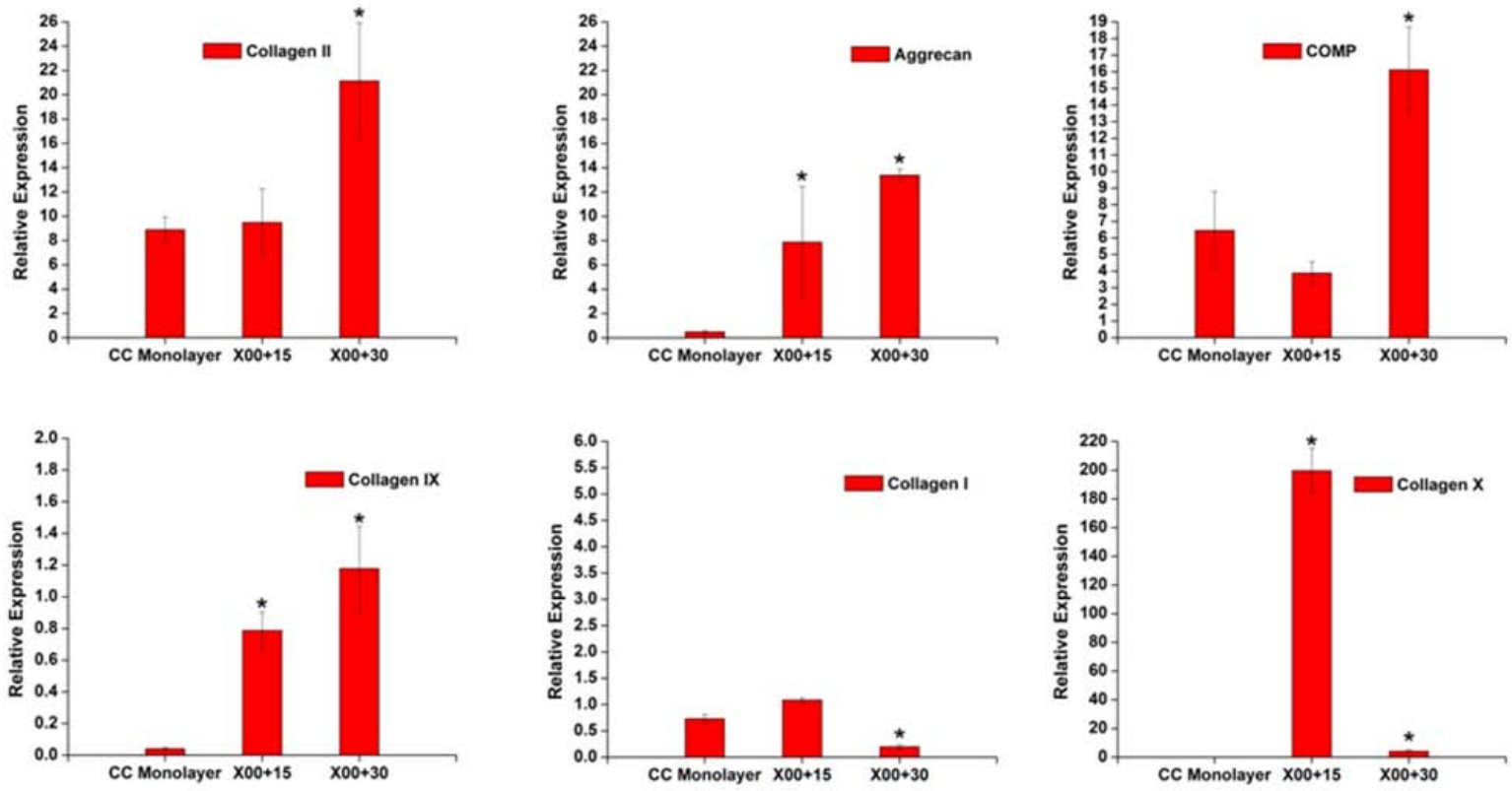


Figure 4.4: Real-time PCR results for gene expression in transfected rabbit chondrocytes (seeding density – 10 million cells/ml in alginate, from group X00) for cartilage markers Collagen II, Aggrecan, COMP, Collagen IX; fibrocartilage marker Collagen I and hypertrophyic marker Collagen X. (*) indicates statistically significant difference from CC Monolayer (Monolayer passage 3 chondrocytes). X00+15 and X00+30: Transfected chondrocytes seeded at Day 0 and collected at Day 15 and 30 respectively.

4.3.4 Gene expression in co-cultured SMSCs

4.3.4.1 SMSCs in co-culture with transfected chondrocytes (TCC seeding density – 5 million cells/ml)

In **Fig. 4.5**, expression of cartilage markers Collagen II, COMP, Aggrecan and Collagen IX from all SMSC groups co-cultured with transfected chondrocytes – V00, V10, V20, VD20 – is more than that in negative control groups SMSC, Co- Non(V) and Co-Nill(V), while it is less than or comparable to that in the positive control groups Manual and CC Monolayer. Collagen I expression is higher in the manually dosed group – Manual, compared to that in rest of the groups. In most of the groups, collagen I expression gets downregulated by 30 days after the start of co-culture. Collagen X expression is higher in all the groups compared to that in CC Monolayer. There is not much of a difference in the expression of Collagen X between the various groups and its expression follows no trend in particular.

The higher expression of chondrocytic markers (Collagen II, IX, COMP, Aggrecan) in all TGF-β3 treated groups (V00, V10, V20, VD20 and Manual) compared to that in non-treated groups suggests that TGF-β3 indeed promotes chondrocytic differentiation in SMSCs. The higher expression of sensitive chondrocytic markers – COMP and Collagen IX – in all TGF-β3 treated groups again supports the experimental design of differentiating SMSCs in gene transferred co-culture systems.

Figure 4.5A **Collagen II**

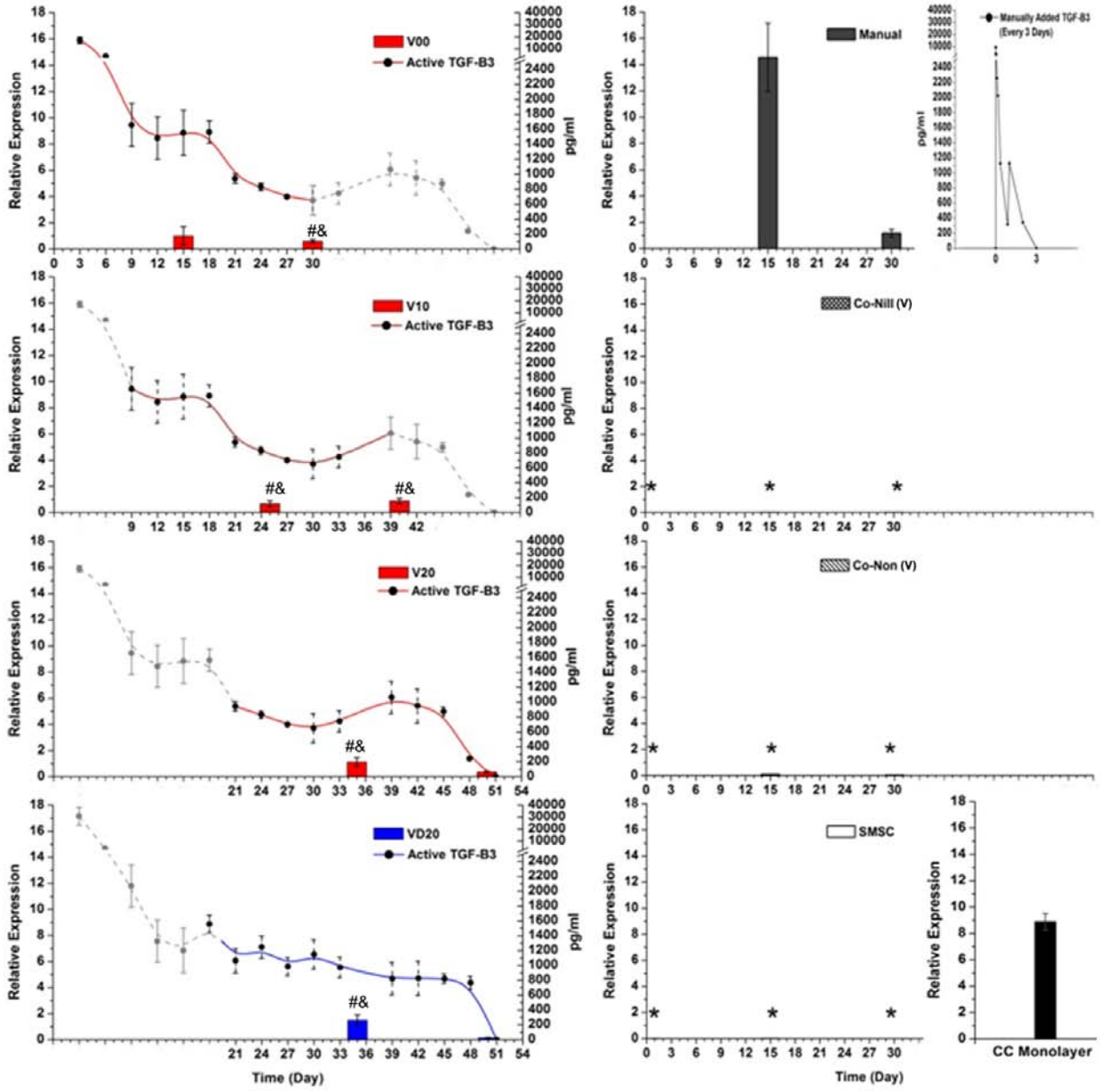


Figure 4.5B **Aggrecan**

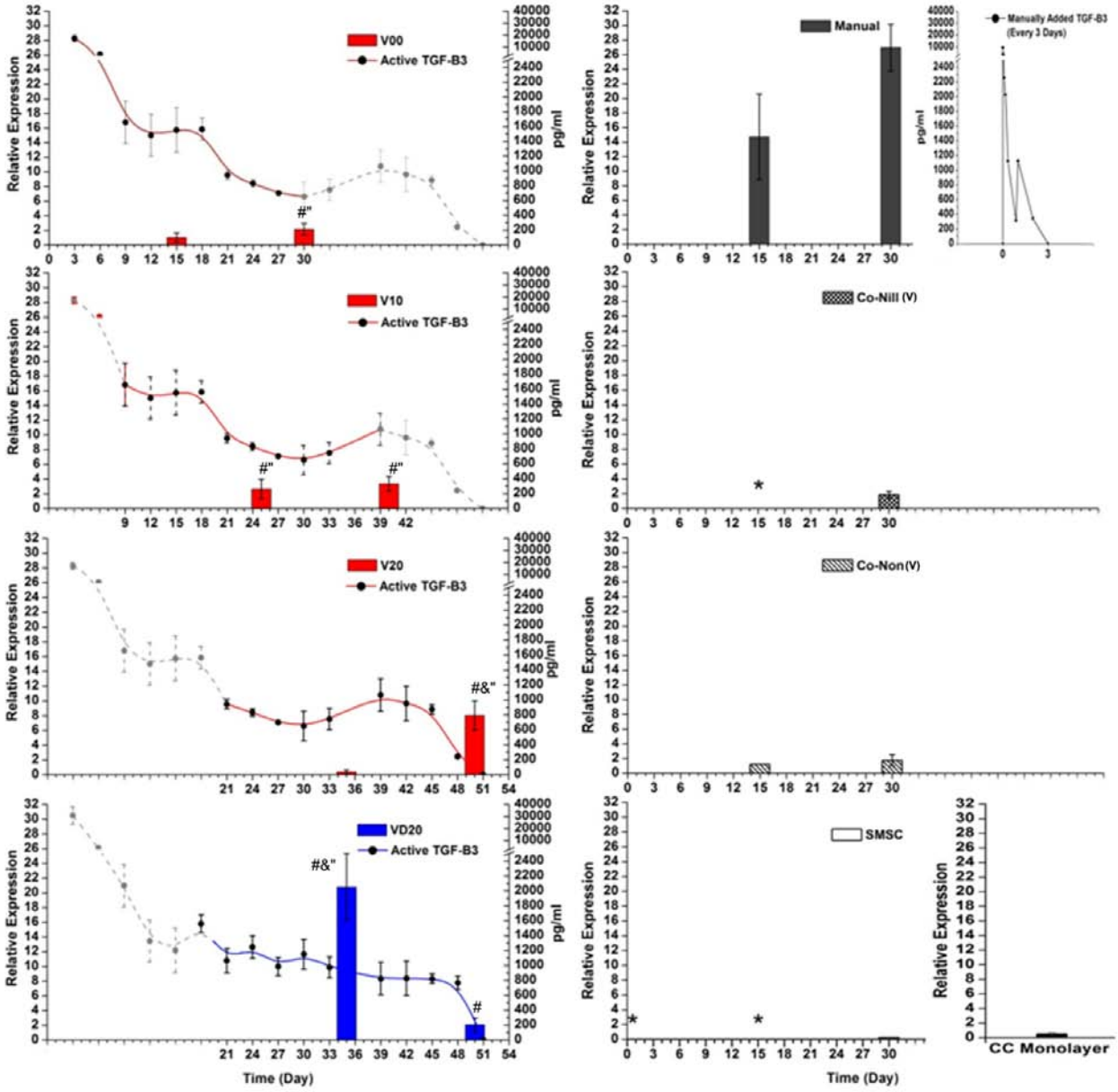


Figure 4.5C **COMP**

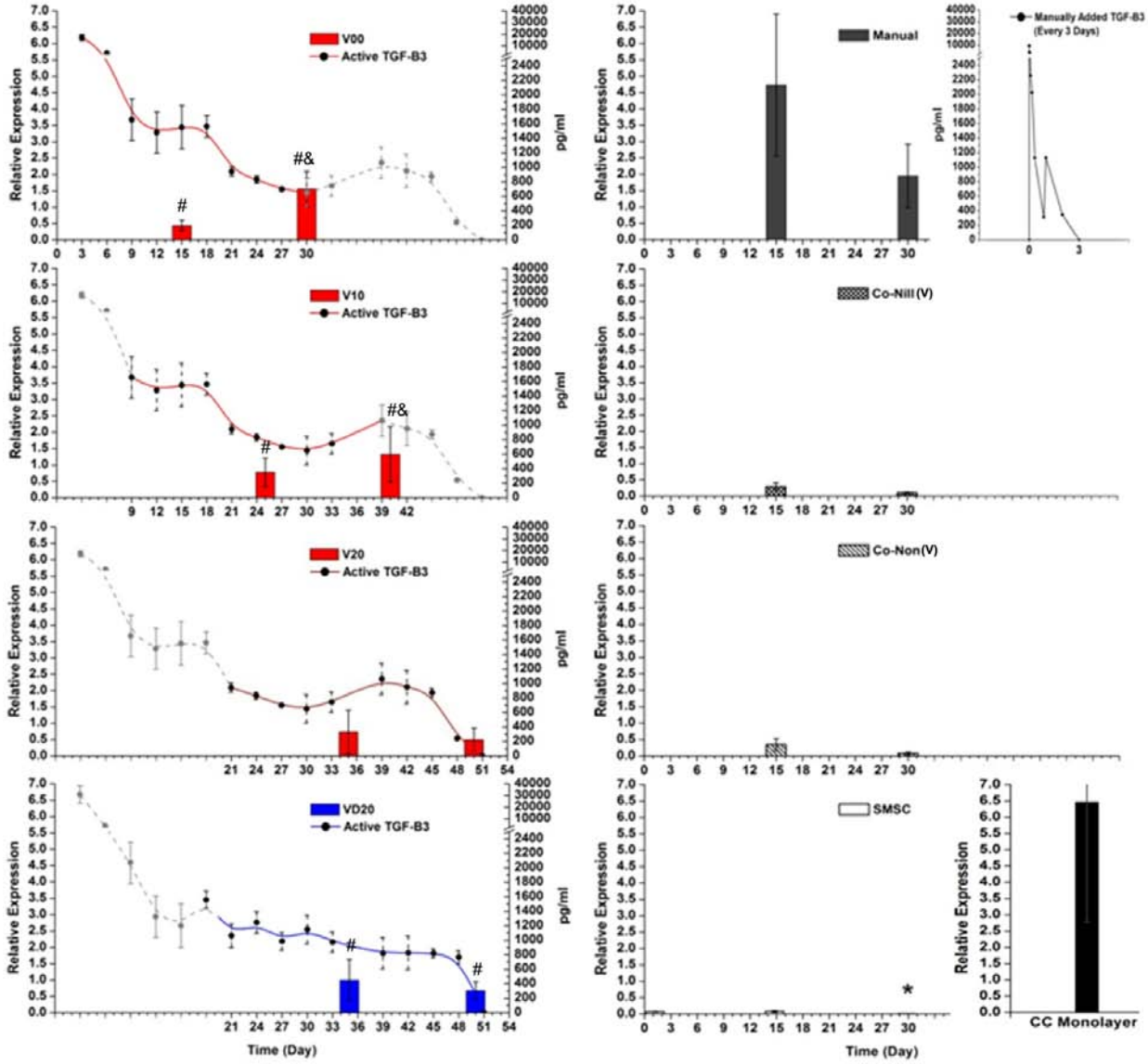


Figure 4.5D **Collagen IX**

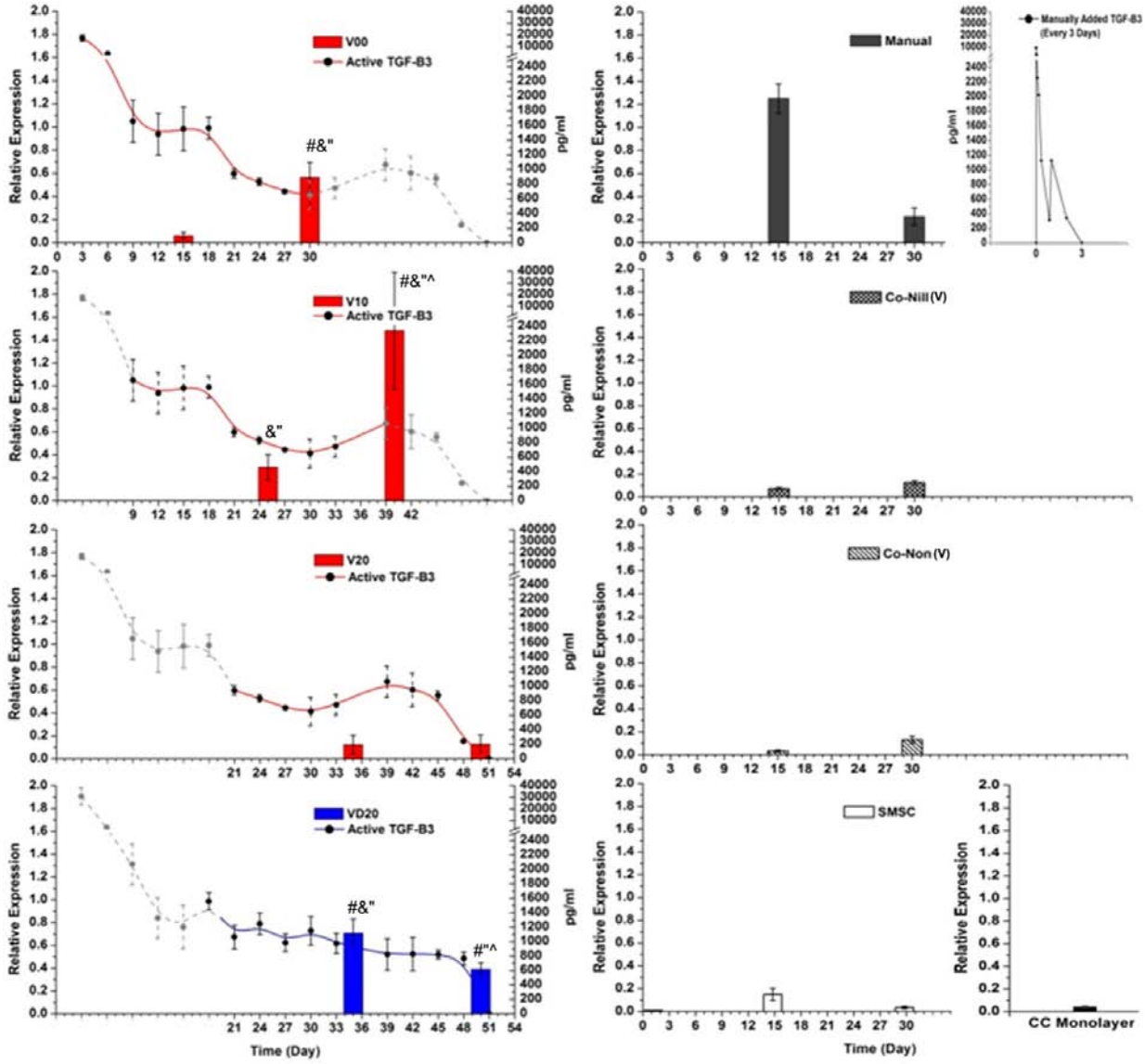


Figure 4.5E **Collagen I**

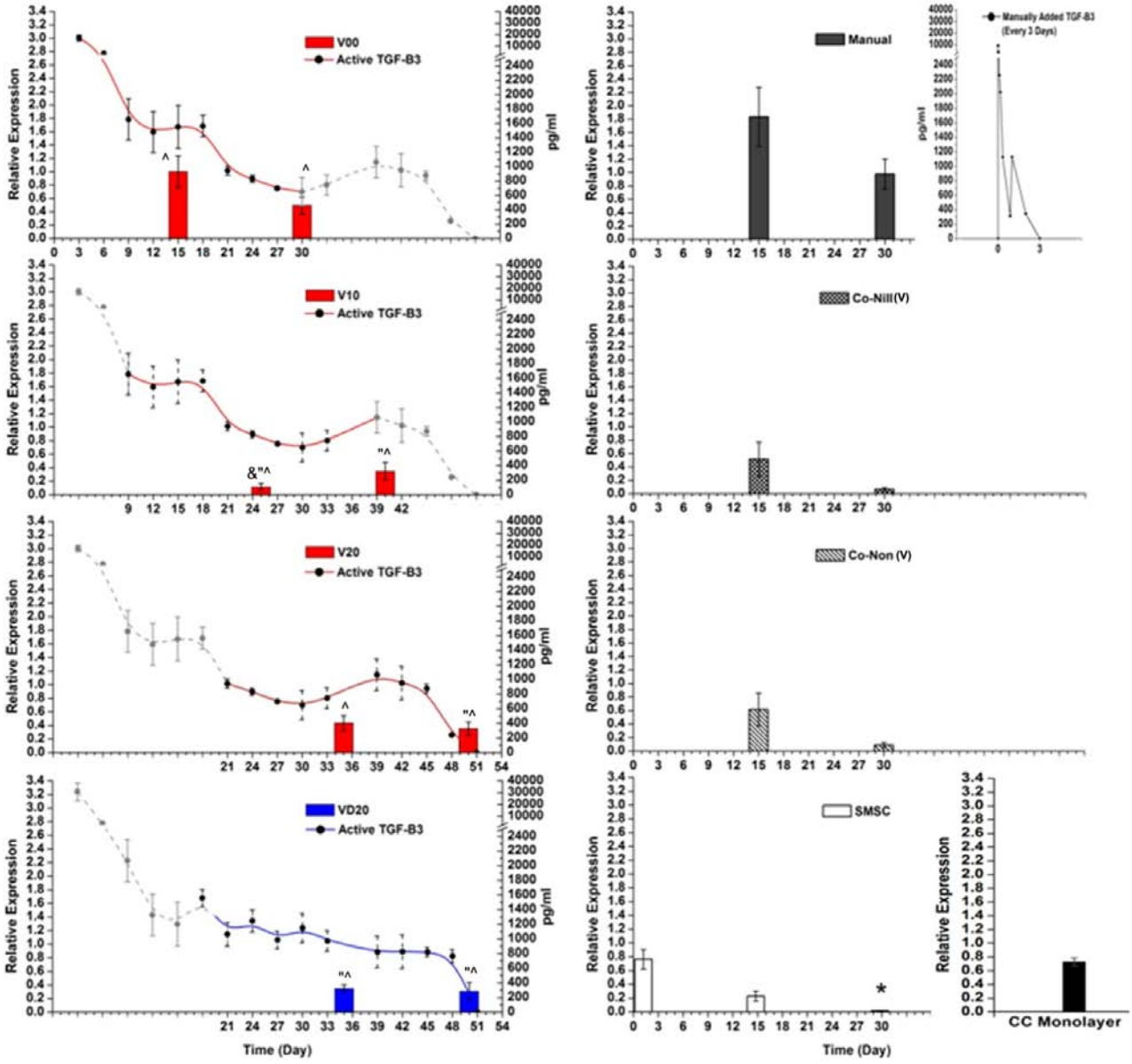


Figure 4.5F **Collagen X**

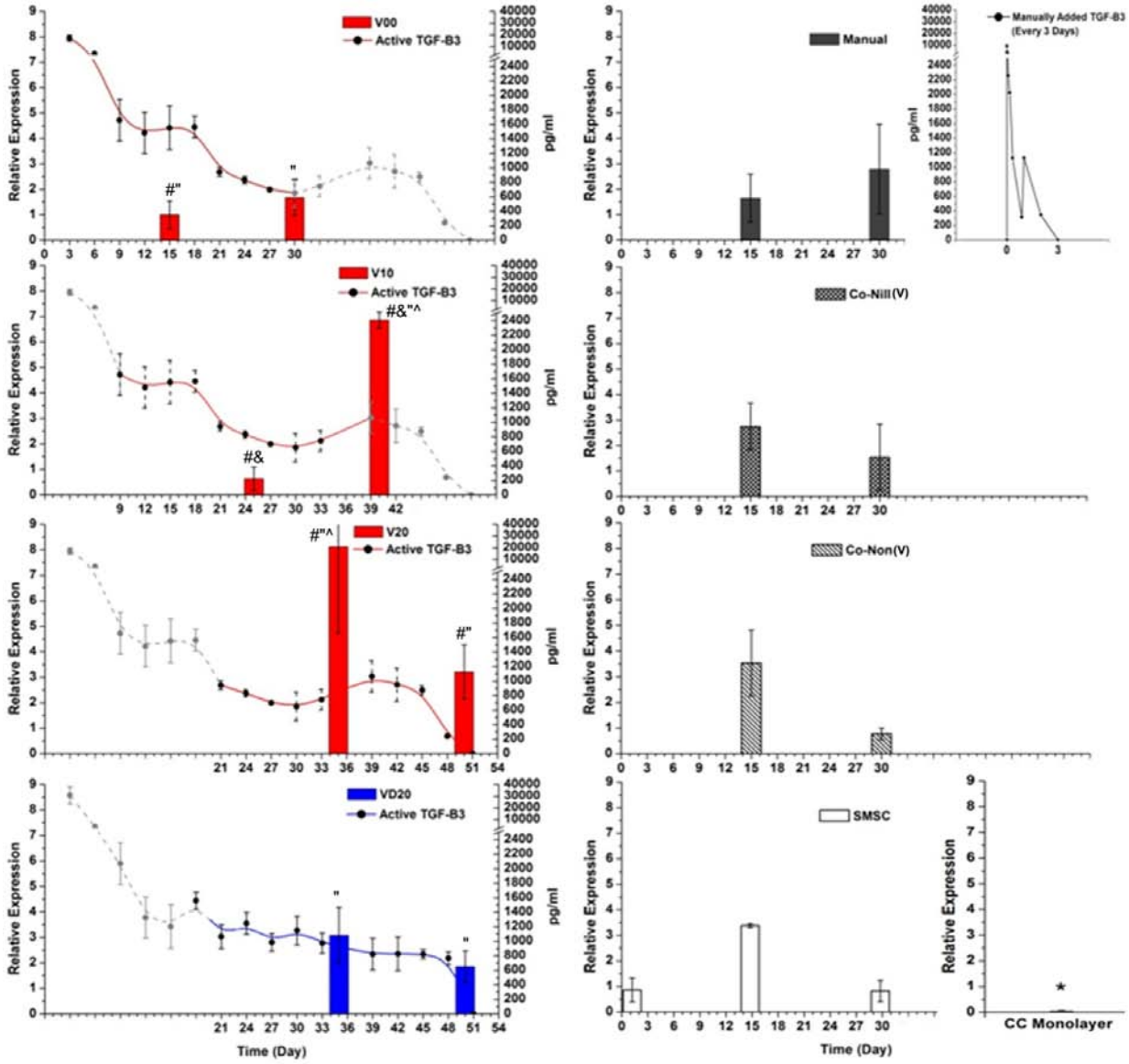


Figure 4.5: Real-time PCR results for gene expression in SMSCs co-cultured with transfected rabbit chondrocytes (seeding density of 5 million cells/ml) for groups V00, V10, V20, VD20. Manual, Co-Nill, Co-Non and SMSC respectively represent SMSCs under manual addition of TGF-β3, SMSCs co-cultured with nil adenovirus transfected chondrocytes, SMSCs co-cultured with non-transfected chondrocytes and SMSCs in medium alone. CC Monolayer is monolayer chondrocytes used as positive control. Data

collected at time points of 15 and 30 days after seeding. All groups are shown with the corresponding TGF-β3 concentrations (measured by ELISA) experienced by the SMSCs during the culture period. **(A)**Collagen II **(B)**Aggrecan **(C)**COMP **(D)**Collagen IX **(E)**Collagen I **(F)**Collagen X.

- indicates significant difference from negative control group **SMSC**

& - indicates significant difference from negative control groups **Co-Non** and **Co-Nill**

“ - indicates significant difference from positive control group **CC Monolayer**

^ - indicates significant difference from positive control group **Manual**

Data for TGF-β3 degradation profile over 3 days when added at a concentration of 10ng/ml in medium as measured by ELISA (shown along with **Manual** group) obtained from our previous study [1].

4.3.4.2 SMSCs in co-culture with transfected chondrocytes (TCC seeding density – 10 million cells/ml)

As seen in **Fig. 4.6**, the expression of chondrocytic markers Collagen II, Aggrecan, COMP and Collagen IX in groups X00, X10, X20 and XD20 is higher or comparable to that in the positive control groups Manual and CC Monolayer and significantly higher than that in the negative control groups Co-Nill(X), Co-Non(X) and SMSCs wherein it is nearly zero for all the cartilage markers. Collagen II (6-9 times higher) and Collagen IX (2-4 times higher) expression in groups X00, X10, X20 and XD20 is significantly higher than that in Manual, while their expression gets downregulated by 30 days after the start of co-culture in groups X20 and XD20. Expression of Aggrecan is highly upregulated in X20+15 and XD20+15 (about 15-20 folds compared to control groups) while it gets downregulated by 30 days after the start of co-culture. The expression of COMP in

groups X00, X10, X20 and XD20 is comparable to that in Manual and CC Monolayer and also seems to get downregulated in X20+30 and XD20+30.

Collagen I expression in groups X00, X10, X20 and XD20 is comparable to or lower than that in the control groups. In all the groups (with the exception of Manual), expression of Collagen I drops below that in CC Monolayer by 30 days after the start of co-culture. Collagen I expression is downregulated in all groups at 30 days after the start of co-culture. Collagen X expression seems to be higher in all the groups that are co-cultured with chondrocytes, particularly in groups X10, X20 and XD20 (about 2-6 folds higher compared to control groups), but nowhere near as high as in TCCs. There is not much of a difference in Collagen X expression among the control groups at Day 30, while all the groups have a higher Collagen X expression compared to CC Monolayer.

Figure 4.6A **Collagen II**

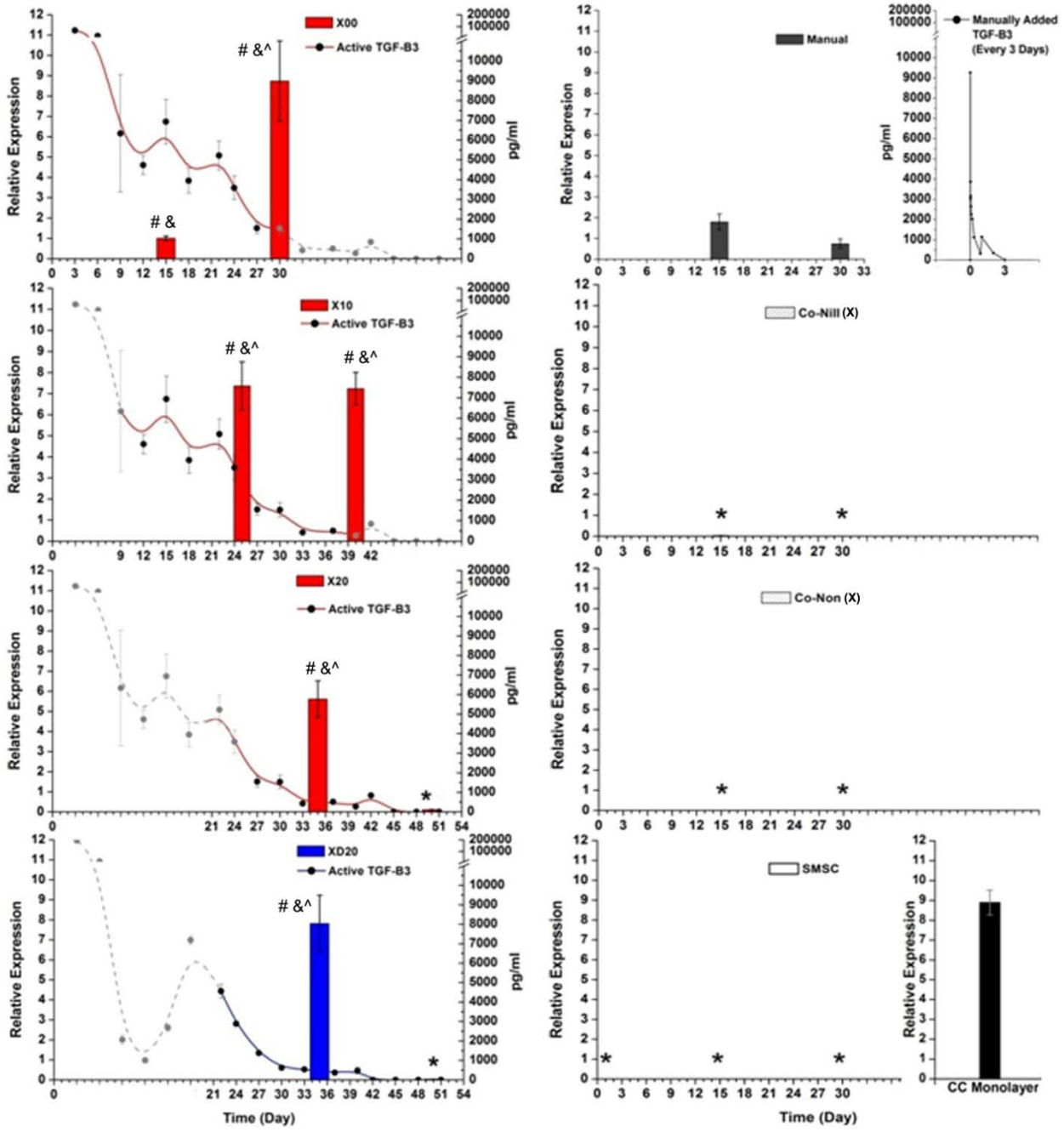


Figure 4.6B **Aggrecan**

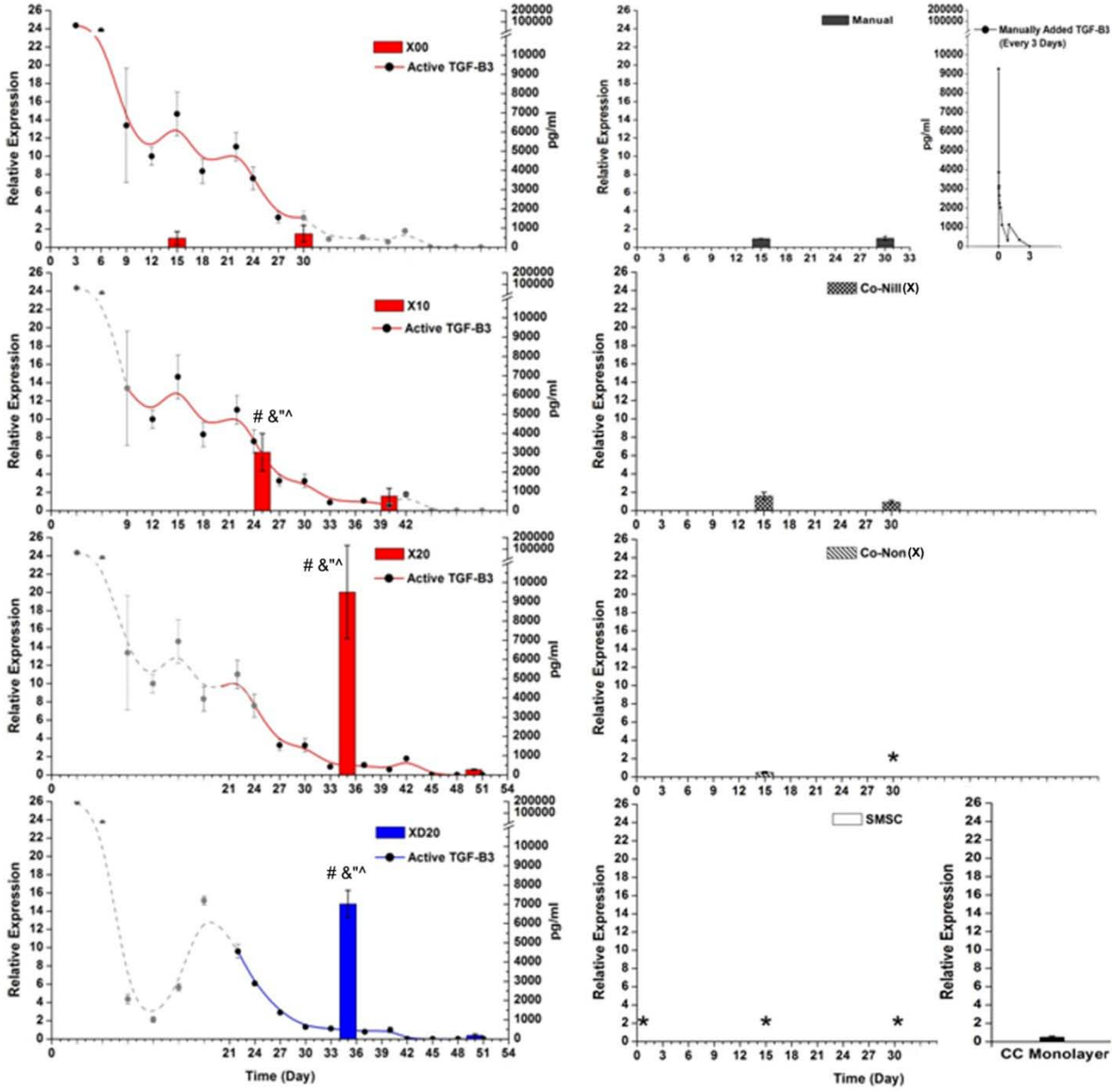


Figure 4.6C **COMP**

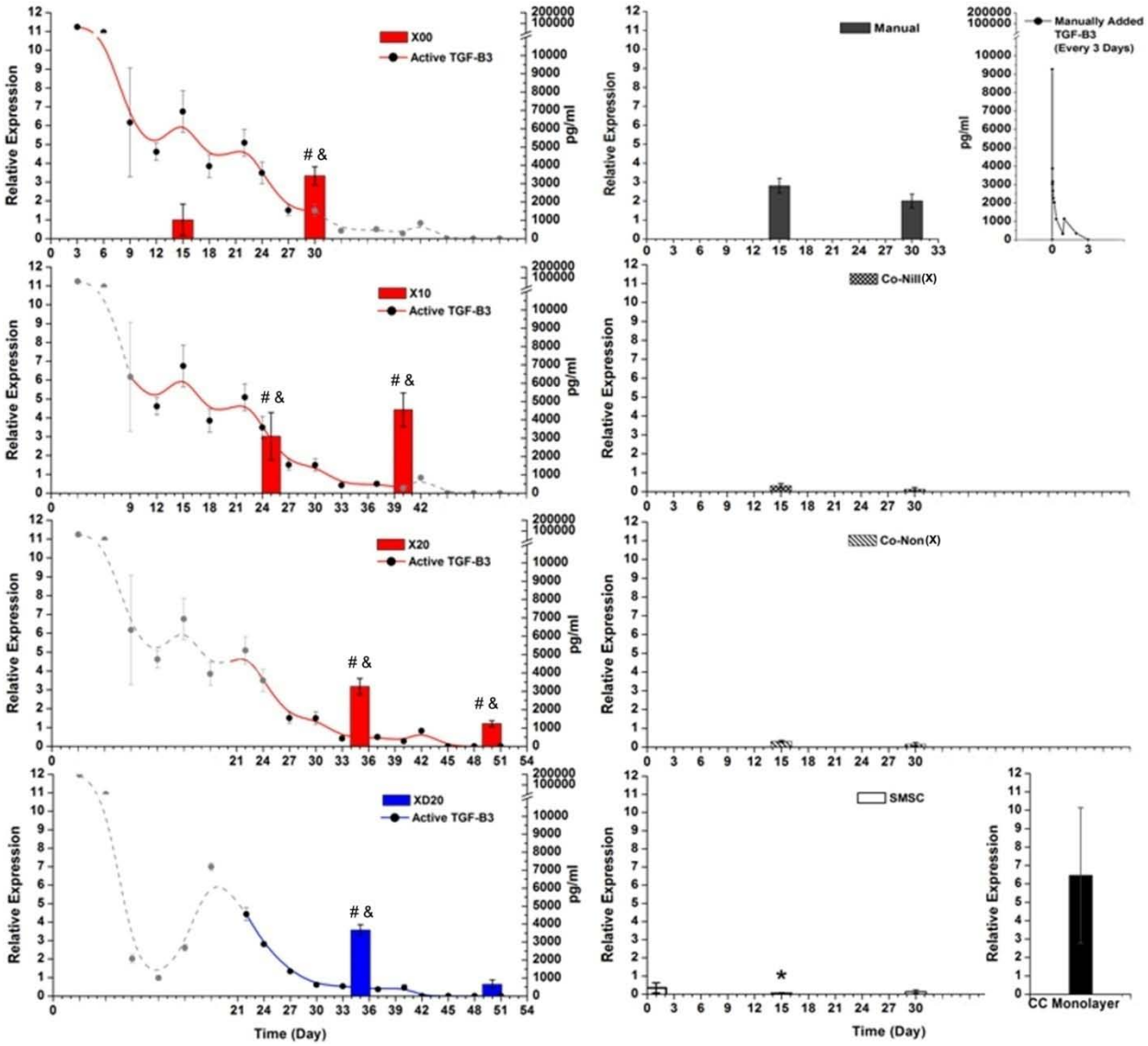


Figure 4.6D **Collagen IX**

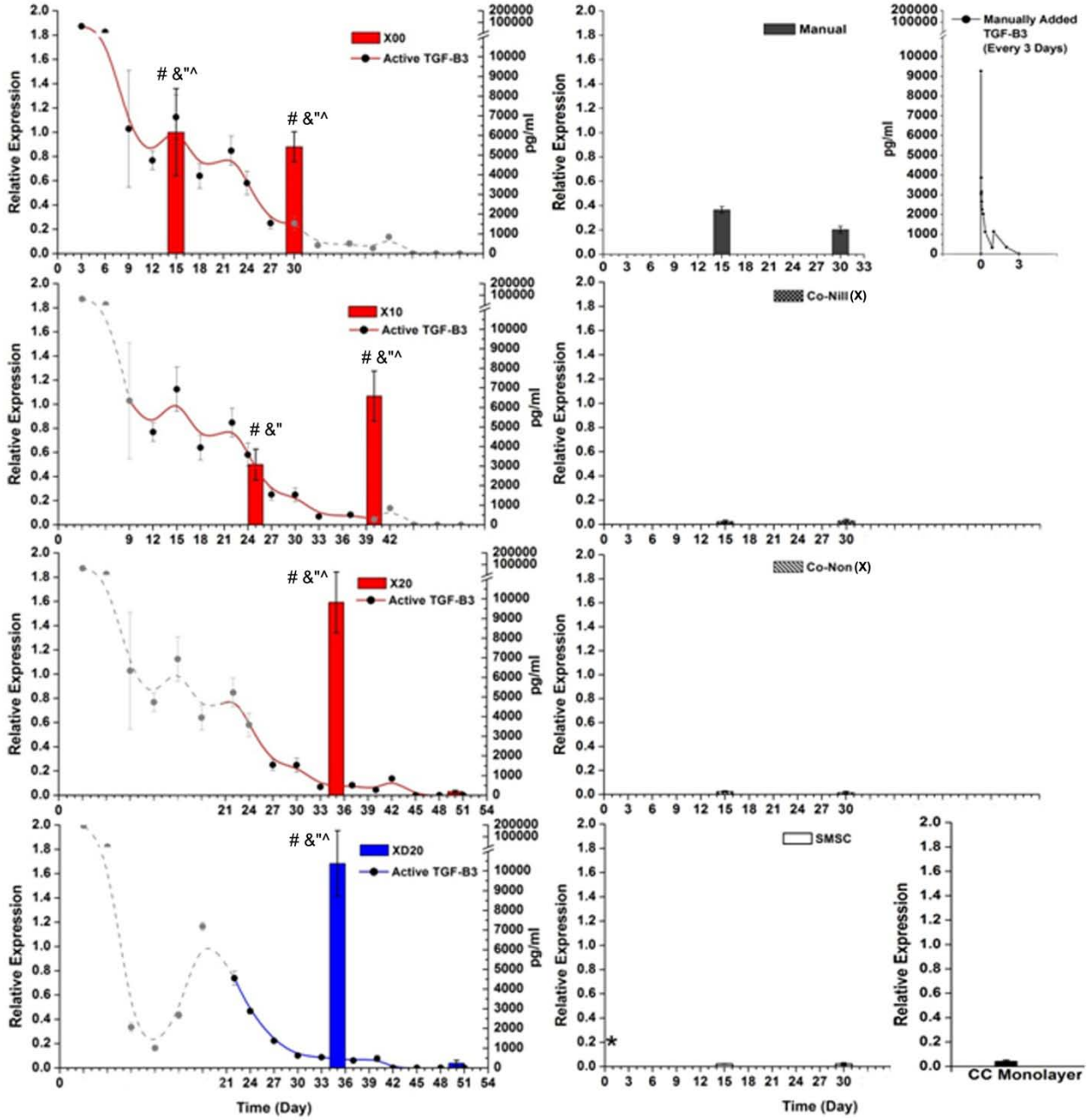


Figure 4.6E **Collagen I**

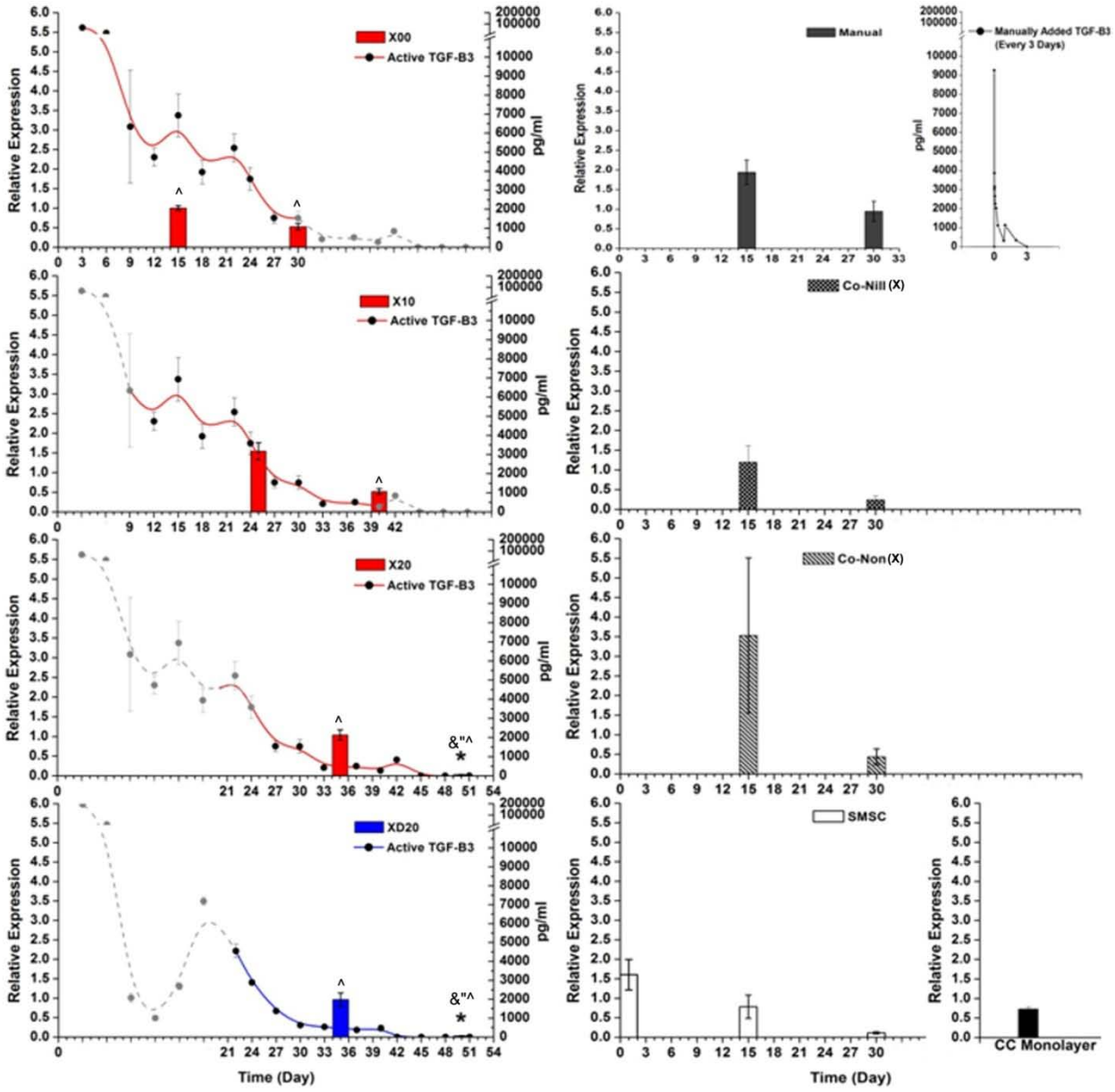


Figure 4.6F **Collagen X**

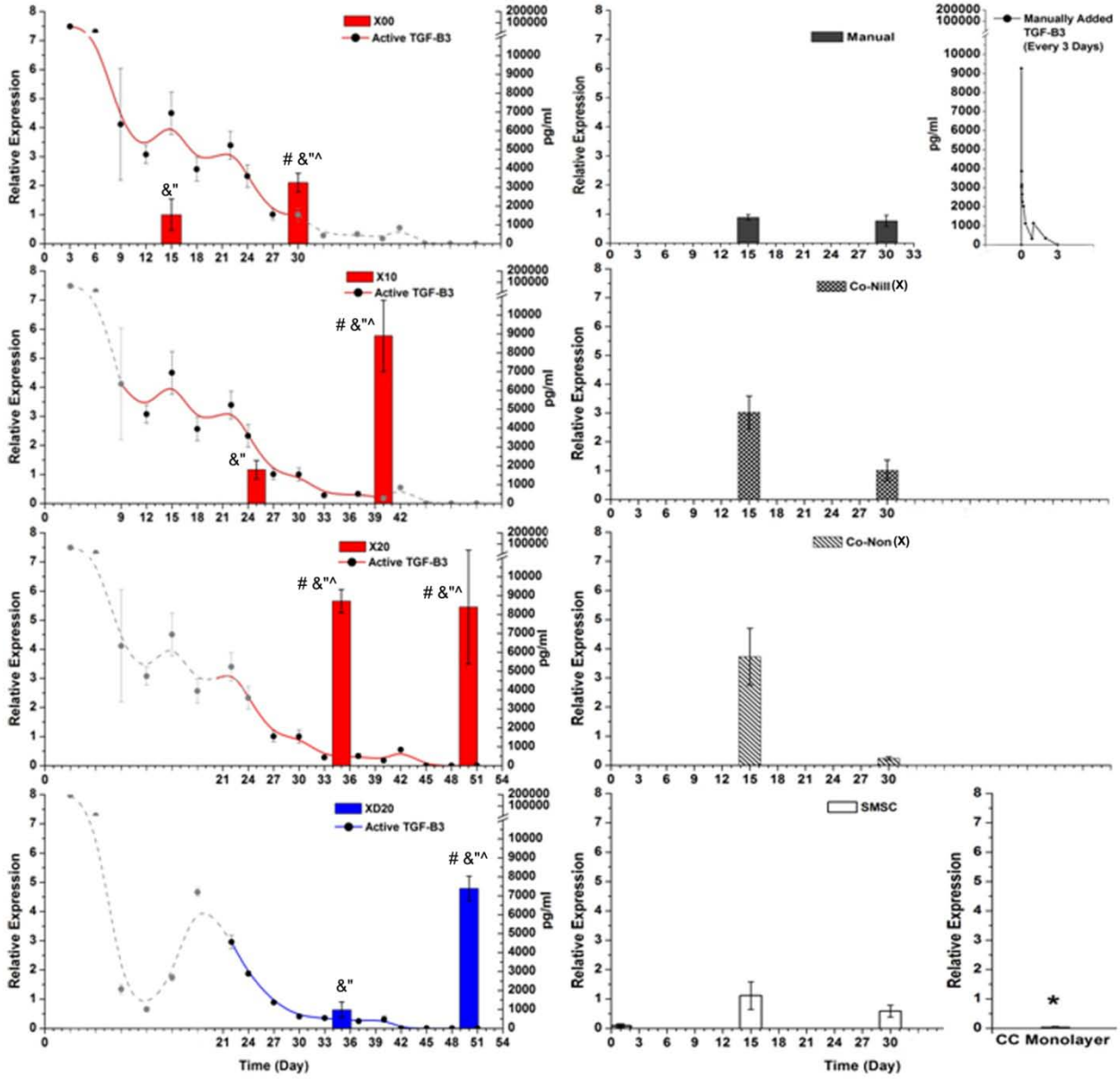


Figure 4.6: Real-time PCR results for gene expression in SMSCs co-cultured with transfected rabbit chondrocytes (seeding density of 10 million cells/ml) for groups X00, X10, X20, XD20. Manual, Co-Nill, Co-Non and SMSC respectively represent SMSCs under manual addition of TGF-β3, SMSCs co-cultured with nil adenovirus transfected chondrocytes, SMSCs co-cultured with non-transfected chondrocytes and SMSCs in

medium alone. CC Monolayer is monolayer chondrocytes used as positive control. Data collected at time points of 15 and 30 days after seeding. All groups are shown with the corresponding TGF-β3 concentrations (measured by ELISA) experienced by the SMSCs during the culture period. **(A)**Collagen II **(B)**Aggrecan **(C)**COMP **(D)**Collagen IX **(E)**Collagen I **(F)**Collagen X.

- indicates significant difference from negative control group **SMSC**

& - indicates significant difference from negative control groups **Co-Non** and **Co-Nill**

“ - indicates significant difference from positive control group **CC Monolayer**

^ - indicates significant difference from positive control group **Manual**

Data for TGF-β3 degradation profile over 3 days when added at a concentration of 10ng/ml in medium as measured by ELISA (shown along with **Manual** group) obtained from our previous study[1]

4.3.5 Immuno/histochemical staining analysis

Histochemical staining for sulfated GAG **(Fig. 4.7A)** shows that groups Manual and X00 have the highest expression of GAG compared to rest of the groups and that they have undergone differentiation into chondrocytes as seen by the morphological features and the presence of lacunae in the matrix. The same can be said for immunohistological staining for Collagen II **(Fig. 4.7B)**. It can also be seen that groups X10 and XD20 have higher expression of Collagen II compared to the control group Co-Nill(X). No significant difference can be seen in the expression of Collagen I between the groups **(Fig. 4.7C)**, which correlates with real-time PCR results.

Thus it can be said that group X00 has undergone chondrogenic differentiation evident by significant extra-cellular matrix (ECM) deposition and morphological changes; comparable to or better than that in the Manual group.

Figure 4.7A

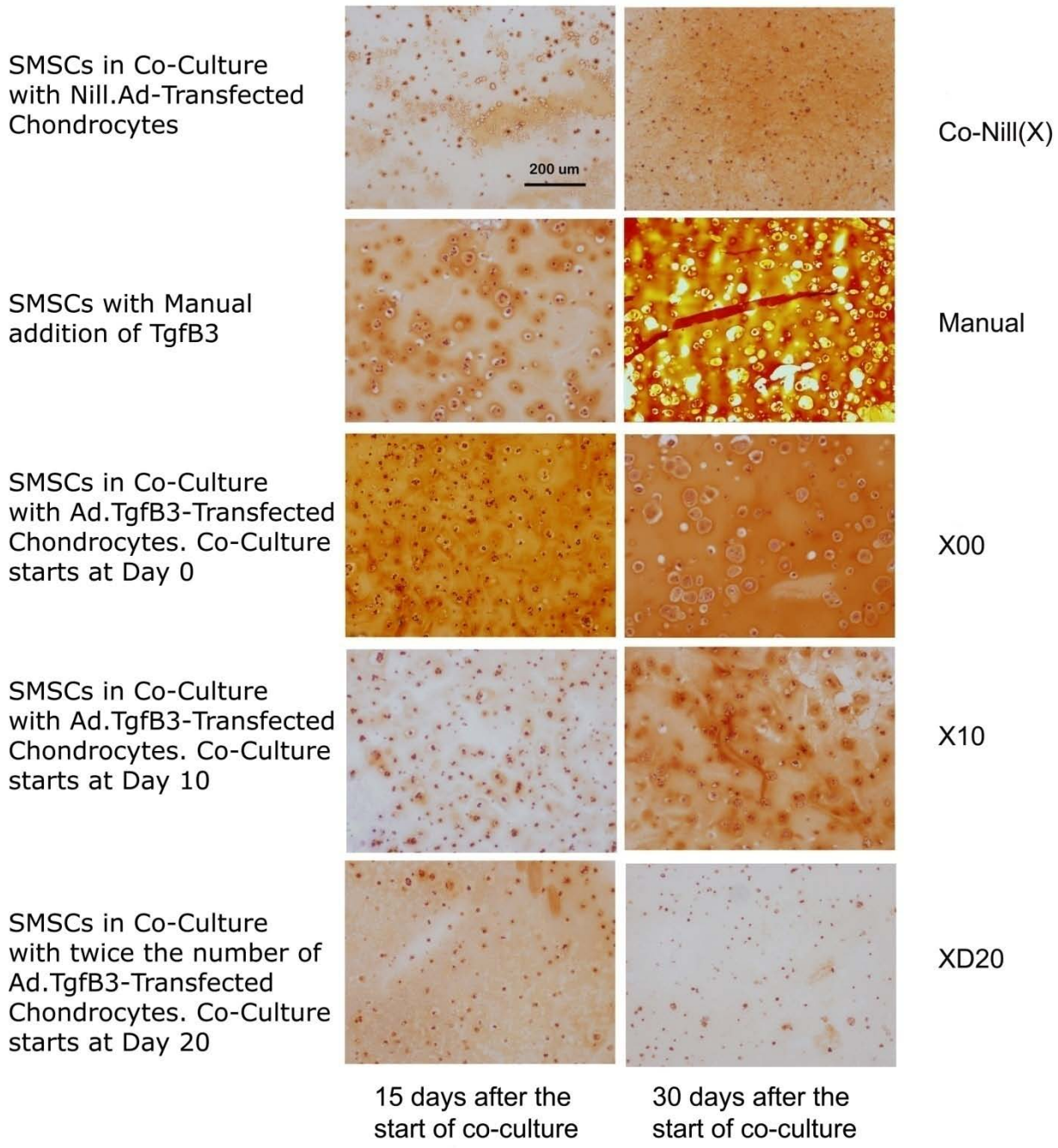


Figure 4.7B

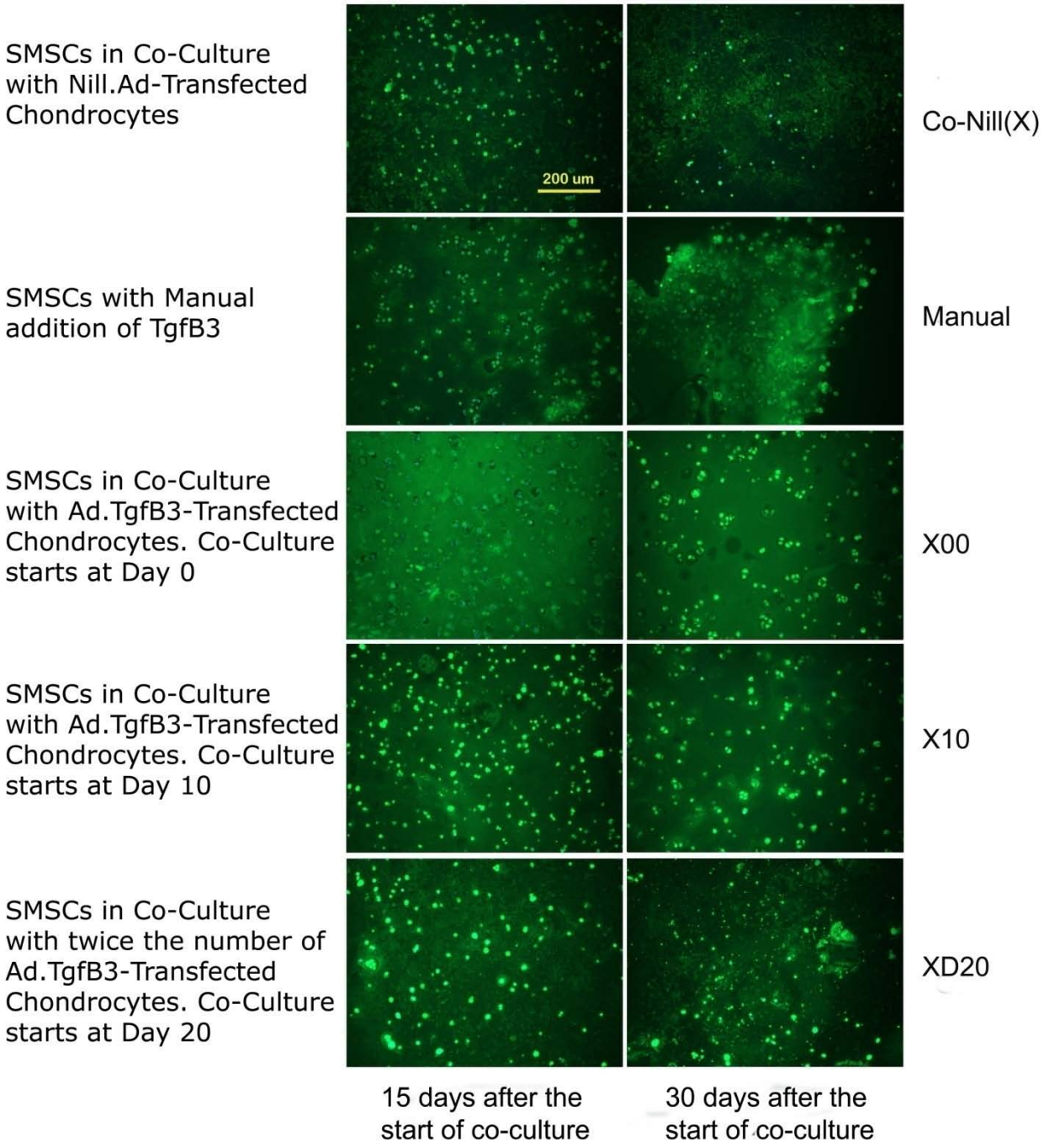

SMSCs in Co-Culture with Nill.Ad-Transfected Chondrocytes
200 um
Co-Nill(X)
SMSCs with Manual addition of TgfB3
Manual
SMSCs in Co-Culture with Ad.TgfB3-Transfected Chondrocytes. Co-Culture starts at Day 0
X00
SMSCs in Co-Culture with Ad.TgfB3-Transfected Chondrocytes. Co-Culture starts at Day 10
X10
SMSCs in Co-Culture with twice the number of Ad.TgfB3-Transfected Chondrocytes. Co-Culture starts at Day 20
XD20
15 days after the start of co-culture
30 days after the start of co-culture

Figure 4.7C

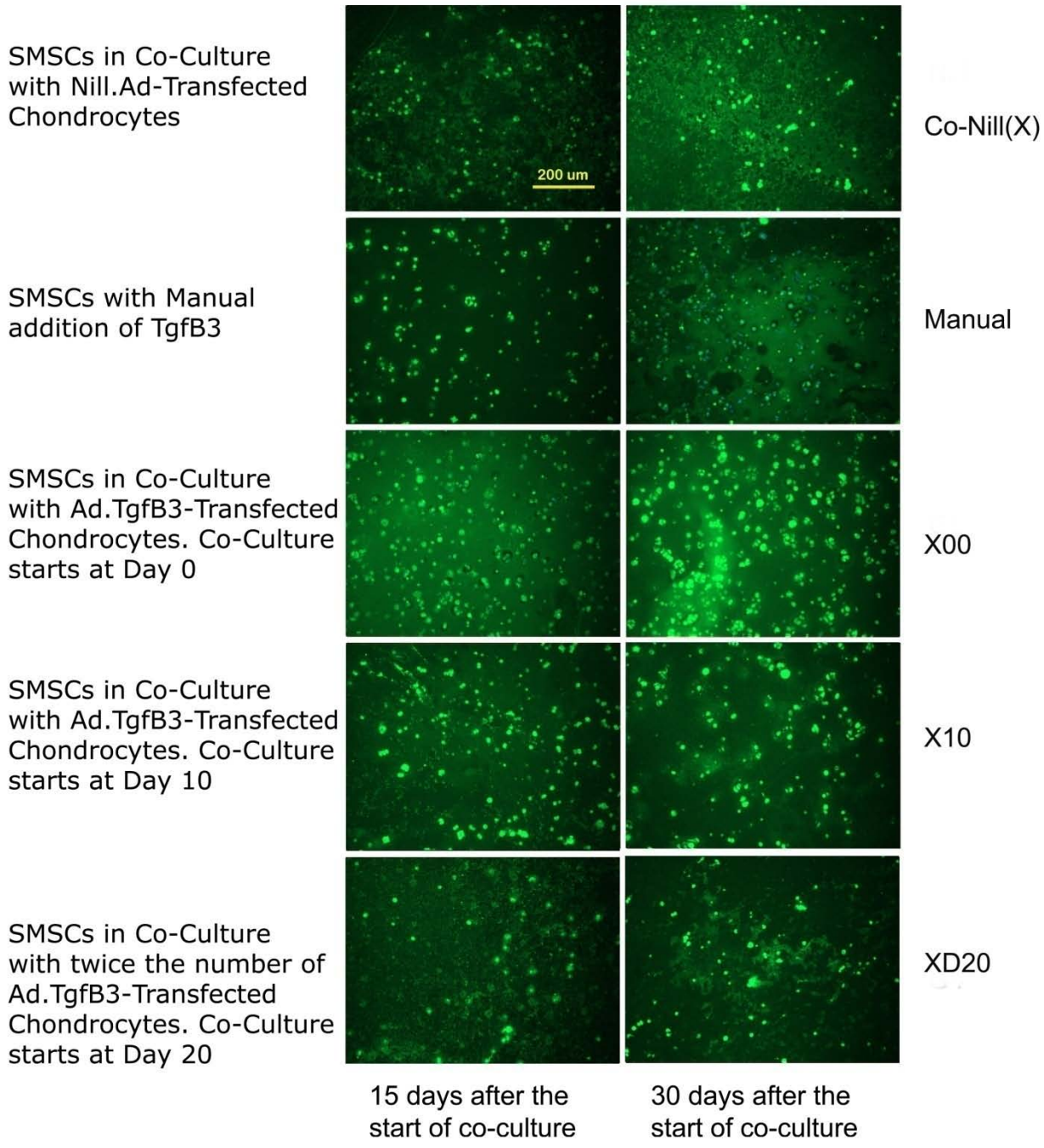


Figure 4.7: Immuno/histochemical staining pictures for SMSCs in co-culture with transfected chondrocytes (seeding density of 10 million cells/ml). **(A)** Histochemical staining pictures for sGAG (orange/red) obtained using Safranin O staining. **(B)**

Immunohistochemical staining pictures for Collagen II. Collagen II expression is indicated in green. **(C)** Immunohistochemical staining pictures for Collagen I. Collagen I expression is indicated in green.

4.4 Discussion and Summary

In this study, rabbit SMSCs were co-cultured with autologous chondrocytes adenovirally transfected with TGF-β3 gene. From the experiment on TGF-β3 expression profile from TCCs **(Fig. 3.10)**, we found that the expression of the GF is more than 100ng/ml for the first one or two days and is between 10-100ng/ml for the next 10-15 days when chondrocytes are transfected at a previously determined optimal MOI of 100-500 pfu/cell. Expression of the GF reaches a stable plateau between 2-10 ng/ml after about 20 days of culture. Such high concentrations of the GF during first 15 days may have unpredictable effects on TPCs in co-culture, while the concentration after Day 20 may be ideal for the differentiation of TPCs [185]; hence different starting points for co-culture (Day 0, Day 10, Day 20) were adopted. The co-culture was made to last for identical period of time (30 days) for each group.

Such a release profile of transgenic GF can be linked to the fate of the TCCs. The TCC viability experiment conducted previously **(Section 3.3.4)** showed that the number of transfected living chondrocytes kept on reducing over time while the number of dead TCCs kept on increasing. The overall cell population and the population of non-transfected chondrocytes remained stable. Real-time PCR analysis in TCCs shows a marked increase in the expression of hypertrophic marker Collagen X at Day 15 which

however gets downregulated by Day 30 in accordance with decreased TGF-β3 expression. Thus over-expression of endogenously produced GFs leads to death and decrease in population in a subset of TCCs. The expression of cartilage markers however significantly increases in rest of the TCCs after 30 days of culture. The expression of fibrocartilage marker Collagen I also reduces by Day 30. This interesting outcome could be due to the combined effects of 3D alginate culture and the presence of TGF-β3 and ultimately proves early passage chondrocytes to be the best TCC candidate.

As indicated in **Fig. 4.5**, the expression of cartilage related markers in SMSCs (TPCs) co-cultured with lower TCC seeding density (5 million cells/ml; hence lower TGF-β3 expression) is generally less than that in SMSCs manually dosed with 10ng/ml of the GF every 3 days (Manual). When TCC seeding density was increased to 10 million cells/ml, the expression of cartilage markers was 2-5 times more in the co-cultured SMSCs (TPCs) than in the manually dosed cells (**Fig. 4.6**). With higher TCC seeding density, chondrogenesis in TPCs was observed to be better when co-culture started from Day 0, compared to that in TPCs where co-culture started from Day 10 or Day 20; the expression of cartilage markers was also significantly higher in groups co-cultured with TCCs compared to control groups co-cultured with non-transfected (Co-Non(X)) or null-adenovirus transfected (Co-Nill(X)) chondrocytes. This result suggests that successful differentiation depends on the presence of high initial GF concentrations.

Other interesting findings in this study are recorded and reported as follows. Collagen II expression in SMSCs co-cultured with either non-transfected (Co-Non(X)) or null

adenovirus transfected (Co-Nill(X)) chondrocytes was almost zero. Collagen I expression in all the groups was downregulated by 30 days after the start of co-culture and was even near to zero in some groups. This down-regulation in Collagen I can be largely attributed to 3D alginate culture [191-193] and to a lesser extent to the presence of TGF-β3 [185]. Collagen X expression was reported to be significantly upregulated in rat MSCs supplemented with TGF-β3 compared to MSCs co-cultured with non-transfected chondrocytes [170]. Interestingly, in this study, Collagen X expression in the Manual group as well as that in groups Co-Non(X) and Co-Nill(X) was found to be lower than that in the transfected co-culture groups X10, X20 and XD20; while at the end of 30 days, there was no significant difference in expression between Manual, Co-Non(X), Co-Nill(X) and SMSC groups. Long term evaluation of hypertrophy in SMSCs co-cultured with transfected chondrocytes at both gene transcription and ECM secretion levels is further required before we can get a clearer picture.

As described above, co-culture was either established by mixing the two populations together [165, 173-174, 176], using well inserts to separate the two populations in a homogenous medium [168-170] or as bi-layered hydrogels [175]. In order to distinguish between TCCs (chondrocytes) and TPCs (SMSCs) used in our study - so as to individually characterize each kind of cell population - we encapsulated each cell type in separate alginate beads and the beads were then separated from each other using a silicon rubber partition which allowed for the exchange of medium. This also allows for a pure cell population of TPCs to be possibly used for transplantation. However, if required by some particular or

practical demands, the two cell populations may also be mixed together in future trials or applications.

Thus the co-culture system leads to successful differentiation of SMSCs into chondrocytes and formation of a cartilage-like tissue *in vivo* as evidenced by the immuno/histochemical staining data and real-time PCR results.

Chapter 5
Conclusions and Future Directions

5.1 Conclusions

In the present work, an adenoviral vector carrying TGF-β3 gene was successfully constructed. Various parameters and protocols for the transfection of chondrocytes (TCCs) with the recombinant adenoviral vector were optimized. An effective transfection MOI and TCC seeding density that could lead to sustained and sufficiently high TGF-β3 expression was experimentally established. Re-differentiation of adenovirally transfected chondrocytes in the presence of self-expressed TGF-β3 was carried out. Finally, the chondrogenesis of co-cultured SMSCs (TPCs) was carried out and the formation of cartilage-like tissue *in vitro* was observed. The following conclusions and findings were reached from the study:

1) It was found that early passage chondrocytes are ideal to be used for re-differentiation as well as for co-culture with the TPCs. When chondrocytes in monolayer culture are passaged for more than four or five times, they start losing their phenotype, turn hypertrophic and start de-differentiating. Thus for the cells to be robust enough to withstand transfection with an adenoviral vector, express abundant GF and at the same time recover their original differentiated native phenotype, it becomes essential to use early passage (Passage 3 or Passage 4) chondrocytes. Sufficient number of early passage chondrocytes can be obtained by passaging primary chondrocytes for a few times to satisfy the demands of the co-culture system.

2) Recombinant GF proteins have extremely short pharmacological half-lives. This fact was confirmed when *in vitro* degradation profile of recombinant TGF-β3 protein was measured and it was found that the half-life was in the order of minutes under normal culture conditions. Thus direct administration of the GF either *in vitro* or *in vivo* would require repeated high dosing. It makes much more sense to have the GF constitutively and endogenously expressed by gene transfer techniques. Accordingly, we obtained constitutive, endogenous over-expression of recombinant TGF-β3 from transfected TCCs. The transgene was over-expressed during the early period of culture (for about one to two weeks) and its expression was found to decrease with time, however the expression reached a plateau (about 3~5ng/ml) by the later stages of culture and was maintained at this value for up to the sixth week. The decrease in expression of TGF-β3 can be attributed to the transient nature of adenoviral expression and to the death of the transfected cells. This constitutive expression of TGF-β3 however proves to be effective in promoting re-differentiation in TCCs and chondrogenesis in co-cultured SMSCs; while at the same time the self-termination of GF supply prevents the unpredictable effects of high, long-term GF presentation in the TPCs. It was also established that both active and latent forms of TGF-β3 get expressed by transfected cells. The TGF-β3 pro-peptide gets enzymatically cleaved to release the active monomer from the inactive, latent form. Both these forms of TGF-β3 are present in the conditioned media from transfected cells, with the amount of latent TGF-β3 being much higher than that of the active form. In future, efforts can be made to transfer only the active form of recombinant TGF-β3 gene to the cells.

3) When adenovirally transduced TCCs encapsulated in alginate are re-differentiated in the presence of self-expressed TGF-β3, it can be seen that the overall chondrocytic status of TCCs is much the same as native cartilage tissue and much better compared to non-transfected chondrocytes encapsulated and cultured under the same conditions. Due to the fixed encapsulation capacity of hydrogel constructs, the total number of chondrocytes remains constant over the culture period. At the same time, the number of transfected living chondrocytes keeps on decreasing with time. This is in accordance with the transient nature of adenoviral transgene expression.

4) Finally, co-culture of SMSC based TPCs with TGF-β3 transfected TCCs (chondrocytes) results in significantly improved and controlled chondrogenesis of the progenitor cells and leads to the formation of a cartilage-like tissue in alginate constructs. For the purpose of this study, the TPCs and TCCs were encapsulated in separate alginate beads. However, in future *in vivo* trials, these two cell populations can be mixed together in a single transplantable construct. This co-culture strategy can be made use of in other applications where the transgenic GF expression needs to be controlled and modulated and the desired cell population needs to be protected from the effects of transfection. It can be used as a way of producing and supplying GFs for the differentiation of stem cells into different lineages.

5.2 Future directions

The current work provides a biological evaluation of the feasibility of the co-culture system in inducing chondrogenesis and, by extension, the efficacy of the system in tissue

regeneration techniques making use of stem cells and GFs. The current work provides a basis for future co-culture studies that could be translated *in vivo*. The co-culture strategy devised in this work is versatile in terms of the materials and cells used and can be extended to other cell types, GFs and cell delivery agents in the future.

TCCs sourced from a target tissue (bone, cartilage, skin, muscles) can be used to provide a modulated appropriate GF supply to corresponding stem cells (bone marrow stromal stem cells, epidermal stem cells, skeletal muscle stem cells) for the regeneration and repair of the respective tissues. As an example, the co-culture system presented in this study can be utilized for bone regeneration by co-culturing BMSCs or ESCs with osteocytes that are transfected with GFs like BMPs.

For the purpose of the current study, the two cell populations – TPCs and TCCs – were separately encapsulated in alginate beads so as to individually characterize each cell type. With successful realization of the co-culture strategy, this is no longer necessary and future efforts can have both the cell populations encapsulated in the same hydrogel. Doing so would significantly reduce *in vitro* culture time, as the GF would be supplied in situ. Also as seen in this system, a timely termination of the GF supply takes place as a result of transient gene expression and gradual decrease in the number of transfected cells in the construct. Thus this system can be used wherever the expression of GF needs to be modulated and the desired cell population needs to be protected from the effects of transfection. The system can be used to produce constitutive, in situ GF supply for the differentiation of stem cells into different cell types.

Future work as a continuation of this study will involve encapsulating GF transfected chondrocytes and MSCs in the same alginate construct and implanting the constructs into partial or full thickness articular cartilage defects in small animal models like rabbit. Wound healing and tissue regeneration will be measured using histology analysis. Safranin-O/Alician Blue staining and immunostaining for collagen II will be used to assess hyaline cartilage formation and growth post treatment as compared to controls. Collagen I staining will indicate formation of fibrocartilaginous tissue or scar tissue. MRI may be used to measure radiographic changes in cartilage growth and tissue formation. Controls will include animal models with induced cartilage defects that receive 1) no cell treatment, 2) treatment with encapsulated MSCs alone and 3) treatment with non-transfected chondrocytes and MSCs encapsulated in the same constructs. Assessment of the fate of individual cell types – chondrocytes or MSCs - can be a potential difficulty in using this system. However using GFP tagged adenoviruses to transfect the chondrocytes can help in identifying the TCCs from the TPCs. This would also help in determining if the MSCs are involved in repopulating the defect or if they induce/enhance tissue repair primarily by paracrine signalling. Formation of fibrocartilaginous tissue is another issue that would need to be addressed. This can be ameliorated by adding a Collagen I silencing RNA into the TGF-β3 encoding adenoviral vector[194]. MSCs have been shown to migrate to tumor tissues and aid in tumor development and propogation, owing to their immunosuppressive properties. They have also been shown to undergo spontaneous transformation under long term culture. As the MSCs would be immobilized in alginate constructs and undergoing cartilaginous differentiation, these issues would not be a major concern, but would still require monitoring. The calcification and vascularisation of

cartilaginous tissue derived from the transplanted constructs could be a potential pitfall of this strategy or any other strategy aiming for *in vivo* cartilage repair and regeneration.

Based on previous *in vivo* studies [41, 77, 81, 83-84], the system can be tested in large animal models (rabbits, goats or cows). A focal defect in the articular cartilage of the animal will be filled with a transplanted co-culture construct and characterized as described above for the efficacy of reconstruction, integration and healing.

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1. **Varshney, R. R.**, Zhou, R. J., Hao, J. H., Yeo, S. S., Chooi, W. H., Fan, J. B., Wang, D. A. Chondrogenesis of Synovium-Derived Mesenchymal Stem Cells in Gene-Transferred Co-Culture System. Biomaterials, 31 (2010), 26: 6876-6891.
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12. Fan, J. B., Gong, Y. H., Ren, L., **Varshney, R. R.**, Cai, D. Z., Wang, D. A. In Vitro Engineered Cartilage Using Synovium-Derived Mesenchymal Stem Cells with Injectable Gellan Hydrogels. Acta Biomaterialia, 6 (2010), 3: 1178-1185.