# Differentiation of Human Mesenchymal Stem Cell into Chonderocyte Like Cells 3D Poly Lactic Acid Glycosaminoglycan (PCL-GAG) Nano Fibre Scaffold

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## ABSTRACT

**Introduction:** Failure of human body tissue and organs is believed to be one of the most important health problems all over the world. The great challenge for tissue engineers is to optimize suitable systems to separate, proliferate and differentiate the cells so that they can set out to create tissue by a harmonic 3-D growth. Therefore, the tissue engineers must provide an environment like the living cells environment.

**Methods:** In this study, to increase efficiency, the nano fiber structure of poly lactic acid modified with glycosaminoglycan. Then the mesenchymal cells of bone marrow cultured on this 3-D structure with differentiated media culture of TGF-ß .To investigate differentiation, the genes expressed in cartilages tissue were used containing collagen gene, aggrecan and SOX9. The expression of gene confirmed by quality method of Reverse Transcriptase PCR then quantitatively examined by Real –Time PCR.

**Results:** The finding showed that using nano fiber scaffold of poly lactic acid modified with glycosaminoglycan leads to the increase of expression of cartilage gene.

**Conclusion:** Our findings indicated that GAG/PCL Nano fiber scaffold enhances the differentiation of mesenchymal stem cells into cartilage.This can help to overcome the limitations of tissue engineering.

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## 1. Introduction

The damages of cartilage tissue considered to be a widespread problem. Lack of repairing cartilage tissue is related to the biology of this tissue. In fact, cartilage tissue lacks neurons and vascularity therefore the repairing mechanism fails to work [1]. Recent studies performed about basic changes in our knowledge of regeneration mechanism of adults` organs. Previously, it was believed that the formation of organs and their repair limited to the active- stem cells of the same tissue or organs. It was specified, for example, not only -hematopoietic stem cells can create mature blood cells but also participate in repairing and modifying tissue of blood, kidney, brain and liver on the basis of a feature called plasticity [2]. Mesenchymal stem cells are type of non-hematopoietic cell residing bone marrow and other skeleton tissues [3].

In the biomaterials field, the major challenge to obtain the controlled or desired cell adhesion and spreading under physiological conditions through materials surface characteristics. As cells adhesion and extra cellular matrix production the topography and biochemical moieties on the surface of nano fibrous scaffold can strongly influence cell function [4].Cells of tissue are arranged and surrounded in a bed of extra cellular arrays. The challenge of tissue engineers is to optimize the scaffold or to create a suitable system for cell to separate, proliferate and differentiate so that it can precede the harmonic 3-D growth of tissue [5].

Biodegradable scaffold including materials such as poly lactic acid, poly glycolic acid, poly lactide ,glycolide , and poly caprolacton used to induce cell growth. Among natural biodegradable scaffolds glycosaminoglycan is a compound of aidaroanic acid, glucoaroanic acid and N-D- glucose amine [6].

Also, it was proved that PCL fibers effective for cell proliferation. The cultures of the mammary cells had a slow growth during the first few days. However compared to the control groups, the PCL enhances the cell growth kinetic rates over time. Mesenchymal stem cells, from the bone marrow, used upon PCL electros pun scaffolds. In early studies about tissue engineering, Polycaprolactone was used [7].

The recognition of glycosaminoglycan function was a remarkable progress in cell biology and genetics study .Glycosaminoglycan consists of 90% dried weight of cell in cartilage tissue. After obtaining plasma membrane, Glycosaminoglycan can be considered as a part of structure playing a role in absorbing molecular signal such as the growth factor and δ interferon and transferring the message form membrane to nucleus during a complex process [8].

As the main member of glycosaminoglycan hyaluronic acid is an anion polysacarid consisting of N-acetyl glucosamine and D-glyconic acid, found in cartilage, joints and vitreous fluid . In tissue engineering , Hyaluronic-acid is used to manufacture polymer scaffold [6].

Scientists seeking to find more optimal methods to produce scaffold to simulate extra cellular matrix structure a potential Scaffold in tissue engineering. In this study, electro-spinning method used to produce nanofiber Scaffold .Recently, this method attracted the attention of tissue engineers, using some natural and artifact polymers were produced .All these structures are suitable for Engineering and pharmaceutical applications [9].

## 2. Materials & Methods

### 2.1. Isolation of MSCs from Bone Marrow Human

After taking the consent from the donors who filled the medical ethics form (Taleghani hospital of Tehran), 10ml aspire sample of bone marrow was taken from a donor in a tube containing unit coagulation heparin in sterile condition. It was transferred to lab at 16-24°c .Then, the sample was mixed with 10 ml of (DMEM) media culture and its mono nucleus cells isolated by concentrationslope method on Faycol based on separation method of mono nucleus cells from bone marrow. Mono nucleus cells cultured in (DMEM) media culture and FBS10% and placed in incubator at 37°c and CO2 pressure 5%. After 48h, the media culture replaced to remove hematopotic cells floated in the media culture. For 14days, every other 3days, the media culture replaced .Then fibroblast-like cells were proliferated and covered the flask floor.

### 2.2. Flow Cytometry Analyses

To evaluate surface markers, the cells were separated form flask bottom by Trepcinate method. The separated cells with media transferred to falcon tube and centrifuged for 10min at 1500RPM. Precipitated cells are suspended in 1 ml of PBS solution; then 50µl of cellular suspension containing $10^6$ cell/ml transferred to test and control tube. 5µl of monocolonal antibody CD34- CD45 –CD44-CD90-CD105-CD166 to control tube and 5µl of antibody IGg-FITC against mouse cells were added to negative control tube, to recognize and remove nonspecific bounds. The tubes were kept in dark at 4°c tempera-

ture for 30 min. After wards add 500ml of PBS and rinse the sample .To fix the cells, add 50µl of paraformaldeid 1% solution to the sample .All samples are prepared for analysis. To analyzed the sampel using flowcytometry apparatus (Partec) under Flowmax software.

## 2.3. Electrospinning Set-up

The electrospinning set-up is represented in figure2- 1. The set-up including a high voltagepower supply, an infusion pump, aplastic syringe (Terumo, inner diameter 13.04 mm), a stainless Steel blunt ended needle (inner diameter 0.6 mm) and a circular rotating grounded collector with a adjustable rotating speed(diameter 7 cm). The syringe was horizontally fixed in the infusion pump.

The Polymer solution was led through a plastic tube to the needle vertically fixed at the centre of an electrode and placed above the collector. Applying a high voltage between the electrode and the collector the polymer solution was electrostatically drawn from the tip of the needle.

To optimize the morphology of the collected fibers, some variable parameters were considered including: theflow rate of the polymer solution (Q), the high voltage (V),the distance between the needle tip and the collector (air gap, h), and the viscosity ( ) of the polymer solution [10].

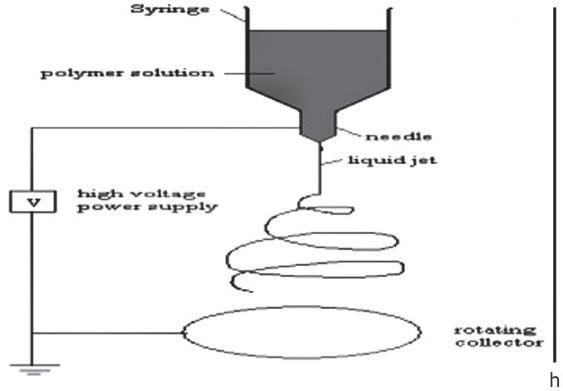



**Figure 1.** Electrospinning set-up

## 2.4. Polymers

Polycaprolactone is a semi-crystalline polymer used to make surgical graft and tissue engineering scaffolds. The main properties made it a popular polymer in the field of tissue engineering including having slow hydrolysis rate compared to polyglycolic acid and polylactic, biodegradable, biocompatible, gradual resorbable with good mechanical integrity. Using oil/water emulsion and solvent evaporation technique PCL microspheres were prepared .

Briefly, 1 g of PCL dissolved in 20 ml chloroform to obtain 5% transparent PCL/CHCl3 solution .(voltage-24KW, distance of DMF is 18 cm ,Debi 0.5 ml/h) under agitation with a rate. To evaporate the organic solvent the agitation lasted for 24 h at room temperature using standard sieves the PCL microspheres were separated and used in the following experiments [11].

## 2.5. Modification of PCL Scaffold

At first, scaffold was plasming by oxygen. Then, it was treated in EDC (3mg/ml) and NHS (3mg/ml)Solution for 10 hours, in temperature room.

Afterward scaffold washed with distilled water and treated in solution consist of the reaction functional group for 24h in 4C° temperature .

## 2.6. Cells Seeding in Scaffolds

Before cell seeding, scaffold prepared in square with dimensions of 1cm ×1cm (L×W) under sterile condition. Scaffold was sterilized in 70% ethanol for 2 h. Then they washed with PBS an incubation in DMEM for 24h. Then the medium was removed and cells were seeded on the scaffold (5-10×$10^3$ cells/$cm^2$) and cultured as described previously. After cell seeding , scaffold incubated under DMEM and FBS 10% for 2 week s. Scanning electron microscopy the surface morphology of scaffold was Characterized [12].

## 2.7. Scanning Electron Microscopy (SEM)

To study the structure of nano fiber and assess the form and morphology of cultivated cells on nano fiber scaffold, electronic microscope SEM of type vega - tescan was used. Scaffold containing cells fixed in glutar aldeid 2.5% for 2 hours, then washed with salt buffer and placed in an increasing slope of ethanol to be dehydrated. Afterwards it was kept in serial of resin and acetone and at last kept in pure resin for 24h at 70°C in an oven. The sample was dissected at 500nm thickness for SEM.

## 2.8. Differentiation Media Culture

After being assured of cell links to scaffold, a differentiation media culture used to proceed containing, DMEM with FBS 10% ,TGF-ß of 10ng/ml concentration ,Ascorbic acid of 50mg/ml (100X) and Dexamethazone of 0.0012g/ml concentrations over 20ml (100X)A

mixture of differentiation media culture added to scaffold-continuing plate at 500ml every other 3days.In this study; four groups were designed for research. The first group was the scaffold-connected cells along with differentiation materials. The second was the scaffold-connected cells along with DMEM media culture containing antibiotic and FBS10% designed to investigate the effect of Nanofiber scaffold without differentiation media.

The third consisted cells in differentiation media which were not on scaffold. This group was designed to investigate the effect of differentiation media effect. And the last was intact mesenchymal cells used as witness and control group in Real-time PCR.

### 2.9. cDNA Synthesis and RT-PCR Experiment

The cells were left idle three weeks so that the differentiation process took place. During three weeks the cell media culture was replaced every other 3 days. Then, using RNX, RNA was extracted and c DNA manufactured through Accupower premix series kit (Bioneer) according to protocol. After the completion of synthesis, the reaction product used for RT-PCR reaction.

Using specific primers, it was set out to express gene of collagen ,agrecan and sox9 which are the differentiation index of cartilage. The basis of RT-PCR reaction is on reverse transcription enzyme and the ability to produce compelementary DNA chain form mRNA. To do the test, Cinagen company kit was used. Thermal cycler machine was Biotak. The product of RT-PCR reaction was electrophoresised on gel agarose 2%.

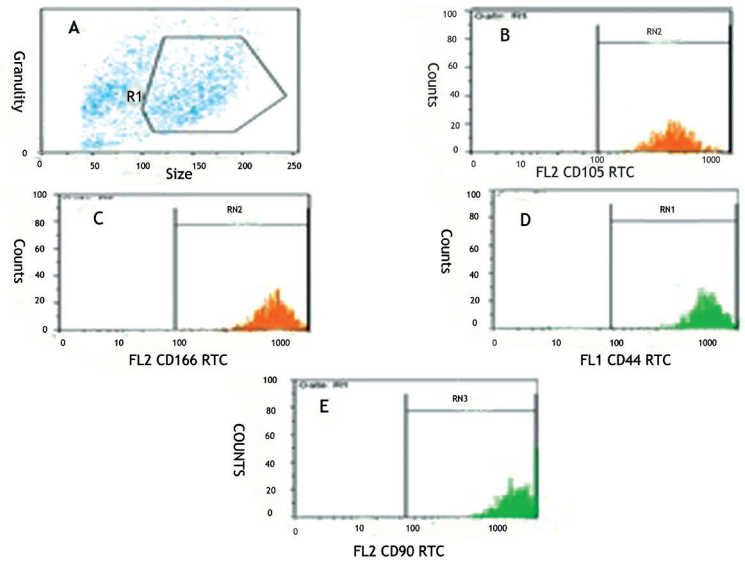
For expression of the gene confirmed by RT-PCR, the quantity investigation carried out Real -Time PCR of genes. This method consisted of diagnosis of SYBER Green bound to DNA molecule. Therefore at the end of elongation stage of each PCR reaction cycle, the radiation rate of fluorescence measured to control the proliferated DNA molecule. In this experiment the kit of SYBER®Premix EX TakaRa was used.

### 2.10. Statistical Analysis

Using SPSS software statistical analysis were carried out .Data were expressed as mean ± SD. Differences were considered statistically signification at $P < 0.05$.

## 3. Results

Confirmation of mesenchymal stem cell nature by flowcytometeryIn this study, CD34, CD45, CD44, CD90, CD105, CD166 markers were investigated. The results of flowcytometry analyses show that the percentage of expressing specific markers of mesenchymal stem cells containing CD90, CD166, CD105, CD44, in cells separated form bone marrow were 96.8% ,98. 8% , 93% and 99.5%, respectively, while specific markers of hematopoietic cells such as CD45 and CD34 in these cells did not have remarkable expression(3.8%,2.8% ,respectively). These results show the quality of separating mesenchymal cells and removal of hematopoietic cells during the separation of mesenchymal cells form bone marrow. The results of flowcytometry confirm this issue that the initial steps of separating mesenchymal cells from bone marrow was done perfectly.



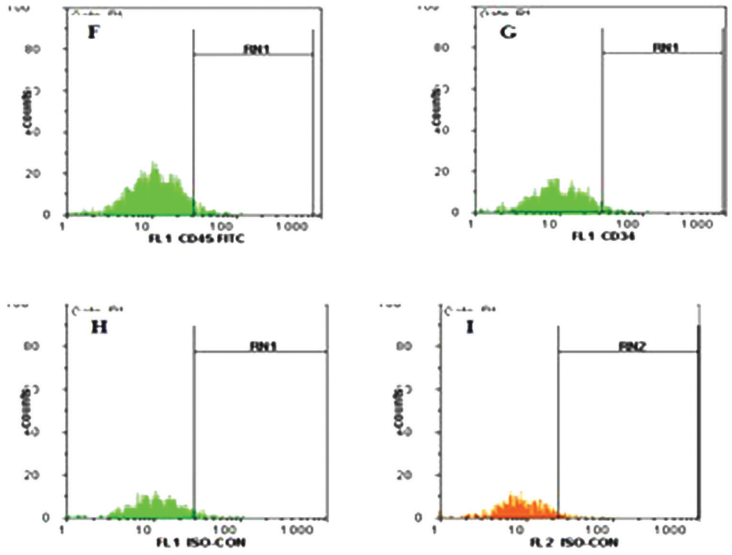



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**Figure 2.** shows the expression of surface markers of bone marrow mesenchymal cells by flowcytometry Graph (A) shows the gate of mono nuclear cells. (B) shows the expression of CD105 antigen (93%). (C) the expression of CD166 antigen (98. 8%), (D) the expression of anti-gene CD44(99. 5%), (E) the expression of anti-gene CD90(96.6%) (F) The expression of anti-gene CD45 (3. 8%), graph (G) the expression of anti-gene CD34 (2. 8%), (H) the negative control of FITC and graph (I) shows the negative control of PE.

### 3.1. Cell Adhesion Assay

Using electronic microscope SEM, the form and morphology of cultured cells on scaffold were investigated. These images in three magnitudes show the cell bound to scaffold and formation of extracellular matrix among them.

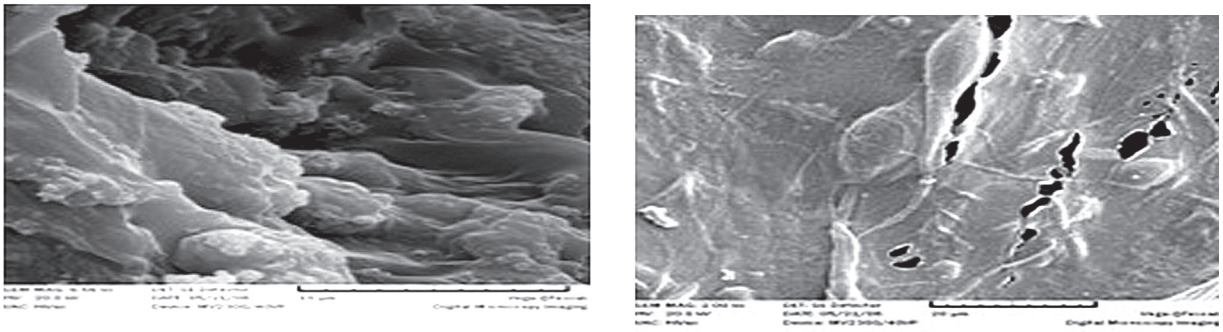

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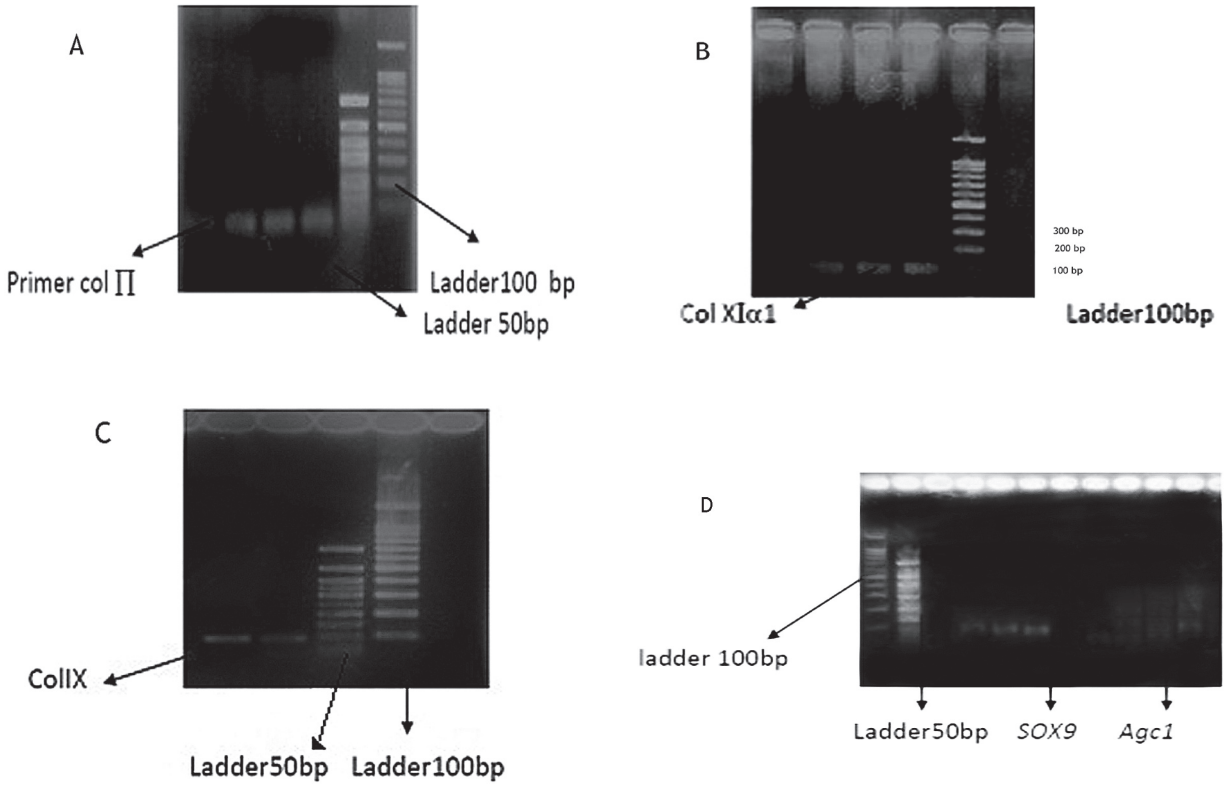
**Figure 3.** The above images are the picture of electronic microscope SEM from nano fiber scaffold after 14days of cultivating mesenchymal cells (A) Picture with magnitude at 10µm shows the cellular ECM , (B) the picture at magnitude 20µM , the empty space between cells is seen a little and mostly filled.

### 3.2. The Analysis of Expression Gene by RT-PCR

To confirm the gene expression after differentiation and extraction of RNA, the gene expression by RT-PCR was investigated .The primers designed in Reverse and Forward manner and the expression of 8 genes related to cartilage in cultivated cells on nano fiber was examined. The results of electrophoresis on the gel Agarose 2% are presented.

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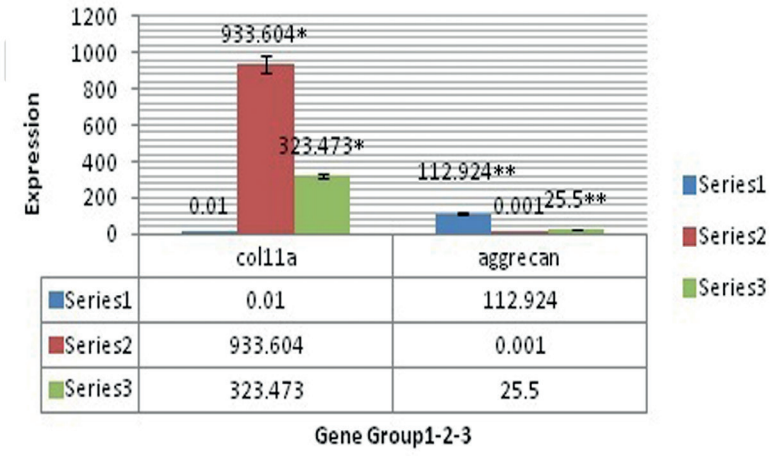
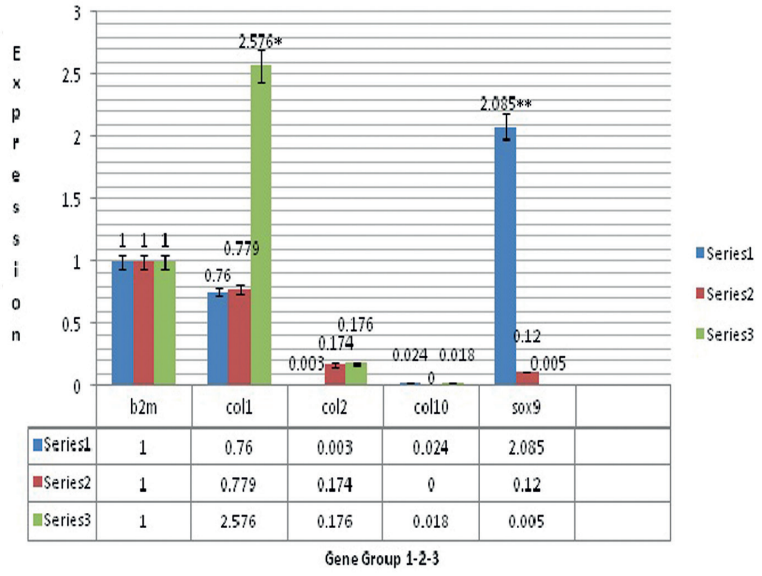
**Figurre 4.** (A) Expression of collagen geneΠ in groups 1, 2, 3 and4 .The size of gene is 85b. (B) Expression of collagen xIa1 gene in groups1, 3 and4.There is no expression in group 2 .The size of gene is 96bp. (C) Expression of gene collagen IX in groups 1 and 4.The size of gene is 77bp. (D) Expression of sox9 gene at 71bp in groups 1, 2 and4 and expression of Aggrecan gene at 190 bp in group 1were observed.

### 3.3. Real Time –PCR

In this step the primer of 8genes was required to find the increase of sample expression versus control sample (the mesenchymal cell on which there is no differentiation). Then the product of cDNA used for different reaction.








**Graf 1.** The value of mRNA from group 1, 2 and 3 . Expression of col11a and aggrecan . b2m ( house keeping) is control in Real time -PCR. Dots and bar represent the mean ± SD. *(P<5%),**(P<5%) Graf- 2 The value of mRNA from group 1, 2 and 3 . Expression of col, col2 ,col9 ,col10 and sox9 . b2m (house keeping) is control in Real time -PCR. Dots and bar represent the mean ± SD. *(P<5%), **(P<5%).

The previous studies on nano fiber of poly lactic acid show the increases of expression of cologne Π and aggrecan after 21days [13].The 1056 genes expressed in extracellular matrix of embryonic cartilage some of which are, at first, expressed extracellular by collagen (60-70%) and , then proteoglycans(10-15%) [14].

In the second group, in which mesenchymal cells were grown on nano fiber scaffold without differentiation media, there was a significant increase in collagen I, but collagen II and sox9 had less expression. There was no significant increase in other genes. Collagen II and sox9 were cartilage tissue markers. Sox9 was a part of DNA bounding proteins. Its activity increased during the differentiation of cartilage. In the researches, gene expression of sox9 was observed [15].

In the third group of mesenchymal cells without nano fiber scaffold with differentiation media culture, there was significant increase in the expression of collagen I, collagen 11a1. collagen II and aggrecan but there were no significant increase in other genes.

## 4. Discussion

There are sevral studies on mesenchyme cells as a replacement cellular source for cartilage tissue engineering , attracted the attention of tissue engineers.

Thes cell are able to differentiate into chondrogenic lineage and maintain their phenotypic stability for multiple passages .Most of the new studies have focused on cell differentiation on a 3 –dimensional (3D) environment. Differentiation of mesenchyme cells to condrocyte mainly depended on expression of SOX9,Aggrecan, collagen and proteoglycans [15].

The purpose of the present study was to modify the nano fiber structure of poly lactic acid with glycosaminoglycan resulting in the increase of mesenchymal cell differentiation to cartilage cell. In the first group with mesenchyme cells on nanofibre scaffold and differentiation media,a significant increase in expression of aggrecan protein and collagen IX was reported . Collagen I had little expression and there were no significant increase in other genes. Aggrecan gene is a member of proteoglycan and an encoded protein seen in extracellular matrix. The increase of expression of mRNA of this gene can be known as a sign of mesenchymal cell differentiation to the cartilage. Collagen of type IX and I observed in the most tissues such as cartilage , bone, tendon and skin.

Comparing these groups, the following conclusions were reported. As mentioned before, Aggrecan is a member of proteoglycan family and can be seen in the extracellular matrix of cartilage while considered to be

the distinctive aspect of cartilage. In the first group of cells in which the cells were grown adjacent to structure-modified nanofibre and TGF-ß, its expression increased 112 times as much as that of in control group .It can be concluded that structure-modified nanofibres have remarkable effect on cell differentiation to cartilage tissue and reinforce the effect of TGF-ß.

Sox9 encodes the protein increased during chondrocyte differentiation. In the first group, in which the cells grown on modified Nano fiber and TGF-ß, the expression increase 2.085 times as much as that of in control group. In the second group in which cells grown adjacent to structure-modified nanofibre without differentiation media, the increase of expression was 0.12 times versus control group. In the third group in which the cells grown only adjacent to TGF-ß, there was a 0.005 times increase. It was conclude that the effect of structurally modified nano fiber on the expression of sox 9was greater than that of TGF-ß on protein expression.

As an extracellular matrix protein collagen of type I is seen in most connective tissues such as skin, tendon and bone. Collagen of type I in the first and second group with nanofibre scaffold had almost the same expression and there was no significant difference between two groups. It was found that TGF-ß induced the gene expression of collagen I. In the third group, there was a remarkable increase in the expression of collagen I. Therefore It is concluded that the differentiation media culture alone had a better effect on the activation of cell signaling of collagen I .Allowing more time to cells grown on nanofibre scaffold collagen may result in greater expression of collagen I belongs to protein of extracellular matrix. Collagen of type II is seen in cartilage and vitreous humor. Disturbance in this collagen may lead to cartilage dystrophy.

There was no significant difference in the gene expression of these three groups. Finally, it can be concluded that structurally modified nano-fibre scaffold with glycose amino- glycan has a positive effect on the differentiation of mesenchyme cells to chondrocyte and improves the conditions of differentiation.

## 5 .Conclusion

In this study, it was concluded is suggested that the modification of PCL with glycosamynoglycan enhance the expression of collagen, aggrecan and Sox9 and induces the mesenchyme cells differentiation to chondrocyte cells . Thus, it is expected that the PCL- GAG scaffold can be useful for promoting scaffold condition in tissue engineering.

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