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PRECONDITIONING WITH TRANSFORMING GROWTH FACTOR ALPHA; A STRATEGY TO ELEVATE CHONDROGENESIS IN HUMAN AMNIOTIC FLUID STEM CELLS

[1,2] Suphakde Julavijitphong, [1] Puttachart Pokathikorn, [1] Suparat Wichitwiengrat,
[3] Supatra Klaymook, [1*]Tatsanee Phermthai

[1] Stem Cell Research and Development Unit, Department of Obstetrics & Gynecology,
Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, 10700, Thailand

[2] Infertility unit, Department of Obstetrics & Gynecology,
Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, 10700, Thailand

[3] Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, 10700, Thailand

* Corresponding author

Abstract: For tissue regeneration, preconditioning of mesenchymal stem cell (MSC) prior to cell transplantation has been noted to activate target gene set and specific molecular functions. An efficient MSC source for tissue regeneration is amniotic fluid-derived MSC (AF-MSC) owing to their potential in tissue repair and paracrine secretion. However, a standard chondrogenic differentiation medium of MSC, which promotes differentiation through a signal of transforming growth factor (TGF)-β1, makes for an unstable and limited outcome on amniotic fluid-derived MSC (AF-MSC) differentiation into chondrogenic lineage. To increase the differentiation outcome of AF-MSC, we investigated chondrogenic differentiation effects of eight growth factors (GFs)/cytokines on AF-MSC through expression of chondrogenesis-related genes, including *Col-I, Col-II, ACAN, RunX2* and *SOX9*. Our work demonstrates that anabolic *Col-II* gene can be increased by stimulation of TGF-α, insulin like growth factor-1 (IGF-1) and stromal derived factor 1 α (SDF-1α) with a concentration-dependency. We found that TGF-α elevated anabolic gene function and declined expression of Col-I, typically present in fibrocartilage, during chondrogenic differentiation of AF-MSC. Moreover, TGF-α was not toxic to micro-environment for AF-MSC differentiation. Substitution of TGF-β1 by TGF-α in the condition medium can enhance over two times expression of *Col-II* and *SOX9* genes in AF-MSC. The current study also demonstrated that single GF/cytokine has a greater effect to elevate chondrogenic differentiation of AF-MSC than the GF/cytokine combinations. We conclude that TGF-α preconditioning improved chondrogenesis in AF-MSC for cartilage regeneration**.**

***Key words*: Mesenchymal stem cell, Chondrogenic, Differentiation**

1. INTRODUCTION

Regenerative medicine has been a promising strategy for degenerative diseases. A treatment for cartilage degeneration is transplantation of active cells to motivate cartilage regeneration. Autologous chondrocyte transplantation is the first treatment of Osteoarthritis, a joint disease causing by cartilage degeneration, using cell-base therapy [1]. This technique achieves for pain reduction and also durable cartilage-like tissue production. However, *in vitro* expansion of autologous chondrocytes has been limited by a long period of manipulation time, cell differentiation during in vitro manipulation and loss of extracellular matrix [1-2]. Allogenic mesenchymal stem cells (MSC) from various tissue sources (for examples; amniotic membrane, amniotic fluid, placenta, umbilical cord and dental pulp) are of interest for cell-base therapy in cartilage regeneration [3-7]. However, appropriate sources of MSC and approaches to manipulate the MSC before cell transplantation are still a challenge.

Amniotic fluid-derived stem cell (AF-MSC) is a pluripotent mesenchymal stem cell derived from fetal tissues during gestation period. AF-MSC has early stemness and is enriched with active gene sets, which results in a differentiation potential for a wide spectrum of tissues. According to its high proliferation potential, multi-lineage differentiation, genetic stability, immunosuppressive, non-tumorigenic

formation in an *in vivo* state and no ethical concerns, AF-MSC is a promising source for therapeutic uses [8-9] and cartilage regeneration [1,7,10-12]. There is evidence of the chondrogenic potential of AF-MSC contributing *collagen type II* (*Col-II*), *SOX9* and a cartilage protein (sulfated glycosaminoglycan; sGAG) in an *in vitro* chondrogenic differentiation. Owing to a number of active genes that enrich in AF-MSC, cell transplantation in therapeutic uses is appropriate for consideration in a precondition of AF-MSC with specific molecular signals stimulation. For therapeutic treatment of cartilage defect, AF-MSC should be preconditioned to activate cartilage-specific molecules and functions. A traditional chondrogenic differentiation protocol and medium were first performed for bone marrow MSC differentiation into chondrogenic lineage [13] and subsequently applied for all MSC sources. The medium stimulates biomolecular activities through transforming growth factor beta signal (TGF-β1 and TGF-β3) leading to chondrogenesis, glycosaminoglycan synthesis in ECM and the transcription of *SOX9* in various sources of MSC.

However, traditional differentiation medium in standard protocol of MSC still has limitation for conversion of AF-MSC into chondrogenic lineages because of the instability of chondrogenic phenotypes. Recently, evidence of chondrogenic differentiation in adipose MSC has been accomplished with an independent TGF-β signal. A report by Zhou *et al.*, 2016 [14] found that adipose-derived MSC enhanced for *Col-II*, *Aggrecan* and *SOX9* expression in chondrogenic differentiation medium supplemented with IGF-1. Moreover, other growth factors that affect MSC proliferation and chondrogenic differentiation have been reported. Solchaga *et al.*, 2005 [15] and Park *et al.*, 2008 [16] described enhancing chondrogenic gene expression and production of GAG and Collagen type II through a stimulation of bFGF signal.

From above information, TGF-β1 might not be an optimal GF to promote chondrogenic differentiation in AF-MSC. To achieve GFs/cytokines effecting chondrogenic differentiation in AF-MSC for precondition culture, we determined chondrogenic stimulating effect of eight different GFs/cytokines in various concentrations and three GFs/cytokines combinations to AF-MSC via anabolic gene expression and toxic effect of growth factor on AF-MSC.

2. MATERIALS AND METHODS

2.1 AF-MSC samples

Three cell lines of amniotic fluid-derived stem cells (AF-MSC), with ethics committee approval, were obtained from Stem Cell Research and Development Unit, Department of Obstetrics & Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University. The cell lines were scaled up under AF-MSC medium, containing minimum essential medium alpha medium (α-MEM; Gibco Invitrogen, CA), 15% fetal bovine serum in embryonic stem cell grade (ES-FBS; Millipore, Billerica, MA), 20% Chang medium (Irvine Scientific, CA), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, MO) at 37˚C in 5% CO2. Medium was changed every three days. Subculture was performed at 70% confluence of culture with a dilution of 1:3. AF-MSC was repeated subculture to passage 10 (P10) for experiments.

2.2 Characterizations for AF-MSC

AF-MSC was investigated for proliferation capacity, phenotypic analysis and differentiation potentials to confirm MSC characteristics before experiment began. Proliferation capacity was observed through population doubling time. Phenotypic analysis of AF-MSC was classified through cell surface markers; CD29, CD34, CD45, CD73 and CD105 using flow cytometer. For cell differentiation potentials, AF-MSC was examined for properties of *in vitro* differentiation into adipogenic and osteogenic lineages. AF-MSC, which reached 70-80% confluence under AF-MSC medium, was shifted to an appropriate differentiation medium. The appropriate differentiation medium for adipogenic contained α-MEM supplemented with 10% ES-FBS (Millipore), 1 µM dexamethasone (Sigma), 5 µg/ml insulin (Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) and 60 µM indomethacine (Sigma). Adipogenesis was assessed with Oil Red O staining on intracellular lipid droplets. The appropriate differentiation medium for osteogenic contained α-MEM supplemented with 10% ES-FBS, 0.1 µM dexamethasone (Sigma), 10 mM glycerol-2-phosphate (Sigma) and 50 µM ascorbic acid (Sigma). Staining for alkaline phosphatase activity was performed to prove osteogenesis.

2.3 Assessment of *in vitro* chondrogenic differentiation of AF-MSC in GFs / cytokines

AF-MSC (n=3) at 70% confluence was shifted from AF-MSC medium to standard chondrogenic differentiation medium. The differentiation medium contained DMEM (high glucose) supplemented with 40 µg/ml L-proline (Sigma-Aldrich), 1% insulin-transferrin-selenium (ITS; Gibco), 100 µg/ml sodium pyruvate (Gibco), 0.1 µM dexamethasone, 50 µg/ml L-ascorbic acid, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and a growth factor. The growth factor, which has normally been used for supplementation in chondrogenic differentiation medium, is a 10 ng/mL TGF-β1. To assess action of various growth factors/cytokines to AF-MSC differentiation into chondrogenic lineage, growth factors (GFs)/cytokines involved in chondrogenic differentiation-were applied as substitution of TGF-β1. Eight GFs/cytokines were used to induce chondrogenic differentiation of AF-MSC, including TGF-β3 (R&D systems, Minneapolis, MN), basic fibroblast growth factor (bFGF), transforming growth factor type alpha (TGF-α), platelet derived growth factor type AA and AB (PDGF-AB, PDGF-BB), insulin like growth factor type1 (IGF-1), stromal cell-derived factor 1α (SDF-1α) and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) (Millipore). To study the dose-dependent effect, each GF/cytokine was varied with concentrations into 0.1, 1, 5 and 10 ng/mL and supplemented in differentiation medium. The chondrogenic differentiation medium supplemented with 10 ng/mL TGF-1α was used as a control medium. AF-MSC was cultured in GFs/cytokines medium for two weeks under 5% CO2 at 37°C. The GFs/cytokines medium was changed twice a week. The cell morphology, population doubling time and proliferation capacity were analyzed through inverted microscope. Molecular activity was observed through expression of chondrogenic related genes using real-time reverse transcription-polymerase chain reaction (real-time RT-PCR).

To study action of growth factor combination to chondrogenic differentiation of AF-MSC, GF/cytokine, which achieved chondrogenic induction effect in AF-MSC, was selected based on concentration-dependency and combined to perform GFs/cytokines cocktails. AF-MSC lines (n=3) were incubated in the cocktail and compared their differentiation effects to control medium, which was supplemented with 10 ng/mL TGF-β1.

2.4 Gene expression analysis

After two weeks under differentiation medium, cells were extracted based on total RNA using Trizol reagent (Invitrogen, CA) according to the manufacturer's instructions. The total RNA was reverse transcribed into complementary DNA (cDNA) by RevertAid First-Strand cDNA Synthesis Kit (Fermentas, EU). Expression of *Aggrecan (ACAN), Collagen type I (Col-I), Collagen type II (Col-II), Transcription factor SOX9 (SOX9)* and *Runt-related transcription factor 2* (*RunX2*) were investigated. Selective primer validations of the amplification efficiency of *ACAN, Col-I, Col-II, SOX9* and *RunX2* genes were provided. The quantitative real-time RT-PCR reaction mixtures contained 5 µl of 5 ng cDNA, 10 µl SYBR Green Master Mix (FastStart SYBR Green Master; Roche Diagnosis, Mannheim, Germany) and 10 pmol of forward and reverse primers. The primers used in real-time RT-PCR are shown in Table 1. The specificity of the real-time RT-PCR product was evaluated by melting curve analysis. The transcript of *ACAN, Col-I, Col-II, SOX9* and *RunX2* genes was quantified by real-time RT-PCR and was reported in comparative threshold cycle (CT). Gene expression performance was calculated using the method also known as the $2^{-\Delta\Delta CT}$ method by a formula: Performance of gene expression = $2^{-\Delta\Delta CT}$ of studied differentiation medium /$2^{-\Delta\Delta CT}$ of a control differentiation medium. The quantities of *ACAN, Col-I, Col-II, SOX9* and *RunX2* were evaluated relative to the quantity of a housekeeping gene Beta-actin (*ACTB*). Analysis of gene expression in AF-MSC lines was performed in triplicate.

Table 1: Primer sequences and annealing temperatures used for real-time RT-PCR analysis

Genes	Primer Sequences (5' → 3')	Product size (bp)	Annealing temperature (˚C)	Accession NO.
ACAN	F: 5'-ACAGCTGGGGACATTAGTGG-3' R: 5'-GTGGAATGCAGAGGTGGTTT-3'	189	57	NM_001135.3
ACTB	F: 5'-ATGTGGCCGAGGACTTTGATT-3' R: 5'-AGTGGGGTGGCTTTTAGGATG-3'	107	57	NM_001101.3
Col-1	F: 5'-AGGACAAGAGGCATGTCTGGTT-3' R: 5'-GGACATCAGGCGCAGGAA-3'	122	57	NM_000088.3
Col-II	F: 5'-GGCAATAGCAGGTTCACGTACA-3' R: 5'-CGATAACAGTCTTGCCCCACTT-3'	79	60	NM_001844.4
SOX9	F: 5'-CCCAACAGATCGCCTACAG-3' R: 5'-TTCTGGTGGTCGGTGTAGTC-3'	97	57	NM_000346.3
RunX2	F: 5'-ATGCTTCATTCGCCTCAC-3' R: 5'-ACTGCTTGCAGCCTTAAAT-3'	156	57	NM_001015051.3

2.5 Statistical analysis

All results were presented as means ± standard derivation (SD). Data analyses were performed using Student's t-test. A significant difference was evaluated by GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). A statistical significance was considered at a value of $P<0.05$.

3. RESULTS

3.1 AF-MSC cell lines

AF-MSC lines used in experiment were verified to authenticate stem cell characteristics before applying them for examinations. AF-MSC cell lines at passages 10 were used for the experiment. All cell lines exhibited typical characteristics of AF-MSC with a high proliferation capacity. They grew rapidly with a low population doubling time (PDT) during *in vitro* culture. The PDT of AF-MSC with an initial density of 4,000 cells/cm^2 showed an average at 1.42 ± 0.21 days with a range of 1.23 to 1.75 days. Typical AF-MSC characteristics were confirmed by cell morphology, phenotypic cell differentiation (CD) markers and multi-lineage differentiation. AF-MSC showed cell morphology in short fibroblastic cell type. The cells exhibited strong positive MSC surface markers, including CD29 (99.4 ± 0.3 %), CD73 (98.93 ± 1.76 %), CD105 (96.26 ± 5.17 %) and showed no signal of hematopoietic CD34 (0.80 ± 0.94 %) and CD45 (0.55 ± 0.28). For multi-lineage differentiation capacities, AF-MSC showed ability to *in vitro* differentiate into adipogenic-lineage by presenting endogenous lipid droplets by Oil Red O staining and osteogenic-lineage by presenting alkaline phosphatase activity.

3.2 Morphologic appearance of AF-MSC cultured under GFs/cytokines medium

At day four of differentiation, AF-MSC in TGF-β3, bFGF and control medium was found as a fibroblastic cell type, whereas, the cells in TGF-α, PDGF-AB, PDGF-BB, RANTES, SDF-1α and IGF-1 medium became short and morphologic changed into epitheloid type without forming of cell cluster. Cell clusters were found in a few areas of control medium (10 ng/mL TGF-β1). Increasing of cell amount,

indicating cell proliferation, was observed in cultures supplemented with bFGF, IGF-1, PDGF-AB and PDGF-BB, but not in RANTES, using inverted microscope.

At day seven of chondrogenic differentiation, cells under TGF-β1, TGF-β3 and control medium were found in fibroblastic type. Cells in TGF-β3 medium showed as a big size and transformed shape in every concentration. The cell cluster was found in TGF-β1 medium at every concentration. TGF-β3 medium at a concentration of 5-10 ng/mL formed many aggregations as macroscopic cells mass. Cells in IGF-1, PDGF-AB and PDGF-BB medium presented an epitheloid morphology and formed aggregations as a thicker layer, whereas, cells that were stimulated with TGF-α, SDF-1α and RANTES at a concentration of 5-10 ng/mL formed a number of aggregates on a two-dimensional surface. Many small free areas were found in cultures (Fig.1A).

At day 14 of chondrogenic differentiation, cells in fibroblastic type under stimulation of 5-10 ng/mL TGF-β1 and TGF-β3 showed aggregates with a multilayer allowing a number of large free areas to take place on culture surface, whereas, cells in bFGF medium grew in a monolayer and turned into necrotic cell dead because of overpopulation. For cells in epitheloid morphology, the cells under a stimulation of TGF-α, SDF-1α and RANTES at a concentration of 5-10 ng/mL formed condensing aggregates and multilayer structure resulting in a number of large free areas on culture dishes. The cells became round shaped and the nucleus was condensed. Cell-cell interaction, thin cell membrane and performing of cell lining with 3-5 single-cells were observed at every concentration of TGF-α and at a concentration of 5-10 ng/mL SDF-1α. Formation of compact multilayer structure was found in cultures stimulated with 5-10 ng/mL of TGF-α and RANTES (Fig.1B).

Our work also observed toxic effect of each GFs/cytokines on AF-MSC. The toxicity of each GFs/cytokines medium at various concentrations was analyzed via cell morphology, and total cell numbers at day 14 of chondrogenic induction (Fig.1C).

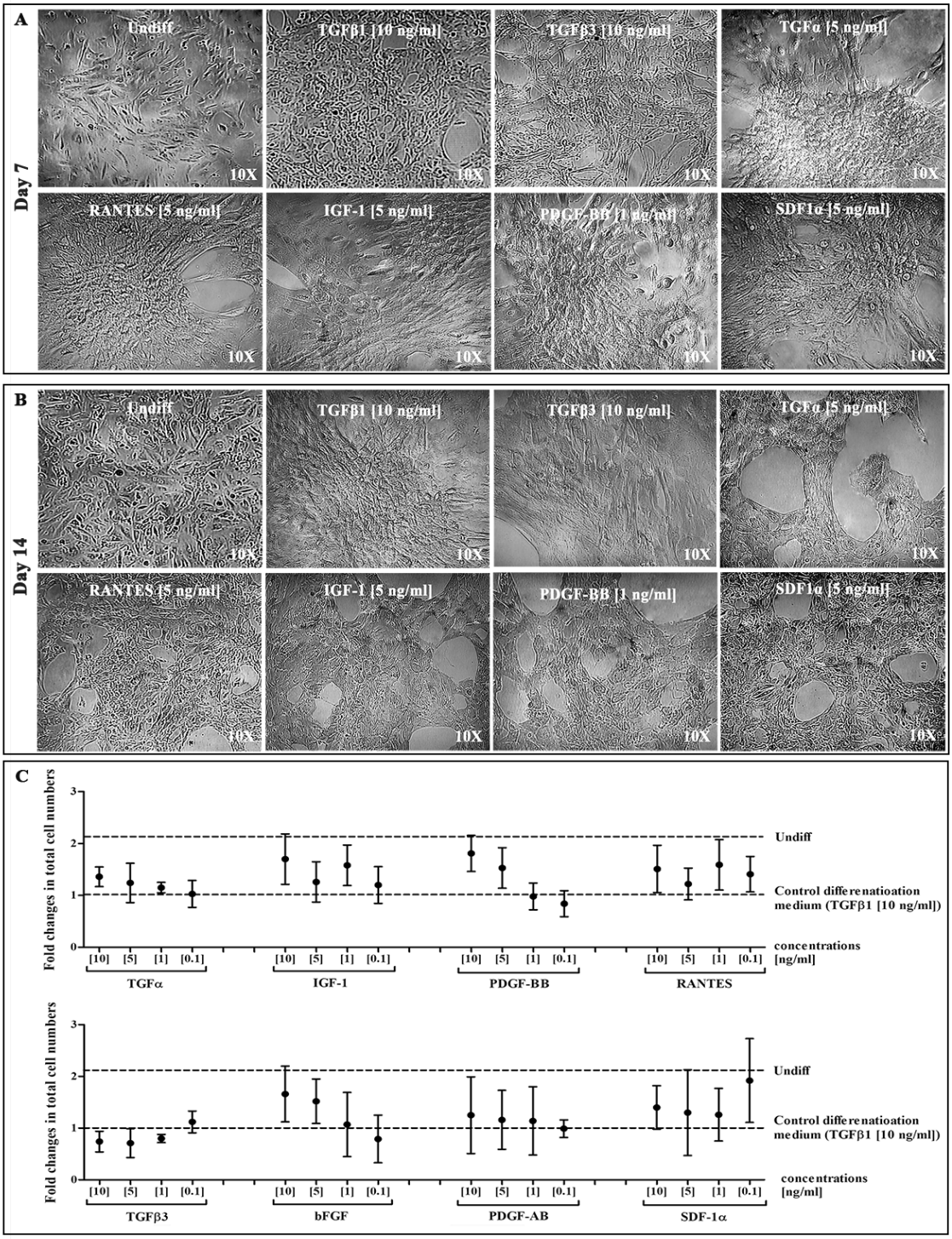

Figure 1: Morphologic appearances and total cell numbers of AF-MSC in GFs/cytokines supplemented culture

Morphologic appearances of AF-MSC, aggregates, multilayer structure as observed at day7 (1A) and day14 (1B) of culture under GFs/ cytokines medium at appropriate concentrations, control medium and AF-MSC medium (undiff) was presented using inverted microscope**.** Amount of cells under GFs/cytokines medium was presented in fold changes (means ± SD) and compared to control medium and undifferentiated cells in AF-MSC medium .The observation was done at day 14 of culture (1C).

3.3 GFs/cytokines induces chondrogenic differentiation in AF-MSC

To explore the role of GFs/cytokines on the chondrogenic differentiation via molecular function, AF-MSC was induced for chondrogenic differentiation with eight GFs/cytokines at different concentrations and investigated for the expression of genes relating chondrogenesis. The expression level was compared with AF-MSC under GFs/cytokines medium, control medium and AF-MSC medium (undifferentiated cell). The comparison study was done by individual cell line. Increasing of *Col-II* and *SOX9* expressions in AF-MSC under GFs/cytokines medium as compared to undifferentiated cell indicates chondrogenic differentiation. Our data showed that three AF-MSC lines presented a consistent pattern of anabolic gene expression. *Col-II* expression of cells under AF-MSC medium (undiff) and control medium showed a comparable level, indicating limitation of chondrogenic differentiation in AF-MSC using standard chondrogenic differentiation medium supplemented with 10 ng/mL TGF-β1. High expression of *Col-II* gene was found in AF-MSC under medium supplemented with TGF-α, IGF-1, PDGF-AB and PDGF-BB at a high concentration. AF-MSC was cultured under medium supplemented with 5-10 ng/mL TGF-α showed a greater than three to five times *Col-II* expression than the culture under control medium. AF-MSC under medium supplemented with 5-10 ng/mL IGF-1 expressed a greater than four to seven times *Col-II* expression than control. For *ACAN* gene,

high expression level was observed in AF-MSC cultured under control medium and medium supplemented with a high concentration of TGF-α, SDF-1α and a low concentration of RANTES and bFGF. For *SOX9* expression, we found TGF-β3 did not stimulate AF-MSC to express *SOX9*, whereas, TGF-α, bFGF and IGF-1 provoked a remarkable increase of *SOX9* mRNA in AF-MSC. The result showed that SDF-1α, TGF-α, bFGF and IGF-1 at a concentration of 5 ng/mL can stimulate a greater than two to four times *SOX9* expression than AF-MSC in control medium. For *Col-I* expression, high expression of *Col-I* gene was found in AF-MSC cultured under TGF-β3 medium. Low *Col-I* expression was observed in AF-MSC under TGF-α and PDGF-BB medium. For RunX2 gene expression, we found that TGF-β, bFGF and PDGF retained low expression of *RunX2*, whereas, TGF-α, IGF-1 and SDF-1α provoked *RunX2* expression in AF-MSC.

Expression levels of *ACAN, Col-I, Col-II, SOX9* and *RunX2* genes in three AF-MSC cell lines were calculated as means ± SD and presented in Fig.2. The average of gene expression level by means ± SD was used for statistical analysis. No statistical significant difference of gene expression analysis was found among different GFs/cytokines and various concentrations.

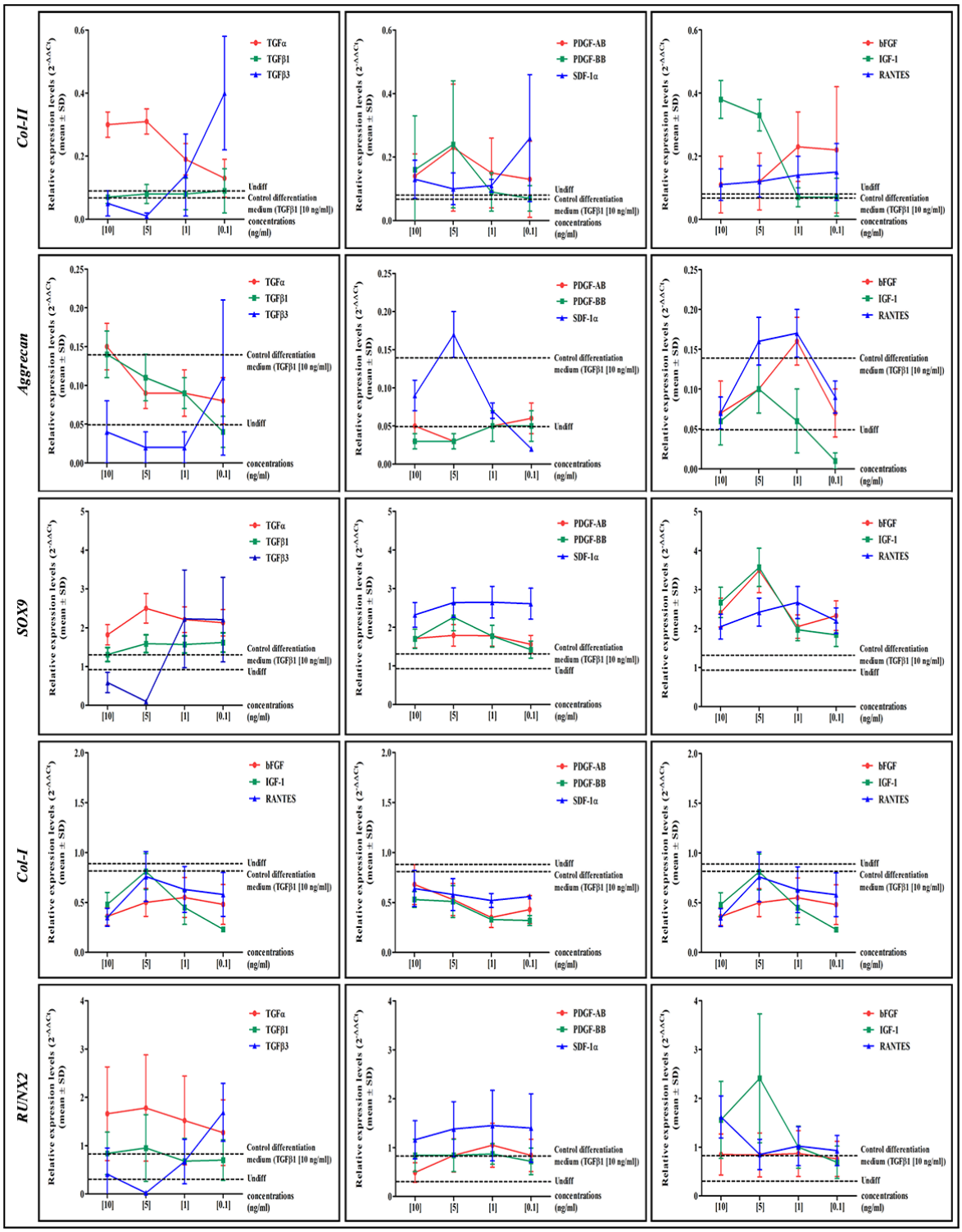

Figure 2: Effects of GFs/cytokines to chondrogenic differentiation in AF-MSC

Relative expression levels $)2^{-\Delta\Delta Ct}$ (of *ACAN, Col-I and -II, RunX2* and *SOX9* genes in AF-MSC cultured under GFs/cytokines medium for 14 days were presented in means ± SD .Greater expressions of *Col-II* and *SOX9* genes occurred by RANTES and TGF-α stimulation, indicating AF-MSC differentiation . Statistical significant difference was not found in AF-MSC under either different GFs/ cytokines medium or different concentrations .

3.4 GFs/cytokines combination induce in vitro chondrogenic differentiation in AF-MSC

The GF/cytokine cocktails were created by a combination of many GFs/cytokines at appropriate concentrations. GFs/cytokines stimulating *Col-II* and *SOX9* gene expression and declining *Col-I* expression were used for cocktail preparation, including TGF-α at concentrations of 5-10ng/mL, PDGF-BB at a low concentration, RANTES at a concentration of 5ng/mL and IGF-1 at a concentration of 5ng/mL. The GFs/cytokines were combined and generated three types of GFs/cytokines cocktails. The cocktails were used for chondrogenic differentiation of AF-MSC. The GFs/cytokines composition of each cocktail has been demonstrated in Table 2.

We found that cell amounts of AF-MSC at day 14 under cocktails one and two were higher than in control medium, indicating a low toxic effect of cocktails one and two on AF-MSC as compared to control (Fig. 3B). AF-MSC morphology under cocktail one and two were found in an epithelial cell type, whereas, AF-MSC incubated under cocktail three and control medium showed as a fibroblastic cell type (Fig. 3A). The cocktail one medium promoted expression of *ACAN*, but not *SOX9* gene expression. Cocktail two medium induced comparable expression of *ACAN*, *Col-II*, *SOX9* and *RunX2* to control medium, but diminishing expression of *Col-I* gene. Cocktail three medium encountered expression of *ACAN*, but declining expression of *Col-II* and *SOX9* genes as compared to control medium (10 ng/mL TGF- β 1 supplementation) (Fig. 3C).

Table 2: GFs/cytokines combination in chondrogenic induction cocktails

Cocktails	Growth factors / cytokines
control	[10 ng/mL] TGF-β1
cocktail 1	[10 ng/mL] TGF-α, [1 ng/mL] PDGF-BB, [5 ng/mL] RANTES, [5 ng/mL] IGF-1
cocktail 2	[5 ng/mL] TGF-α, [1 ng/mL] PDGF-BB
cocktail 3	[5 ng/mL] TGF-α, [1 ng/mL] PDGF-BB, [5 ng/mL] RANTES, [10 ng/mL] TGF-β3

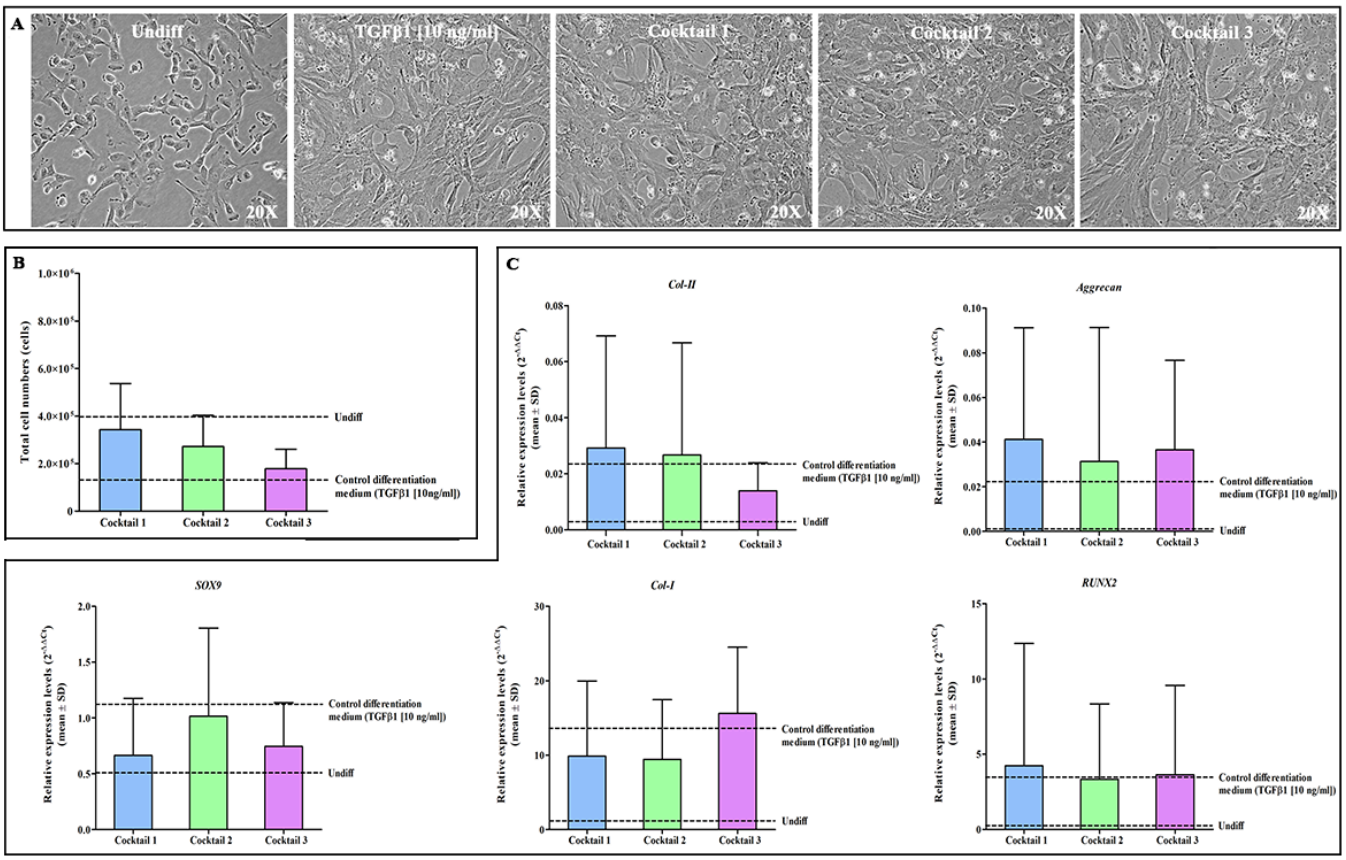

Figure 3: Differentiation effects of GFs/cytokines combinations in AF-MSC

At day14 of culture, AF-MSC cultured under control medium, AF-MSC medium)undiff (and GF/cytokine cocktails were observed and analyzed for morphologic appearance**) 3A(**, cell amount as presented in means ± SD**) 3B(** and chondrogenic gene expression using quantitative real time PCR as presented in relative expression levels)$2^{-\Delta\Delta Ct}$) (**3C.(**

4. DISCUSSION

For MSC transplantation in cell therapy, it is better to precondition MSC to activate target gene set and specific molecular functions prior to cell implantation. The condition medium to induce MSC to chondrogenic lineage is majorly affected by growth factors. TGF-β1 has been normally used as growth factor for traditional chondrogenic differentiation medium for MSC. However, a limitation of TGF-β1 medium was found in chondrogenic differentiation of AF-MSC. To provide chondrogenic precondition medium for AF-MSC, our study investigates chondrogenic differentiation effects of eight GFs/cytokines to AF-MSC in monolayer culture via expression of chondrogenic

gene set. We also determined toxic effect of GFs/Cytokines on micro-environment of AF-MSC via cell morphology, peeling off of necrotic cell during culture and cell amount in long period of culture (14 days). Our work presents that chondrogenic differentiation of AF-MSC is more effective to develop via independent-TGF-β1 signal. We are the first report that TGF-α, which has been reported to induce catabolic activity in articular cartilage, becomes a strategic growth factor to activate genes involving anabolic function and chondrogenic differentiation in AF-MSC. A preconditioning of AF-MSC with TGF-α stimulated medium can provoke anabolic gene activation with a less micro-environmental toxicity on AF-MSC. We also found that GFs/cytokines, including IGF-1, RANTES and SDF-1α have an effect in upregulating expression of anabolic genes of chondrocyte; *ACAN, Col-II* and *SOX9*. Our results suggest that TGF-α is an effective preconditioned growth factor for AF-MSC to chondrogenic lineage.

Transforming growth factor-alpha (TGF-α) is a member of the epidermal growth factor (EGF) family. TGF-α is not structurally and genetically related to TGF-β. Two ligands of TGF-α act through EGF receptors and activate a tyrosine kinase signaling pathway to function in cell proliferation, differentiation and development. Previous studies revealed that TGF-α has an effect of stimulating proliferation of chondrocyte and formation of articular chondrocyte cluster, but inhibits chondrogenesis and down-regulates expression of *ACAN, Col-II, SOX9* while increases *MMP13* in articular cartilage [17]. On the contrary, we found that TGF-α presented a strong chondrogenic differentiation effect by up-regulating anabolic genes, including *ACAN, Col-II* and *SOX9* in AF-MSC. The discrepancy of studies about effect of TGF-α on anabolic chondrogenic gene expression, as reported in AF-MSC and articular chondrocyte, is still informative. However, it has evidences that TGF-α has a role in various functions through different signal pathways in different cell types. Chen *et al*., 2012 [18] reported that TGF-α is important to maintain pluripotency and self-renewal in human embryonic stem cell (ESC) via p44/42 MAPK pathway, but not the PI3K/Akt. They found that knockdown or neutralization of TGF-α protein in human ESC culture led to increased expression of early differentiation markers and lower attachment rate of human ESC to feeder cells. They also presented that TGF-α has no proliferative effect in human ESC. Whereas, Heo *et al*., 2008 [19] revealed that TGF-α has a role in increasing the proliferation effect and promotes DNA synthesis in mouse ESC through the p44/42 MAPK, PI3K/Akt and Notch/Wnt signaling pathway, but no effect in maintaining pluripotency of mouse ESC. In bone marrow MSC, TGF-α was found to be an effective inducer for VEGF production via MAP kinase (MEK) and PI3K [20]. For chondrocyte, Appleton *et al*., [17] described that TGF-α can alter morphology of chondrocyte through reorganization of actin cytoskeleton and provoke stress fibers. For AF-MSC, we found that TGF-α has an effect on cell proliferation and increases the expression of *ACAN, Col-II* and *SOX9* in chondrogenic differentiation of AF-MSC. With the existence of AF-MSC, it might indicate a spatiotemporal fate happening in an intermediate stage of biological development causing the source of AF-MSC, which is obtained from fetal route in gestation period and considered as a cell in an intermediate stage. In the developmental period, it has been found that a number of spatiotemporal signal pathways depend on microenvironment and a specific niche [21] and alteration of epigenomic activities, such as gene imprinting and modification of histones [22]. However, a signal transduction pathway respective to TGF-α in AF-MSC is of interest.

Our data showed expression together with anabolic *Col-II* and *SOX9* genes and a hypertrophic *RunX2* gene in AF-MSC cultured under TGF-α, SDF-1α and IGF-1 medium. *RunX2* gene expression and protein is temporal and is spatial expressed through multiple signaling pathways. The study of Jonason *et al*., 2009 [23] exhibited that *RunX2* not only acts in hypertrophic chondrocytes, It promotes chondrocyte maturation. *RunX2* accelerates *Col-IIa-1*-expressing in differentiating chondrocytes and leads to maturation of chondrocytes that normally do not differentiate into hypertrophic chondrocytes. Rich *et al*., 2008 [24] studied a gene expression profile in an in vitro chondrogenic differentiation of human adipose-derived stromal cells using TGF-β3 induction. They found increasing of *Sox9* transcription factors, *Col-IIa-1, Col-Xa-1, RunX2* and *Osterix* mRNA expression in chondrogenic differentiation of human adipose MSC. They presented a significant up-regulation in expression of osteogenesis-related transcription factors *RunX2* in an in vitro chondrogenesis of human adipose-derived stromal cells.

TGF-β1 and TGF-β3 are members of the TGF-β superfamily. TGF-β1 can induce expression of *ACAN, Col-II* and *SOX9* genes in MSC derived from various sources as well as AF-MSC, but TGF-β3 is not an effective inducer of chondrogenesis in AF-MSC. Our result is consistent to the finding of Kolambkar *et al*., 2007 [12], who studied differentiation potential of AF-MSC in forms of pellet and alginate hydrogel. They cultured AF-MSC for three weeks in TGF-β3 medium and found that induced AF-MSC has lower *ACAN* and *Col-II* expression than those in TGF-β1 medium. Although, TGF-β3 did not afford chondrogenic differentiation of AF-MSC, evidence confirmed that TGF-β3 enhanced MSC chondrogenesis in some MSC sources, such as MSC from umbilical cord tissue [25] and adipose tissue [11].

A study on the effect of RANTES to chondrocyte has been reported by Alaaeddine *et al*., in 2001 [26]. They found that normal cartilage could release RANTES at a low level. Elevation of RANTES releasing can be observed in OA cartilage in respond to IL-1β or IL18. They found that RANTES recruits inflammatory cells and contributes joint inflammation via inducing the expression of IL-6 and iNOS. They also presented that RANTES has a negative effect to GAGs formation in cartilage. Inconsistent with that report, our results found that RANTES is another cytokine that yields a great chondrogenic differentiation effect and also increases expression of glycosaminoglycan in AF-MSC. It might be an effect of an MSC source in an intermediate route. Study on the effect of IGF-1 and SDF-1α, in a previous study by Zhou *et al*., in 2016 [14] stated the achievement of IGF-1 to chondrogenic differentiation of MSC. They reported that MSC derived from adipose tissue could be induced to chondrogenic lineage via signal of IGF-1[14]. Our work consistently showed the potential of IGF-1 and SDF-1α to elevate the expression of *Col-II* and *SOX9* genes in AF-MSC.

In this study, we also induced chondrogenic differentiation in AF-MSC with GFs/cytokines combination, which was provided as cocktail medium. The cocktail was enriched by GFs/cytokines, which were effective to chondrogenic differentiation in AF-MSC at appropriate doses in our study. We found that using a single GF has a greater stimulating effect to anabolic genes expression than the GFs/Cytokines combination. Although, PDGF-BB has not promoted genes involving anabolic function in chondrogenic differentiation, it

was added as a composition in GFs/cytokines cocktails by reason of its action to inhibit apoptosis in chondrocyte as revealed by Montaseri *et al*., in 2011[27].

In this present work, we found a tiny expression of *ACAN* in every growth factor supplementation and even in standard chondrogenic differentiation medium. However, a positive signal of GAG formation was found by alcian blue staining in every culture. It can be a result of low density of cells clusters and aggregations on monolayer culture [28]. The study by Zuliani *et al*., 2018 [10] presented that AF-MSC differentiation with micromass technique in three-dimensional culture has greater effectiveness than AF-MSC differentiation under monolayer culture. They found that micromass culture of AF-MSC at day21 showed a significantly higher expression of *ACAN*, *Col-I* and *SOX9* genes than the cells in monolayer culture.

However, one of the aims of our study was to observe a toxic effect and a micro-environmental stress of GF/cytokine on AF-MSC through determination of cell morphology and cell behavior during culture periods. We conducted our work using monolayer culture. Moreover, an objective of study directs to preconditioning the AF-MSC in order to activate chondrogenic genes in cell preparation for clinical transplantation.

In our experiment, we found that another concern is unique characters of AF-MSC in each cell lines. In the present study, an AF-MSC line was derived from a clonal cell establishment using a "starter cell" isolation method [29]. With this method, each AF-MSC clonal line is established from a single MSC cell in amniotic fluid. Cells in amniotic fluid are possible to obtain from various fetal organs, such as fetal lung, kidney and amniotic membranes. Cells in amniotic fluid, which originate from a different route, are governed by a difference in epigenetic programming [22]. Thus, since AF-MSC can have their own unique characteristics among different clonal cell lines, we found that one of four studied cell line has no response to chondrogenic induction with any GFs/cytokines and even with TGF-β1-standard differentiation medium, but prefer to achieve induction into neurogenic lineage (unpublished data). Three of four cell lines can be induced into chondrogenic lineage, but showed a wide range of standard deviation (SD) of gene expression levels. This finding is evidence to support that AF-MSC lines from clonal derivation are able to be characterized and designed for organs or disease-specific treatment strategies. For this study, we examined gene expression levels of AF-MSC in different medium and data interpretation of gene expression was determined within each cell line, which was used in cultures under GF/cytokine and medium.

CONCLUSIONS

This present study confirmed that AF-MSC is an effective mesenchymal stem cell source considered for cell-based therapy in cartilage regeneration. We suggest that TGF-α is an effective GF for preconditioning AF-MSC to activate chondrogenic gene function. Preconditioning AF-MSC with TGF-α results in motivating *ACAN*, *Col-II*, *SOX9* and *RunX2* genes and retaining a low *Col-I* expression in AF-MSC.

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CORRESPONDENCE

Tatsanee Phermthai, PhD, Obstetrics and Gynecology Department, Faculty of Medicine, Siriraj Hospital, Mahidol University, 2 Prannok Road, Siriraj, Bangkoknoi, Bangkok, 10700, Thailand. E-mail: bsuteevun_1@yahoo.com

ETHICAL STATEMENT

The study protocol was approved by the Siriraj Institutional Review Board, Mahidol University (IRB No. Si 441/2011). Informed consent was confirmed by the IRB.

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