The Effects of TNF-α on Osteogenic Differentiation of Umbilical Cord Derived Mesenchymal Stem Cells

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Mesenchymal stem cells (MSCs) are multipotent stem cells which are able to differentiate into various lineages including osteoblasts, adipocytes and chondrocytes. They can be isolated from several tissues including bone marrow, adipose tissue, placenta and umbilical cord. Although MSCs could be differentiated into osteoblasts under appropriate culture condition, their osteogenic differentiation capacity is still not very efficient. Previous studies reported that TNF-α could promote osteogenic differentiation of bone marrow derived MSCs by triggering NF-κB signaling pathway. However, the effect of TNF-α on the osteogenic differentiation ability of umbilical cord derived MSCs has not been investigated. This study aimed to examine the effect of TNF-α on osteogenic differentiation of umbilical cord derived MSCs (UC-MSCs). The results demonstrated that TNF-α has osteopromotive effect for umbilical cord derived MSCs as evidenced by more matrix mineralization and alkaline phosphatase staining. Interestingly, UC-MSCs cultured in osteogenic differentiation medium supplemented with TNF-α had significantly increase expression of Osteocalcin, the marker of mature osteoblasts,when it was compared to UC-MSCs cultured in osteogenic differentiation medium without TNF-α ($p<0.05$). On the contrary, the UC-MSCs cultured in osteogenic differentiation medium supplemented with TNF-α had significantly lower levels of Runx2 and Osterix (the markers of immature osteoblasts) than UC-MSCs cultured with osteogenic differentiation medium without TNF-α. The present study suggested that TNF-α promotes osteogenic differentiation of UC-MSCs. The data add a possibility for the use of UC-MSCs as an alternative source for cell replacement therapy in bone defect.

Keywords: *Mesenchymal stem cell, Umbilical cord, TNF-α, Osteogenesis*

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Mesenchymal stem cells (MSCs) are multipotent stem cells which can be isolated from several tissues such as bone marrow, adipose tissue, placenta and umbilical cord. MSCs gained importance in regenerative medicine, due to their ability to differentiate not only into mesodermal lineage but also endodermal[1] and ectodermal lineages[2,3]. The capacity to differentiate into osteoblast endows a great promise to the use of MSCs as a cell replacement therapy for treating bone defects[4]. However, the low efficiency of osteogenic differentiation of MSCs has limited their use in clinical practice. Enhancement of osteogenic differentiation of MSCs is therefore essential.

Tumor necrosis factor-alpha (TNF-α), pro-inflammatory cytokines, has a key role in skeletal disease. It promotes bone formation by mature osteoblasts and increased osteoclastic resorption[5]. Previous studies reported the use of TNF-α as an osteopromotive mediator for bone regeneration[5,6]. Recent insight into the critical role TNF-α in bone regeneration has suggested a new direction in the design of tissue engineering constructs. Although the osteogenic differentiation of mesenchymal stem cells has been extensively studied, most of the studies was conducted using bone marrow derived MSCs[7,8]. However, the harvest of bone marrow is a highly invasive procedure. In addition, the number, differentiation potential, and maximal life span of MSCs derived from bone marrow decline with increasing age of donor[9]. Recently, umbilical cord has been suggested

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as an alternative source of MSCs for both research and clinical application[10-12]. The present study focused on characterizing the effects of TNF-α on osteogenic differentiation of MSCs derived from umbilical cord.

Material and Method

Cell isolation and culture

The present study was approved by the Human Ethics Committee of Thammasat University No. 1 (Faculty of Medicine; MTU-EC-DS-1-061-57). All subjects participated in the study after giving written informed consents at Thammasat University Hospital. The umbilical cords were minced into small pieces and digested with 1.6 mg/ml collagenase XI (Sigma-Aldrich, USA) and 200 mg/ml deoxyribonuclease I (Sigma-Aldrich, USA) for 4 h at 37°C. Subsequently, the cells were washed twice with washing buffer and cultured in completed medium containing Dulbecco's Modified Eagle's Medium (DMEM; GibcoBRL, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA), 2 mM L-glutamine (GibcoBRL, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. The medium was changed every 3-4 days. The plastic-adherent fibroblast-like cells or so-called UC-MSCs (about 80-90% confluence) were sub-cultured using 0.25% trypsin-EDTA (GibcoBRL, USA) and replated at density of $1x10^4$ cell/cm^2 for further expansion.

Characterization of culture cells

UC-MSCs ($5x10^5$ cells) were re-suspended in 50 µl of phosphate buffer saline (PBS) and incubated with 2.5 µl of antibodies against human antigens, including fluorescein isothiocyanate (FITC) conjugated CD45 antibody (dilution ratio 1:20, AbDSeroTec), FITC-CD90 antibody (dilution ratio 1:20, AbDSeroTec), FITC-CD 105 antibody (dilution ratio 1:20, AbDSeroTec), Phycoerythrin (PE) conjugated CD34 antibody (dilution ratio 1:20, AbDSeroTec) and PE-CD73 antibody (dilution ratio 1:20. AbDSeroTec) for 30 min at 4°C in the dark. After washing with PBS, the cells were fixed with 1% paraformaldehyde in PBS. At least 10,000 labeled cells were acquired and analyzed using flow cytometry (FACScalibur™, Becton Dickinson, USA) and CellQuest® software (Becton Dickinson, USA).

Osteogenic differentiation assay

UC-MSCs were plated into 35-mm tissue culture dishes and cultured in completed medium at 37°C for 24h. Subsequently, the cells were cultured in osteogenic differentiation medium containing DMEM supplemented with 10% FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 0.1 µM dexamethasone (Sigma, USA) and 50 µg/ml ascorbic acid (Sigma, USA) in the absence/presence of the 10 ng/ml tumor necrosis factor-alpha (TNF-α; Peprotech, USA). On day 7 of culture, 10 mM β-glycerophosphate (Sigma, USA) was added into each cultured dishes. Cells were harvested on 14 of cultures for measuring alkaline phosphatase (ALP) activity using 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBT/BCIP; Sigma, USA) as a substrate. Cells cultured in completed medium served as a control and were treated in pararell to the experiment.

Quantitative real time PCR for measuring mRNA expression

After treating with TNF-α, the cells were harvested on day of cultures for RNA extraction using PureLink™ RNA MiniKit (Invitrogen, USA). For cDNA synthesis, 1 µg of total RNA for each sample were reverse transcribed to cDNA using Superscript III Reverse Transcriptase (Invitrogen, USA). The synthesized cDNAs were subjected to quantitative real time PCR (qRT-PCR) analysis using the StepOnePlus™ Real-Time PCR System (Applied Biosystems; USA). The PCR condition was performed using 40 cycles of amplification (denaturation at 95°C for 10s, annealing at 60°C for 10s and extension at 72°C for 20s) after an initial activation at 95°C for 10 min. Melting curves were assessed to ensure single products were quantified. The quantitation was based on normalizing the gene of interest to the invariant control gene (GAPDH). The data were analyzed by comparative $^{\Delta\Delta}$CT method using StepOne™ Software version 2.2 (Applied Biosystems; USA) and presented as the relative mRNA level. Primers were specified in Table 1.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). For statistical analysis, a Mann-Whitney test was used. A difference between experimental groups was considered to be significant when $p<0.05$.

Results

The characteristic of UC-MSCs derived from umbilical cord

The UC-MSCs were cultured in DMEM supplemented with 10% FBS. After plating, the cells exhibited as spherical shape cells and only a few cells attached to the surface of the culture flasks (Fig. 1A).

The media was changed every 3-4 days and the non-adherent cells were removed. The adherent cells were appeared as spindle-shaped cells (Fig. 1B). Further passages of these MSCs were done whenever the cell density reached 90% confluence (Fig. 1C). The cells could be propagated up to 20 passages before stopped growing and reached senescence.

Immunophenotype of UC-MSCs

After culturing for 3 passages, the UC-MSCs were harvested and examined the expression of cell surface markers using flow cytometry. The results indicated that UC-MSCs expressed typical surface markers of MSCs, including CD73, CD90, and CD105, and did not expressed hematopoietic cell markers, including CD34 and CD45 (Fig. 2).

The effect of TNF-α on matrix mineralization and alkaline phosphatase expression

To determine the effect of the TNF-α on the osteogenic differentiation of UC-MSCs, the cells were cultured in osteogenic differentiation medium in the presence or absence of TNF-α for 14 days (Fig. 3). After induction for 7 days, the deposition of matrix mineralization was observed in UC-MSCs cultured with osteogenic differentiation medium in the absence of TNF-α (Fig. 3E). Interestingly, treatment with TNF-α during osteogenic differentiation increased the matrix mineralization (Fig. 3H). In addition, UC-MSCs treated with TNF-α for 14 days exhibited higher matrix mineralization (Fig. 3I) as compared to UC-MSCs treated with TNF-α for 7 days. The result of alkaline phosphatase staining also indicated the higher

Table 1. The primers and the product size

Gene	Sense primer (5'-3')	Anti-sense primers (3'-5')	Product size (bp)
Runx 2	ACAACCACAGAACCACAAG	TCTCGGTGGCTGGTAGTGA	105
Osx	TGAGGAAGAAGCCCATTCAC	ACTTCTTCTCCCGGGTGTG	198
OCN	TGCAAAGCCCAGCGACTCT	AGTCCATTGTTGAGGTAGCG	129
GAPDH	GTCAACGGATTTGGTCGTATTG	CATGGGTGGAATCATATTGGAA	193

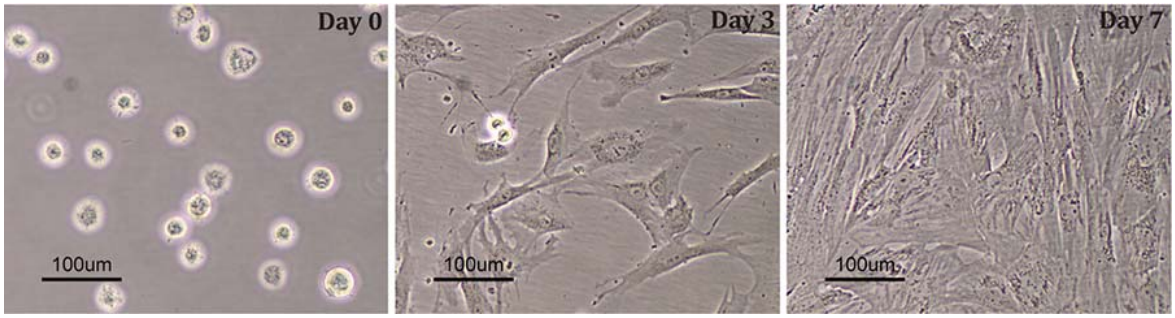

Fig. 1 Morphology of mesenchymal stem cells isolated from umbilical cord. Scale bar = 100 μm.

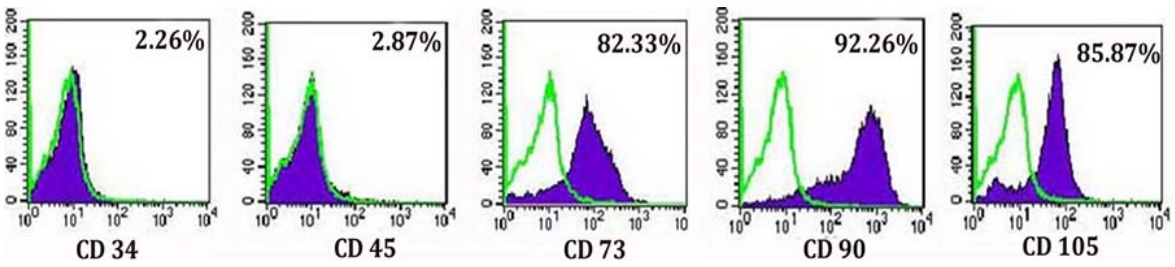

Fig. 2 Immunophenotype of mesenchymal stem cells isolated from umbilical cord.

expression of alkaline phosphatase in UC-MSCs treated with TNF-α for 14 days as compared to the untreated and control groups respectively (Fig. 4).

The effect of TNF-α on the expression of osteogenic-lineage genes

Quantitative real-time PCR was used to measure the effect of TNF-α on the expression of osteogenic lineage genes, *Runt-related transcription factor 2 (Runx2)*, *Osterix (Osx)* and *Osteocalcin (OCN)* (Fig. 5). The results demonstrated that treatment of UC-MSCs with TNF-α over the whole period of 14 days of osteogenic differentiation significantly increased *OCN* mRNA levels as compared to UC-MSCs cultured in osteogenic differentiation medium without TNF-α ($p<0.05$). In contrast, UC-MSCs cultured in osteogenic differentiation medium supplemented with TNF-α had a significantly lower expression level of *Runx2* and

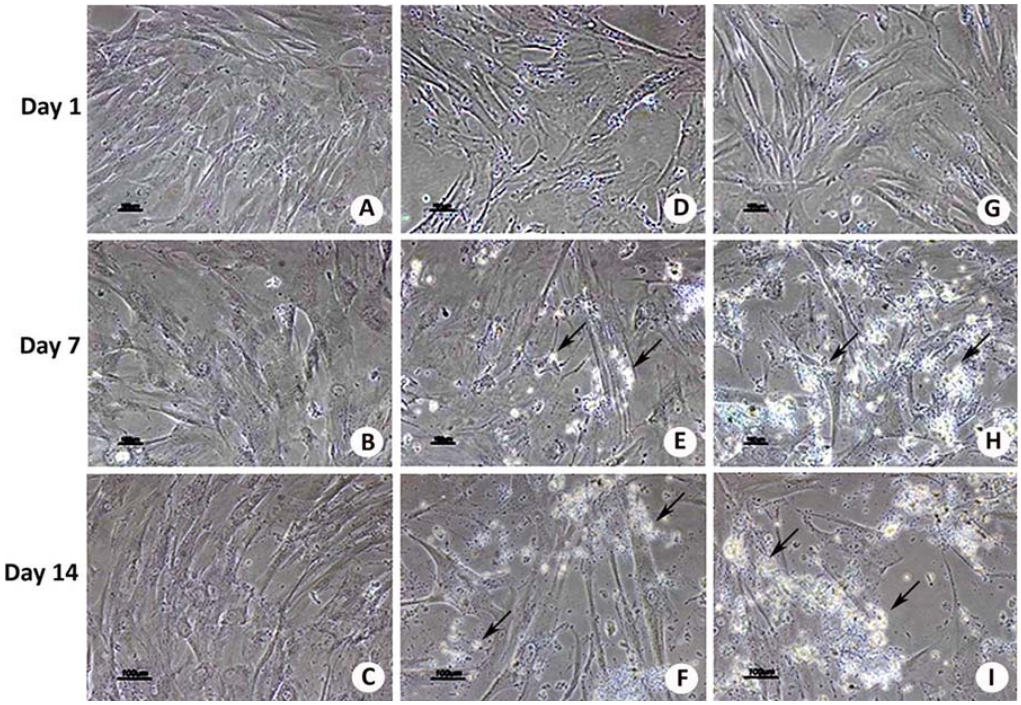

Fig. 3 The characteristic of UC-MSCs cultured in osteogenic differentiation medium with (G, H, I) or without (D, E, F) TNF-α at day 1, 7 and 14. UC-MSCs cultured in completed medium served as a control (A, B, C). Arrow indicated the calcium deposition during osteogenic differentiation. Scale bar = 100 μm.

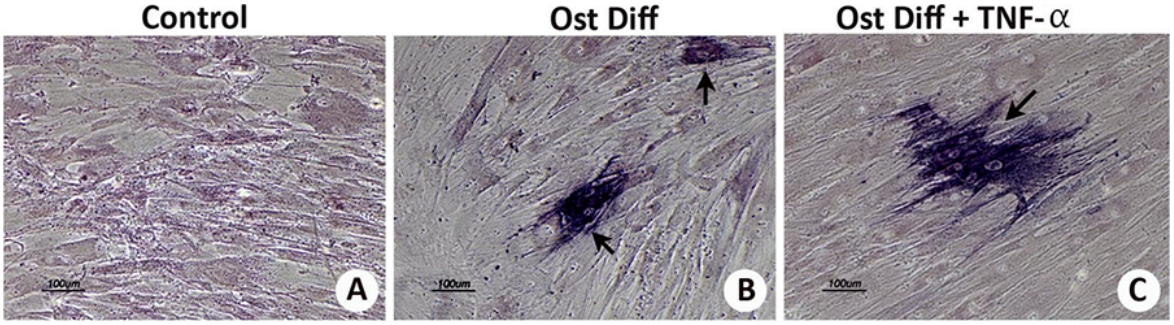

Fig. 4 Alkaline phosphatase staining of UC-MSCs after cultured in osteogenic differentiation medium with or without TNF-α for 14 days. UC-MSCs cultured in completed medium served as a control. Arrow indicated the osteoblast those were positive for alkaline phosphatase staining. Scale bar = 100 μm.

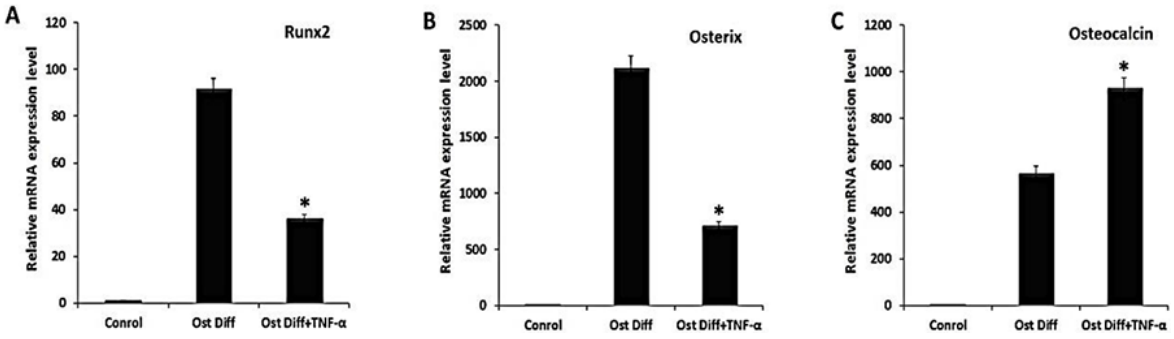

Fig. 5 Quantitative real time PCR showed the expression of *Runx-2*, *Osterix* and *Osteocalcin* of UC-MSCs after cultured in osteogenic differentiation medium with or without TNF-α for 14 days. UC-MSCs cultured in completed medium served as a control.

Osx mRNA than UC-MSCs cultured in osteogenic differentiation medium without TNF-α ($p<0.05$).

Discussion

Mesenchymal stem cells (MSCs) are mostly derived from bone marrow stroma and can differentiate into various lineages such as osteoblasts, adipocyte, and chondrocyte[13,14].

The multi-lineage differentiation potential of MSCs has been extensively studied and culturing conditions for in vitro differentiation has been established. Although much progress has been made regarding the osteogenic differentiation process in the last decade, the restricted quantity and quality of functional osteocytes are the main obstacles for the therapeutic application. Exploratory for the new mediators that are able to enhance the osteogenic differentiation capacity of MSCs is thus necessary.

This is the first study demonstrated that TNF-α treatment could enhance the osteogenic differentiation of UC-MSCs as demonstrated by the increased deposition of matrix mineralization to the extracellular matrix, the enhanced expression of alkaline phosphatase activity and the expression of specific mRNA involved in osteogenic differentiation.

After culturation, the cells isolated from umbilical cord were examined for the characteristics of MSCs. The results indicated that cells isolated from umbilical cord displayed the characteristic of MSCs according to the criteria of International Society for Cellular Therapy[15]. They adhered to cultured flask with spindle shape morphology and expressed specific cell surface makers for MSCs including the positivity for CD73, CD90, CD105 and negativity for CD34 and CD45.

Treatment with TNF-α in creased matrix mineralization as evidenced on day 7 and day 14 after osteogenic induction. This result is similar to the previous study which indicated that continuous delivery of TNF-α to bone marrow derived MSCs cultured on three-dimensional biodegradable electrospun microfiber meshes stimulates the deposition of mineralized matrix[16]. TNF-α could induce MSCs derived from bone marrow to undergo osteogenic differentiation by triggering NF-κB signaling pathway[6]. Thereafter, NF-κB stimulated the regulators of osteogenesis and resulted in enhanced expression of osteogenetic proteins, including bone morphogenetic protein2 (BMP-2) and alkaline phosphatase, and also enhanced mineralization of the extracellular matrix[6].

The quantification of mRNA involved with osteogenesis revealed that TNF-α declined *Runx2* and *Osx* expressions whereas stimulated *OCN* expression. The results was supported by the previous study which demonstrated that TNF-α decrease the expression of *Runx2* by interfering with the DNA binding of S mad proteins instead of inhibiting phosphorylation of Smad1/5/8 or nuclear translocation of the Smad1/Smad4 complex[17]. TNF-α decreases *Runx2* expression, suggested that *Runx2* was not involved in mineralization of MSCs[5]. *Runx2* can interact with other genes, such as *Osx* and upregulate the expression of *OCN*[19]. *Osx* is a specific osteogenic transcription factor which is required for the differentiation of pre-osteoblasts into fully functioning osteoblasts[20]. *Runx2* may commit cells to osteogenesis though mediating *Osx* gene[21]. *OCN* is the bone-specific gene exhibiting the osteoblastic phenotype. It is the late bone marker during the osteogenic differentiation and mineralization[22]. Previous study reported that osteoblasts did not exhibit major changes in *Runx2* expression during in vitro differentiation even though

expression of *OCN* and alkaline phosphatase is dramatically increased[23]. During development *Runx2* expression precedes osteoblast differentiation and *OCN* expression by several days[18]. In the present study, the expression levels of *Runx2* and *Osx* were decreased, whereas the expression of down stream genes such as *OCN* and alkaline phosphatase were significantly increased, it might be possible that TNF-α promotes the osteogenic differentiation of UC-MSCs. *Runx2* and *Osx* may be expressed in early stage but declined in late stage, therefore detection of mRNA expression at various time points might get more information on the osteopromotive effect of TNF-α on osteogenic differentiation of UC-MSCs.

In summary, the present study demonstrated that TNF-α could enhance the osteogenic differentiation capacity of UC-MSCs and could potentially be used as a therapeutic agent for treating patients with bone defect in the future.

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Potential conflicts of interest

None.

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ผลของ TNF-α ต่อการเจริญพัฒนาเป็นเซลล์กระดูกของเซลล์ต้นกำเนิดมีเซนไคม์จากสายสะดือ

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เซลล์ต้นกำเนิดมีเซนไคม์เป็นเซลล์ที่มีศักยภาพในการเจริญพัฒนาเป็นเซลล์ได้หลายชนิด เช่น เซลล์กระดูก เซลล์ไขมัน เซลล์กระดูกอ่อน เป็นต้น เซลล์ต้นกำเนิดเหล่านี้สามารถคัดแยกได้จากเนื้อเยื่อต่างๆ เช่น ไขกระดูก ไขมัน รก และสายสะดือ ถึงแม้เซลล์ต้นกำเนิดมีเซนไคม์จะสามารถเจริญพัฒนาเป็นเซลล์กระดูกได้แต่ประสิทธิภาพในการเจริญพัฒนายังไม่ดีนัก มีรายงานก่อนหน้านี้ว่า TNF-α สามารถกระตุ้นการเจริญพัฒนาของเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกให้เจริญเป็นเซลล์กระดูกได้โดยผ่านทาง NF-κB อย่างไรก็ตาม ยังไม่มีการศึกษาถึงการใช้ TNF-α ในการกระตุ้นเซลล์ต้นกำเนิดมีเซนไคม์จากสายสะดือให้เจริญเป็นเซลล์กระดูก การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของ TNF-α ต่อการเจริญพัฒนาเป็นเซลล์กระดูกของเซลล์ต้นกำเนิดมีเซนไคม์จากสายสะดือ ผลการศึกษาพบว่า TNF-α สามารถกระตุ้นการเจริญพัฒนาของเซลล์ต้นกำเนิดมีเซนไคม์จากสายสะดือให้เจริญเป็นเซลล์กระดูกได้ วัดได้จากการเพิ่มขึ้นของ matrix mineralization และการแสดงออกของ alkaline phosphatase นอกจากนี้ยังพบว่า เซลล์ต้นกำเนิดมีเซนไคม์จากสายสะดือที่เพาะเลี้ยงในน้ำยากระตุ้นการเจริญพัฒนาเป็นเซลล์กระดูกที่มีส่วนผสมของ TNF-α มีการเพิ่มขึ้นอย่างมีนัยสำคัญของการแสดงออกของจีน Osteocalcin ซึ่งเป็นจีนที่แสดงถึงการเจริญพัฒนาเป็นเซลล์กระดูกอย่างสมบูรณ์ ($p<0.05$) ในทางตรงกันข้ามการแสดงออกของจีน Runx2 และ Osterix ซึ่งเป็นจีนที่แสดงถึงการเจริญพัฒนาของเซลล์กระดูกในระยะเริ่มต้นนั้นลดลงอย่างมีนัยสำคัญ ($p<0.05$) ผลศึกษาในครั้งนี้แสดงให้เห็นว่า TNF-α ส่งเสริมการเจริญพัฒนาเป็นเซลล์กระดูกของเซลล์ต้นกำเนิด มีเซนไคม์จากสายสะดือ ข้อมูลที่ได้จากการศึกษาครั้งนี้จึงเป็นการเพิ่มทางเลือกในการรักษาโรคที่มีความผิดปกติของกระดูก โดยการใช้เซลล์ต้นกำเนิด มีเซนไคม์จากสายสะดือได้ในอนาคต