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第五生長分化因子對人類牙髓細胞的影響及其訊息傳導機制

Effects of Growth/Differentiation Factor-5 on human dental pulp cell and its signaling

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中文摘要

實驗目的: 第五生長分化因子 (Growth/Differentiation factor-5, GDF-5) 與許多組織的發育與修復有很大的相關性，如軟骨、韌帶、椎間盤、骨頭與皮膚。本篇研究注重於第五生長分化因子對於人類牙髓細胞在形態表現、細胞增生與生存能力、細胞分化及膠原蛋白生成的影響，並進一步探討第五生長因子於細胞增生與分化相關的訊息傳導路徑。

實驗方法: 使用不同濃度的第五生長分化因子刺激人類牙髓細胞，在某些實驗中則先加入特定訊息抑制劑做前處理以探索相關訊息傳導路徑。利用光學顯微鏡觀察細胞形態；以MTT測定牙髓細胞之存活能力；利用鹼性磷酸酶染色與定量分析來檢測細胞分化程度；以膠原蛋白定量分析來做膠原蛋白之定量；使用反轉錄鏈聚合反應印證細胞分裂相關基因的表現；另外利用免疫螢光抗體技術觀察單核及雙核細胞的比例。

實驗結果: 第五生長分化因子可增加人類牙髓細胞的存活能力；影響與細胞增生相關基因 (促進Cyclin B1的表現，抑制p21的表現)；提高雙核細胞的比例。SB431542 (ALK-4/5/7之特殊訊息抑制劑) 可些微逆轉第五生長分化因子所誘發的細胞增生現象，而Noggin (骨形成蛋白質之拮抗劑) 及U0126 (ERK之抑制劑) 則無法。在細胞分化方面，在第五生長分化因子作用下，鹼性磷酸酶的染色程度及定量表現會有顯著下降的趨勢；使用之抑制劑，包含Dorsomorphin (ALK-2/3/6之特殊訊息抑制劑)、SB431542、U0126和SB203580 (p38 kinase之抑制劑)，都無法改善第五生長分化因子所誘發的細胞分化抑制現象。在細胞間質生成方面，第五生長分化因子對於膠原蛋白的含量沒有顯著影響。

結論: 人類牙髓細胞受到第五生長分化因子的刺激下，會促進細胞增生、抑制細胞分化，但對膠原蛋白之形成無影響。在細胞增生方面的訊息傳導路徑可能與ALK-4/5/7部分相關，但在細胞分化方面則尚未找到相關之訊息傳導路徑。

關鍵字: ALK-4/5/7、細胞增生及分化、人類牙髓細胞、第五生長分化因子

Abstract

Aim: Growth/Differentiation factor-5 (GDF-5) is a multifunctional protein and close related to the development and repair of multiple tissues, including cartilage, tendon, intervertebral disk, bone and skin. The purpose of our study is to investigate whether GDF-5 influences the morphological changes, cell proliferation and viability, cell differentiation and collagen formation of human dental pulp cells in vitro within the period of 5 or 10 days. Furthermore, the related signal transduction pathways were also been evaluated.

Materials and Methods: Primary-cultured human dental pulp cells were treated with different concentration of GDF-5 (0-500 ng/ml). In some experiments, cells were pretreated with different specific signaling inhibitors 30 minutes before adding GDF-5 for investigating the signaling of GDF-5. Morphology of pulp cells was observed under light microscopy (100X). Cell proliferation was evaluated by MTT assay. Cell differentiation was evaluated by alkaline phosphatase (ALP) staining and ALP quantitative assay. Changes in mRNA expression of cell mitosis-related genes (Cyclin B1, CDC2, CDC25C and p21) were determined by reverse-transcriptase polymerase chain reaction (RT-PCR). Collagen content was determined by Sircol Collagen assay. Besides, immunofluorescence assay was used to observe the percentages of binuclear and mononuclear cells.

Results: Human dental pulp cells were spindle with extended cellular processes with/without GDF-5 treatment. Under the treatment by various concentrations of GDF-5: cell viability was up-regulated significantly in dose-dependent manner; Cylin B1 mRNA expression was stimulated, but the expression of p21 mRNA was inhibited; the percentage of binuclear cells was increased. In the inhibitory experiment of MTT assay, SB431542 (ALK-4/5/7 specific inhibitor) could slightly prevent the GDF-5-induced declination in cell proliferation. In cell differentiation: GDF-5 declined the ALP activity of human dental pulp cells; four inhibitors, including Dorsomorphin (ALK-2/3/6 specicic inhibitor)、SB431542、U0126 and SB203580 (p38 inhiibitor) could not reverse the effect of GDF-5 on ALP activity. GDF-5 did not affect the collagen content significantly.

Conclusion: GDF-5 demonstrated proliferative property in human dental pulp cells, but had inhibitory effect on cell differentiation and no effect on collagen formation. Signal transduction of GDF-5 in human dental pulp cells is a complex system. GDF-5 could induce cell proliferation through the activation of ALK-4/5/7 partially, but the signal pathway of GDF-5-induced cell differentiation is still unknown. These events are crucial in the mechanism of pulpal repair and regeneration.

Keywords: ALK-4/5/7, cell proliferation and differentiation, human dental pulp cells, GDF-5

Chapter I Literature Review

1.1 Reparative mechanism of pulpal-dentin complex

Dentin and pulp are derived from the dental papilla, whose cells migrate to the first branchial arch from within the ectomesenchyme of the cranial neural crest. The tissues remain closely associated during development and throughout the life of an adult tooth and are most commonly referred to as the pulpodentin complex. Pulpodentin complex is a specialized structure which contains mixture of cells and extracellular matrix (Goldberg and Smith 2004). [Table 1]

Dental pulp consists of nerves, vessels, connective tissue fibers, extracellular matrix, fibroblasts, and immature cells. Therefore, the pulp tissue has important functions to sustain teeth providing: (1) nutrient supply for dentin/pulp metabolism to keep the mechanical properties, (2) innervation and serve sensory organ for prevention of deep caries, (3) reactionary/reparative dentin formation during pulpal wound healing process after noxious stimuli such as caries, operative procedure, attrition, abrasion and trauma and (4) immunological response to bacterial infiltration (Dental Pulp 2002).

Because of the brittle deficiency of non-vital tooth, it is important to understand the repair mechanism of pulp and develop precautions to preserve the vitality of the

pulp. Therefore, the areas of odontoblasts biology and dentinogenesis, pulp biology, and the formation of reactionary and reparative dentin have been extensively reviewed during the last three decades (Lesot et al. 1993; Linde and Goldberg 1993; Ruch et al. 1995; Smith and Lesot 2001).

Odontoblast is the major cellular component in dentin and it plays an important role in dentin formation during tooth development and repair. Odontoblasts are tall and columnar secretory cells. They are aligned along the periphery of the pulp on the formative surface of dentin and vary throughout their life cycle both in size and in cytoplasmic organelles, and these changes are closely related to their functional activity (Dental pulp 2002). The cell-rich layer of Höhl is a rich capillary plexus underlying the odontoblasts layer. The cells of the Höhl layer can not only function to support odontoblasts activity, but also express some transcription factors and differentiate into new odontoblasts for reparative dentinogenesis (Kitamura et al. 1999; Mitssiadis et al. 1999).

The dental pulp responses differently depending on the intensity of irritation, and the differences are closely related to odontoblasts. Reactionary dentinogenesis is defined as a tertiary dentin matrix secreted by surviving postmitotic odontoblast cells in response to a milder stimulus. When the irritation becomes more intensive, which cause the death of odontoblasts, the recruitment, proliferation and differentiation of adjacent

mesenchymal cells into new odontoblasts is crucial to generative reparative dentin.

Healing and repair process in reparative dentinogenesis are the result of successive and interrelated processes including proliferation, chemotaxis, and differentiation of dental pulp cells into odontoblasts that leads to reparative dentin formation (Dental pulp 2002). Repair of dental pulp is a complicated process. Growth factors such as transforming growth factor beta (TGF-βs), bone morphogenic proteins (BMPs), growth/differentiation factors (GDFs), insulin growth factors (IGFs), fibroblast growth factor (FGF), and platelet derived growth factors (PDGFs), etc. may take part in this complicated process.

Together, the long-term goal of operative dentistry and endodontics is the preservation and restoration of function of entire tooth including dental pulp. Because pulpodentin complex poses potential of tissue regeneration and repair, the scientist comes up with an idea that maybe we can use some drugs or materials to preserve pulp vitality, promote pulp repair and induce dentinogenesis. This is the major concept of vital pulp therapy, and this idea becomes popular around the dentist. In addition, tissue engineering is another hot topic today. It may be a prospective alternative treatment using the concept of tissue engineering to preserve pulp, including use of stem cells, cytokines and various artificial scaffolds, and administration of some growth factors. The triad is critical for both tissue engineering and regeneration of tooth.

Together, previous studies constitute the mile stones of our present knowledge. However, despite this apparent wealth of information, many assumptions and questionable hypotheses still prevail.

1.2 Growth/Differentiation Factor-5 (GDF-5)

1.2.1 General concept of GDF-5

GDF-5, also called as BMP-14 and cartilage derived morphogenetic protein (CDMP)-1, is closely related to the BMP family and is a member of the TGF-β superfamily. TGF-β superfamily are multifunctional regulators which posses a wide range of biological activities and influence a variety of cell types, including monocytes, epithelial, mesenchymal and neuronal cells. Therefore, they regulate cell proliferation, differentiation, chemotaxis, apoptosis, synthesis of the extracellular matrix and immune responses (He et al. 2004). The biological functions of GDF-5 will be introduced in 1.2.3.

GDF-5 was first identified in 1994 and expressed predominantly in the precartilaginous mesenchymal condensation and the cartilaginous cores of the developing long bone (Storm et al. 1994; Thomas et al. 1996).

1.2.2 GDF-5 peptide structure

The mature and functional form of GDF-5 is a homodimer of two 120 amino-acid

polypeptide chain (monomers) linked by a single disulfide bond. Each GDF-5 monomer is expressed as the carboxyl-terminal part of a precursor polypeptide, which also contains a 27 amino-acid signal peptide and a 354 amino-acid propeptide. This precursor undergoes intracellular dimerization, and upon secretion it is processed by a furin-type protease. rhGDF-5 is a 27.0 kDa homodimeric disulfide-linked protein consisting of two 120 amino acids.

As previous studies, the peptide structures of the TGF-β family members are highly similar, and this superfamily comprises a large group of structurally related proteins that are secreted as dimers and then cleaved after an Arg-X-X-Arg site to release biologically active carboxyl-terminal domains containing seven highly conserved cysteines. Furthermore, as discussed by Storm et al., molecules in any given BMP subfamily share 74-92% of their mature carboxyl-terminal signaling region amino acid sequence, whereas members of different subfamilies share only 40-60% (Storm et al. 1994) .In GDF-5, its carboxyl-terminal TGF-β domains is 82% identical, and is most closely related to BMP-5, BMP-6, and osteogenic protein-1 (Chang et al. 1994).

1.2.3 The biological effects of GDF-5

Since the first isolation and purification from newborn calf cartilage, GDF-5 has been studied more than twenty years. GDF-5 has been demonstrated to promote mesenchymal cell recruitment and skeletal processes such as endochondral ossification,

synovial joint formation, tendon/ligament development, and odontogenesis (Nishitoh et al. 1996; Morotome et al. 1998; Buxton et al. 2001; Archer et al. 2003; Coleman & Tuan 2003; Settle et al. 2003; Shimaoka et al. 2004). Moreover, mutations in the murine GDF-5 gene resulted in abnormal skeletal development, known as brachypodism (Storm et al. 1994). Mutations in the human GDF-5 gene were shown to be associated with recessive human acromesomelic chondrodysplasia Hunter-Thompson type (Thomas et al. 1996) and chondrodysplasia Grebe type (Thomas et al. 1997). The phenotypes were very similar to the murine brachypodism.

More recent in vitro results revealed that GDF-5 significantly increased the proliferation of primary osteoblasts, periosteum cells, and connective tissue fibroblasts (Yoshimoto et al. 2006). Furthermore, the osteogenic potential of recombinant human (rh) GDF-5 has been intensively examined *in vitro* and *in vivo* for a decade. The *in vitro* response to rhGDF-5 resulted in osteogenic differentiation in various cell types, such as pluripotent mesenchymal precursor cell C2C12 (Yeh et al. 2005), adipose-derived stromal cells (Shen et al. 2006), bone marrow mesenchymal stem cells (Shimaoka et al. 2004) and periosteum-derived cells (Gruber et al. 2001).

In conclusion, on the basis of reviewing the previous studies [Table 2], it is clear that GDF-5 is closely related to the development and repair of multiple tissues, including cartilage, tendon, intervertebral disk, skin, and bone (Mikic 2004). In the

healing processes examined (i.e., tendon repair and fracture healing), a delay is encountered which could be explained by the influence of GDF-5 on cell recruitment, proliferation, differentiation, and migration, as well as the process of angiogenesis.

1.2.4 The role of GDF-5 in tooth development and differentiation

TGF-β superfamily members play inductive and regulatory roles in tooth development and repair. The expression of mRNA for TGF-β1, TGF-β2, BMP-2, BMP-4, BMP-6, BMP-7, and activin, is confirmed by in situ hybridization studies, RT-PCR, and Northern blot analyses in tooth germ and adult pulp (Roberts et al. 1991, Vaahtokari et al. 1991, Nakashima et al. 1994, Thesleff et al. 1996).

Until 1997, GDF-5 mRNA is identified for the first time in both dental papilla and follicle at the root-forming stage of odontogenesis (Oida et al. 1997; Morotome et al. 1998; Sena et al. 2003). In adult human teeth, the expression of GDF-5 and its receptor mRNA is found in periodontal ligament. In PDL-derived cells, rhGDF-5 stimulates cell proliferation and glycosaminoglycan synthesis, but inhibits osteoblastic differentiation, which may be one mechanism by which PDL is maintained around roots (Nakamura et al. 2003).

More recently, the effect of GDF-5 on the growth and differentiation of porcine dental papilla- and follicle-derived cells is investigated. The results reveal that GDF-5 regulates differentiation of both dental papilla and follicle during odontogenesis

(up-regulation effect in dental papilla-derived cells in a dose-dependent manner, and down-regulation effect in dental follicle-derived cells), co-operatively with other growth factors such as BMP-2 (Sumita et al. 2010). However, the role of GDF-5 in adult dental pulp remains largely unknown.

1.3 Signaling transduction pathways of GDF-5

1.3.1 Receptors of GDF-5

In most of cells, there are three types of TGF-β receptors, and they are classified as type I, II, and III (Tai et al. 2008). As a member of TGF-β superfamily, GDF-5 transduces its signals through two types of serine/threonine kinase receptors, termed type I and type II receptors.

In animal studies, dental pulp tissue express six different type I receptors (termed activin receptor-like kinases (ALK)-1 to -6) and two different type II receptors (TGF-β receptor II (TβR-II) and BMP receptor II (BMPR-II)).

GDF-5 share BMP type I receptors with BMP-2, but the affinities to the receptors differ. GDF-5 binds to the BMP type IB receptor (BMPR-IB, also termed ALK-6) with high affinity, but binds to BMP type IA receptor (BMPR-IA, also termed ALK-3) with low affinity (Sebald et al. 2004; Nickel et al. 2005). Moreover, GDF-5 preferentially binds to ALK-6 in ROB-C26 osteoprogenitor cells (Nishitoh et al. 1996), GDF-5 and

ALK-6 interaction has been shown to be important in chondrogenesis of mouse distal limb formation (Baur et al. 2000). Both of ALK-6 and ALK-3 can induce phosphorylation of Smad-1, Smad-5 and Smad-8, meanwhile, ALK-4/5/7 promotes the phosphorylation of Smad-2, and Smad-3 [Figure 1-1].

1.3.2 Smad pathway

At first, the ligands bind to type II receptor and activate the type II receptor. Then the GS domain of the type I receptor is phosphorylated. Dimers initiate signaling by binding to both type I and type II receptors and induced the formation of heterotetracomplexes. The heterotetracomplexes promotes the phosphorylation of two Ser residues in the motif Ser-Ser-X-ser of the C-terminal region of an intracellular protein called receptor- regulated Smad (R-Smad), made up of Smad- 1/2/3/5/8.

Two phosphorylated R-Smad molecules form a heterotrimeric complex with Smad 4, a common-partner Smad (Co-Smad). The R-Smad/Co-Smad complexes then translocate into the nucleus and regulate transcription of target genes by interaction with various transcription factors and transcriptional co-activators or co-repressors. The third class of Smads, inhibitory Smad (I-Smad, including Smad-6/7), negatively regulates signaling by R-Smad and Co-Smad.

Of the eight Smad proteins identified in mammals, Smad-1/5/8 can activated by BMP type I receptors, whereas Smad-2/3 are activated by activin and TGF-β type I

receptors. Smad-1/5/8 are structurally highly similar to each other, and functional differences between them have not been fully determined. Smad-1/5, but not Smad 8, are efficiently activated by BMP-6 and BMP-7, whereas all Smad-1/5/8 are activated by BMP-2 (Aoki et al. 2001). However, the interaction between the Smad pathway and GDF-5 is still not fully understood. The only clue is in the Smad-1/5/8 phosphorylation study of Xu et al., rhGDF-5 could not stimulate an obvious Smad-1/5/8 phosphorylation in the nascent human mesenchymal stem cells (HMSCs) but induced the Smad-1/5/8 phosphorylation in the rhTGFβs treated HMSCs (Xu et al. 2006).

1.3.3 Mitogen activated protein kinase (MAPK) pathway

TGF-β & BMPs are shown to modulate cellular activities via Smad-independent pathways. Signaling mechanisms including MAPK pathways are recently found to mediate the biological effects of TGF-β and BMPs (Sowa et al. 2002, Lebrin et al. 2005). The MAPK pathway involves three different cascades: and extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase (p38) [Figure 1-2].

Previous studies have shown that GDF-5 induced the phosphorylation of ERK1/2 and p38 in chondrogenic ATDC5 cell line (Nakumura et al. 1999), activated ERK1/2 in human umbilical vein smooth muscle cells (Chen et al. 2006), and induced osteogenic differentiation of human ligamentum flavum cells through activation of ERK1/2 and

p38 MAPK (Zhong et al. 2010). However, the relationship between GDF-5 and MAPK pathway has not been clarified in dental pulp cells yet.

1.4 The role of cyclin-dependent kinase 1 (CDK1), CDC25C, cyclin B1, p21 in cell proliferation

1.4.1 Cell cycle

The cell cycle or cell-division cycle means the series of events that takes place in a cell leading to its division and duplication [Figure 2]. The cell cycle consists of four distinct phases: G_1 phase, S (synthesis) phase, G_2 phase, and M (mitosis) phase. Activation of each phase is dependent on the proper progression and completion of the previous one. When cells have temporarily or reversibly stopped dividing, the state of quiescence called G_0 phase.

1.4.2 The role of cyclins and cyclin-dependent kinases (CDKs)

Cyclin-dependent kinases (CDKs) are a family of protein kinases first discovered for their role in regulating the cell cycle (Nigg et al. 1995). CDKs are relatively small proteins, with molecular weights ranging from 34 to 40 kDa, and contain little more than the kinase domain. Animal cells contain at least nine CDKs, four of which, CDK1, 2, 3, and 4, are directly involved in cell cycle regulation. The known CDKs, their cyclin partners and their function in human are listed in table 3.

By definition, a CDK binds a regulatory protein called a cyclin. Without cyclin, CDK has little kinase activity; only the cyclin-CDK complex is an active kinase. The concentration of cyclins varies in a cyclic fashion during the cell cycle. They are produced or degraded as needed in order to drive the cell through the different stages of cell cycle. There are two main groups of cyclins: G_1/S cyclins and G_2/M cyclins. G_1/S cyclins, including cyclin A, cyclin D, and cyclin E, is essential for the control of the cell cycle at the G_1/S transition. G_2/M cyclins is important for the control of the cell cycle at the G_2/M transition. G_2/M cyclins accumulate steadily during G_2 and are abruptly destroyed as cells exit from mitosis (at the end of the M-phase). The typical G_2/M cyclins is cyclin B, and it reacts with CDK1 (as known as cell division control protein 2(CDC2) homolog) to regulate progression from G_2 to M phase.

Different combinations of the cyclin and CDK determine the different downstream proteins. The mechanism of cyclin-CDK interaction is shown in Figure 3. Once the cell receives a pro-mitotic extracellular signal, G1 cyclin-CDK complexes become active to prepare the cell for S phase. It promotes the expression of transcription factors that induce the expression S-phase cyclins and enzymes necessary to DNA replication. Active S-phase cyclin-CDK complexes phosphorylated proteins that make up the pre-replication complexes assembled during G_1 phase on DNA replication origins. Mitotic cyclin-CDK complexes are synthesized but inactivated during S phase and G_1

phase. The complex promotes the initiation of mitosis by stimulating downstream proteins involved in chromosome condensation and mitotic spindle assembly.

1.4.3 CDC25C

CDC25C activates cyclin dependent kinase by the removal of phosphates from residues in the CDK active site. M-CDK complex activates CDC25C, which is the activator of CDK1. The switch-like behavior forces entry into mitosis is irreversible. CDC25A to C are known to control the transitions from G_1 to S phase and G_2 to M phase.

1.4.4 p21

p21 also known as CDK inhibitor 1 or CDK-interacting protein 1 is a potent CDK inhibitor. There are two families of genes that prevent the progression of the cell cycle: the *cip/kip* family and the INK4a/ARF(Inhibitor of Kinase 4a/ Alternative Reading Frame). The *cip/kip* family consists of the genes of p21, p27, and p57. They stop the cell cycle in G_1 phase by binding to and inactivating the cyclin-CDK complexes.

The p21 protein inhibits the activity of cyclin E/CDK2 and cyclin D/CDK4 complexes and functions as a regulator of G_1 phase. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G_1 phase arrest in response to a variety of stress stimuli. p21 can also interact with proliferating cell nuclear antigen (PCNA), a DNA

polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair.

1.5 Alkaline phosphatase (ALP) and its role in tissue mineralization

Alkaline phosphatases (ALPs) are a glycosylated, membrane bound enzymes that are present in nearly all living organisms. ALPs are cell surface glycoproteins that hydrolyze a variety of monophosphate esters. In humans, there are at least four types of genetically different iso-enzymes, namely, tissue-nonspecific alkaline phosphatase (TNAP), intestinal alkaline phosphatase, placental alkaline phosphatase, and placentallike alkaline phosphatase (Goseki-Sone et al. 2002). Intestinal alkaline phosphatase, and placental alkaline phosphatase are tissue-specific and relatively thermostable. TNAP are thermolabile iso-enzyme, characteristic for liver/bone/kidney and present in most cell types. Placental-like phosphateses are present at low levels in adult germ cells (Chang et al. 1994).

The bone isoenzyme has long been thought to play a role in mineralization of bone and cartilage (Robinson 1923; Beertsen and van den Bos 1992). In dental follicale, this activity is also used as an early marker of cementoblast/osteoblast differentiation (Zhao et al. 2002; Morsczeck 2006). Likewise, as a marker of odontoblast differentiation, Goggins and Fullmer (1967) reported that ALP activity is greatest in the

pre-odontoblastic cell-rich zone near the lateral and occlusal walls of the rat molar pulp chamber. Furthermore, ALP is likely a prerequisite for differentiation and specialization of pulp cells in vivo (Miller et al. 1976). They suggest this is because ALP activity is greater in the pre-odontoblastic layer than in the mature odontoblastic layer and greater in pulp cells just prior to differentiation into odontoblasts. ALP is an enzyme which can be used in detection of mineralized tissue formation in cells. ALP as well as osteopontin (OPN), osteocalcin (OCN), osteonectin, and other specific markers of dentin differention are protein markers presented at early stage of pulpal cell differentation (Narayanan et al. 2001).

Taken together, ALP activity is an appropriate early indicator of cementoblast/osteoblast and odontoblast differentiation (Nuki and Bonting 1961; Yoshiki and Kurahashi 1971; Tonomura et al. 2007). ALP activity was gradually increased, reached the peak on day 14 and then declined during the differentiation of both human and rat dental pulp cells.

Chapter II The Purpose of the Study

The purpose of our study is to investigate whether GDF-5 influences the morphological changes, cell proliferation and viability, cell differentiation, and collagen formation of human dental pulp cells *in vitro*. In addition, this study also tests the signaling transduction pathways in GDF-5-induced cell proliferation and differentiation.

Chapter III Materials and Methods

3.1 Materials

3-(4,5-dimethyl-thiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (MTT), alkaline phosphatase (ALP) staining assay reagents, dimethylsulfoxide (DMSO) and dorsomorphin are purchased from Sigma (Sigma Chemical Company, St. Louis, MO, USA). Noggin and Recombinant GDF-5 are obtained from PeproTech Inc. (NJ, USA). U0126 is purchased from Promega (Promega Corporation, Madison, Wisconsin, USA). SB431542 and SB203580 are from Tocris (Tocris bioscience, St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin are from Gibco (Life Technologies, Grand Island, NY, USA). The SuperScript TM III First-Strand DNA synthesis system for RT-PCR is purchased from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA). RNA isolation kit, and NucleoSpin RNAII are purchased from Macherey-Nagel (Macherey-Nagel Inc, Easton, PA, USA). Tris Base is from Amresco (Amresco Inc, Solon, Ohio, USA). Sircol collagen assay kits are from Biocolor (Belfast, N. Ireland, UK)

3.2 Culture of human dental pulp cells

Human dental pulp cells were cultured by an explant technique and characterized as previous studies (Chan et al. 2005, Tai et al. 2008). Briefly, human dental pulp cell were derived from the extracted human third molars or premolars of patients under 25 y/o of age with proper informed consent before providing samples. Teeth were split with a hammer to get vital pulp tissue. Pulp tissues were cut into 1 x 1 x 1 mm^3 small pieces by a surgical knife and cultured in DMEM containing 10% FBS and 1X penicillin/streptomycin in culture dishes. The medium were changed every 2 or 3 days. Pulp cells were cultured at 37℃, in a humidified atmosphere with 95% air and 5% CO2. When pulp cells were growing from the explants reached near confluence, they were trypsinized and subcultured at a ratio of 1:3. The cell passage numbers from 3 to 8 were used for all experiments in this study.

3.3 Morphology of human dental pulp cells

Briefly, 5 x 10^3 pulp cells were incubated onto 24-well culture plate in DMEM containing 10% FBS. Twenty-four hours later, the culture medium was changed by serum-free DMEM and various concentrations of GDF-5 were added, depending on the experimental conditions. The concentrations of GDF-5 were ranged from 0, 1, 10, 50, 100, 250 ng/ml. 5 days after GDF-5 treatment, the morphological changes of human

dental pulp cells were observed and photographed under a phase contrast microscope (Olympus IX71, Olympus America Inc.).

3.4 MTT assay

MTT assay is a rapid colorimetric assay for cellular growth and survival. It can be applicated for evaluation of cell proliferation and cytotoxicity. In short, human pulp cells were incubated onto 24-well culture plate in DMEM with 10% FBS. (In condition I, 5 x 10^3 human pulp cells per well is indicated; in condition II, the cell number alters to 1 x 10^4 human pulp cells per well.) After twenty-four hours for attachment of cells on the plate, the culture medium was replaced by serum-free DMEM and various concentrations of GDF-5 were added, depending on the experimental conditions. (I) The concentration of GDF-5 was ranged from 0, 1, 10, 50, 100, 250 ng/ml. (II) Cells were individually pretreated with 1 or 5μM of U0126 (ERK inhibitor), 0.5or 1μM of SB431542 (ALK-4/5/7 specific inhibitor), or 500 or 1000 ng/ml of Noggin (BMP antagonist) for 30 minutes. Then 250 ng/ml of GDF-5 was added for co-incubation.

Cells were cultured under the same condition for another 5 days. Then, 20ul of MTT reagent was added into each well and incubated at 37℃ for 2 hours. After 2-hour incubation, the cultured medium was decanted and the produced formazan was dissolved in 150 ul of DMSO. Then, 100ul of dissolved formazan solution from each

well was transferred to 96-well plate. The amount of dissolved formazan was monitored by the readings against blank sample (DMSO) at OD 540 nm using a Dynatech Microwell plate reader (Dynatech Microwell, Dynatech, Alexandria, Virginia).

Cell viability = (sample value of OD540nm-blank) / (negative control value of OD540nm- blank) X 100%

3.5 Alkaline phosphatase (ALP) staining

ALP activity of pulp cells was determined. Briefly 1 x 10^5 human pulp cells were inculated onto 24-well culture plate in DMEM containing 10% FBS. Twenty-four hours later, medium was changed and according to experimental conditions various concentrations of GDF-5 were added. (I) The concentration of GDF-5 was ranged from 0, 10, 50, 100, 250, 500 ng/ml. (II) Cells were individually pretreated with 1 or 5 μM of U0126 (ERK inhibitor), 0.5 or 1 μM of SB431542 (ALK-4/5/7 specific inhibitor), 1 or 5 μM of SB203580 (p38 inhibitor), 1 or 5 μM of Dorsomorphin (ALK-2/3/6 specific inhibitor; AMPK inhibitor) for 30 minutes. Then 250 ng/ml of GDF-5 was added for co-incubation.

Cells were cultured at 37℃, in a humidified atmosphere with 95% air and 5% CO_2 for another 5 days or 10 days. (In condition I, the 5-day and 10-day experiments were both done, and the cultured medium was changed in 5th day. In condition II, only

the 5-day experiment was done.) Then, the medium was collected and cells were washed with PBS three times. ALP activity of human dental pulp cells was evaluated by histochemical staining using azo-dye coupling method as described before (Chan et al., 2005; Tai et al., 2008). After removal of PBS, cells were fixed by 2% of paraformaldehyde/PBS under room temperature for 20 minutes. Fresh stock substrate solution was prepared [Figure 4]. The fixation medium was removed and cells were rinsed with PBS containing Mg^{2+} and Ca^{2+} ions [Figure 5]. Then cells were flooded with 1000μl of stock substrate. At least, 1000ul/ well of incubation solution [Figure 6] were added. Pulp cells were incubated under room temperature for 15 min in the dark. The ALP staining of cells were observed and photographed.

3.6 Quantitative assay of Alkaline phosphatase activity

3.6.1 Cell lysate collection

1 x 10^5 human pulp cells were seeded onto 24-well culture plate in DMEM containing 10% FBS. After twenty-four hours for attachment of cells on the plate, the medium was changed and according to experimental conditions various concentrations of GDF-5 were added. (I) The concentration of GDF-5 was ranged from 0, 10, 50, 100, 250, 500 ng/ml. (II) Cells were individually pretreated with 1 or 5 μM of U0126 (ERK inhibitor), 0.5 or 1 μM of SB431542 (ALK-4/5/7 specific inhibitor), 1 or 5 μM of

SB203580 (p38 inhibitor), 1 or 5 μM of Dorsomorphin (ALK-2/3/6 specific inhibitor; AMPK inhibitor) for 30 minutes. Then 250 ng/ml of GDF-5 was added and co-incubated.

Pulp cells were cultured at 37℃, in a humidified atmosphere with 95％ air and 5 ％ CO_2 for another 5 days or 10 days. (In condition I, the 5-day and 10-day experiments were both done, and the cultured medium was changed in 5^{th} day. In condition II, only the 5-day experiment was done.) Then, the medium was collected and cells were washed with PBS twice.

First, 0.5％ Triton X-100 solution (extraction buffer) was prepared: 250 ul Triton and 80 ul 1M $MgCl_2$ were mixed in 40 ml ddH_2O. After aspirating the PBS to dry, 250 ul of extraction buffer was added immediately to each well. Then, place the culture plate on ice in the shake for 30 minutes. After 30-minute incubation, the cell lysate was collected for protein measurement.

3.6.2 Alkaline phosphatase activity measurement

p-Nitrophenol standard solution was prepared in 1.5 ml eppendorfs into various concentrations. Add each standard solution with 2.5 ul in volume to standard well of 96-well plate. 50 ul stock substrate (phosphatase substrate) was added into blank and sample wells; 50 ul water was added into standard wells. Then, 50 ul alkaline buffer was added to each well. The culture plate was covered with Parafilm® to avoid the

reaction of CO_2 and incubated at 4℃ for 30 minutes. After 30-minute incubation, 12.5 ul of 0.5% triton was added into blank and standard wells; 12.5 ul cell lysate was added into sample wells. Finally, the culture plate was incubated at 37℃ for 45 minutes. The reaction was quenched by adding 1 N NaOH and the absorbance at 405 nm was read on the plate reader.

3.7 Collagen content assay

Sircol™ Collagen Assay kit. (Biocolor Ltd., Newtownabbey, Northern Ireland) is a quantitative dye-binding method that has been designed for analysis of acid-soluble collagens extracted from mammalian tissues and collagens released into culture medium by mammalian cells during *in vivo* culture. Synthesis of collagen by pulp cells in this experiment was measured by Sircol Collagen Assay kit.

1 x 10^5 human pulp cells were seeded onto 24-well culture plate in DMEM containing 10% FBS. Twenty-four hours later, according to experimental conditions the medium was changed and various concentrations of GDF-5 were added. (I) In condition I, we used DMEM containing 10%FBS as changing medium, and GDF-5 with various concentrations (0, 10, 50, 100, 250, 500 ng/ml) were added. (II) In condition II, serum-free DMEM was used as changing medium, and 0-500 ng/ml GDF-5 were added; meanwhile, serum-containing DMEM without GDF-5 was used as positive control

group.

Pulp cells were cultured at 37℃, in a humidified atmosphere with 95％ air and 5 ％ CO_2 for another 5 days or 10 days. (In condition I, the 5-day and 10-day experiments were both done, and the cultured medium was changed in 5^{th} day and the various concentrations of GDF-5 were added. In condition II, only the 5-day experiment was done.)

Then, culture medium was collected and the pulp cells were washed with PBS twice. 50 ul of 0.5 M acetic acid was added into each well for cell dissolving. Then, pulp cells were stained by 200 ul of Sircol dye reagent (Sirius Red) in a slight shaking motion for 30 minutes. Sirius Red is an anionic dye with sulphonic acid chain groups. These groups may react with the side chain groups of the basic amino acids present in collagen. After 30 minutes, the dye reagent was decanted. The complex of Sirius Red binding to collagen was extracted with 200 ul of alkaline reagent for 10 minutes. Alkaline reagent contained 0.5N sodium hydroxide, which released Sircol dye form the collagen-dye complex. 100 ul of the reagent solution from each well was taken into 96-well plate. Type I collagen solution was used for calibration of standard curve [Table 4]. Standard type I collagen solution was prepared in 1.5 ml eppendorfs into various concentrations, dissolved in 50 ul of 0.5M acetic acid and stained by 200 ul of Sircol dye reagent. After 30-minute incubation, these standard samples were centrifuged at

5000rpm for 10 minutes. The clear lysate was removed and the dye-collagen was dissolved in 200 ul of alkaline reagent. 100 ul of the reagent solution from each eppendorf was loaded into 96-well plate. The optical density of Sirius Red was read against blank at a wavelength of 540 nm by Dynatech Microwell plate reader.

3.8 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2 x 10^6 human pulp cells were seeded onto 10-cm culture dishes with 10 ml of DMEM containing 10% FBS. After twenty-four hours for attachment of cells on the plate, the culture medium was replaced by serum-free DMEM and the cells were exposed to various concentrations of GDF-5 (0, 10, 50, 100, 250 ng/ml) for 48 hours. RT-PCR (Reverse Transcription Polymerase Chain Reaction) method was used to evaluate the gene expression (Cyclin B1, Cdc2, Cdc25c, p21,) of human dental pulp cells after 48-hour incubation.

3.8.1 Isolation of total RNA

Macherey-Nagel RNA isolation kits were used to isolate total cellular RNA. First of all, the culture medium was completely removed and cells were washed with PBS twice. Then, 3.5 ul of β-mercaptoethanol and 350 ul RA1 was added into each cultured dishes for cell lysis. The cells were detached from dish with a cell scraper, and then collect the lysate. Cell lysate was filtered in the NucleoSpin Filter units with attached

filtered centrifuge tube and then centrifuged at 14,000 rpm for 1 minute to obtain the cleared lysate. 350ul of 70% ethanol was added to the cleared lysate. Then, the total 700 ul of mixture solution from the filter centrifuge tube was collected and placed into NucleoSpin RNA column with centrifuge tube and it was centrifuged at 10,000 rpm for 30 seconds. The obtained product in the RNA column was added with 350 ul of Membrane Desalting Buffer and centrifuged at 14,000 rpm for 1 minute. The DNase I was mixed with 90 ul of DNase I Reaction Buffer to obtain the DNase I Reaction Mixture, and 95ul of the mixture was added to the RNA column with a centrifuge tube. Then they were incubated at room temperature for at least 15 minutes.

After 15 minutes of incubation, 200ul of RA2 Buffer was added and centrifuged at 10,000 rpm for one minute. The flowthrough was discarded, and the centrifuge tube was replaced by a new one. 600 ul of RA3 Buffer was added, and the mixture was centrifuged at 10,000 rpm for one minute. The flowthrough was discarded again. Then, 250 ul of RA4 Buffer was added and centrifuged at 14,000 rpm for two minutes. Finally, the RNA column with centrifuge tube was placed into a new nuclease-free 1.5 ml microcentrifuge tube. RNA was eluted with 60ul of RNase –free water, centrifuged at 14,000 rpm for 1 minute, and stored under –20℃.

3.8.2 RNA quantitation

DEPC (diethylpyrocarbonate) water and RNA samples were obtained. After RNA

samples were centrifuged, 1,000ul of DEPC water and 10 ul of isolated RNA were mixed in eppendorfs. Hitachi U-2001 spectrophotometer was used to measure the absorbance value of the mixed RNA solution at wavelengths of 260 nm and 280 nm. The ratio OD260 nm/ OD 280 nm must be > 1.8, which indicates an adequate RNA quality and purity. The absorbance values and the ratio of OD260 nm and OD280 nm can be used not only to assess the concentration and purity of RNA but also to measure the amount of RNA necessary for reverse transcription.

3.8.3 Reverse Transcription (RT)

Invitrogen SuperScript ™ III First Strand Synthesis System was used for Reverse Transcription Polymerase Chain Reaction (RT-PCR) [Figure 7]. An appropriate amount of RNA for each group according to the results of RNA quantification could be determined. DEPC water should be added to each test sample in order to make up the total volume to 64 ul. 8 ul of Random Hexamer and 8 ul of 10 mM dNTP was added. Then, the samples were incubated in a PCR machine (Perkin Elmer 4800, City of Industry, CA, USA).All the sample tubes in the PCR machine were kept at 65℃ for 5 minutes and followed by at least 2 minutes of soaking period at 4℃. The cDNA Synthesis Mix in total volume of 80 ul should be prepared: 16 ul of 10X RT Buffer, 16 ul of $MgCl_2$, 16 ul of 0.1M dithiothreitol (DTT), 28 ul of DEPC water and 4 ul of SuperScript ™ III RT were added into each sample. These samples reacted at 25℃ for

10 minutes, at 50℃ for 50 minutes, at 85℃ for 5 minutes and were soaked at 4℃ for at least one minute. Then, 2ul of Rnase H was added to each sample. The samples were again placed into the PCR machine and run at 37℃ for 20 minutes and a final soak should be at 4℃ for one minute. At last, the RT samples could be used for PCR amplification immediately or stored at -20℃.

3.8.4 Polymerase Chain Reaction (PCR)

3 ul of the cDNA product of each sample was used for PCR amplification in a reaction volume of 50 ul. In addition to the cDNA product, the total reaction volume of five samples (GDF-5 0, 10, 50, 100, 250 ng/ml) was comprised of 33.2 ul of 10X Taq buffer combined with Prime Taq^{TM} DNA Polymerase (Genet Bio, Chungnam, Korea), 24 ul of 2.5mM dNTP, 9 ul of 25mM MgCl2, 12 ul of each specific primer, and DEPC water. All samples were placed into the PCR machine. The samples were denatured at 94℃ for 5 minutes at first, and then run for 20~30 cycles. Each cycle comprised of 1 minute at 94℃ for denaturing, 1 minute at 55℃ for annealing, and another 1 minute at 72℃ for elongation. The samples were finally run at 72℃ for 7 minutes for elongation and soak at 4℃ after all cycles were completed.

Specific primers used in this experiment were Cyclin B1 (317bp), CDC2 (288bp), CDC25C (456bp), p21 (450bp), and β-actin (BAC) (218 bp) as control [Table 5].

The agarose gels for electrophoresis were prepared. The 1.8% agarose gel was

composed of 1.8g of agarose and 100 ml of 0.5X TBE buffer. After mixing, the solution was heated in a microwave oven until agarose was completely dissolved. 5 ml of 0.625 mg/ml ethidum bromide (EtBr) was added and mixed. The solution was then placed into the gel holders. It took about one hour for gel formation.

15 ul of PCR product was mixed with 3 ul of DNA loading dye and loaded into each well of the gel and 6ul of DNA marker mixed with 3 ul of DNA loading dye was also loaded. After the electrophoresis process was completed, the gels were removed from the holder for photo and semi-quntification under UV camera and the AlphaEaseFC (Alpha Innotec Corp, San Leandro, CA, USA) software program.

3.9 Cell mitosis: Immunofluorescence assay

5 x 10^4 human dental pulp cells were seeded onto 6-well culture plate, where 18x18 mm sterile coverslips were placed, in DMEM containing 10% FBS. After 24-hour incubation, culture medium was replaced by serum-free DMEM and the cells were exposed to various concentrations of GDF-5 (0, 10, 50, 100, 250 ng/ml). In the positive control group, the medium was only replaced by DMEM containing 10％ FBS. Then, 2 ug/ml of cytochalasin B(D) was added into each well. These pulp cells were cultured for 2 days.

Cytochalasins are fungal metabolites that have the ability to bind to actin filaments

and block polymerization and the elongation of actin. As a result of the inhibition of actin polymerization, cytochalasins can change cellular morphology, inhibit cellular processes such as cell division (cytokinesis), and even cause cells to undergo apoptosis. **Cytochalasin B** is a cell-permeable mycotoxin. It inhibits cytoplasmic division by blocking the formation of contractile microfilaments (Theodoropoulos et al. 1994). It inhibits cell movement and induces nuclear extrusion. Addition of Cytochalasin B into culture medium was for observation of nuclear division. During the stage of cell mitosis, there were more nuclei inside each cell when cytoplasmic division was inhibited. In another word, the radios of mononuclear and binuclear cells may imply the potential for cell proliferation.

After 2-days incubation with different concentrations of GDF-5, culture medium was removed, and pulp cells were washed by PBS three times and fixed by 4% paraformaldehyde in PBS for 15 minutes. Then, cells were washed by PBS three times again. 2% PBST (2% Tween 20 in PBS) was added into each well and reacted with pulp cells for 15 minutes. Later, cells were washed by PBS three times again. 0.5% BSA (bovine albumin from serum) was added onto these coverslips for blocking and incubated for 45 minutes. After that, cells were washed by PBS four times. Prepare primary antibody (Rhoadimine phalloidin, Introvigen) in 1:100 ratio (v/v). Pulp cells on the coverslips were incubated with primary antibody at room temperature for 2 hours.

Then cells were washed with PBS four times and cultured with secondary antibody (donkey anti-goat antibody, Santa Cruz Biotechnology) for 1 hour. Next, 4-times of PBS wash was performed again. After PBS wash, pulp cells were stained with DAPI solution (0.25 ug/ul) in 1:500 ratio (v/v) for 30 minutes. Finally, coverslips were sealed by 90％ glycerol on the glass slide. Pulp cells were observed and photographed under a fluorescence microscope (Olympus TH4-100 and Olympus IX71, Olympus America Inc.). DAPI has an excitation maximum at 345 nm and an emission maximum at 455 nm.

3.10 Statistical analysis

Three or more separate experiments were performed. Difference between control and experimental groups are analyzed by One-way ANOVA and post hoc Tukey test using the SPSS 8.0 software for Windows. A p value < 0.05 was considered to have significant difference between groups.

Chapter IV Results

4.1 Morphological observation on human dental pulp cells

Dental pulp cells are spindle shape with extended cellular processes when observed under a phase-contrast microscope [Figure 8]. After 5-day incubation with different concentrations of GDF-5 (0, 1, 10, 50, 200, 250 ng/ml) in serum-free DMEM, the morphology of human dental pulp cell did not markedly changed. However, the numbers of dental pulp cells with GDF-5 treatment were much higher than those without GDF-5 treatment, especially in the groups of higher concentration of GDF-5 (100, 250 ng/ml).

4.2 Effects of GDF-5 on cell viability of human dental pulp cells--- MTT assay (condition I)

In MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) can be metabolized into formazan salt by mitochondria dehydrogenase of viable cells. So MTT assay can evaluate cell viability through calculating the amount of purplish-blue formazan salt at a wavelength of 540nm.

Estimated cell viability = (sample value of OD540nm- blank value) / (negative control value of OD540nm-blank value) X 100%

In condition I of this experiment, MTT assay was performed after 5-day incubation of pulp cells after treatment by different concentration of GDF-5 (0, 1, 10, 50, 100, 250 ng/ml) in serum-free DMEM [Table 6 & Figure 9]. Cell viability of human dental pulp cells was dose-dependently promoted under the treatment of GDF-5 and the difference was significant between the control and concentration of 10-250 ng/ml.

4.3 Effects of GDF-5 on cell viability of human dental pulp cells and its modulation by Noggin, SB431542, and U0126--- MTT assay (condition II)

In this experiment, in order to identify by which pathway that GDF-5 up-regulates the cell viability in human dental pulp cells, U0126 (ERK inhibitor), SB431542 (ALK-4/5/7 specific inhibitor) and Noggin (BMP antagonist) were used independently or in combination with GDF-5 (250 ng/ml) to see whether they may affect the cell proliferation of human dental pulp cells.

At concentration of 250 ng/ml, GDF-5 stimulated the cell viability of human dental pulp cells as analyzed by condition I of MTT assay experiment. Pulp cells treated only with Noggin (500 & 1000 ng/ml) showed moderate increase in cell viability in comparison with the normal control group. However, Noggin could not reverse the up-regulatory effect caused by GDF-5, but slightly enhanced the cell viability [Figure

10-1].

SB431542 (0.5 & 1 uM) itself did not affect the pulp cell in cell viability comparing with the normal control group. However, SB431542, as an ALK-4/5/7 specific inhibitor, was able to slightly attenuate the GDF-5-induced increase of cell viability ($p>0.05$) [Figure 10-2].

Pulp cell treated only with U0126 (1 & 5 uM) showed slightly increase in cell viability. However, in cells pretreated with U0126 before administration of GDF-5 (250 ng/ml), U0126 was not effective to prevent the GDF-5-induced rise of cell viability [Figure 10-3].

4.4 Effects of GDF-5 on expression of cell cycle related genes (Cyclin B1, CDC2, CDC25C, p21) of human dental pulp cells--- RT-PCR

Cyclin B1 reacts with CDK1 (CDC2), forming the cyclin B1-CDK1 complex that initiates the G_2/M transition. CDC25C is highly conserved during evolution and it plays a key role in the regulation of cell division. p21 inhibits the activity of cyclin E-CDK2 and cyclin D-CDK4 complexes and functions as a regulator of S phase. These gene expressions indicated the state of cell cycle.

RT-PCR was performed after human dental pulp cells were incubated with different concentrations of GDF-5 (0, 10, 50, 100, 250 ng/ml) in serum-free DMEM for

48 hours. Cyclin B1 mRNA expression of pulp cells were markedly increased, especially in GDF-5 250 ng/ml group. Expression of CDC2 and CDC25C mRNA were not altered obviously. However, p21 mRNA expression was declined in GDF-5-treated groups [Figure 11].

4.5 Effects of GDF-5 on ALP activity of human dental pulp cells---ALP staining and ALP quantitative assay

ALP is an enzyme that is popularly used for evaluation of mineralized tissue forming capacity of cells. At different stages of pulpal cell differentiation, ALP, osteopontin (OPN), osteocalcin (OCN), osteonectin and specific markers of dentin differentiation such as dentin sialoprotein (DSP), dentin matrix protein 2 (DMP2) are significantly expressed (Narayanan et al. 2001).

In experiment of ALP staining, Naphthol AS phosphate binds to the side chain of ALP molecules at pH 9.1. Then, fast blue BB salts are coupling agents that turn the ALP-positive-cell blue (Burstone, 1958). ALP activity of pulp cells may be indicated by blue staining after 5 or 10-day incubation with different concentrations of GDF-5 (0, 10, 50, 100, 250, 500 ng/ml) in DMEM containing 10% FBS.

After 5-day and 10-day incubation, human dental pulp cell without GDF-5 treatment showed positive response to ALP staining. Comparison of the ALP activity

between 5-day-cultured and 10-day-cultured experiment, the 10-day-cultured experiment showed generally higher ALP activity than the 5-days-cultured experiment. Moreover, the level of ALP seemed to be decreased from 10 to 500 ng/ml in a dose dependent manner, especially in 10-day-cultured experiment [Figure 12a].

In both 5-day-cultured and 10-day-cultured experiment of ALP quantitative assay, GDF-5 decreased the concentration of p-Nitrophenol obviously [Figure 12b]. There are significant differences in groups of GDF-5 10, 50, 100, 250, 500 ng/ml, comparing to the control group. In the 5-day-cultured experiment, the most declination of ALP activity was the GDF-5 250 ng/ml group; in the 10-day-cultrued group, GDF-5 declined the ALP activity of human dental pulp cells dose-dependently.

4.6 Effects of GDF-5 on ALP and its modulation by Dorsomorphin, SB431542, SB203580, and U0126 ---ALP staining and ALP quantitative assay

According to above experiment, GDf-5 down-regulated the ALP activity of the human dental pulp cells, and the most declination of ALP activity was at concentration of 250 ng/ml in the 5-day-cultured experiment. In order to identify by which pathway that GDF-5 down regulates the ALP activity in human dental pulp cells, Dorsomorphin (ALK-2/3/6 specific inhibitor), SB431542 (ALK-4/5/7 specific inhibitor), U0126 (ERK

inhibitor), and SB203580 (p38 inhibitor), were used independently or in combination with GDF-5 (250 ng/ml) to see whether they may affect the ALP activity of human dental pulp cells.

At concentration of 250 ng/ml, GDF-5 declined the ALP activity of human dental pulp cells as analyzed ALP staining and ALP quantitative assay. Pulp cells treated only with Dorsomorphin (1 uM) showed moderate increase in ALP activity and pulp cells treated only with Dorsomorphin (5 uM) showed slightly decrease in ALP activity, in comparison with the normal control group. However, Dorsomorphin could not reverse the down-regulatory effect caused by GDF-5 [Figure 13-1].

SB431542 (0.5 & 1 uM) itself did not affect the pulp cell in ALP activity comparing with the normal control group. However, SB431542, as an ALK-4/5/7 specific inhibitor, still could not attenuate the GDF-5-induced decrease of ALP activity [Figure 13-2].

Pulp cell treated only with U0126 (1 & 5 uM) showed slightly decrease in ALP activity. However, in cells pretreated with U0126 before administration of GDF-5 (250 ng/ml), U0126 was not effective to prevent the GDF-5-induced declination of ALP activity [Figure 13-3].

SB203580 (1 & 5 uM) itself showed inhibitory effect on ALP activity, especially at concentration of 5 uM ($p<0.05$). However, SB203580 still could not reverse the

down-regulatory effect caused by GDF-5 [Figure 13-4].

Take together, all inhibitor used in this part of experiment (either ALP staining or ALP quantitative assay) could not prevent the GDF-5-induced declination of ALP activity.

4.7 Effects of GDF-5 on collagen formation of human dental pulp cells--- Sircol assay

Sircol assay of this experiment is designed for analysis of acid-soluable collagens extracted from human dental pulp cells. After pulp cells were incubated with GDF-5 in DMEM containing 10% FBS for 5 or 10 days, Sircol assay was performed. Furthermore, in addition to exclude the effect of serum on collagen formation, condition II experiment of Sircol assay (pulp cells were cultured with GDF-5 in serum-free DMEM for 5 days) was also performed.

In condition I experiment, GDF-5 at various concentrations in DMEM containing 10% FBS had no effect on the collagen content of human dental pulp cells after 5-day incubation or 10-day incubation [Figure 14-1]. In condition II experiment, pulp cells incubated in DMEM containing 10% FBS without GDF-5 treatment, as positive control group, showed significant higher collagen content than normal control group. However, GDF-5 still had no effect on the collagen content of human dental pulp cells [Figure

14-2].

4.8 Effects of GDF-5 on cell mitosis--- immunofluorescence assay

By treatment with Cytochalasin B and different concentrations of GDF-5, human dental pulp cells underwent nuclear division but not cytoplasmic division. The percentages of mononuclear and binuclear cells may indicate tendency to cell proliferation.

In our experiment, the percentage of binuclear cells was significantly higher in group of GDF-5 10, 50, 100, 250 ng/ml than that in negative control group [Figure 15]. On the contrary, the percentage of mononuclear cells was significantly lower in these experimental groups comparing to that in negative control groups. Furthermore, the group incubated in DMEM containing 10% FBS, as positive control group, showed highest percentage of binuclear cells and lowest percentage of mononuclear cells. Take together, GDF-5 increased the percentages of binuclear cells and decreased that of mononuclear cells after the treated pulp cells were incubated in serum-free DMEM for 48 hours.

Chapter V Discussion

5.1 Effects of GDF-5 on cell viability and proliferation of human dental pulp cells

In the previous studies, GDF-5 could significantly increase the proliferation of primary osteoblasts, periosteum cells, and connective tissue fibroblasts (Yoshimoto et al. 2006). In an *in vitro* experiment of PDL-derived cells, GDF-5 exerted a significant effect on cell proliferation in dose-dependent manner (Nakamura et al. 2003). However, GDF-5 had little proliferative effect on dental papilla-derived cells and dental follicle-derived cells (Sumita et al. 2010).

In our studies, morphological observations under light microscopy showed that morphology of pulp cells did not alter markedly in the presence of GDF-5. All human dental pulp cells expressed spindle shape with long process either GDF-5 addition or not. However, the cell density in randomly selected field was much higher in the experimental groups than that in the control group, especially in the higher concentration groups.

In MTT assay, the activity of mitochondria dehydrogenase was measured to evaluate cell viability of pulp cells. In this experiment, GDF-5 could dose-dependently promote the cell viability of human dental pulp cells in serum-free DMEM. These

results coincided with the observations of cell numbers. The more pulp cells proliferated, the more cell viability expressed. These results revealed that GDF-5 may stimulate human pulp cell proliferation and play an important role in tissue development and repair of dental pulp tissue.

In the cell cycle, cyclin and cyclin-dependent kinases (CDKs) determine a cell's process. Different combinations of cyclins and CDKs lead to different downstream proteins. Cyclin B reacts with CDK1, forming cyclin B-CDK1 complex that initiates the G_2/M transition. Cyclin B-CDK1 complex activation causes breakdown of nuclear envelope and initiation of prophase. CDC25 activates CDK by the removal of phosphates from residues in the CDK active site. It directs dephosphorylation of cyclin B-bound CDK1 (CDC2) and triggers entry into mitosis. RT-PCR results of the present study demonstrated Cyclin B1 gene expression of pulp cells were increased markedly. This phenomenon indicated human dental pulp cells incubated with GDF-5 in serum-free DMEM had potential to cause initiation of cell cycle progression and cell mitosis. This can partly explain why GDF-5 stimulates pulp cell proliferation.

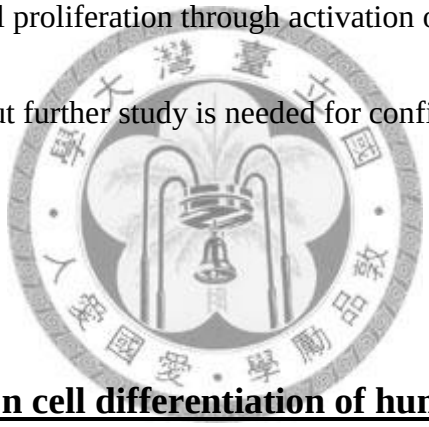
Moreover, we also detected the gene that prevents the progression of cell cycles. p21 inhibits the activity of cyclin E/CDK2 and cyclin D/CDK4 complexs and functions as a regulator of S phase. Furthermore, growth arrest by p21 can promote cellular differentiation. In the present study, p21 mRNA expression was decreased after GDF-5

treatment. This findings of mRNA expression demonstrated that GDF-5 also acted on the inhibitor (p21) in the cell cycle. Beside, the results of immunofluorescence for mitotic index showed more binuclear cells in groups of higher concentrations of GDF-5. It may imply that human dental pulp cells under GDF-5 treatment possess higher potential for cell proliferation. Take together, in this study, GDF-5 promoted cell proliferation, cell viability, and expression of the genes related to cell mitosis of human dental pulp cells.

5.2 Signaling transduction pathway of GDF-5 on cell viability and proliferation

In the previous studies, GDF-5 could transduce it signals through Smad pathway or MAPK pathway. In Smad pathway, the close relationship between ALK-6 (BMPR-IB) and GDF-5 had been already demonstrated (Nishitoh et al. 1996; Baur et al. 2000; Sebald et al. 2004), and ALK-6 could subsequently activate Smad-1/5/8. However, rhGDF-5 could only induced Smad-1/5/8 phosphorylation in rhTGF-β treated human mesenchymal stem cell (Xu et al. 2006). In MAPK pathway, GDF-5 had been proved to activate ERK and p38 pathway in ATDC5 chondrogenic cells and human ligmentum flavum cells, but only activated ERK in human umbilical vein smooth muscle cells (Nakumura et al. 1999; Chen et al. 2006; Zhong et al. 2010)

In previous statement, we found that GDF-5 could stimulate human dental pulp cell proliferation and cell viability. Then, different signaling inhibitors were used to identify the signaling transduction pathway of GDF-5-induced cell proliferation in human dental pulp cells. In this preliminary study, only SB431542, an ALK-4/5/7 specific inhibitor, slightly reversed the up-regulatory effect of GDF-5. Noggin (BMP antagonist) and U0126 (ERK inhibitor) both could not prevent the effect of GDF-5, and Noggin slightly enhanced the GDF-5-induced cell proliferation. These results replied that GDF-5 may induce cell proliferation through activation of ALK-4/5/7 partially in human dental pulp cells. But further study is needed for confirmation of the actual and detail signaling pathway.



5.3 Effects of GDF-5 on cell differentiation of human dental pulp cells

ALP is a phosphatidylinositol-linked membrane glycoprotein that catalyzes the hydrolysis of monophosphate esters at alkaline environment (Balcerzak *et al.* 2003). Expression of ALP is considered to be not only an early marker for tissue mineralization but also as a parameter for osteoblast differentiation and osteogenic properties. Elevation of ALP activity is essential for the differentiation and possible specialization of dental pulp cells *in vivo* (Yoshiki and Kurahashi 1971) and odontoblast differentiation of cells *in vitro* (Sloan et al. 2001). Besides, dental pulp cells and

periodontal ligament cells exhibit ALP activity, suggesting the functional activities of these cells to form mineralized tissue.

In the previous studies, GDF-5 could promote osteogenic differentiation in various cell types, such as pluripotent mesenchymal precursor cell C2C12, adipose-derived stromal cells, bone marrow mesenchymal stem cells and periosteum-derived cells (Gruber et al. 2001; Shimaoka et al. 2004; Yeh et al. 2005; Shen et al. 2006). In PLD-derived cells, GDF-5 significantly inhibits ALP expression in dose-dependent manner. More recently, GDF-5 had been showed that it increased ALP activity dose-dependently in dental papilla-derived cells and decreased ALP activity in dental follicle-derived cells (Sumita et al. 2010).

In our experiment, human dental pulp cells exhibited marked alkaline phosphatase activity from cytochemical staining, indicating their capacity to form mineralized tissue. After 5-day and 10-day incubation with various concentrations of GDF-5 in DMEM containing 10% FBS, ALP activity of pulp cells declined significantly. The 10-day-cultured experiment showed generally higher ALP activity than the 5-days-cultured experiment, and the declination caused by GDF-5 treatment was much more obvious in 10-day incubation. These phenomenons coincide with the results of ALP quantitative assay. Therefore, we suspect that GDF-5 may play a role in specific stages of pulp cell mineralization. And the effect of GDF-5 could be tissue-specific,

depending on the state of differentiation and the microenvironment in which it is located.

5.4 Signaling transduction pathway of GDF-5 on cell differentiation

In condition I of ALP experiment, GDF-5 declined significantly the cell differentiation of human dental pulp cell. However, the signaling pathway of GDF-5 in pulp cells is still unknown. So, we used four signaling inhibitor, including Dorsomorphin (ALK-2/3/6 specific inhibitor), SB431542 (ALK-4/5/7 specific inhibitor), U0126 (ERK inhibitor) and SB203580 (p38 inhibitor) to identify the signaling transduction pathway of GDF-5-induced cell differentiation in human dental pulp cells.

However, in this preliminary experiment, all inhibitors could not reverse GDF-5-induced cell differentiation. So, it may imply that GDF-5 down-regulate ALP activity via other signaling transduction pathway. Besides, there were some difficulties to be excluded or solved. One was the incubation time: only 5-day incubation was not enough to identify the effect of signaling inhibitor on GDF-5-induced ALP declination. 10-day experiment may be needed for further confirmation. Another was the dose of inhibitor used in this experiment: some inhibitors, such as U0126 and SB203580, had slight to moderate inhibitory effect on ALP activity which would affects the judgment. So, the suitable concentration of inhibitors is needed to be effective and poisonless in

the further study.

5.5 Effects of GDF-5 on collagen formation of human dental pulp cells

Several studies demonstrated that GDF-5 plays an important role in the development and repair of multiple tissues, including cartilage, tendon, intervertebral disk, skin, and bone (Mikic et al. 2004). Besides, GDF-5 affected synthesis of extracellular matrix constituents associated with chondrogenesis (Heidaran et al. 2000). However, little knowledge or studies was on the effect of GDF-5 on collagen formation of human dental pulp cells. The only clue was that GDF-5 could stimulate glycosaminoglycan synthesis, but had no effect in gene expression of collagen α2 (I) in PDL-derived cells.

Sircol collagen assay has been successfully used to measure the solute fibrillar collagen contents in different experimental models (Ciapetti et al. 1996, Tang et al. 1998). In our study, the concentrations of collagen content (mean value) were similar among the control and experimental groups when the pulp cells were incubated in serum-containing or serum-free DMEM. Even prolong the incubation time to 10 days, the effect still could not be identified. These results indicated that GDF-5 has no effect on collagen formation in human dental pulp cells.

Chapter VI Conclusion

1. GDF-5 did not markedly alter the morphology of human dental pulp cells. However, GDF-5 increased the cell number and cell viability dose-dependently. The findings of mRNA expression demonstrated that GDF-5 acted on the checkpoint proteins (cyclin B1) and the inhibitor (p21) in the cell cycle. Besides, in experiment of immunofluoscence, GDF-5 could increase the percentage of binuclear cells significantly in dose-dependent manner.
2. Pretreatment of Noggin (BMP antagonist) and U0126 (ERK inhibitor) did not reverse the GDF-5-induced cell proliferation. However, SB431542 (ALK-4/5/7 specific inhibitor) could slightly attenuate the effect of GDF-5 on cell proliferation.
3. GDF-5 down-regulated ALP activity of human dental pulp cells significantly.
4. Pretreatment of Dorsomorphin (ALK2/3/6 specific inhibitor), SB431542, U0126 and SB203580 (p38 inhibitor) did not reverse the GDF-5-induced declination of cell differentiation.
5. In serum-containing or serum-free environment, GDF-5 had no effect on collagen formation in human dental pulp cells.

In conclusion, growth/differentiation factor-5 demonstrated proliferative property in human dental pulp cells, but had inhibitory effect on cell differentiation and no effect on collagen formation. Signal transduction of GDF-5 in human dental pulp cells is a complex system. GDF-5 could induce cell proliferation through the activation of ALK-4/5/7 partially, but the signaling pathway of GDF-5-induced cell differentiation is still unknown. These events are crucial in the mechanism of pulpal repair and regeneration.

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Tables

Table 1: Cells and extracellular matrix components found in dentin and pulp

TABLE
Cells and Extracellular Matrix Components (ECM) Found in Dentin and Pulp

	Dentin	Pulp
Cells	Odontoblasts exclusively	Fibroblasts (pulpoblasts), vascular cells, pericytes, neural cells, histiocytes/ macrophages, dendritic cells, lymphocytes, mast cells
Collagens	Types I and I trimer (98%) Types III (1-2%) and V (1%) (90% of the dentin ECM)	Type I (56%) Types III (41%) and V (2%); Type VI (0.5%) associated with microfibrillin
Non-collagenous proteins	(10% of the dentin ECM)	
	Phosphorylated matrix proteins (SIBLINGs): DSPP > DSP and DPP DMP-1, BSP, OPN, MEPE	 none BSP, OPN
	Non-phosphorylated matrix proteins: Matrix GLA protein, osteocalcin, osteonectin	Fibronectin Osteonectin (in tooth germs)
	Proteoglycans (SLRPs) CS/DS PGs: decorin-biglycan (CS-4 81%, CS-6 14%, CS/DS 2%) KS PGs: lumican, fibromodulin, osteoadherin	Versican CS-4 and -6, 60%; DS, 34%; KS, 2% Hyaluronic acid
	Amelogenin 5-7 kDa Growth factors: TGF-β, ILGF-I and -II, FGF-2, VEGF, PDGF	 BMPs Types IA and II receptors for TGF-β, activin, and BMPs
	Metalloproteinases: collagenase (MMP-1), gelatinases (MMP-2 and -9), stromelysin-1 (MMP-3), enamelysin (MMP-20), MT1-MMP, TIMP-1 to -3	MMPs: collagenases, gelatinases, stromelysin-1 TIMPs
	Alkaline phosphatase Serum-derived proteins: αHS_2-glycoprotein, albumin, lipoproteins	 Fibronectin of serum origin
	Phospholipids: Membrane phospholipids (66%) Extracellular-mineral-associated phospholipids (33%)	 Membrane and ECM phospholipids

Adapted from

Michel Goldberg, Anthony J. Smith. CELLS AND EXTRACELLULAR MATRICES OF DENTIN AND PULP: A BIOLOGICAL BASIS FOR REPAIR AND TISSUE ENGINEERING. Crit Rev Oral Biol Med 2004; 15(1):13-27

Table 2: Effects of GDF-5 on skeletal tissues and processes

Reference	Tissue/process	Model used	Main findings
Landauer (1952)[27]	General description of mutant skeletal phenotype	*bp* Mouse	Reduced foot size due to fewer phalanges and abnormally shaped metatarsals and metacarpals; shorter long bones; proximal bones more affected than distal ones within a given limb
Grünenberg and Lee (1973)[20]	Anatomy and development of mutant skeletal phenotype	*bp* Mouse	*Brachypodism* likely due to abnormalities in prechondrogenic limb blastema
Storm *et al.*, 1994[48]		Degenerate PCR; chromosome mapping; examination of *Gdf5* sequence in *bp* mutations	Isolation of *Gdf-5*, 6, and 7 for first time; demonstration that *brachypodism* caused by mutations in *Gdf5*; *bp* mutations caused by frameshift resulting in premature stop codon before mature signaling portion of GDF-5 (i.e., functional null mutation)
Chang *et al.* (1994)[6]	Chondrogenesis and endochondral ossification	Human embryonic development	CDMP-1 and CDMP-2 (*a.k.a.* GDF-5 and 6) purified from newborn calf cartilage; both induce cartilage and bone in ectopic assays; in human embryos, CDMP-1 found primarily in prechondrogenic cores of developing long bones while CDMP-2 restricted to hypertrophic chondrocytes
Polinkovsky *et al.* (1997)[40]		Genetic studies in individuals with hereditary brachydactyly	Autosomal dominant brachydactyly type C in humans caused by mutation in CDMP-1; dominant phenotypes may also arise because of haploinsufficiency
Thomas *et al.* (1996)[50]		Genetic studies in individuals with hereditary chondrodysplasia	Mutation in hCDMP-1 is associated with recessive human acromesomelic chondrodysplasia, Hunter–Thompson type
Thomas *et al.* (1997)[51]		Genetic studies of individuals with hereditary chondrodysplasia	Chondrodysplasia Grebe Type (CGT) due to CDMP-1 mutation; protein is not secreted and is inactive
Duke and Elmer (1977)[10]	Chondrogenesis	*bp* Mouse limb mesenchyme rotation culture	E12 *bp* mesenchymal cells exhibit delay in chondrogenesis and appear to be more adhesive than controls
Duke and Elmer (1978)[11]	Chondrogenesis	*bp* Mouse limb mesenchyme fragment fusion studies	Interface of fused E12 distal postaxial *bp* limb bud fragments showed *bp* mesenchyme to be more adhesive than controls; chondrogenesis delayed in *bp* fragments
Duke and Elmer (1979)[12]	Chondrogenesis	Ultrastructural analysis of E12 and E13 *bp* limb blastema	E12 *bp* fibular blastemal cells exhibit abnormal morphology with small spiky microvilli, extensive intercellular contacts, and deficient matrix; tibial blastemal cells appeared normal; cellular appearance at E13 appeared more normal than on E12
Francis-West *et al.* (1999)[19]	Chondrogenesis	Retroviral overexpression of GDF-5 during chick limb bud development	Overexpression of GDF-5 results in increased length of skeletal elements due largely to increased numbers of chondrocytes; *in vitro* micromass cultures showed that GDF-5 increased chondrogenesis in a dose-dependent manner; GDF-5 enhanced cell adhesion in prechondrogenic stages; once differentiated into chondrocytes, GDF-5 can increase proliferation
Erlacher *et al.* (1998)[16]	Chondrogenesis	Adult bovine and human articular cartilage explants	CDMPs 1 and 2 present in adult bovine and human articular cartilage; treatment with CDMPs can stimulate metabolic activity of chondrocytes *in vitro*
Elmer and Selleck (1975)[13]	Chondrogenesis	*In vitro* cyto-differentiation of E12 *bp* hindlimb mesoderm	High-density *bp* E12 mesenchymal cells exhibit abnormalities in cell aggregation and do not show normal increases in GAG synthesis associated with chondrogenesis
Hewitt and Elmer (1976)[22]	Plasma membrane	*bp* Limb bud mesenchymal cell reactivity with concanavalin-A	Cells from *bp* limb buds express a temporal delay in reactivity with the plant lectin concanavilin-A; suggests that *bp* cells may have abnormal pattern of carbohydrates on cell surface
Hewitt and Elmer (1978)[23]	Plasma membrane	Lectin-induced agglutination in *bp* limb bud mesenchyme	Normal and *bp* limb bud cells vary in agglutination with age, but in a different temporal sequence; *bp* cells appear to be more adhesive than normal; differences also exist in lateral mobility of ConA and wheat-germ agglutinin (WGA) binding sites on the cell membrane
Elmer and Wright (1983)[14]	Plasma membrane	*In vitro* studies of *bp* postaxial hindlimb cells during chondrogenesis	Abnormal expression of GlcNAc glycoproteins in plasma membrane of *bp* cells which occurs some time between E12 and E13

Reference	Tissue/process	Model used	Main findings
Elmer *et al.* (1988)[15]	Plasma membrane	Biochemical analyses of purified plasma membrane from *bp* embryonic limb cells	Decrease in cell-surface enzyme that transfers galactose to its GlcNAc substrate, galactosyltransferase, in *bp* cells
Storm and Kingsley (1999)[47]	Chondrogenesis and joint development	Recombinant GDF-5 protein administered to developing chick and mouse limbs *in vitro*	GDF-5 is necessary and sufficient for cartilage development and for restriction of joint formation to specific locations
Merino *et al.* (1999)[33]	Chondrogenesis and joint development	Developing chick autopod	As in the mouse, *Gdf5* is expressed in sites of future joint formation in developing chick digital rays; GDF-5 coated beads implanted at digit tips promote cartilage growth; beads implanted in interdigital regions inhibit joint formation in adjacent digits
Storm and Kingsley (1996)[46]	Joint development	Mice with single and double mutations in *Gdf5* and *Bmp5*	*Gdf5* transcripts are expressed in stripes in future locations of synovial joints of limb; mice with mutations in both *Gdf5* and *Bmp5* show abnormalities not seen in either single mutant alone
Settle *et al.* (2003)[43]	Joint development and skeletal patterning	Mice with single and double mutations in *Gdf5* and *Gdf6*	*Gdf6* and *Gdf7* are expressed in different subsets of developing joints; GDF-6-deficient mice exhibit abnormalities in wrist and ankle, middle ear, and coronal sutures; mice lacking both *Gdf5* and *Gdf6* show additional defects beyond those seen in individual mutations alone
Volenec *et al.* (2002)[53]	Cartilage repair	Full thickness articular cartilage defects in goats	Collagen/hyaluronan matrices in combination with GDF-5 promote healing
Tsumaki *et al.* (1999)[52]	Endochondral ossification	Targeted expression of recombinant CDMP-1 in mice	Transgenic mice exhibited chondrodysplasia, larger hypertrophic zone and smaller proliferative zone, CDMP-1 increased number of chondroprogenitor cells and accelerated differentiation into hypertrophy
Nakamura *et al.* (1984)[38]	Growth plate	*brp* Mouse	Decreased rate of chondrocyte division in the growth plates of homozygous *brp/brp* mice (3-week-old, mixed gender)
Mikic *et al.* (2003)[37]	Growth plate	*bp* Mouse	GDF-5 deficiency is associated with a 14% reduction in proximal tibial growth rate and 25% longer hypertrophic phase duration (5-week-old females)
Chhabra *et al.* (2003)[8]	Fracture healing	*bp* Mouse	One- to 2-week delay in fracture healing of femur in 16-week-old female mice based on measures of callus size, DNA/wet weight and GAG/DNA, as well as histologic grading
Spiro *et al.* (2001)[45]	Spinal fusion	Rabbit posterolateral spinal fusion model	rhGDF-5 in combination with mineralized collagen matrix can enhance osteoinductive and osteoconductive properties of each
Simank *et al.* (2001)[44]	Avascular necrosis	Partial femoral head AVN model in sheep	GDF-5 in combination with absorbable carrier enhances bone regeneration
Yamashita *et al.* (1997)[57]	Angiogenesis	Chick chorioallantoic membrane and rabbit cornea assays; *in vitro* effects of GDF-5 on bovine aortic endothelial cells (BECs)	GDF-5 induces angiogenesis in both chorioallantoic membrane and rabbit cornea assays; GDF-5 induces plasminogen activator activity and accelerated BEC migration via chemotaxis
Li *et al.* (2003)[28]	Intervertebral disk (IVD)	*bp* Mouse	Nucleus pulposus from 16-week-old female *bp* mice are smaller, contain less GAG/DNA and less aggrecan and col II mRNA; no differences in col I production; *in vitro* treatment of *bp* IVD cells from nucleus with recombinant *Gdf5* resulted in elevated col II and aggrecan gene expression
Walsh *et al.* (2001)[54]	Intervertebral disk (IVD)	Mechanically induced degeneration in murine caudal disks	Single injection of GDF-5 (3.5 mg/ml) into nucleus pulposus resulted in increased cell density in inner annulus and increased chondrocyte presence in same region 1 week later; no effect on nucleus
Walsh *et al.* (2002)[55]	Intervertebral disk (IVD)	Mechanically induced degeneration in murine caudal disks	Single injection of GDF-5 (1 μg/ml) resulted in increased disk height and expansion of annular fibrochondrocytes 4 weeks later; multiple injections did not enhance this effect
Wolfman *et al.* (1997)[56]		Ectopic administration of protein in rodents	Both intramuscularly and subcutaneously, implanted GDF-5, -6, and -7 protein resulted in neotendon and ligament formation (no cartilage or bone seen)
Hotten *et al.* (1996)[24]		Ectopic administration of rhGDF5 in rodents; in vitro effects on rat limb mesenchyme	Intramuscular administration of rhGDF5 induced cartilage and bone formation in rodent muscle tissue; rhGDF5 stimulated mesenchyme aggregation and chondrogenesis in rat limb bud cells
Mikic *et al.* (2001)[36]	Achilles tendon	*bp* Mouse	8-week-old male *bp* Achilles tendons contain less collagen/DNA, are weaker and more compliant, and fail in tendon midsubstance (vs. via avulsion); slight trend towards smaller collagen fibrils with irregular shape

Clark *et al.* (2001)[9]	Tail tendon	*bp* Mouse	8-week-old male *bp* tail tendon fascicles subject to stress-relaxation tests relax more slowly and to a lesser extent than controls; no differences in quasi-static properties, GAG/DNA, or collagen/DNA; increased prevalence of irregularly shaped collagen fibrils with shift towards smaller diameter
Chhabra *et al.* (2003)[7]	Tendon repair	*bp* Mouse	8-week-old male *bp* mice exhibit 1- to 2-week delay in Achilles tendon healing based on achievement of peak cellularity, GAG/DNA, and collagen/DNA, changes in collagen fibril size, and revascularization of repair tissue; greater fatty tissue presence in *bp* tendon regenerate at all time points examined (up to 6 weeks); mechanical strength differences (normalized to internal sham values) present at 5 weeks, gone by 12 weeks
Aspenberg and Forslund (1999)[1]	Tendon repair	Rat Achilles tenotomy accompanied by calf muscle denervation	Implantation of GDF-5 or -6 in collagen sponge carrier resulted in a dose-dependent increase in tendon structural strength after 2 weeks
Rickert *et al.* (2001)[41]	Tendon repair	Rat Achilles tenotomy	GDF-5 coated sutures resulted in larger tendons at 1, 2, and 4 weeks; greater structural strength after 2 weeks; presence of chondrocytes noted in tendon regenerate after 4 weeks
Rickert *et al.* (2003)[42]	Tendon repair	Rat Achilles tenotomy	Adenovirus-mediated GDF-5 gene transfer resulted in stronger tendon regenerates at 4 and 8 weeks due to enhanced proliferative response; no differences in elastic modulus of tissue
Tashiro *et al.* (1999)[49]	Ligament repair	Rat medial collateral ligament transection	Implantation of recombinant human GDF-5 in fibrin gel (3 μg/μl) resulted in structurally stronger repair tissue at 3 and 6 weeks and larger diameter collagen fibrils at 6 weeks
Battaglia *et al.* (2002)[3]	Skin	*bp* Mouse	Dorsal skin samples from adolescent male *bp* mice are 10% thinner; collagen fibrils are less densely packed (10% lower area fraction); qualitative increase in fibril shape irregularity; stress-relaxation tests revealed that *bp* skin samples relaxed more slowly and to a lesser extent than controls
Mikic *et al.* (2000)[35]	Cortical bone	*bp* Mouse	8-week-old male *bp* femora are structurally weaker and more compliant than controls when tested in torsion; no differences in material property of maximum effective shear stress, but significantly lower effective shear modulus of elasticity; 6% lower ash content; no differences in collagen content

Adapted from Mikic, B. *Ann Biomed Eng* 2004; 32(3): 466-76

Table 3: The known CDKs, their cyclin partners, and their function in human and consequences of deletion in mice

CDK	Cyclin partner	Function	Deletion Phenotype in Mice
Cdk1	Cyclin B	M phase	None. ~E2.5.
Cdk2	Cyclin E	G1/S transition	Reduced size, imparted neural progenitor cell proliferation. Viable, but both males & females sterile.
Cdk2	Cyclin A	S phase, G2 phase	
Cdk3	Cyclin C	G1 phase?	No defects. Viable, fertile.
Cdk4	Cyclin D	G1 phase	Reduced size, insulin deficient diabetes. Viable, but both male & female infertile.
Cdk5	p35	Transcription	Severe neurological defects. Died immediately after birth.
Cdk6	Cyclin D	G1 phase	
Cdk7	Cyclin H	CDK-activating kinase, transcription	
Cdk8	Cyclin C	Transcription	
Cdk11	Cyclin L	?	Mitotic defects. E3.5.
?	Cyclin F	?	Defects in extraembryonic tissues. E10.5.

Adapted from Satyanarayana A. & Kaldis P. *Oncogene. 2009 Aug 20;28(33):2925-39*

Table 4: Protocol for Sircol Collagen Assay standard sample preparation

		Concentration (mg/ml)	Type I Collagen 1 mg/ml (µl)	0.5M acetic acid (µl)	Dye reagent (µl)
Blank		0	0	50	200
Collagen Standard	1	0.008	1.6	48.4	200
	2	0.016	3.2	46.8	200
	3	0.0325	6.3	43.7	200
	4	0.0625	12.5	37.5	200
	5	0.125	25	25	200
	6	0.2	40	10	200

Table 5: PCR Primer Sense Sequences, Antisense Sequence, Base Pairs

H-BAC-A	AAGAGAGGCATCCTCACCCT	218bp	Jeng et al. 2000
H-BAC-B	ATACATGGCTGGGGTGTTGAA	218bp	Jeng et al. 2000
H-cyclinB1-A	AAGAGCTTTAAACTTTGGTCTGGG	317bp	Nuchic Acid Res. 28:4410 2000
H-cyclinB1-B	CTTTGTAAGTCCTTGATTTACCATG	317bp	Nuchic Acid Res. 28:4410 2000
H-cdc2-E	GGGGATTCAGAAATTGATCA	288bp	JBC 271:28469-77
H-cdc2-F	TGTCAGAAAGCTACATCTTC	288bp	JBC 271:28469-77
H-cdc25c-C	CCTGGTGAGAATTCGAAGACC	456bp	Mol Carci 36:171-82 2003
H-cdc25c-D	GCAGATGAAGTACACATTGCATC	456bp	Mol Carci 36:171-82 2003
H-p21-WAF1 /CIP1-A	GAGCGATGGAACTTCGACTTTGTC ACC	450bp	Urologic Oncology 5:71-77 2000
H-p21-WAF1 /CIP1-B	CTGAGACTAAGGCAGAAGATGTAG AGCG	450bp	Urologic Oncology 5:71-77 2000

Table 6: MTT assay for various concentration of GDF-5 treatment

Original data of GDF-5

NC	GDF-5 1 ng/ml	GDF-5 10 ng/ml	GDF-5 50 ng/ml	GDF-5 100 ng/ml	GDF-5 250 ng/ml	Blank
0.26	0.339	0.35	0.421	0.456	0.478	0.07
0.277	0.365	0.405	0.44	0.512	0.549	0.07
0.227	0.344	0.344	0.407	0.378	0.463	0.07
0.349	0.379	0.425	0.424	0.438	0.433	0.05
0.366	0.404	0.439	0.4	0.449	0.462	0.05
0.388	0.424	0.402	0.44	0.485	0.479	0.05
0.403	0.448	0.582	0.469	0.49	0.546	0.05

% of control

	NC	GDF-5 1 ng/ml	GDF-5 10 ng/ml	GDF-5 50 ng/ml	GDF-5 100 ng/ml	GDF-5 250 ng/ml
	100	141.58	147.37	184.74	203.16	214.74
	100	142.51	161.84	178.74	213.53	231.40
	100	174.52	174.52	214.65	196.18	250.32
	100	110.03	125.42	125.08	129.77	128.09
	100	112.03	123.10	110.76	126.27	130.38
	100	110.65	104.14	115.38	128.70	126.92
	100	112.75	150.71	118.70	124.65	140.51
mean	100	129.15	141.01	149.72	160.32	174.62
Standard error	0	24.70	24.54	41.93	41.47	54.96

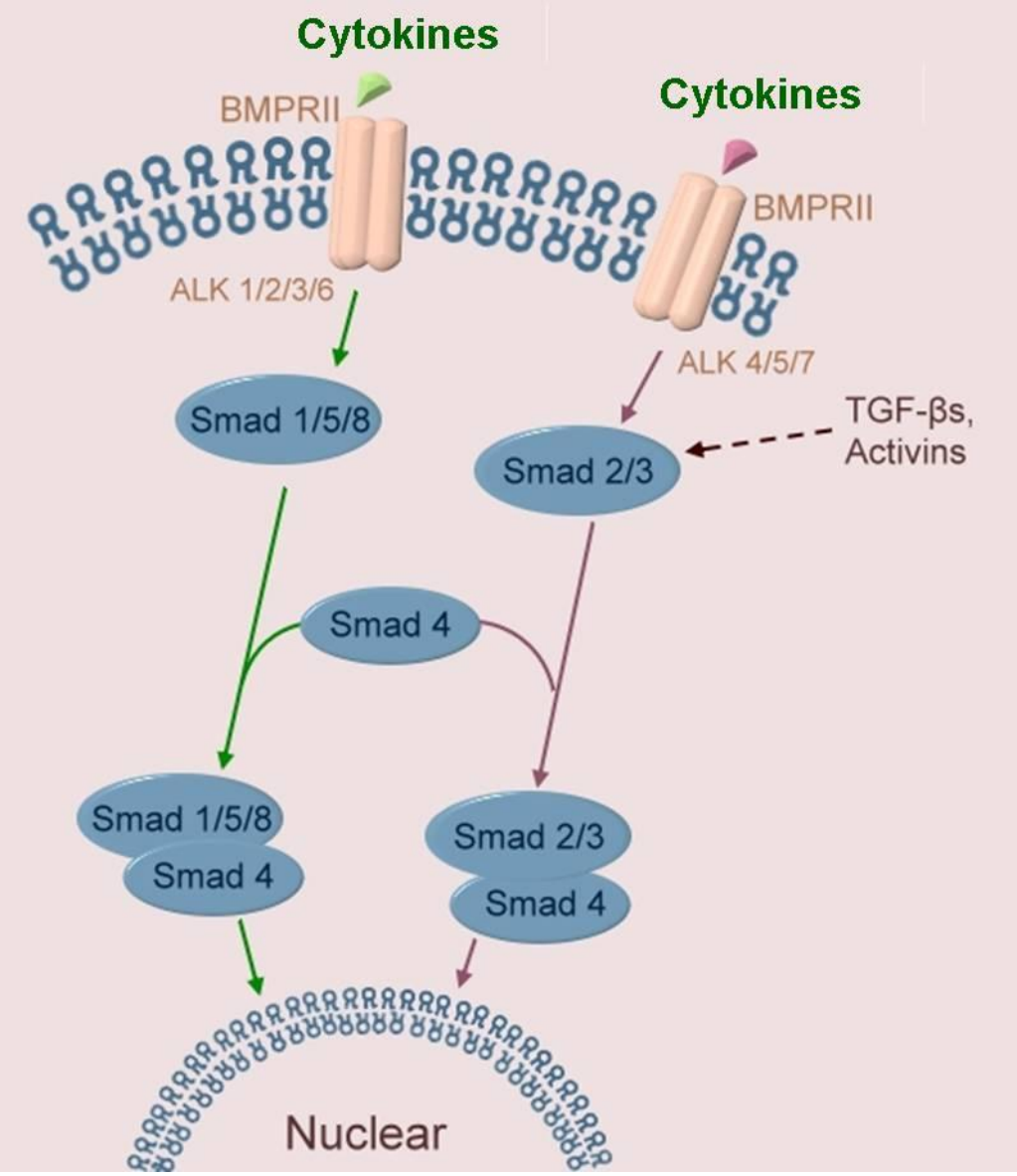


Figure 1-1: Signaling transduction pathway--- Smad pathway

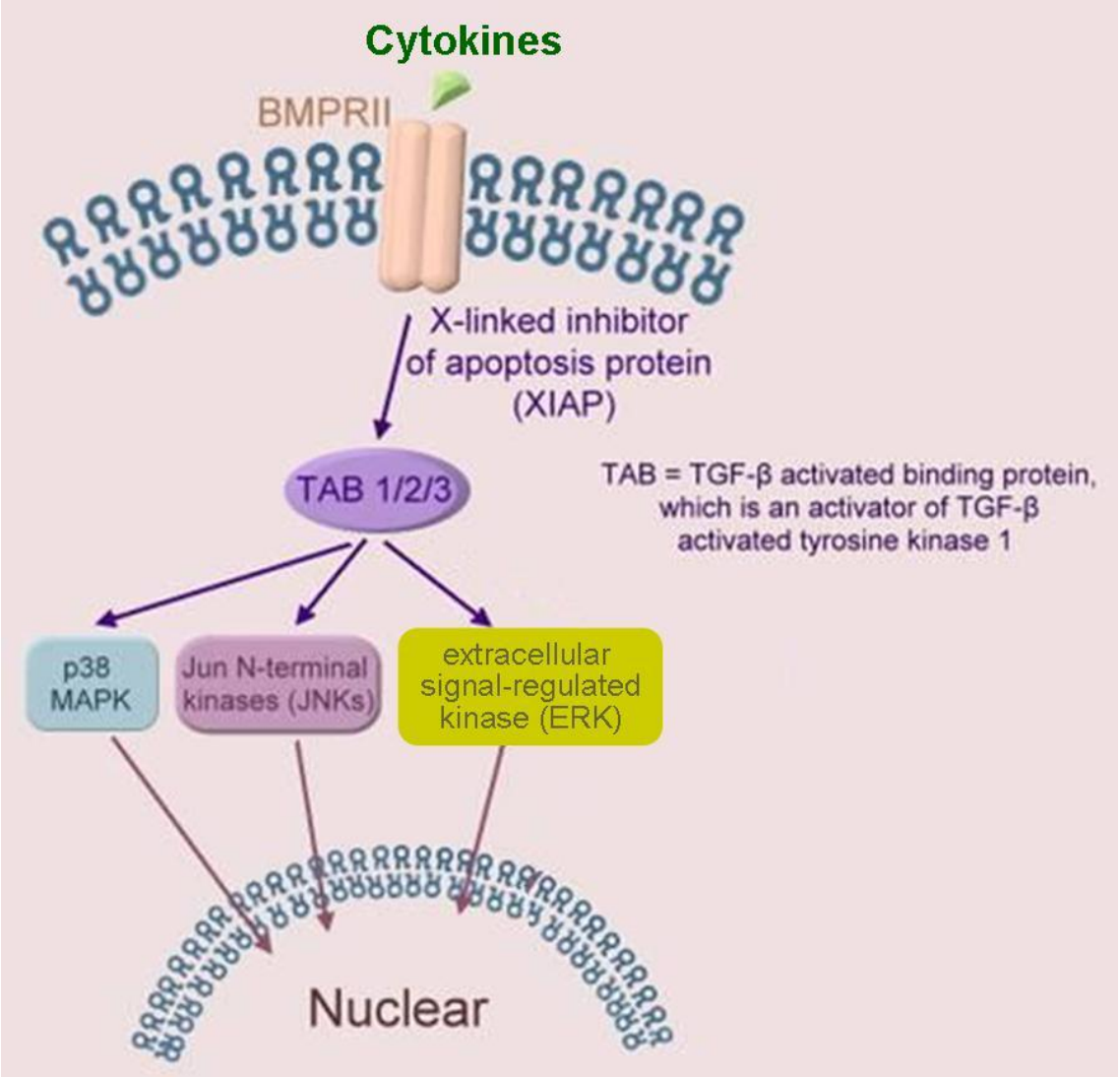


Figure 1-2: Signaling transduction pathway---MAPK pathway

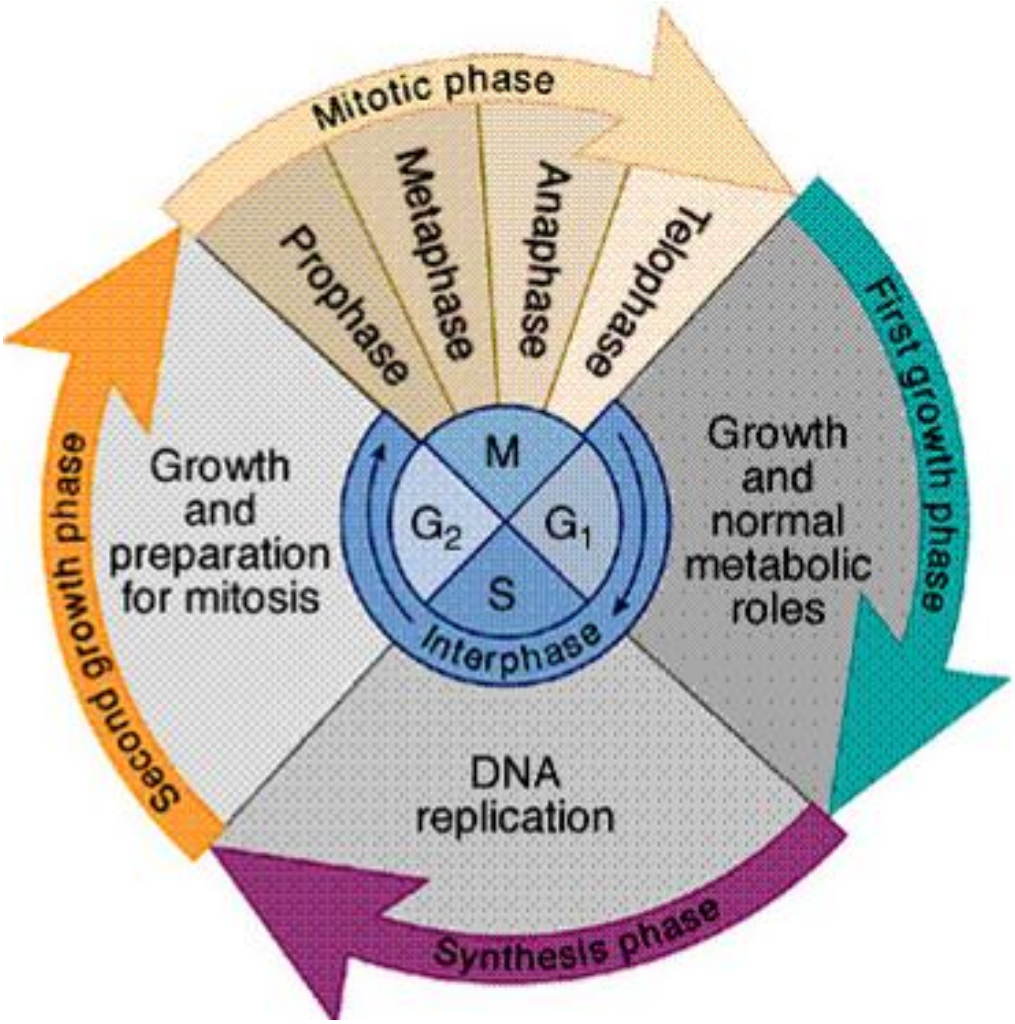


Figure 2: Cell cycle

Adapted from http://adasilva.glogster.com/chapter-10-bio/

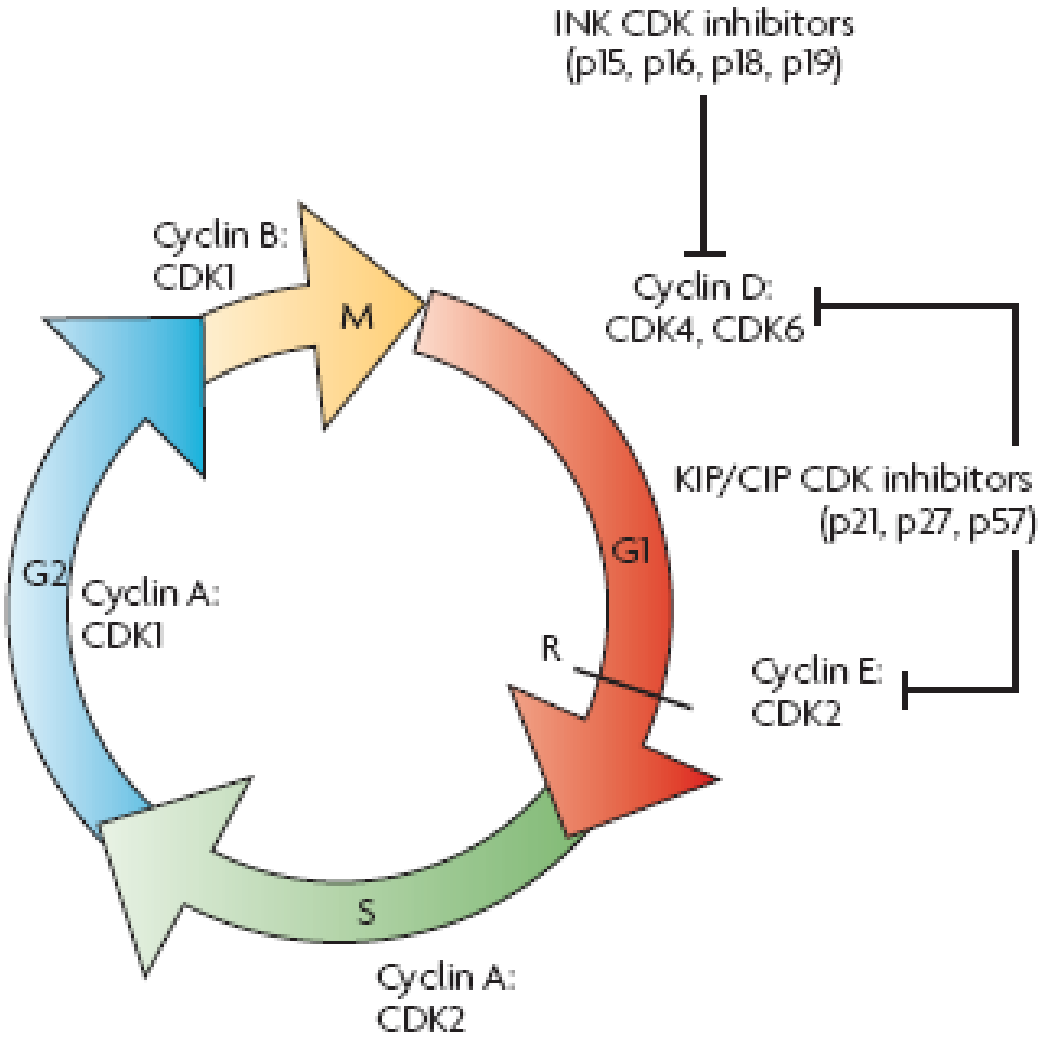


Figure 3: Checkpoints of cell cycle

Adapted from Dehay C. & Kennedy H. *Nature Reviews Neuroscience 2007; 8: 438-450*

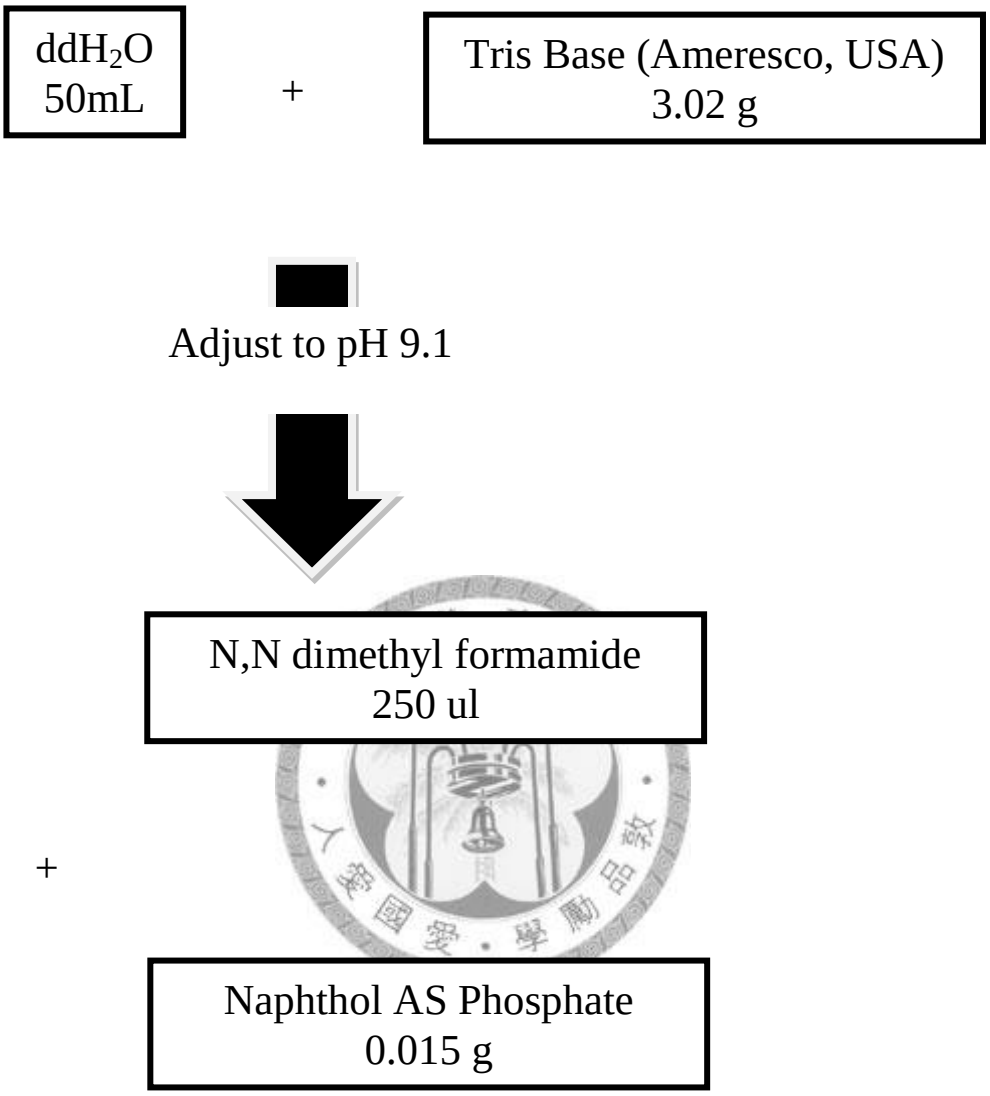


Figure 4: Protocol for stock substrate solution in Alkaline Phosphatase (ALP) Staining

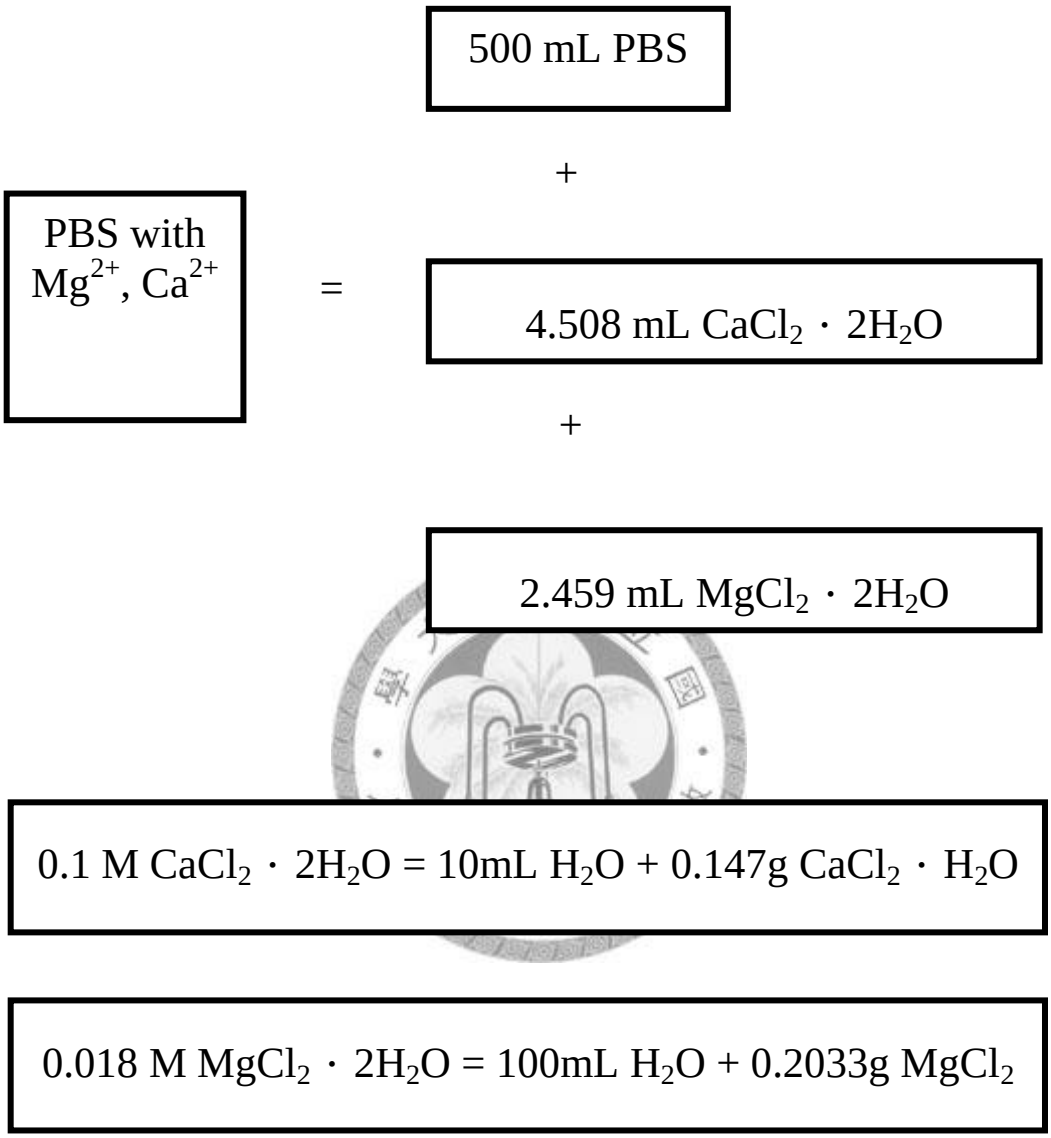


Figure 5: Protocol for preparation of PBS with Mg^{2+}, Ca^{2+} for Alkaline Phosphatase (ALP) Staining

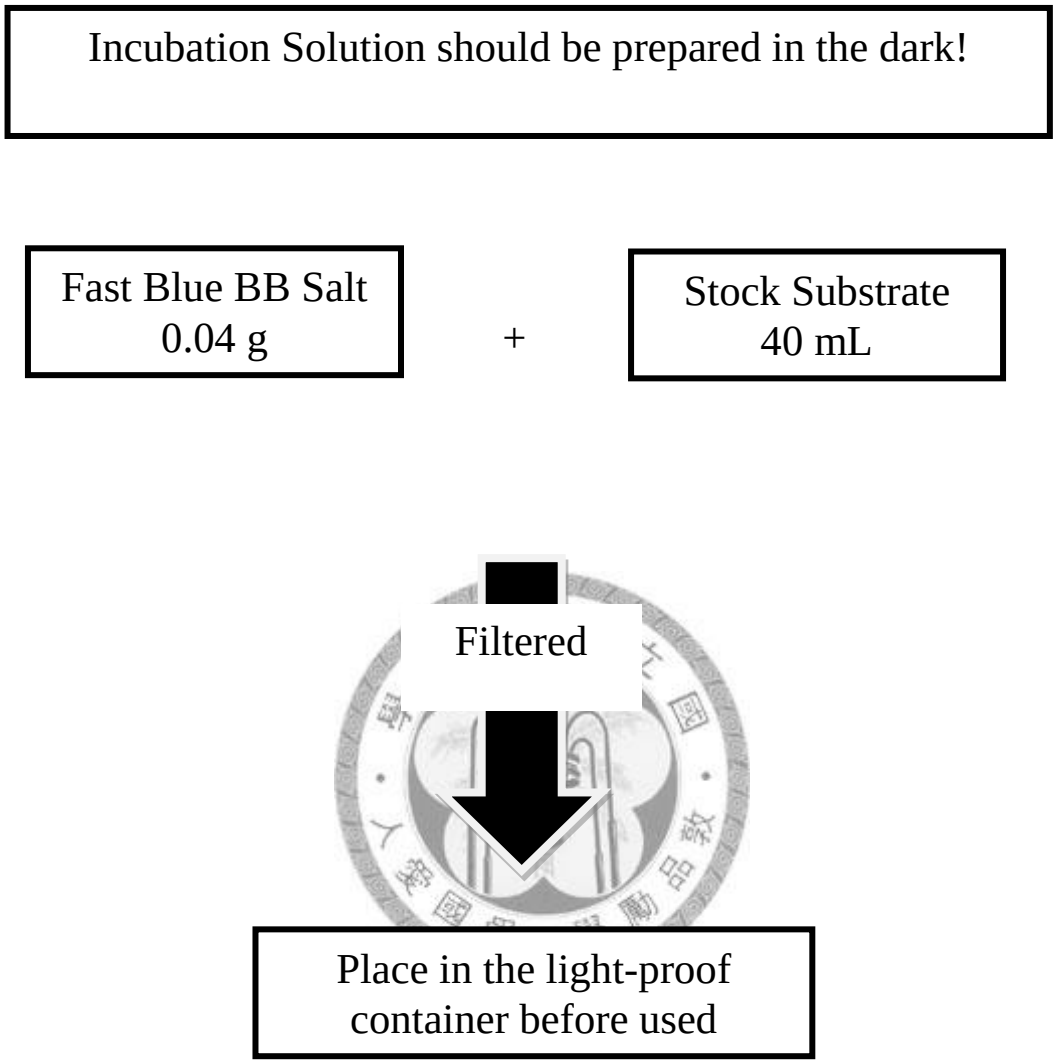


Figure 6: Protocol for preparation of incubation solution for Alkaline Phosphatase (ALP) Staining

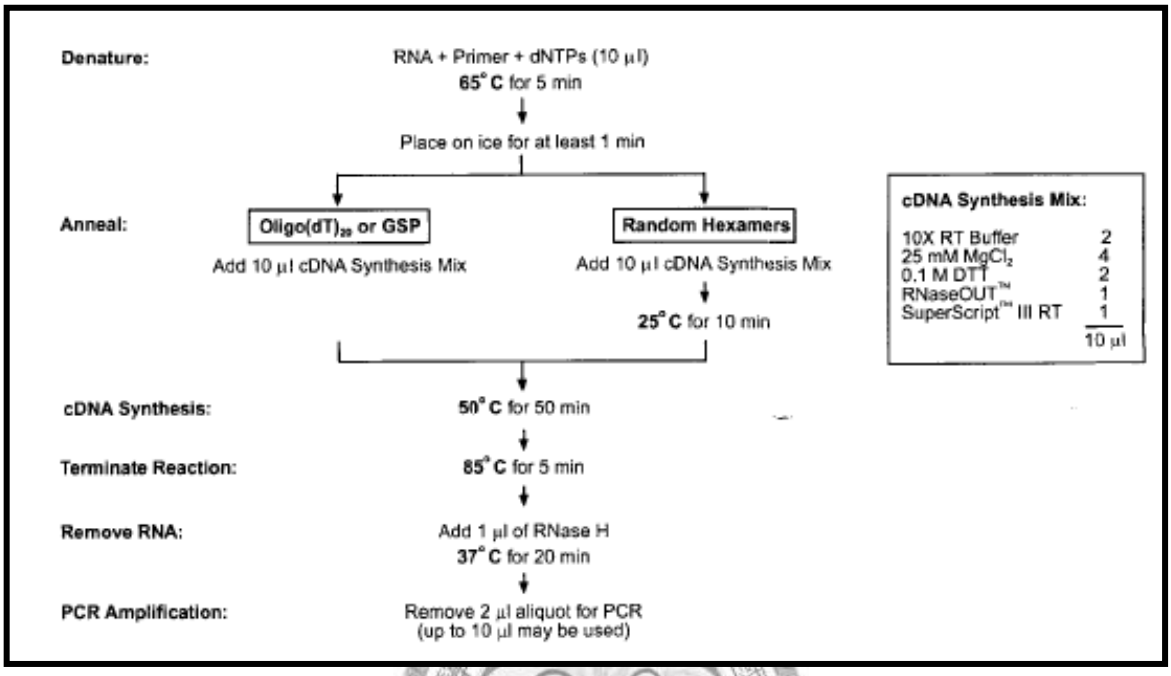
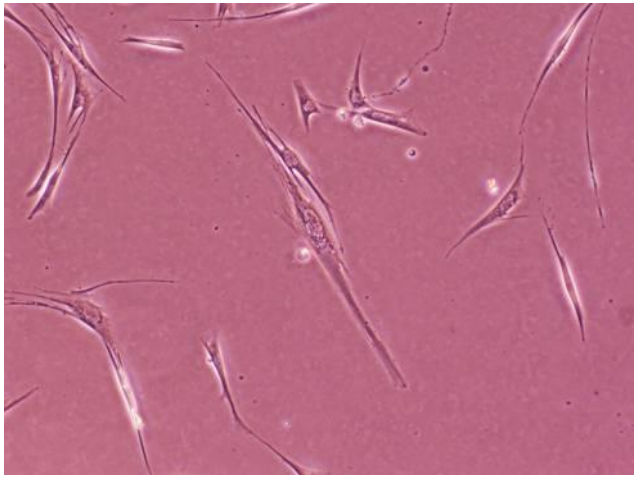
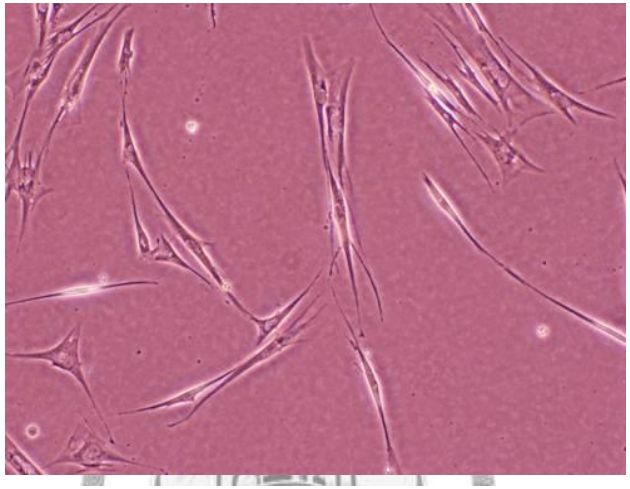


Figure 7: Protocol for total RNA isolation using Invitrogen SuperScript ™ III First Strand Synthesis System

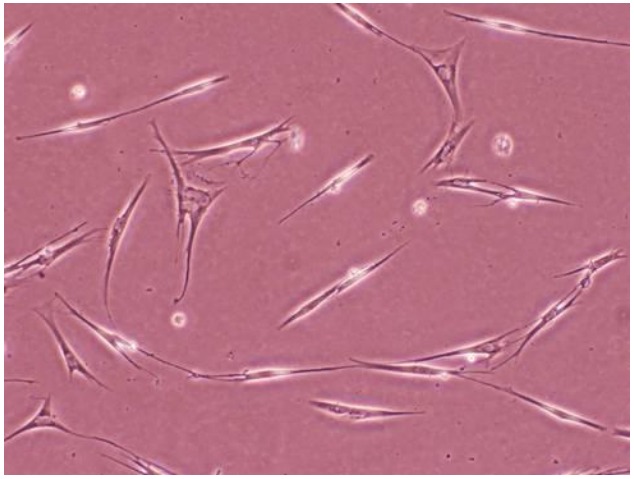
Human dental pulp cells treated with various concentration of GDF-5 in serum-free DMEM for 5 days



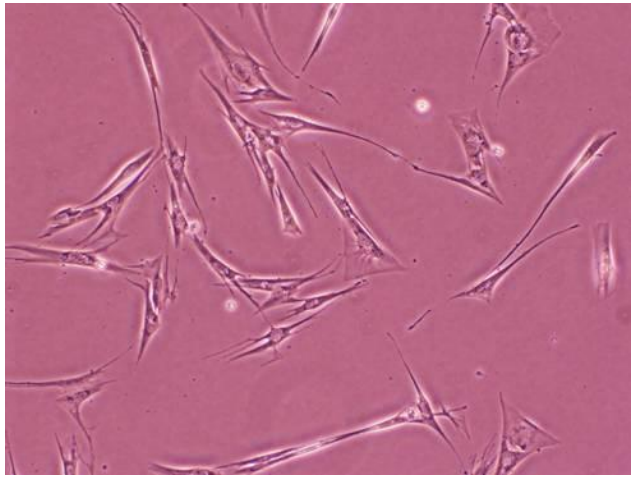
Negative control (NC)



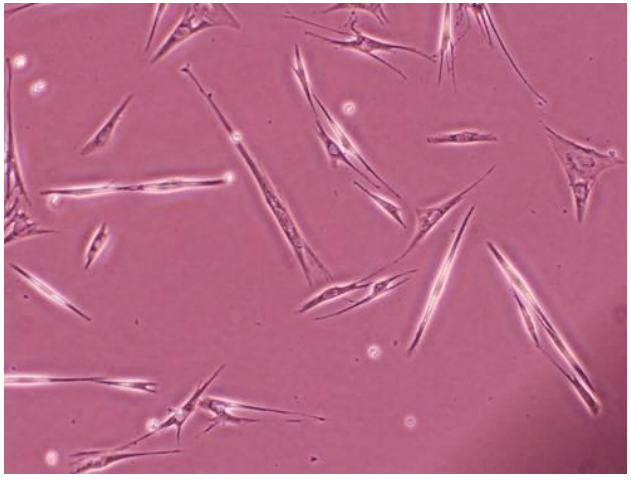
GDF-5 1ng/ml



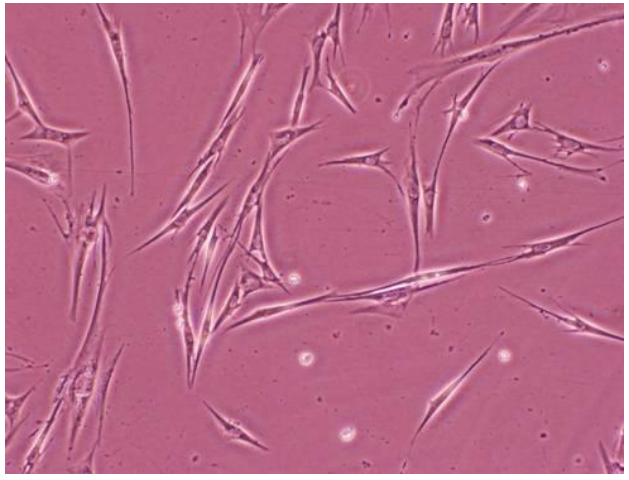
GDF-5 10ng/ml



GDF-5 50ng/ml



GDF-5 100ng/ml

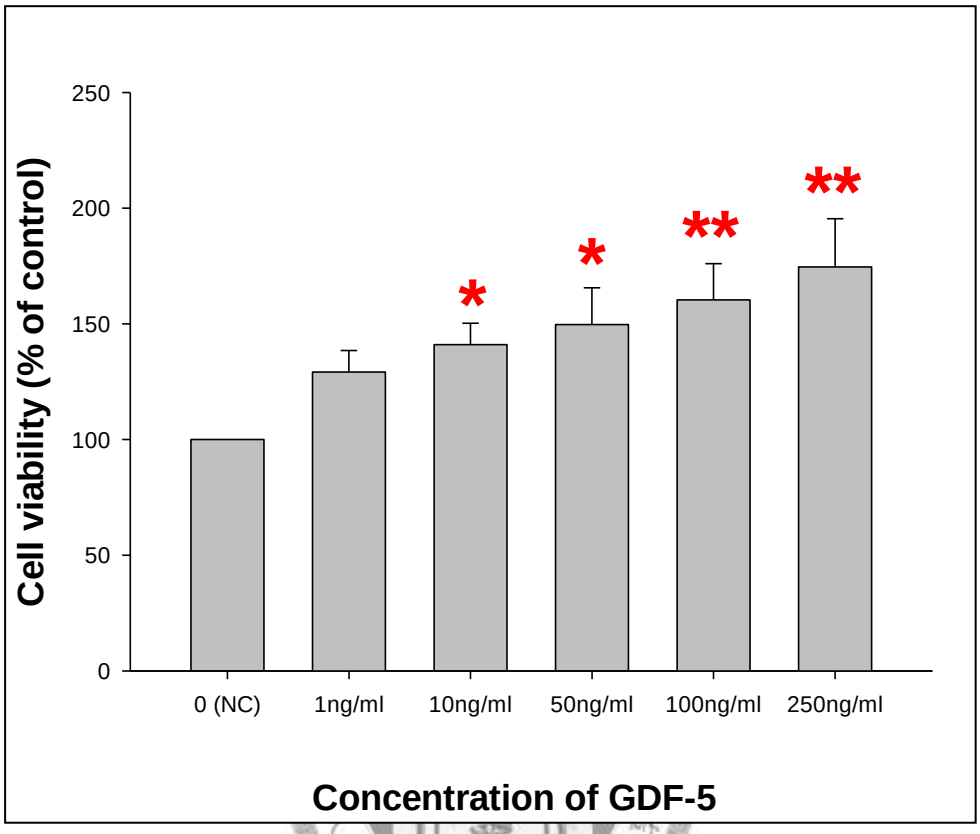


GDF-5 250ng/ml

Figure 8: Morphology of human dental pulp cells after 5 days treatment with GDF-5

Human dental pulp cells cultured in serum-free DMEM with various concentration of GDF-5 (0, 1, 10, 50, 100, 250 ng/ml). Cells were evaluated under phase contrast microscope. Dental pulp cells are spindle shape with extended cellular processes. The morphology of the dental pulp cell did not altered under the treatment of GDF-5 after 5 days incubation. However, the number of pulp cells treated with GDF-5 was higher than those without GDF-5 treatment, especially in the groups of higher concentration of GDF-5. (100X in magnification)

MTT- Pulp cells incubated with GDF-5 for 5 days



(*: p<0.05 comparing with NC group)

(**: p<0.01 comparing with NC group)

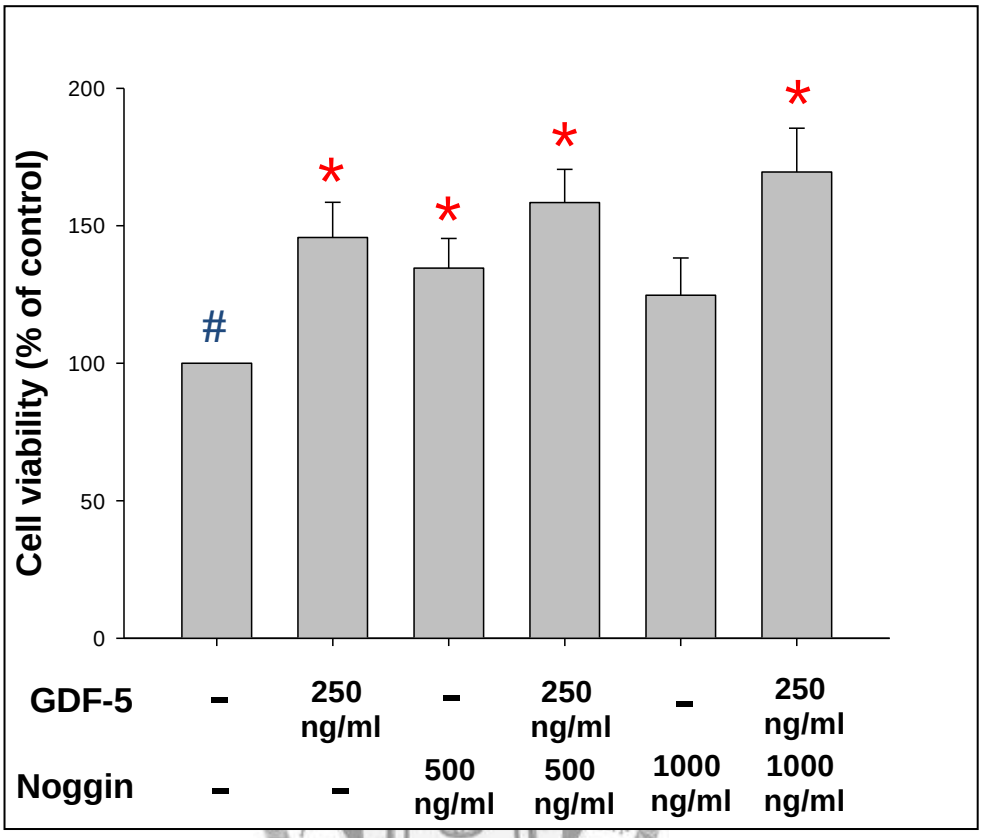
N=7

Mean ± SD

Figure 9: MTT assay: GDF-5 up-regulates dental pulp cell viability after 5-day incubation

Cell viability of human dental pulp cells was increased significantly in dose-dependent manner under the treatment of GDF-5 in serum-free DMEM.

MTT- Pulp cells incubated with GDF-5 & Noggin



(*: p<0.05 comparing with NC group)

(#: p<0.05 comparing with GDF-5 250ng/ml group)

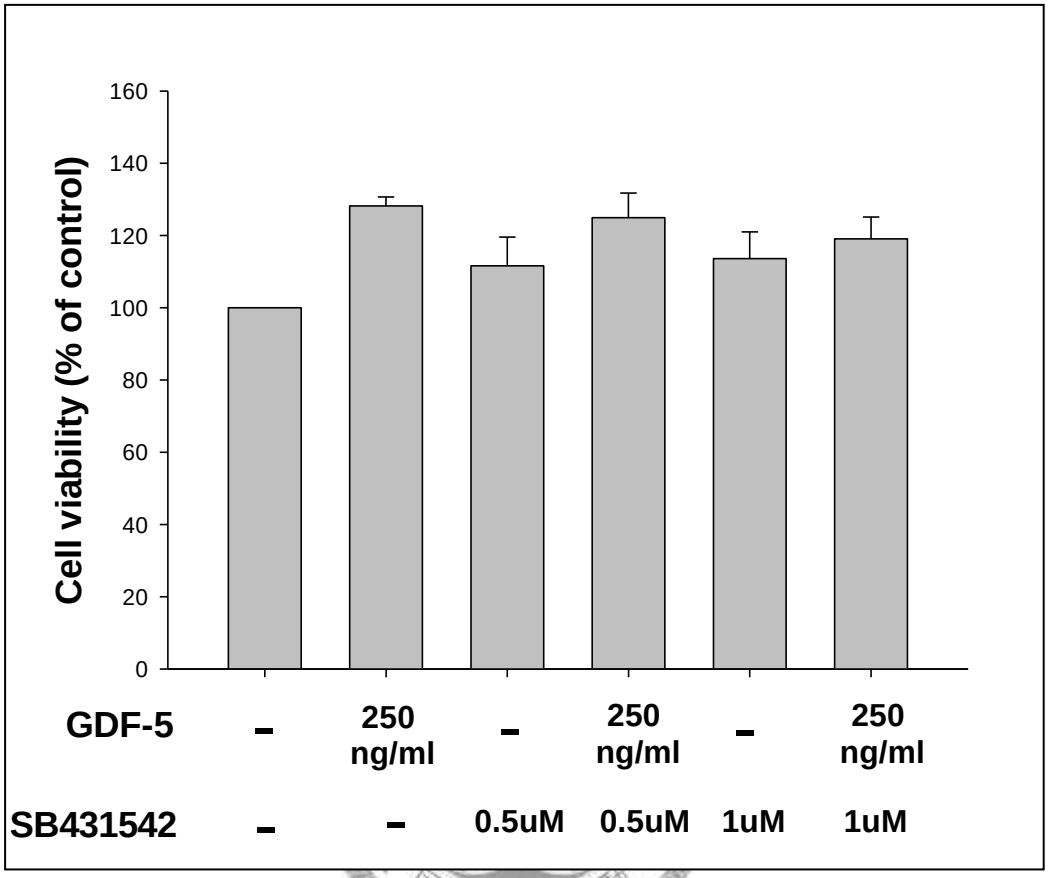
N=3

Mean ± SD

Figure 10-1: MTT assay: Pretreatment of Noggin (500 & 1000 ng/ml) had no effect on GDF-5-induced cell proliferation

5 days incubation of pulp cells under the treatment of GDF-5 250 ng/ml & Noggin 500 ng/ml and 1000 ng/ml. The cell viability was significantly induced under the treatment of GDF-5 250 ng/ml. Noggin could not block the effect of GDF-5, but slightly up-regulated the cell viability.

MTT- Pulp cells incubated with GDF-5 & SB431542



(*: p<0.05 comparing with NC group)

(#: p<0.05 comparing with GDF-5 250ng/ml group)

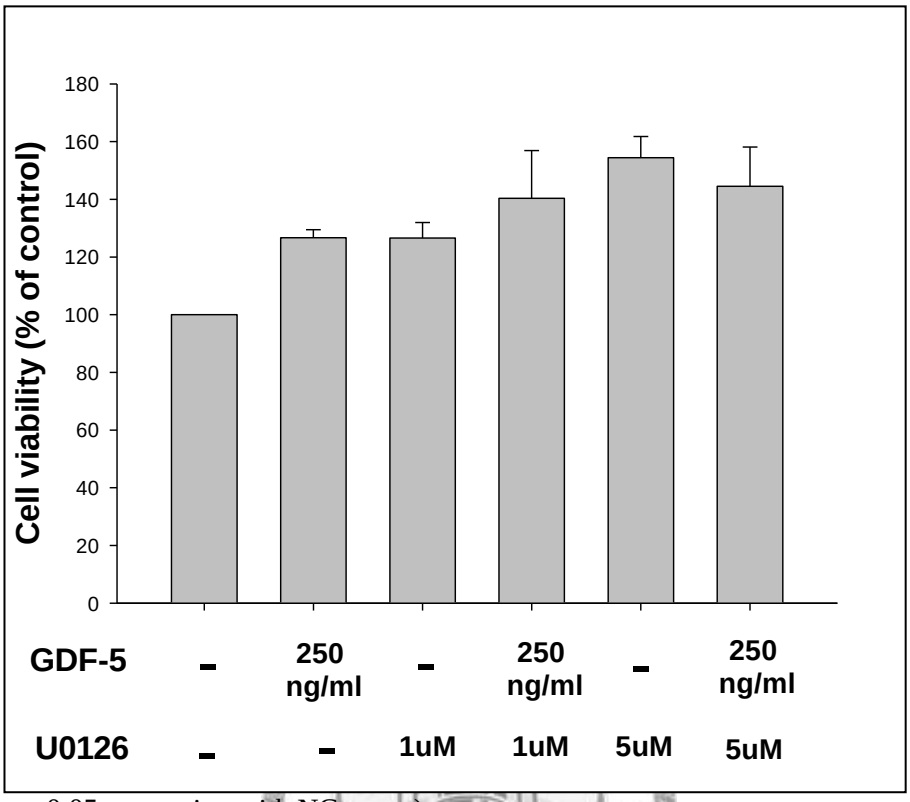
N=5

Mean ± SD

Figure 10-2: MTT assay: Pretreatment of SB431542 (0.5 & 1 uM) and it modulation of GDF-5

5 days incubation of pulp cells under the treatment of GDF-5 250 ng/ml & SB431542 0.5 uM and 1 uM. The up-regulation in cell viability of GDF-5 can be slightly reversed by SB431542 1μM.

MTT- Pulp cells incubated with GDF-5 & U0126



(*: p<0.05 comparing with NC group)

(#: p<0.05 comparing with GDF-5 250ng/ml group)

N=4

Mean ± SD

Figure 10-3: MTT assay: Pretreatment of U0126 (1 & 5 uM) had no effect on GDF-5-induced cell proliferation

5 days incubation of pulp cells under the treatment of GDF-5 250 ng/ml & U0126 1 uM and 5 uM. Pulp cell treated only with U0126 (1 & 5 uM) showed slightly increase in cell viability. However, in cells pretreated with U0126 before administration of GDF-5 (250 ng/ml), U0126 was not effective to prevent the GDF-5-induced rise of cell viability

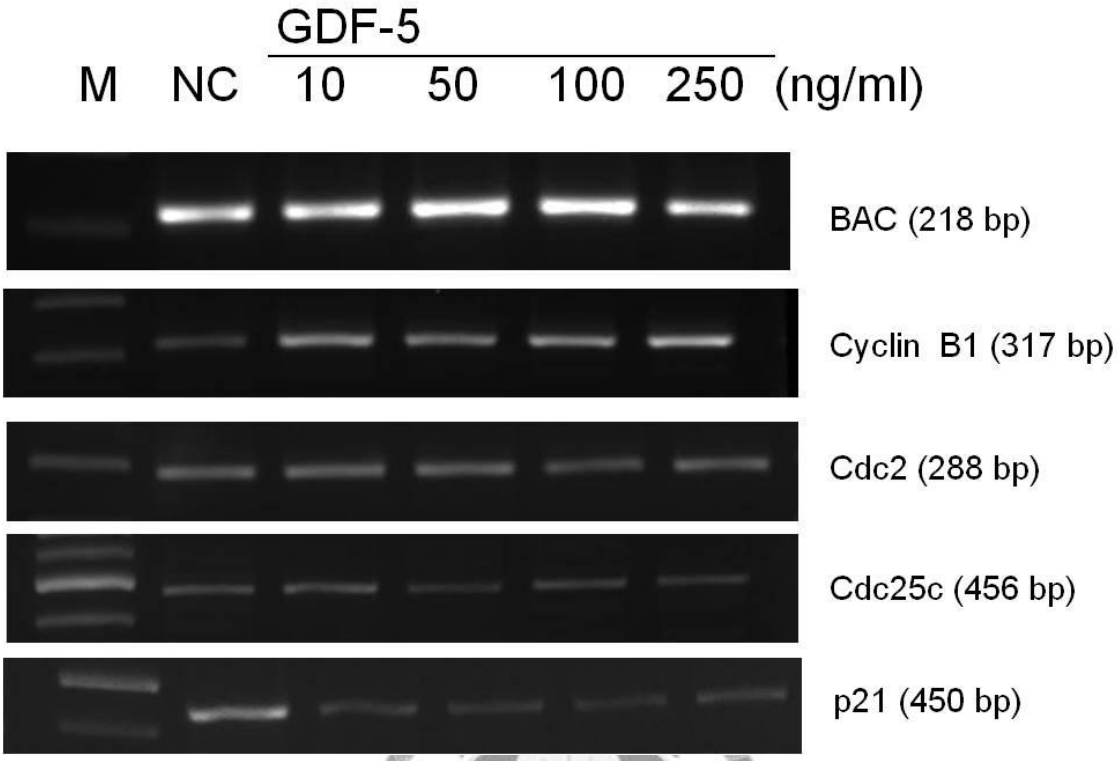
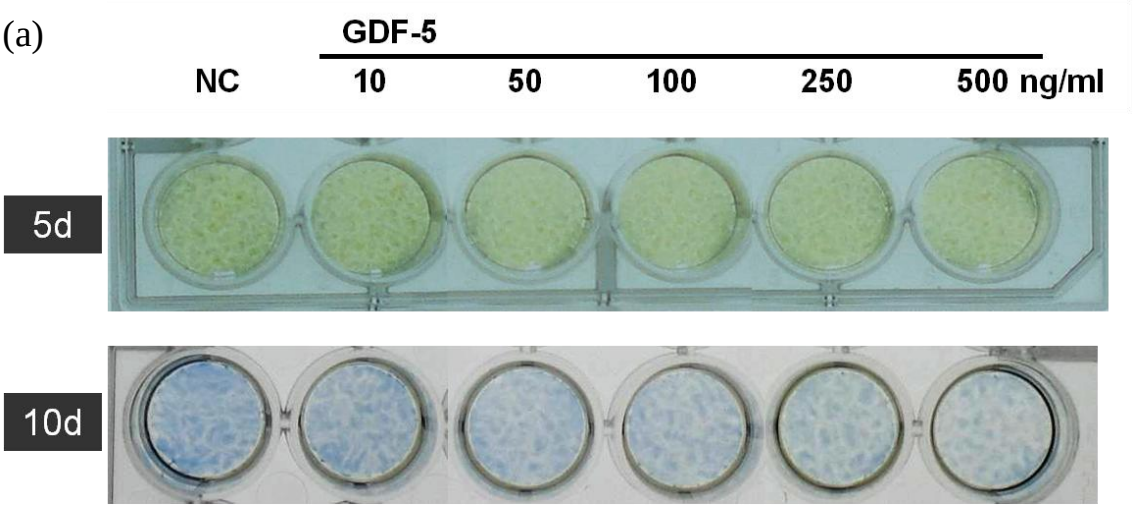


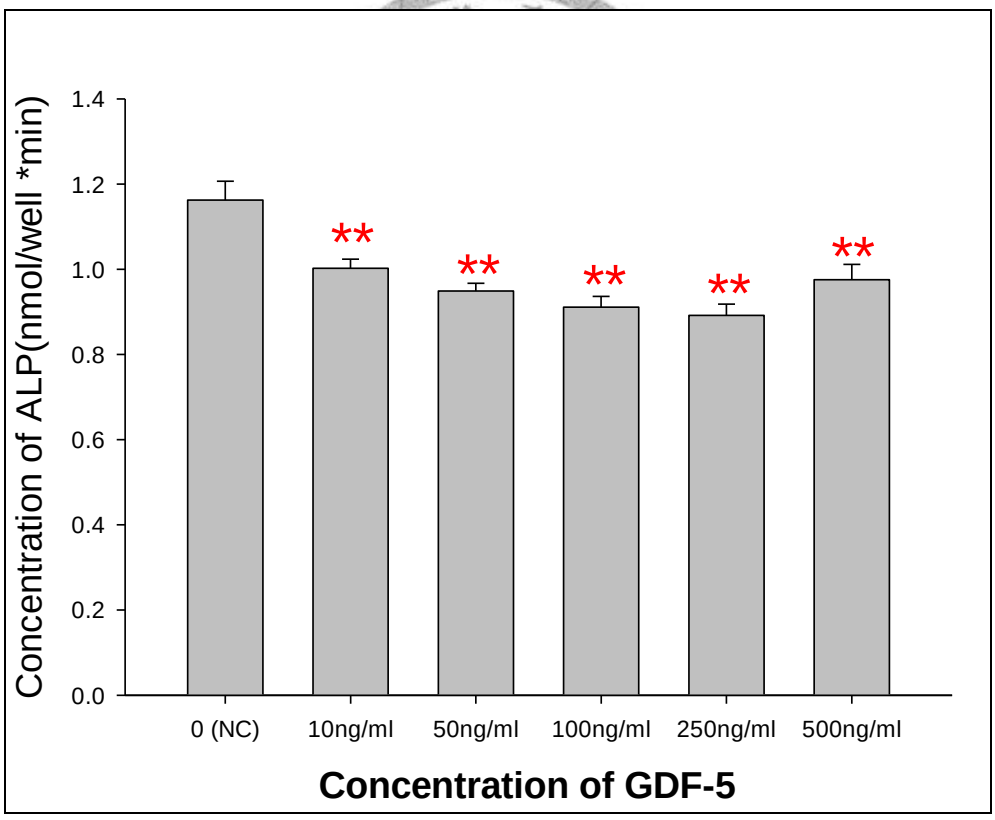
Figure 11: RT-PCR results of Cyclin B1, CDC2, CDC25C, and p21

Humen dental pulp cells were incubated with various concentration of GDF-5 in serum-free DMEM for 48 hours. Cyclin B1 mRNA expression of pulp cells was markly increased, especially in GDF-5 250 ng/ml group. Expression of Cdc2 and Cdc25c mRNA were not altered obviously. However, p21 mRNA expression was declined in GDF-5-treated groups.

(a)



(b)

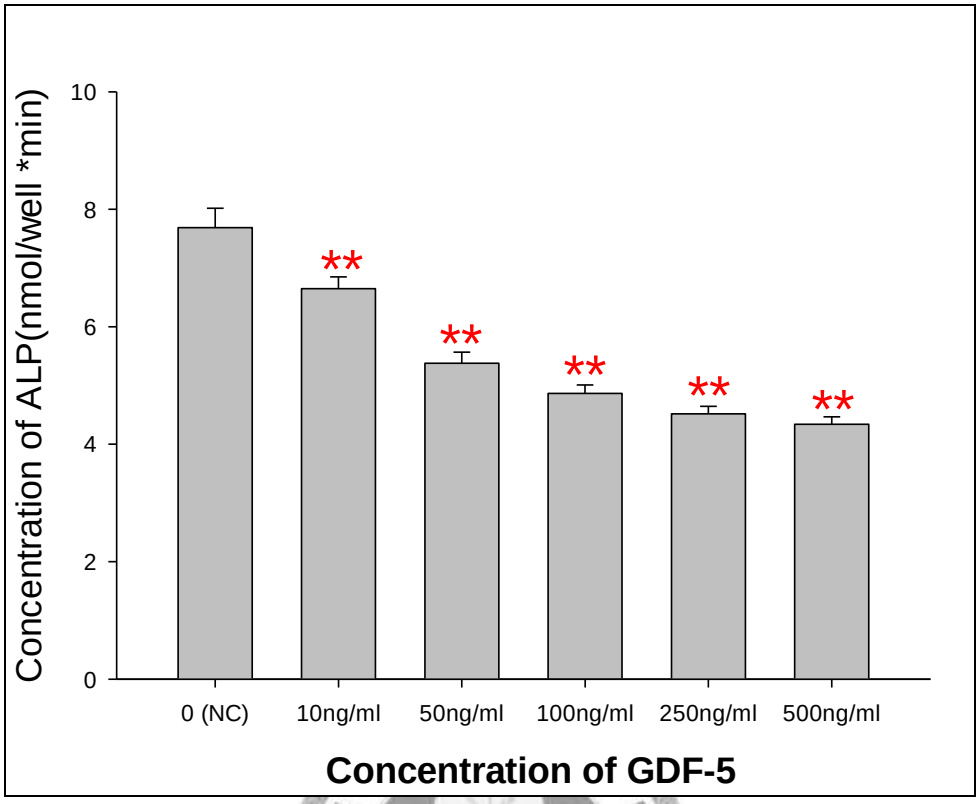


5-day incubation group

(**: $p<0.01$ comparing with NC group)

N=10

Mean ± SD



10-day incubation group

(**: p<0.01 comparing with NC group)

N=10

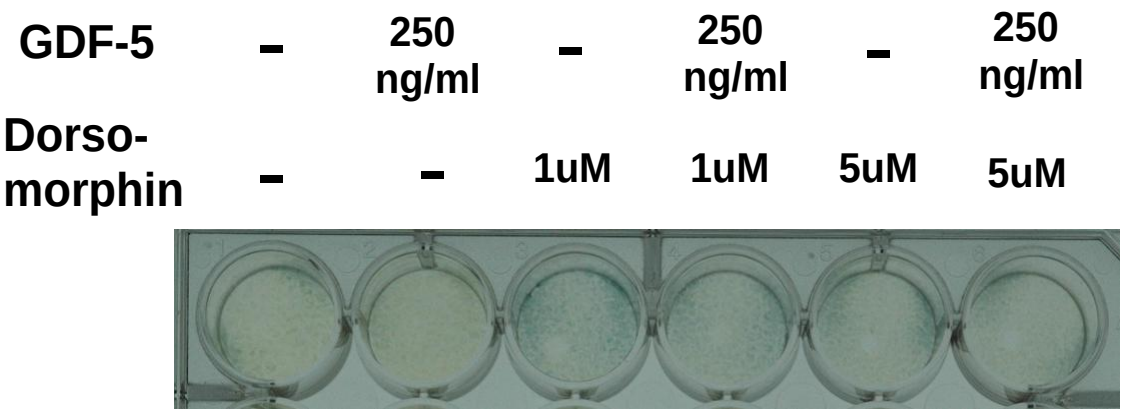
Mean ± SD

Figure 12: (a) ALP staining and (b) ALP quantitative assay of human dental pulp cells treated with or without GDF-5 in DMEM containing 10% FBS for 5 days and 10 days.

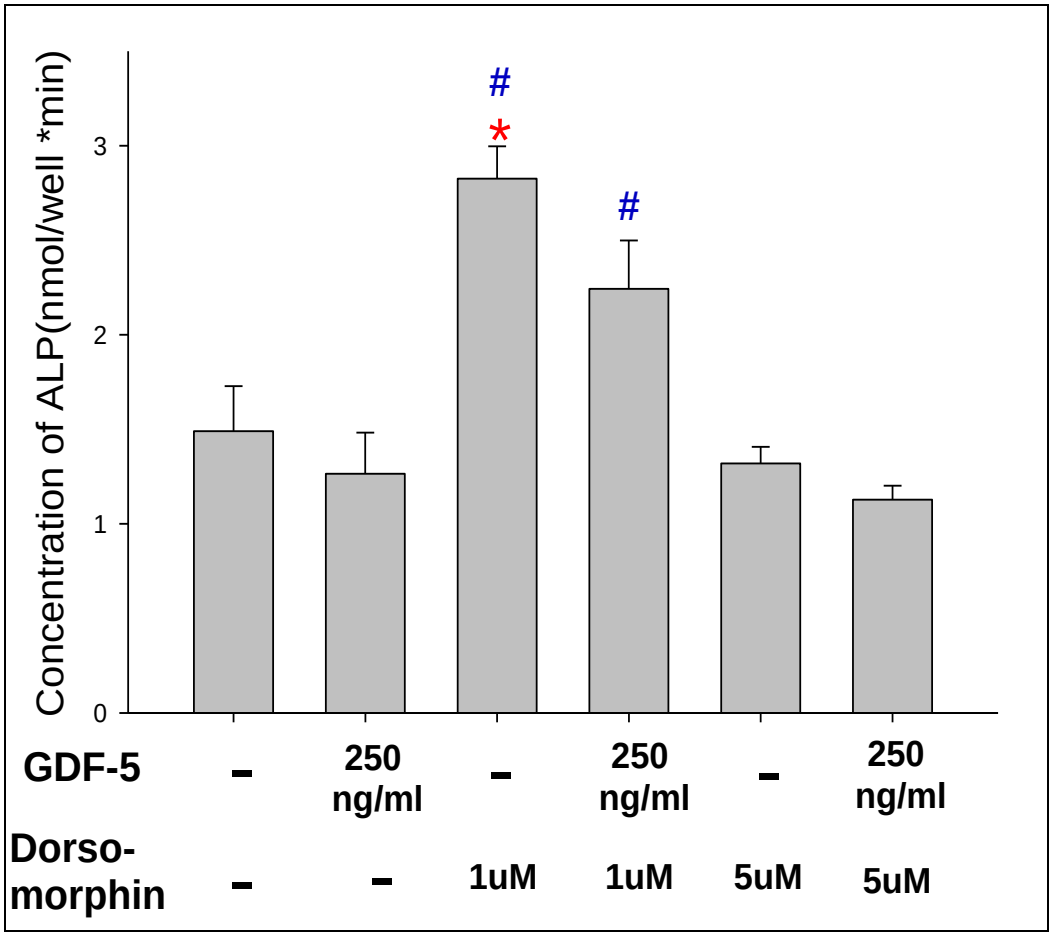
(a) After 5-day and 10-day incubation, human dental pulp cell without GDF-5 treatment showed positive response to ALP staining. Moreover, the level of ALP seemed to be decreased from 10 to 500 ng/ml in a dose dependent manner, especially in 10-day-cultured group.

(b) In both 5-day-cultured and 10-day-cultured experiment of ALP quantitative assay, GDF-5 decreased the concentration of p-Nitrophenol obviously. There are significant differences in groups of GDF-5 10, 50, 100, 250, 500 ng/ml, comparing to the control group. In the 5-day-cultured group, the most declination of ALP activity was the GDF-5 250 ng/ml group; in the 10-day-cultrued group, GDF-5 declined the ALP activity of human dental pulp cells dose-dependently.

(a)



(b)



(*: p<0.05 comparing with NC group)

(#: p<0.05 comparing with GDF-5 250ng/ml group)

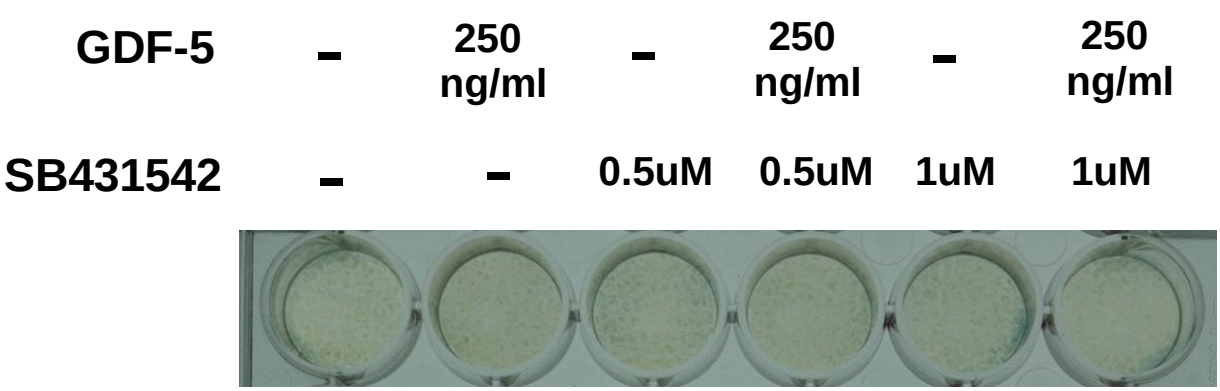
N=6

Mean ± SD

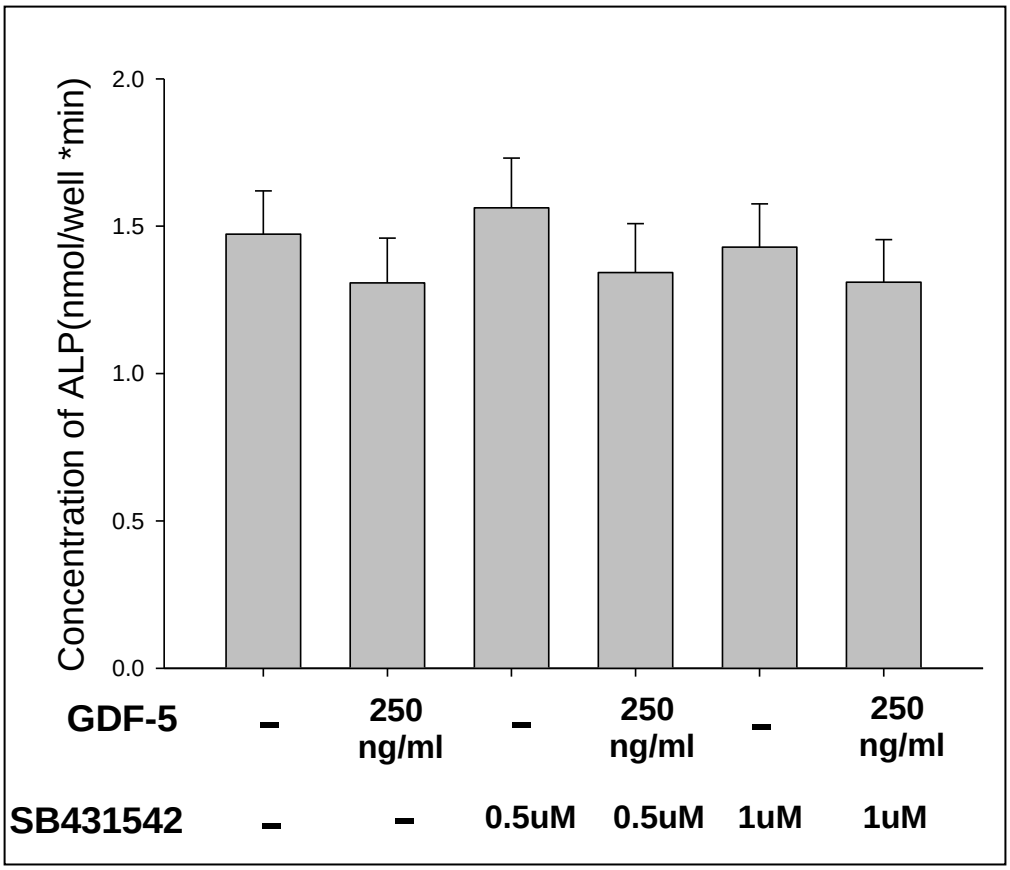
Figure 13-1: (a) ALP staining and (b) ALP quantitative assay of human dental pulp cells treated by GDF-5 (250 ng/ml) with or without pretreatment of dorsomorphin (1 & 5 uM) in DMEM containing 10% FBS for 5 days.

5 days incubation of pulp cells under the treatment of GDF-5 250 ng/ml & Dorsomorphin 1 uM and 5 uM in DMEM containing 10% FBS. At concentration of 250 ng/ml, GDF-5 declined the ALP activity of human dental pulp cells as analyzed ALP staining and ALP quantitative assay. Pulp cells treated only with Dorsomorphin (1 uM) showed moderate increase in ALP activity and pulp cells treated only with Dorsomorphin (5 uM) showed slightly decrease in ALP activity, in comparison with the normal control group. However, Dorsomorphin could not reverse the down-regulatory effect caused by GDF-5.

(a)



(b)



(*: p<0.05 comparing with NC group)

(#: p<0.05 comparing with GDF-5 250ng/ml group)

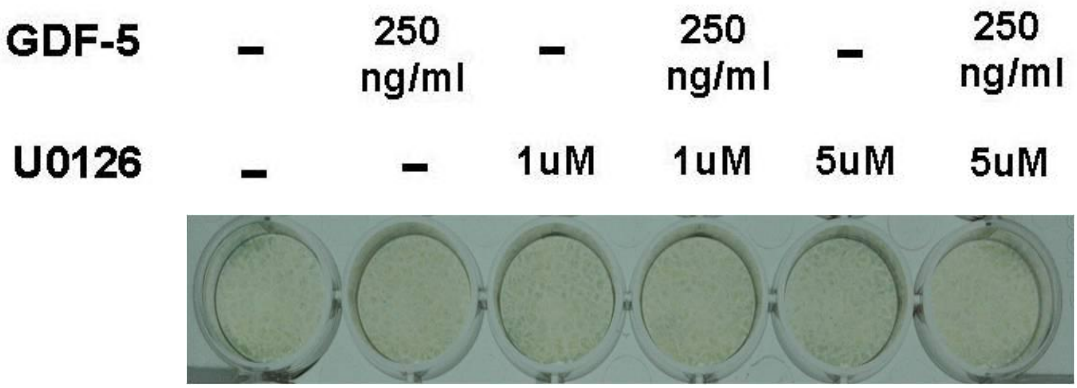
N=8

Mean ± SD

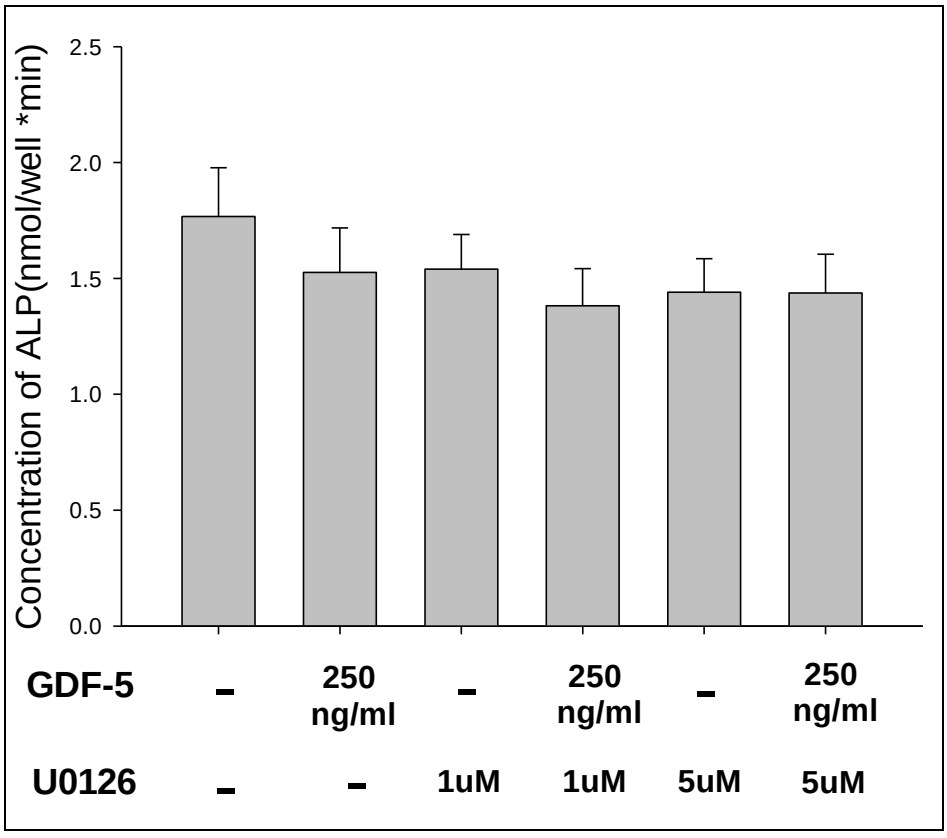
Figure 13-2: (a) ALP staining and (b) ALP quantitative assay of human dental pulp cells treated by GDF-5 (250 ng/ml) with or without pretreatment of SB431542 (0.5 & 1 uM) in DMEM containing 10% FBS for 5 days.

5 days incubation of pulp cells under the treatment of GDF-5 250 ng/ml & SB431542 0.5 uM and 1 uM in DMEM containing 10% FBS. SB431542 (0.5 & 1 uM) itself did not affect the pulp cell in ALP activity comparing with the normal control group. However, SB431542, as an ALK-4/5/7&Smad-2/3 specific inhibitor, still could not attenuate the GDF-5-induced decrease of ALP activity

(a)



(b)



(*: p<0.05 comparing with NC group)

(#: p<0.05 comparing with GDF-5 250ng/ml group)

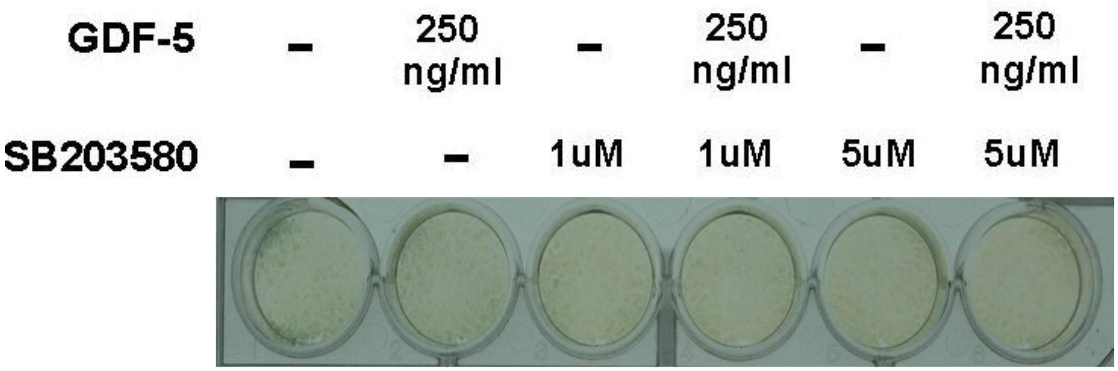
N=6

Mean ± SD

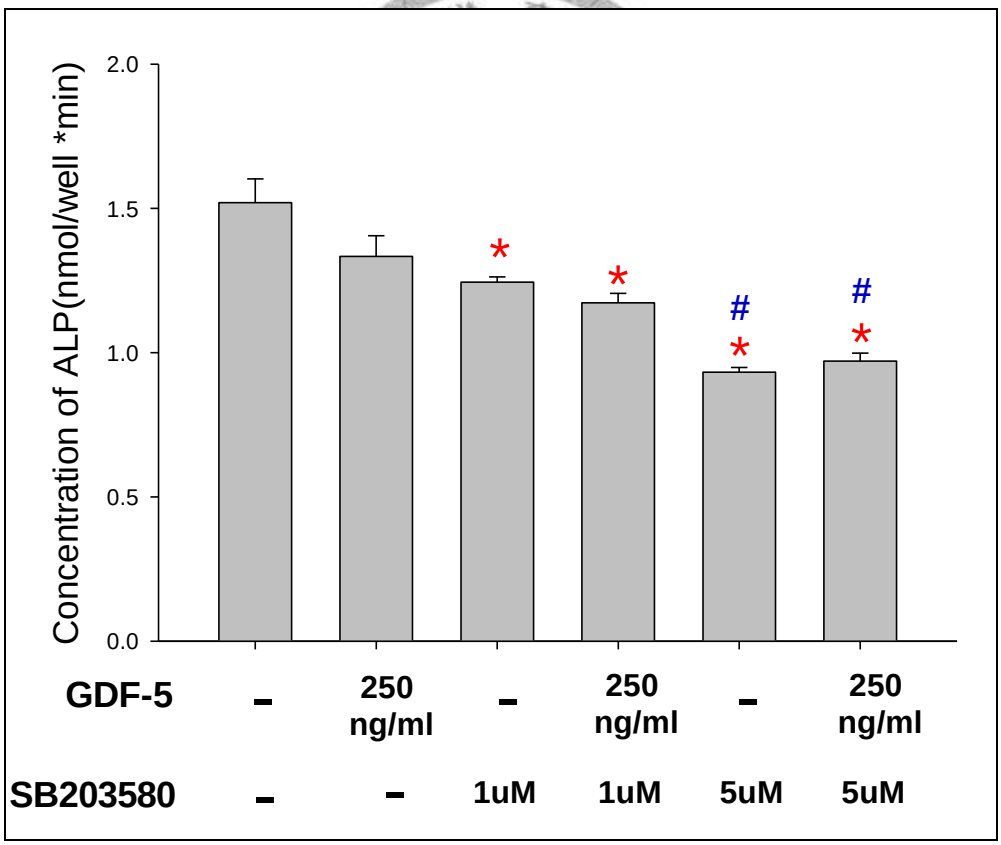
Figure 13-3: (a) ALP staining and (b) ALP quantitative assay of human dental pulp cells treated by GDF-5 (250 ng/ml) with or without pretreatment of U0126 (1 & 5 uM) in DMEM containing 10% FBS for 5 days.

5 days incubation of pulp cells under the treatment of GDF-5 250 ng/ml & U0126 1 uM and 5 uM in DMEM containing 10% FBS. Pulp cell treated only with U0126 (1 & 5 uM) showed slightly decrease in ALP activity. However, in cells pretreated with U0126 before administration of GDF-5 (250 ng/ml), U0126 was not effective to prevent the GDF-5-induced declination of ALP activity.

(a)



(b)



(*: p<0.05 comparing with NC group)

(#: p<0.05 comparing with GDF-5 250ng/ml group)

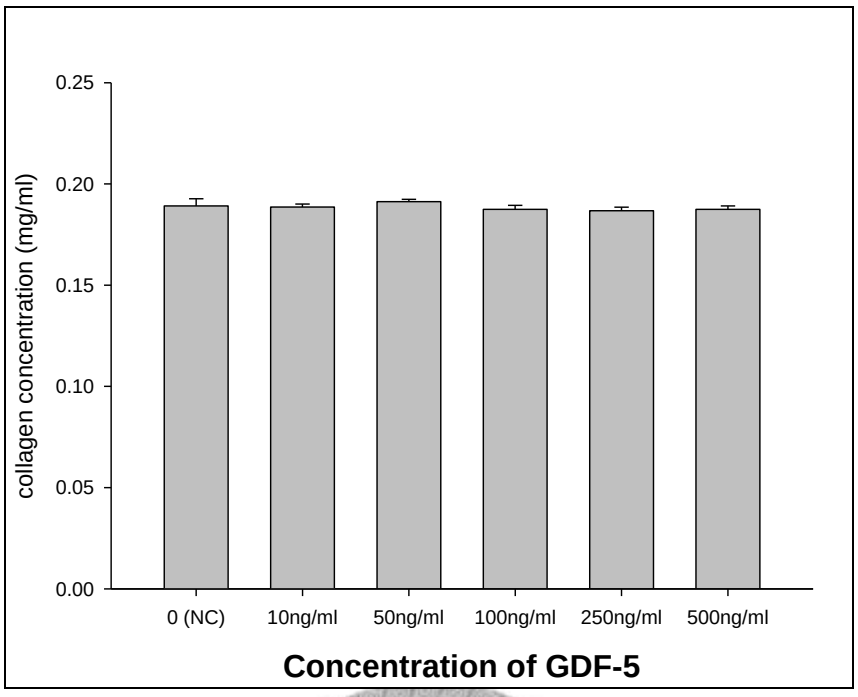
N=6

Mean ± SD

Figure 13-4: (a) ALP staining and (b) ALP quantitative assay of human dental pulp cells treated by GDF-5 (250 ng/ml) with or without pretreatment of SB203580 (1 & 5 uM) in DMEM containing 10% FBS for 5 days.

5 days incubation of pulp cells under the treatment of GDF-5 250 ng/ml & SB203580 1 uM and 5 uM in DMEM containing 10% FBS. SB203580 (1 & 5 uM) itself showed inhibitory effect on ALP activity, especially at concentration of 5 uM ($p<0.05$). However, SB203580 still could not reverse the down-regulatory effect caused by GDF-5.

(a)



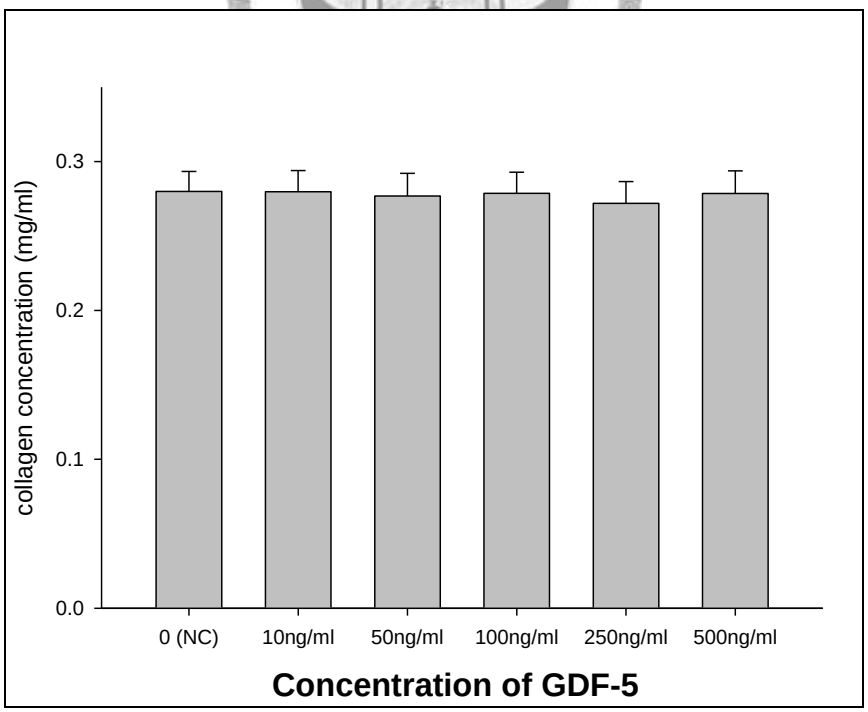
5-day incubation group

(*: p<0.05 comparing with NC group)

N=7

Mean ± SD

(b)



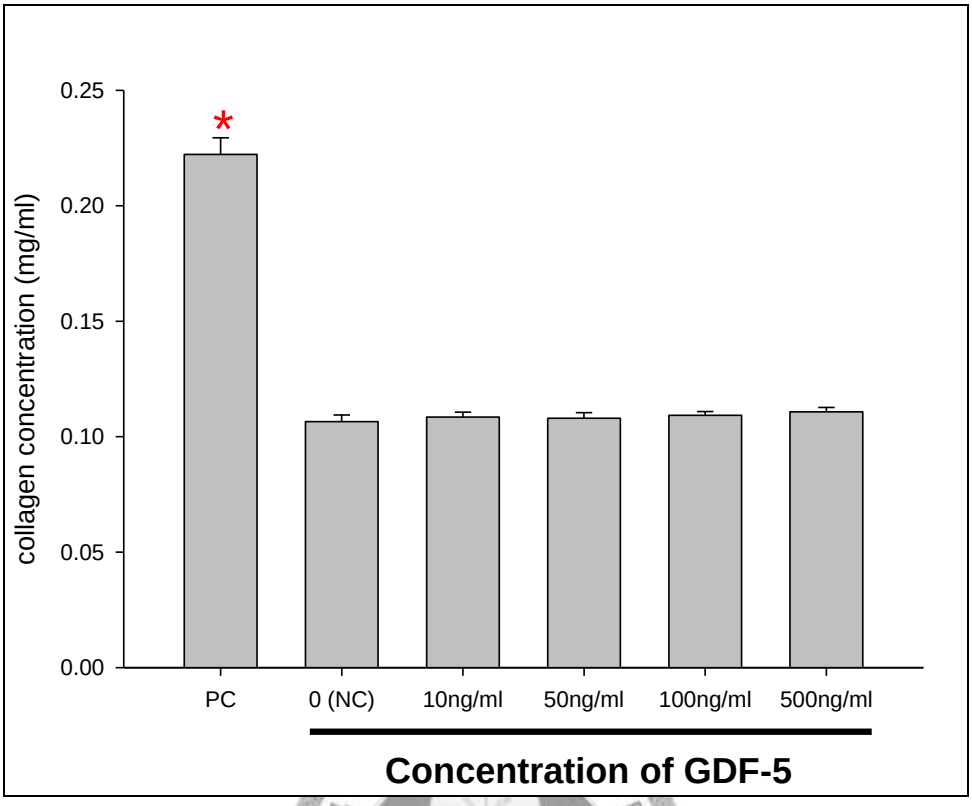
10-day incubation group

(*: p<0.05 comparing with NC group)

N=12 Mean ± SD

Figure 14-1: Sircol collagen assay---condition I experiment

Human dental pulp cells were incubated with various concentrations of GDF-5 in DMEM containing 10% FBS for (a) 5 days or (b) 10 days. Neither 5-day incubation nor 10-day incubation, GDF-5 had no effect on the collagen content of human dental pulp cells.



5-day incubation in serum-free DMEM

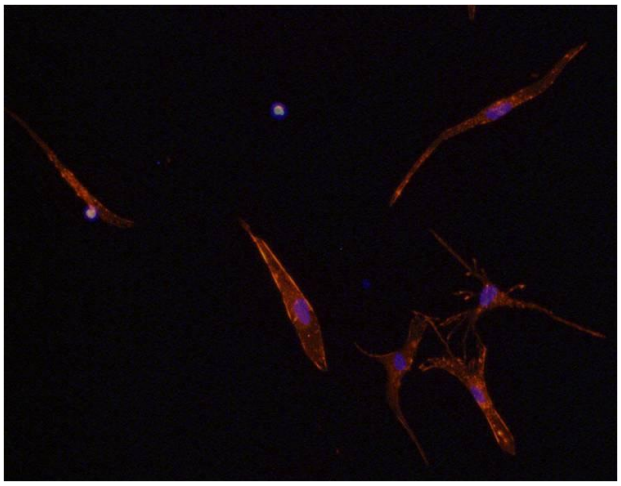
(*: $p<0.05$ comparing with NC group)

N=12

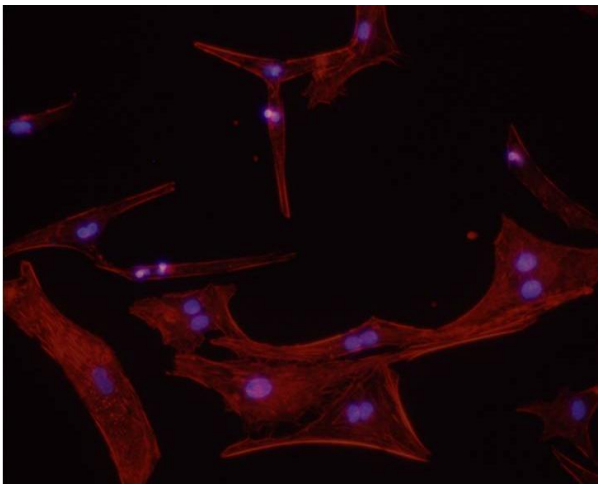
Mean ± SD

Figure 14-2: Sircol collagen assay---condition II experiment

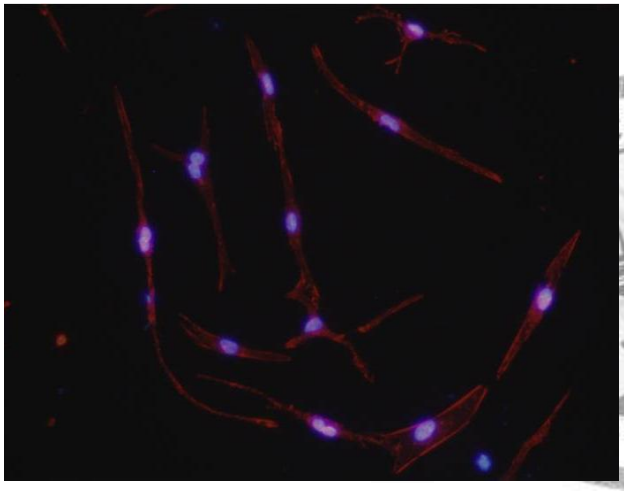
Human dental pulp cells were incubated with various concentrations of GDF-5 in serum-free DMEM for 5 days, and pulp cells which were incubated in DMEM containing 10% FBS was positive control group. Positive control group showed significant higher collagen content than normal control group. However, GDF-5 still had no effect on the collagen content of human dental pulp cells.



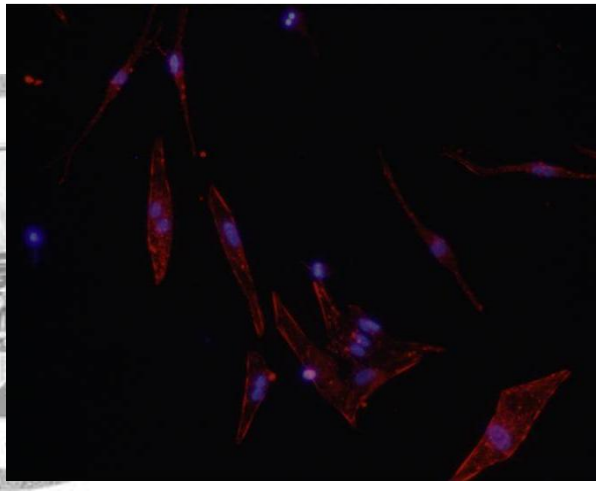
Negative control



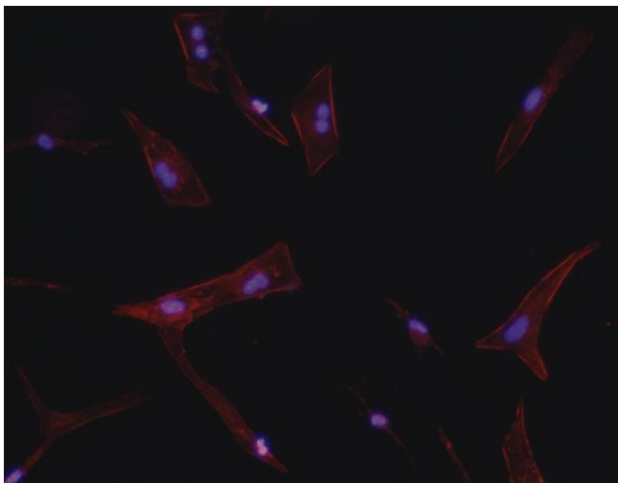
Positive control



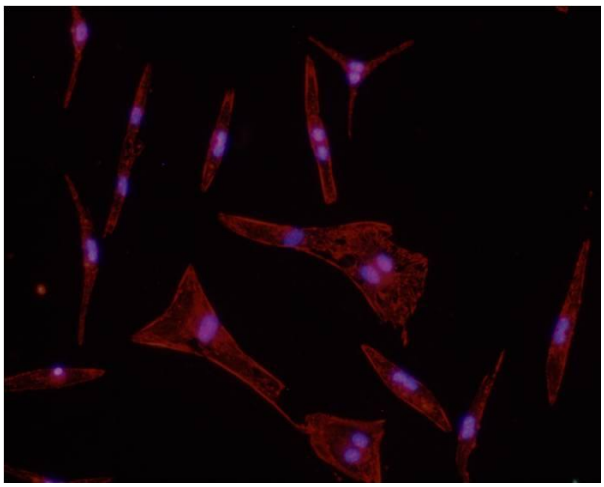
GDF-5 10 ng/ml



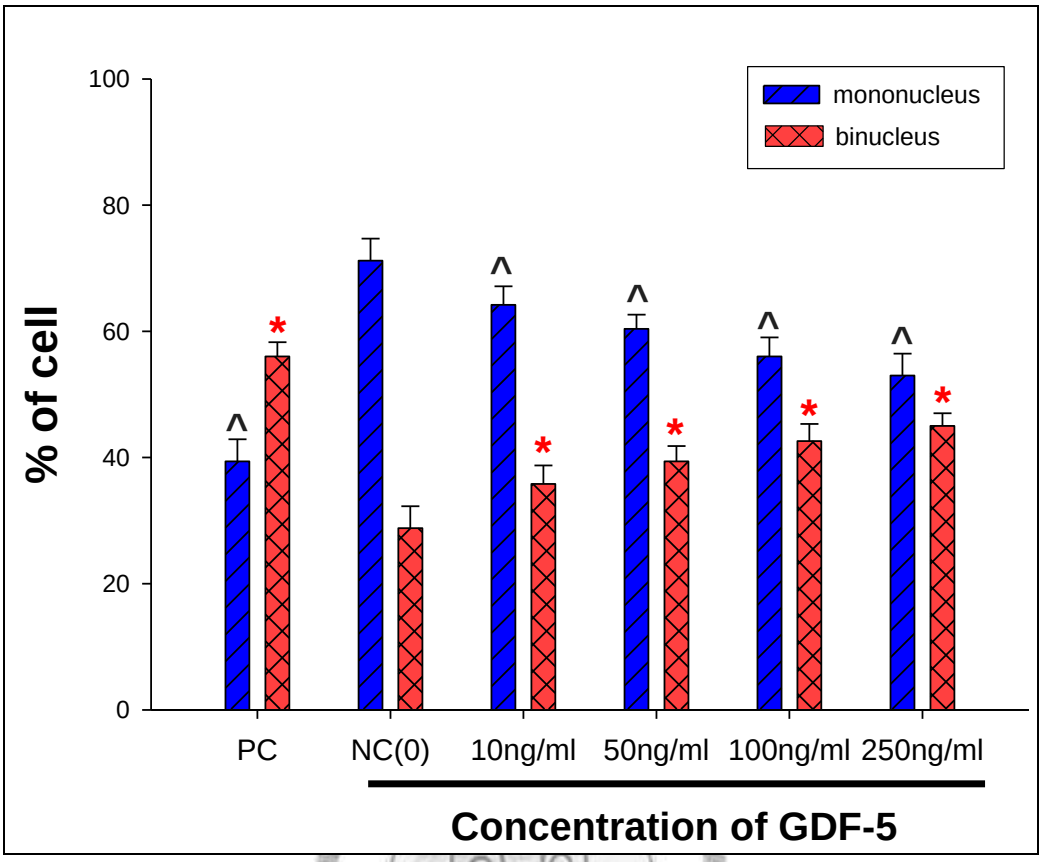
GDF-5 50 ng/ml



GDF-5 100 ng/ml



GDF-5 250 ng/ml



(^: p<0.05 comparing with NC group in mononucleus group)

(*: p<0.05 comparing with NC group in binucleus group)

N=5

Mean ± SD

Figure 15: Cell mitotic index---immunofluorescence assay

In our experiment, the percentage of binuclear cells was significantly higher in group of GDF-5 10, 50, 100, 250 ng/ml than that in negative control group. On the contrary, the percentage of mononuclear cells was significantly lower in these experimental groups comparing to that in negative control groups. Take together, GDF-5 increased the percentages of binuclear cells and decreased that of mononuclear cells after the treated pulp cells were incubated in serum-free DMEM for 48 hours.