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Effects on osteoblast-differentiation in mesenchymal stem cell by Floridoside from Rhodophyta, *Laurencia undulata*

By

Romauli Juliana Napitupulu

A thesis submitted in partial fulfillment of the requirements
for the degree of

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in Department of Chemistry, The Graduate School,
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February 2011

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홍서실로부터 분리한 Floridoside에 의한 mesenchymal stem cell의 조골분화효과

Advisor: Prof. Se-Kwon Kim

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Abstract

Floridoside [α-D-galactopyranosyl-(1-2)- L-glycerol] from edible red alga *Laurencia undulata*, has known as an abundant marine biological resources. In the presents study, floridoside of *Laurencia undulate* have shown to promote the osteoblast differentiation in D1 mouse bone marrow mesenchymal cells. Floridoside noted to enhance phenotype markers such as alkaline phosphate (ALP), type I collagen, osteocalcin, osteopontin and mineralization. Our results indicate that floridoside stimulates osteoblast differentiation at various stages, from maturation to terminally differentially differentiated osteoblats. Induction of differentiation by floridoside is associated with increased bone morphogenetic proteins-2 (BMP-2) production. Induction of differentiation by floridoside is associated with the increased activation mRNA expression and protein expression of associated BMP/Smad pathway such as p-Smad 1/5/8, Runx2 and Osterix. This results suggests that floridoside exerts promoting effect on osteogenetic differentiation, which plausibly function via the BMP pathway. Considering to be effective candidates for applications in food and pharmaceutical fields as natural marine antioxidants.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ALP	alkaline phosphatase enzyme
ARS	alizarin red staining
ASC	ascorbic acid
BMP-2	bone morphogenetic protein-2
Col1	collagen type I
DEX	dexamethasone
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbant assay
FBS	fetal bovine serum
MSC	mesenchymal stem cell
MTT	3-(4,5-dimethyl-2 yl)-2,5-diphenyltetrazolium bromide
OC	osteocalcin
ODM	osteogenic medium
OP	osteopontin
OSX	osterix
pNPP	p-nitrophenyl phosphate
RT-PCR	reverse transcription polymerase shain reaction
Runx-2	runt-related transcription factor-2
SDS	sodium dodysilsulphate
TGF-β1	transforming growth factor-beta 1
β-GP	β-glycerophosphate

1. Introduction

Bone is a mineralized connective tissue in vertebrates that supports body weight, enables locomotion of the organism, protects internal organs from external forces, and maintains mineral homeostasis. Bone mass dramatically increases during embryonic and early postnatal development and again at puberty. In humans, bone mass peaks around age 20, and is maintained at a certain level throughout life. An abrupt decrease in mass of the skeleton or of an individual bone results in fractures, underscoring the importance of the maintenance of bone mass during bone remodeling (Consensus, 1993; Raisz, 2005). Remodeling constitutes the lifelong renewal process whereby the mechanical integrity of the skeleton is preserved. It implies the continuous removal of bone (bone resorption) followed by synthesis of new bone matrix and subsequent mineralization (bone formation). When resorption and formation of bone are not coordinated and bone breakdown overrides bone building, osteoporosis results (Ducy, Schinke & Karsenty, 2000; Goltzman, 2002; Teitelbaum, 2000). The maintenance of normal, healthy bone requires the coupling of bone formation to bone resorption, with intercellular communication between osteoblasts and osteoclasts integral to the achievement of a balance between the two processes. Furthermore, bone remodeling is an integral part of the calcium homeostatic system (Eriksen *et al.*, 1993) that also involves the parathyroid glands, intestinal system and the kidneys.

Current drugs used to treat osteoporosis include bisphosphonates, calcitonin and estrogen. These drugs are all bone resorption inhibitors, which maintain bone mass by inhibiting the function of osteoclasts (Rodan and Martin, 2002). It is desirable, therefore, to have satisfactory bone building agents, such as teriparatide, that would stimulate new bone formation and correct the imbalance of trabecular microarchitecture that is characteristic of established osteoporosis (Berg *et al*., 2003; Ducy *et al*., 2000). Since new bone formation is primarily a function of the osteoblast, agents that act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation of the osteoblasts can enhance bone formation (Ducy *et al*., 2000; Lane and Kelman, 2003).

1.1. What are mesenchymal stem cells?

The study of adult type stem cells is an interesting subject, because of the insights they offer into the understanding of tissue repair and regeneration, and also they represent a possible alternative to embryonic stem (ES) cells in various therapeutic applications. Although haematopoietic stem cells are the best characterised, adult organisms contain several other types such as neural, epithelial and mesenchymal stem cells (MSCs).

"Mesenchyme" designates the developing loose connective tissue of an embryo, mainly derived from the mesoderm, and giving rise to a large part of the cells of the connective tissue in the adult. The definition is generally extended to include connective tissue cells in adult tissues such as (myo)fibroblasts, bone, cartilage, fat, tendon, muscles, and nerve tissue. The interest in this cell type is attracted by their ability to maintain their own population and to proliferate and differentiate in various directions, which provides new possibilities for their use in experimental studies of general biology of multipotent stem cells and in medical practice for therapy of various diseases (Molchanova *et al*, 2008). Mesenchymal stem cells represent a subset of precursor cells that adhere to the stem cell definition, *i.e.*, they are capable of self-renewal (ability to generate at least one daughter cell with characteristics similar to the initiating cell), multilineage differentiation from a single cell; and *in vivo* functional reconstitution of the tissues to which they give rise (Verfaillie, 2002).

Mesenchymal stem cells were first studied for their role in supporting haemopoiesis, where they provide signals for differentiation and proliferation of haemopoietic stem cells and their progeny through direct cell–cell interactions and secretion of growth factors and chemokines (Cherry *et al*., 1994; Guerriero *et al*., 1997; Moreau *et al*., 1993). *In vitro* growth of MSCs depend on the composition of the medium (Wang *et al*., 1990, Kuznetsov *et al*.,1997) and inoculation density (Colter *et al*., 2000); it is different for different animal species (Bianco *et al*., 2001; Kim *et al*., 2007). According to other authors, the MSCs can undergo 8 to 40 and even more passages (Bruder *et al*., 1997; Phinney *et al*., 1999, Bianco *et al*., 2001; Tropel *et al*., 2004; Weber *et al*., 2007); however,

subculturing gradually changes their proliferation and differentiation potential (Bruder *et al*., 1997; De Bari *et al*., 2001; Zhou *et al*., 2005; Vacanti *et al*., 2005).

Bone marrow MSCs can differentiate into the bone cells, cartilage and adipose tissues (Friedenstein *et al*., 2006; Owen, 1988; Caplan, 1991) and also of tendons, ligaments, endothelium, skeletal and cardiac muscle (Wakitani *et al*., 1995; Xu *et al*., 2004; Antonitsis *et al*., 2007; Jackson *et al*., 2007). There is an evidence for their "nonorthodox" differentiation into nonmesenchymal derivatives, such as hepatocytes (Wang *et al*., 2004; Xie *et al*., 2006; Aurich *et al*., 2007), lung epithelium (Prockop *et al*., 2003; Paunescu *et al*., 2007), glial and neuron-like cells (Hermann *et al*., 2004; Deng *et al*., 2006) (Fig. 1).

Considerable success has been achieved in the clinical use of MSCs. For example, MSCs may be used for supporting tissue regeneration (Guhathakurta *et al*., 2009), correcting congenital disorders (e.g. osteogenesis imperfecta (Horwitz *et al*., 2002) and controlling chronic inflammatory diseases (Constantin *et al*., 2009; Newman *et al*., 2009), and have even employed as vehicles for the delivery of biological agents (Anker *et al*., 2003) and as probes in the biocompatibility test of new implant materials.

In the present study, we used D1-cells, a mesenchymal stem cell line cloned from Balb/c mouse bone marrow stromal cells (Dahir *et al*., 2000). The D1-cells can sustain itself in long term culture and it is multipotential, as demonstrated by the capacity for the osteogenic and adipocyte differentiation *in vivo* and *in vitro*.

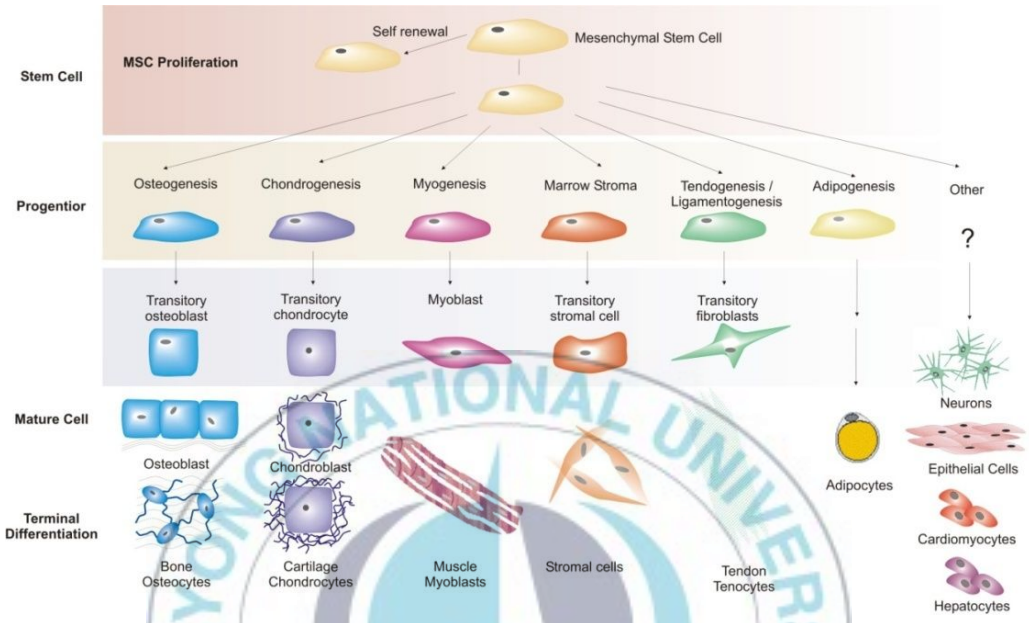

Figure 1: MSC multilineage differentiatin potential. MSCs are able to undergo extensive prior to differentiation into a range of mesenchymal tissue and cell types, including bone, cartilage, muscle, stroma, tendon and adipose and evidence has also suggested they have greater plasticity in an ability to differentiate into non-mesenchymal tissue inscluding liver, heart, skin and nervous tissues.

1.2. Osteogenic differentiation

Bone diseases are consider as major socioeconomic issues. The World Health Organization has acknowledged this fact by declaring the years 2000–2010 "The Bone and Joint Decade". The development of innovative bone-healing strategies is a prerequisite for the successful treatment of a variety of patients suffering from local bone defects caused by trauma, tumour, infection, degenerative joint disease, congenital crippling disorders or periprosthetic bone loss. Furthermore, bone graft material is frequently needed for spinal fusion, joint revision surgery, corrective osteotomy procedures and bone reconstruction in the field of oral and maxillofacial surgery. Bone grafting is one of the most common orthopedic procedures with autologous bone graft providing osteoinductive growth factors, bone-forming cells and structural support for new bone ingrowth. However, the use of autologous bone graft is associated with the disadvantages of limited graft availability and donor site morbidity, *e.g.* pain, infection, pelvic fractures or neurovascular injury. The implantation of sterilized bone allograft material–usually derived from femoral heads during joint replacement procedures–as a widely used alternative bone-filling material may result in failure rates of up to 30% due to insufficient osteointegration of the graft, requiring further surgical intervention (Sorger *et al*, 2001).

The ideal engineered cellular bone graft would need to exhibit the following features: (1) the presence of osteogenic cells to populate the new bone, (2) an appropriate extracellular matrix to provide an osteoconductive scaffold, (3) osteoinductive growth factors to provide signals to the resident cells, and (4) an adequate blood supply to support cell growth and function (Goldberg, 2000). Insufficient bone-healing therefore remains a challenging issue. In this context, innovative cell-based strategies using MSCs are promising for both site-specific and systemic bone regeneration.

1.3. Morphology and differentiation markers

The process of bone nodule formation as studied in rat calvaria populations has been subdivided into three developmental stages: proliferation, extracellular matrix development and maturation, and matrix mineralization. Mature osteoblast cells constitute a lineage of highly differentiated cells which differ substantially in their properties at different stages of development. Thus, depending on the stage of functional differentiation, they are represented by a distinctive phenotype, morphology and biosynthetic activity. There are two kinds of osteogenic mechanisms, firstly, most other types of differentiation usually accompanied by phenotypic alterations of cells (such as changes in cell shape or development of intracellular structures specific for a particular differentiation type), cultured osteoblasts remain phenotypically similar to fibroblasts until the very terminal stages of their differentiation. Only completely differentiated osteoblasts become polygonal and produce a self-mineralized organic matrix (Aubin and Liu, 1996). Secondly, the differentiation of osteoblasts is based on a fairly simple genetic program, because most proteins expressed by these cells can also be expressed by other cells (*e.g.*, by fibroblasts or chondrocytes).

Osteoblasts are rich in alkaline phosphatase enzyme (ALP) (Heath and Reynolds, 1990). Immediately after the down-regulation of proliferation, proteins associated with the osteoblast phenotype are detecxted such as alkaline phosphatase. With progression into the mineralization stage, all cells become positive for alkaline phosphatase. They synthesize and secrete type I collagen, glycoproteins such as osteopontin (rich in sialic acid; N- and O-linked oligosaccharides) and osteocalcin (contains glutamic and aspartic acid residues), cytokines and growth factors into a region of unmineralized matrix (osteoid) between the cell body and the mineralized matrix. At the molecular level, osteogenic differentiation of MSCs is controlled by interactions between distinct hormones and transcription factors.

Collagen type I is the main component of bone matrix; it relates to further MSCs differentiation to an osteoblast. Type I collagen, composed of two α1 chains and one α2 chain, has a unique triple

helical structure formed by these chains, and is the most abundant protein in the human body and in bone (Stryer, 1988). It is essential for bone integrity due to the production of mutated type I collagen or a reduced amount of normal type I collagen by a mutation in its corresponding genes COL1A1 and COL1A2 causes an abnormality of bone structure called osteogenesis imperfecta (Byers and Cole, 2002; Sakai *et al*., 2002).

Consistent with high levels of osteopontin expression in the later osteoblast developmental sequence are the calcium binding properties of this acidic glycoprotein containing O-phosphoserine. The Vitamin K-dependent protein, osteocalcin (Lian and Friedman, 1978), in contrast to OP, is mainly expressed post-proliferatively with the onset of nodule formation. Moreover, expression of osteocalcin in the osteoblast development sequence, suggests that it is a marker of the mature osteoblast, which is consistent with a possible role for the synthesis and binding of osteocalcin to minerals in the coupling of bone formation to resorption.

Other osteoblast-related genes such as bone sialoprotein (BSP) (Nagata *et al*., 1991), osteopontin (OP), and osteocalcin (Owen *et al*., 1990) are induced following the onset of mineralization. OP is expressed during the period of active proliferation (at 25% of maximal levels), decreases post-proliferatively, and then is induced again at the onset of mineralization achieving peak levels of expression. Futhermore, OP functions as a factor promoting cell attachment as well as spreading of fibroblasts and osteoblasts to the substratum.

In addition to the expression of matrix components, the osteoblast differentiation process is both determined and reflected by the expression of certain hormones and cytokines. Runt-related transcription factor-2 (Runx-2) effectuates the expression of bone-specific genes, *e.g*., osterix (Osx), collagen type1 alpha-1 (Col1a1), osteocalcin (OC) and bone sialoprotein (BSP), by binding to the promoters of these genes (Ducy *et al*., 1999; Kern *et al*, 2001; Nakashima *et al*., 2002). Generally, Runx-2, ALP, Col1a1, transforming growth factor-beta 1 (TGF-β1), osteonectin (ON) and bone morphogenetic protein-2 (BMP-2) are known to be early markers of osteoblastic differentiation, whereas OC and osteopontin (OPN) are expressed later in the differentiation process (Long *et al*.,

2001; Spector *et al*., 2001; Young *et al*., 2003; Zhu *et al*., 2001).

1.4. Differentiation protocols

Growth factors are proteins whose binding to cell surface receptors initiates multiple effects involved in various aspects of cell functioning, from reproduction and differentiation to apoptosis. The molecule of a growth factor binds to the extracellular N-terminal domain of the receptor and activates the tyrosine kinase of the cytoplasmic C-terminal enzymatically active domain. This cytoplasmic domain contains numerous sites of autophosphorylation that can bind various adaptor proteins and effector molecules. This binding activates them and changes their functions. The signal mediated by these molecules and transferred into cell nucleus changes the activity of genes responsible for cell proliferation and differentiation (Zinchenko and Dolgacheva, 2003; Perona, 2006).

The classical method for osteogenic differentiation of MSCs in vitro involves incubating a confluent monolayer of MSCs with combinations of dexamethasone (dex), beta-glycerophosphate (β-GP) and ascorbic acid (asc) for several weeks. Dex is a synthetic glucocorticoid and has been reported to be an essential requirement for osteoprogenitor cell differentiation in MSCs (Leboy *et al*., 1991; Herbertson and Aubin, 1995). While MSCs that were cultured in basal medium without osteogenic supplements express increased levels of ALP, they fail to express mineralized ECM as well as other osteogenic markers such as Col1 (Hildebrandt *et al*., 2009). Although the precise mechanisms of action of dex on stem cell differentiation and skeletal function are not known, it is supposed that dex induces transcriptional effects.

The presence of both calcium and phosphate ions is essential for matrix mineralization. β-GP, which is enzymatically hydrolyzed by alkaline phosphatase, serves as a crucial source of inorganic phosphate (Chung *et al*., 1992). Chung *et al*. showed that cultivation of osteoblast-like cells in culture medium containing β-GP leads to mineral formation, lactate generation, increased ALP activity, as well as protein and phospholipid synthesis, indicating enhanced osteogenic differentiation. Usually 5–10 mM β-GP is used for osteogenic differentiation of MSCs.

Asc plays an important role as a cofactor for the hydroxylation of proline and lysine residues in collagens, which are the most abundant group of ECM proteins in the body (Kielty *et al*., 1993; Prockop *et al*., 1995). One difficulty concerning the handling of asc is its instability in solution, especially under standard culture conditions (pH 7.5, 37 ^{o}C, humidified atmosphere, 5% CO_2) . Thus it is recommended to use the long-acting vitamin C derivative ascorbic acid 2-phosphate (Asc-2-P) which was found to be stable under conventional culture conditions (Hata and Senoo, 1989).

1.5. Regulation of osteogenic differentiation via BMP-2 signaling pathway

BMPs, which belong to the TGF-β superfamily, are essential to osteogenic differentiation (Chen *et al*., 2004). Its regulators such as BMP-2 and BMP-4 are known as stimulators in the osteoblastic differentiation of bone marrow-derived stromal cell lines (Yamaguchi *et al*., 1996). Signaling by BMP proteins is mediated through heterodimerization of types I and II serine/theronine kinase receptors, both of which have subcategories. The molecules of BMP were the first factors identified as inducers of ectopic bone formation in the muscle (Wozney *et al*., 1988). Activation of BMP receptors initiates the BMP signaling pathway (mediated by the molecules of Smad1, Smad5 and Smad8) and the MAPK-signaling pathway (Phimphilai *et al*., 2006). In addition to Runx2 the Osterix gene encoding the homonymous transcription factor is a target gene of the BMP2 signaling pathway (Komori *et al*., 1997; Nakashima *et al*., 2002). Runx2 and Osx are downstream regulators of the BMP pathway (Celil and Campbell, 2005; Lian *et al*., 2006; Ryoo *et al*., 2006). Runx2 is known as the master osteogenic transcription factor that takes part in the process of osteoblast maturation (Komori *et al*., 1977), and it plays a critical role in OCN expression (Ducy *et al*., 1997). On the other hand, Osx is a novel zinc finger-containing transcription factor which is specifically expressed in the developing bones. In a previous study, absence of bone formation was observed in a mice model after Osx null-mutation (Nakashima *et al*., 2002). In fact, BMP-2 induces the expression of Runx2, which then regulates the expression of Osx in osteoblastic differentiation (Nakashima *et al*., 2002).

Activation of BMP receptors initiates the BMP signaling pathway (mediated by the molecules of Smad1, Smad5, and Smad8) (Phimphilai *et al*., 2006). The Smad1, Smad5, and Smad8 are recognized by BMP type I receptor (Sykaras *et al*., 2003; Nohe *et al*., 2004).

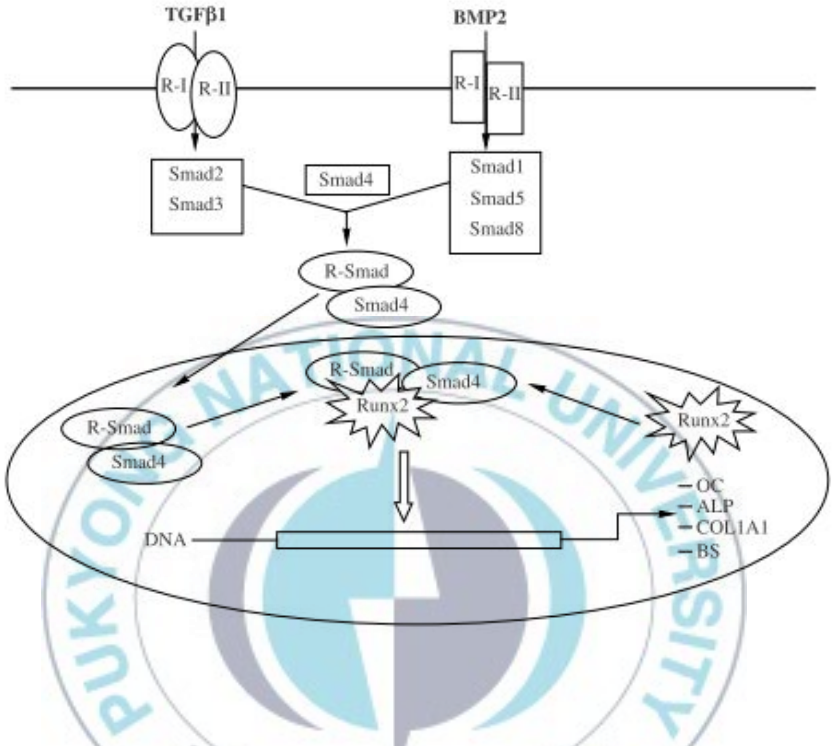

Figure 2: Role of TGF-β1 and BMP2-siganaling pathways in Runx2-mediated regulation of osteogenic differentiation. Runx2 regulates the expression of osteocalcin (OC), alkaline phosphate (ALP), type I collagen (COL1A1), and bone sialoprotein (BS). R-I and R-II designate two types of TGF-β1- and BMP2-binding receptors.

1.6. Floridoside as an agent of bone remodeling in osteoblast differentiation responses

Marine algae have attracted much attention as a source of antioxidative preparations, which could find applications in the food technology, cosmetic industry, and biomedicine. Marine algae have been consumed in Asia since ancient times, which are rich in vitamins, minerals, dietary fibers, proteins, polysaccharides, and various functional polyphenols (Jung *et al*., 2010). Moreover, seaweeds are considered to be a rich source of antioxidant. Hence, many types of seaweeds have been examined to identify novel and effective antioxidant compounds, as well as to elucidate the mechanisms of cell proliferation, anti-inflammation and apoptosis (Heo *et al*., 2006; Nahas *et al*., 2007; Kim *et al*., 2008).

Floridoside, which isolated from the Rhodophyceae *Laurencia undulate,* is considered as the main cytoplasmic carbohydrate, except in *Ceramiales taxa* where it is replaced by digeneaside (2-O-a-D-mannopyranosyl-Dglyceric acid) (Kirst, 1980; Reed, 1990). It takes an important part in most of the red algae as a photosynthetic reserve product and, very likely, as an intracellular osmotic regulator too. (Anthony *et al*., 2008, Karsten *et al*., 1995). Floridoside[α-D-galactopyranosyl-(1-2)-L-glycerol] , which is the terminal galactopyranosyl-(1-2) is responsible for the human xenograft rejection due to the presence of natural anti-gal antibodies. These antibodies are mainly immunoglobulins of type M and G (IgM and IgG) with strong capacity to activate the classical complement pathway. It was thought that the complement system might play a role in many diseases with an immune component, such as various forms of arthritis and bone formation. Results evidences confirm that the glycosides are the isomers with a different linkage; the galactose group and glycerol residue can donate a hydrogeon ion easily, and then excited hydroxyl groups can attract electrons easily. Hence, these isolates are more effective with respect to antioxidant activity.

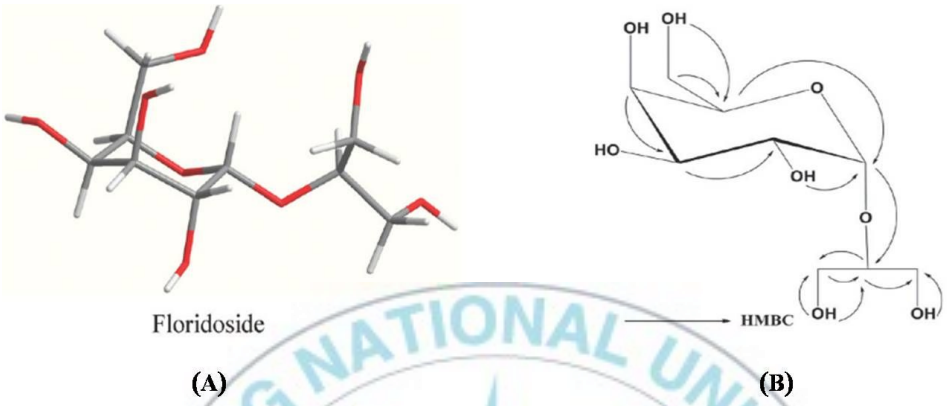

Figure 3: (A) Chemical structure of Floridoside[α-D-galactopyranosyl-(1-2)-L-glycerol] and (B) HMBC correlations of floridoside

Research objectivities

The objective of the present study is to evaluate the effects of floridoside from Rhodophyta, *Laurencia undulata* on osteoblast-differentiation in mesenchymal stem cells. Moreover, we aimed to investigate the signaling pathways responsible for the enhancing effect of floridoside on the osteogenic differentiation of D1 cells, mesenchymal stem cells.

2. Materials and Methods

2.1. Materials

D1-cells, which are mesenchymal stem cell line cloned from bone marrow cells of Balb/mice (Diduch *et al.*, 1993, Dahir *et al.,* 2000) purchased from American Type Culture Collection (Manassa, VA, USA). Cell culture media [Dulbelco's modified Eagle's minimal essential medium (DMEM)], penicillin/streptomycin, fetal bovine serum (FBS) and other materials required for culturing cells were purchased from Gibco BRL, Life Technologies (Grand Island, NY). The MTT reagent [3-(4,5-dimethyl-2yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma Chemical Co. The EZ-Cytox Cell viability assay kit (WST reagent) was purchased from Daeillab Service Co., Ltd (Seoul, Korea). Dye solution Picric acid, 98% moistened with ~33% were purchased from Pancreac Quimica Sau (Barcelona-Spain) and Direct Red 80 (Sirius Red-$C_{45}H_{26}N_{10}Na_6O_{21}S_6$) were purchased from Trade TCT Mark (Tokyo, Japan) used for collagen assay. Procollagen Type I C-Peptide (PIP) EIA Kit purchased from Takara Bio Inc (Japan). Primary and secondary antibodies used for Western blot analysis were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Cell Signaling Tech. (Danvers, MA). Other chemicals and reagents used were of analytical grade commercially available.

2.2. Cell Culture

D1-cell-derived MSCs were maintained in DMEM containing 10% FBS and antibiotics (100 U/ml penicillin and 100μg/ml streptomycin). Cells were seeded at $1x10^4$ cells/cm^2 and maintained in culture for 3 days in a humidified 5% CO_2 atmosphere at 37 ^{0}C. The cells were seeded at a density of $1x10^4$ cells per cm^{-2} in a six-well plate and the experiments were performed after cells had reached about 80% confluence.

2.3. Differentiation

D1-cells can be induced into osteoblasts, adipocytes and chondrocytes. To induce osteogenic differentiation, culture media were changed at 3 days to ODM (DMEM supplemented with 50 μg/ml ascorbic acid, 10^{-7} M dexamethasone, and 10 mM β-glycerolphosphate (all from Sigma Aldrich, MO). After culture for another 3 days, one group was cultured only for DMEM as a blank and ODM as a control, while another group was cultured for ODM plus floridoside (0.1, 1 or 10 μM). Cells were then analyzed in 7, 14 and 21 days later. Dexamethasone is a glucocorticoid steroid capable of stimulating osteogenic differentiation of MSCs at its higher concentration (Marion and Mao, 2006). In addition to its stimulatory effects on cell proliferation, ascorbate further facilitates osteogenic differentiation, including collagen biosynthesis. β-Glycerophosphate is critical to stimulate calcified matrix formation in combination with the effects of dexamethasone and ascorbate. Media was replaced every 3 days. The concentration and exposure period of floridoside were varied according to different parts of the experiments. In general, the culture medium was change three times a week and the D1-cells were kept under 5% CO_2 at 37^0C in a humidified condition. At indicated intervals (*i.e.* day 7, day 14 and day 21), cells were collected for the following experiments: (1) MTT assay; (2) alkaline phosphate (ALP) activity; (3) Mineralization assay, (4) RT-PCR and (5) Western Blot. Independent triplicate test were conducted in each experiment.

2.4. Cell Viability Assay (MTT Assay)

Cell viability was determined by measuring 3-(4,5-dimethyl-2 yl)-2,5-diphenyltetrazolium bromide (MTT) reagent dye absorbance of a living cells as described by Hansen *et al*. (1989). The cells were grown in 96-well plates at density of $5x10^3$ cells/well incubated for 3 days. After the treatment with floridoside (0.1, 1 or 10 μM) for 3, 5 and 7 days. Floridoside were dissolved in 10% DMSO and sterilized by being filtered through a 0.22 μm filter membrane. The final concentration

of DMSO in culture media was kept below 0.1%. The plates were washed with fresh medium and added 100µl of MTT (1 mg/ml) and incubated for 4h. Finally, DMSO (100 µl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using GENios microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan salt. Viability of cells was quantified as a percentage compared to of control (OD of treated cells-OD of blank/OD of control-OD of blank x 100) and dose response curves were developed. The data were expressed as mean from at least three independent experiments and $p<0.05$ was considered significant.

2.5. Cell proliferation assay (WST reagent)

Cell proliferation by floridoside was measured by EZ-Cytox cell viability assay kit (WST reagent). Briefly, cells were plated in 96-well culture plates ($8x10^3$ cells/well). After 24 h incubation, the cells were treated with floridoside (0.1, 1, 10 µM) for 3,5 and 7 days. Ten microliters of WST test solution, was then added to each well. After 1-3 h of incubation, absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 420~480nm and a reference wavelength of 650 nm.

2.6. Alkaline phosphatase (ALP) activity

Osteogenesis of D1-cells was induced by ODM containing either various concentration of floridoside (0.1,1, and 10 µM). Cells were seeded into 24-well plates at a density of $1x10^4$ cells/well and cultured for 24 h. The ODM was added to the wells, and incubation continued for 7, 14 and 21 days. The ODM will be changed and the cells were then washed with PBS twice and lyzed with the lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl , and 1% Triton X-100 for 1 h at 37 ^{0}C. P-nitrophenyl phosphate (pNPP; Sigma-Aldrich) was used as the substrate to measure the intracellular alkaline phosphatase activity. Supernatant (50 µl) of lysate was incubated with 100 µl of enzyme assay buffer (pNPP substrate solution) containing 15 mM pNPP, 250 mM carbonate buffer

(pH 10.3) and 1.5 mM magnesium chloride for 30 min at 37 ^{0}C. Absorbance was measured at 405 nm and the 4-nitrophenol (Sigma-Aldrich) released by the samples was determined.

2.7. Analysis of mineralization

The degree of mineralization was determined in the 12-well plates using Alizarin Red (Sigma Chemical, St. Louis, MO, USA) staining after 7 days treatment. Briefly, cells were fixed with 70% (v/v) ethanol for 1 h and were then stained with 40 mM Alizarin Red S (ARS) in deionized water (pH=4.2) for 15 min at room temperature. After removing Alizarin Red S solution by aspiration, cells were incubated in PBS for 15 min at room temperature on an orbital rotator. Orange red staining indicated the position and intensity of the calcium deposits. Then the cells were rinsed once with fresh PBS, and subsequently destained for 15 min with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH=7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured using a microplate reader.

2.8. Measurement of collagen amount

Collagen assay is based on the observation of Sirius Red in saturated picric acid selectively binds to fibrillar collagens type II, specially to the {Gly-X-Y}n helical structure. Media were replaced and collected every 2-3 days over a period of 21 days. The conditioned media in presence of ODM and treated with various concentration of floridoside (0.1, 1, 10 μM) were analyzed for the release of collagen degradation products using ELISA. Briefly, 100 μl of conditioned cell culture media were added into 1ml of the dye solution and mix gently at room temperature for 30 minutes. The dye solution was centrifuged, discarded the supernatant, and removed the unbound dye with 1 ml of 0.1 M HCl. Then 1 ml of 0.5 M NaOH was added after all of the bound dye has been dissolved, within 5 minutes, the samples are ready for measurement. Absorbance were determined at 550 nm on ELISA microplate reader (Tecan Austria GmbH, Austria).

2.9. Levels of type I procollagen

Cells were treated with various concentrations of floridoside for 7 and 14 days. The type I pro-collagen assay, which measures the propeptide portion of the molecule and reflects the synthesis of the mature form of the protein, was carried out using Prolagen-C kit as described in the manufacturer's protocol (Takara Bio Inc.). The type I pro-collagen levels obtained were normalized to total protein concentrations, as determined by BCA protein assay.

2.10. Reverse transcription (RT)-PCR analysis

Cells were harvested on day 7, 14 and 21 of osteogenic induction after treated with floridoside at different concentrations (0.1, 1, 10 μM). Total RNA was collected using TRIZOL® reagent (Invitrogen, USA) and centrifuged at 13,000 x *g* for 15 min at 25 ^{o}C following the addition of chloroform. Supernatant was separated and isopropanol was added at 1:1 ratio. RNA pellet was obtained by centrifugation for 10 min at 10000 rpm. After washing with 70% ethanol, extracted RNA was solubilized in diethylpyrocarbonate-treated RNase-free water and incubation for 10 min at 60 ^{o}C. RNA concentrations were quantified by measuring the absorbance at 260 nm using the GENios® microplate reader (Tecan Austria GmbH). Equal amounts of RNA (1 μg) were reverse transcribed in a mastermix containing 1 x reverse transcripatase (RT) buffer, 1 mM dNTPs, 500 ng of oligo(dT) 15 primers, 140U of murine moloneyleukaemia virus (MMLV) reverse transcripatase and 40U of RNase inhibitor, for 45min at 42 ^{0}C. Polymerase chain reaction (PCR) was carried out in an automatic Whatmanthermocycler (Biometra, Kent, UK) to amplify Osteocalcin (OCN), Osteopontin (OPN), ALP, Type I collagen, Bone morphogenetic protein 2 (BMP-2), Bone morphogenetic protein 4 (BMP-4), Runx2, Osx mRNA. Each transcript was identified using specifics forward and reverse primers as manufacturer's instruction (Promega, Madison, WI, USA). The condition for amplifications cycles were 95 ^{0}C for 30s, 55 ^{o}C for 30s, and 72 ^{o}C for 1 min for 25 cycles. After polymerase chain reaction (PCR) products electrophoresed on 1.5% agarose gels in 1x TAE buffer for

30 min at 100 V were visualized by ethidium bromide staining using AlphaEase® gel image-analysis software (Alpha Innotech, San Leandro, CA, USA).

2.11. Western blot analysis

Total cellular protein was prepared using whole cell extraction buffer [50 mMTris –HCl (pH 7.5), 0.4% Nonidet P-40, 120 mMNaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 μg/ml of leupeptin, 3 mM NaF and 1mM DTT] at 4 ^{0}C for 30 min. Cell debris were removed by centrifugation followed by quick freezing of the supernatants. Total protein was quantified with BCA Protein Assay Kit (Pierce). The appropriate amount of protein was diluted with SDS-PAGE sample buffer, boiled at 100 ^{0}C for 5 min and clarified by centrifugation. Equal amounts of protein (20 μg) were then loaded onto 10% Tris-Glycine gels and electrophoresed at 100 V for 90 min. The gels were transferred to nitrocellulose membrane using the manufacturer's recommended protocol. The membranes are blocked using 5% (w/v) non-fat dried milk in Tris-buffered saline containing 0.001% (v/v) Tween 20. The membranes were incubated with primary antibody overnight using antibodies against the targets listed: BMP-2/4, BMP-3, Runx2, Osterix, Collagen type 1, p-38, p-p38, Erk1/2 and β-tubulin (Santa Cruz Biotechnoloogy Inc., CA) and p-Smad 1/5/8 (Cell Signaling Technology). After removing unbound primary antibody by washing, secondary antibody (Bio-Rad) was used at 1:4000 dilution. Final chemiluminescence detection is based on the ECL+kit (Amersham Pharmacia Biosciences) per the manufacturer's protocol. Blot bands were visualized using an LAS3000® Luminescent image analyzer and protein expression was quantified by Multi Gauge V3.0 software (Fujifilm Life Science, Tokyo, Japan).

2.12. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). Data are expressed as mean ± SEM. A p-value of less than 0.05 was regarded as statistically significant were analyzed by Dunnett's test.

Table 1: Gene-specific primers sequences used for RT-PCR

Gene	Direction	Sequence
osteocalcin	sense	5′-TAG-GGC-AGC-ACA-GGT-CCT-AA-3′
	antisense	5′AAG-CAG-GAG-GGC-AAT-AAG-GT-3′
osteopontin	sense	5′-TGC-ACC-CAG-ATC-CTA-TAG-CC-3′
	antisense	5′-CTC-CAT-CGT-TCA-TCA-TCA-TCG-3′
ALP	sense	5′ ACA-CCA-GAC-ACA-AGC-ATT-CC-3′
	antisense	5′-GCC-TTT-GAG-TTT-TTG-GTC-A-3′
type I collagen	sense	5′-CAG-TCG-CCT-TCA-CCT-ACA-GCA-3′
	antisense	5′-GGT-GGA-GGG-AGT-TTT-ACA-CGA-3′
BMP-2	sense	5′-GGA-CCC-GCT-CTG-TTC-TAG-TG-3′
	antisense	5′-GCT-TCT-GCT-TCA-GGC-CAA-AC-3′
BMP-4	sense	5′-TGA-GCC-TTT-CCA-GCA-AGT-TT-3′
	antisense	5′-CTT-CCC-GGT-CTC-AGG-TAT-CA-3′
Runx2	sense	5′-CCC-AGC-CAC-CTT-TAC-CTA-CA-3′
	antisense	5′-TAT-GCA-CTG- CTG - CTG -GTC-TG-3′
Osterix	sense	5′-CTT-TGC-CAG-TGC-CTA-GTT-CC-3′
	antisense	5′-GCT-CAG-GAA-GAC-GAA-AGG-TG-3′
GAPDH	sense	5′-TCC-ATG-ACA-ACT-TTG-GTA-TCG-3′
	antisense	5′-TGT-AGC-CAA-ATT-CGT-TGT-CA-3′

3. Results and Discussion

3.1. Cell cytotoxic effects of floridoside on D1-cells

The viabilities of floridoside were carried out on D-1 cells differentiated into osteoblats. MTT assays, which is used to measure the metabolic activity of the mitochondria of cells based on principle that living cells are capable of reducing light color tetrazolium salts into an intense color formazan derivative (Carmichael *et al*., 1987). Figure 4, showed that floridoside increased cellular ploriferation without any evidence of cytotoxicity. Li *et al*., reports that floridoside possess significant antioxidant capacity (Li *et al*., 2009). Until now, there are no other bioactivities provided for floridoside. Therefore, we used the floridoside to investigate their potential on enhancing the productions of gene, proteins and signaling pathway related to osteogenesis during osteoblats differentiation *in vitro* (Fig. 4).

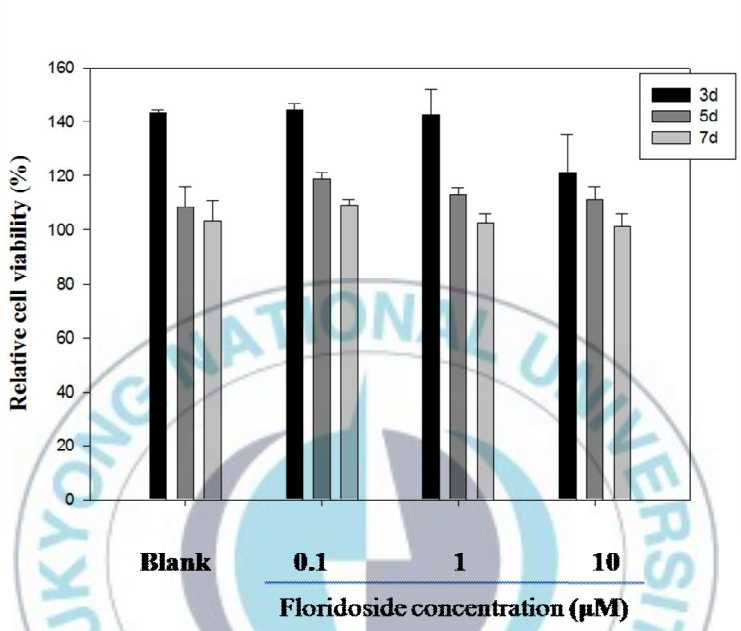

Figure 4: Cytotoxic effects of floridoside derived from *Laurencia undulata* on D1-cells. Cells were grown at the density of $1x10^5$ cells/well and different concentrations of each samples were treated for various time intervals (3, 5 and 7 days). MTT solution was added to each well and incubate for 3 h and cell viabity was assessed, as described. Results of three independent experiments were averaged and are shown as percentage cell viability compared with the viability of untreated control cells. Each value is the mean ± S.D. of three independent experiments.

3.2. Floridoside had no effect on the ploriferation of D1-cells

We first determined the effect of floridoside on the cell ploriferation of D1-cell line by WST assay reagent. Our results showed that floridoside did not exihibit significant effects on cell proliferations at the concentrations used (0.1, 1, 10 μM) after 3, 5 and 7 days of treatment in D1-cells. Cultures grown in control conditions showed a gradual increase in the cell proliferation till day 7.

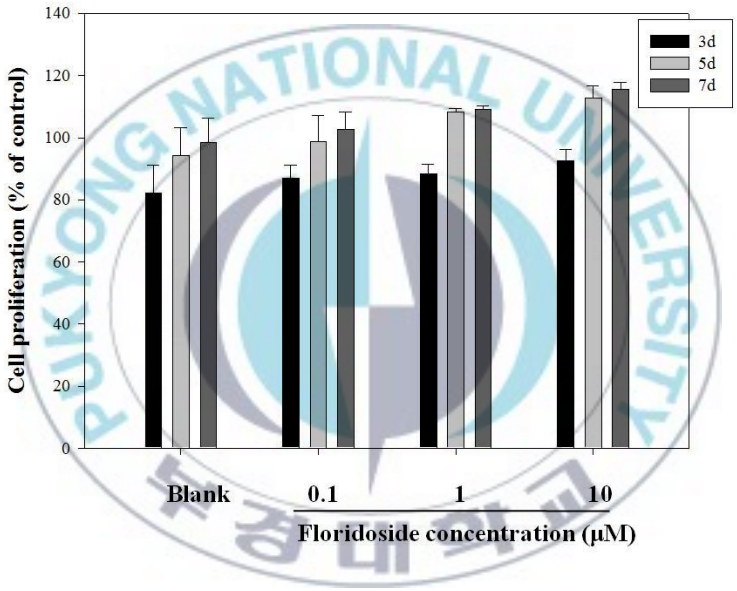

Figure 5: Effect of floridoside on the cell proliferation of D1-cells. Adherent cells that proliferated in 96-well plates were incubated with different concentrations (0.1, 1 and 10 μM) of floridoside for various time intervals (3,5 and 7 days). Cell proliferation was determined by WST assay. Each value is the mean ± S.D. of three independent experiments. Standard deviations were less than 10%.

3.3. Effect of floridoside on osteogenic differentiation in D1-cells

Culture of expanded mesenchymal stem cells (MSCs) seeded on biomaterials may represent a clinical alternative to autologous bone graft in bone regeneration. For the characterization of MSC plasticity, their ability to differentiate in vitro into osteoblasts is currently treated as the gold standard. This, in combination with the advantages that MSCs have no immunogenicity and can be easily isolated from different tissues and expanded in vitro, enables MSCs to be a promising source of stem cells. When being differentiated into osteoblasts, MSCs transform from a fibroblastic to a cuboidal shape, produce extracellular ma trix (ECM), mainly composed of collagen type I, and in a later stage form aggregates or nodules that can be stained positively by alizarin red S (ARS) staining. Increased expression of alkaline phosphatase (ALP) and calcium accumulation are observed in MSCs during osteogenic differentiation. At the molecular level, osteogenic differentiation of MSCs is controlled by interactions between distinct hormones and transcription factors.

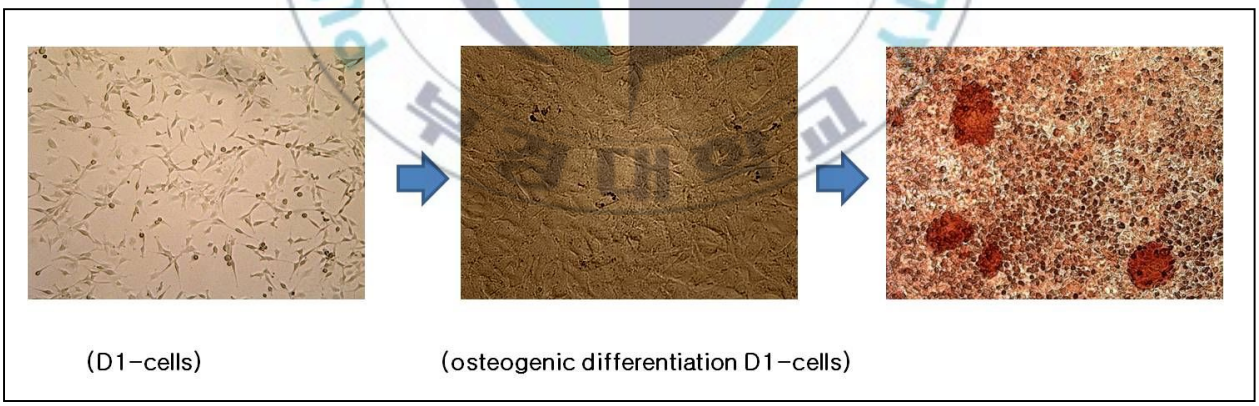

Figure 6: Differentiation of bone marrow cell into osteoblast D1 bone marrow cell were differentiated into osteoblast in the medium containing DMEM supplemented with 50 µg/ml ascorbic acid, 10^{-7} M dexamethasone, and 10 mM β-glycerolphosphate for 21 days.


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3.4. Effect of floridoside on maturation and differentiation markers, ALP activity in D1-cells

Differentiation of pluripotent progenitor D1-cells into osteoblats is a crucial step of osteogenesis. ALP is an ectoenzyme which act as a marker for cells undergoing differentiation to form preosteoblats and osteoblasts (Farley *et al*., 1983). Differentiated osteoblats exhibit elevated ALP activity, which is correlated with high levels of enzyme expression (Tang *et al*., 2007a). ALP is considered to be important in producing bone minerals and as marker for cells that are undergoing differentiation forming preosteoblasts and osteoblasts. Generally, ALP activities were expressed after *in vitro* osteogenic induction for 7 days, while later ALP staining was seen after 14 to 21 days of osteogenic induction (Abdallah *et al*., 2004). Potential effects of floridoside on osteogenic differentiation of D1-cells cultured for 7,14 and 21 days were thus assessed by measuring the ALP activity normalized to total protein content. ALP activity production was due to the enhancing of protein synthesis. In the control cultures, the ALP activity of D1-cells at the first week of experiment was significantly higher than that at the second week of experiment. This results showed that cells had entered the mineralization stage and the ALP activity had declined at the second week of experiment. The results from the first week experiment were compared among the samples, the ALP activity of D1-cells could be decreased by floridoside treatment. Floridoside inhibited the ALP activity of D1-cells in a time dependent manner without evident of dose dependent manner. Figure 7. shows the ALP activity in D1-cells cultured with different concentrations of floridoside.

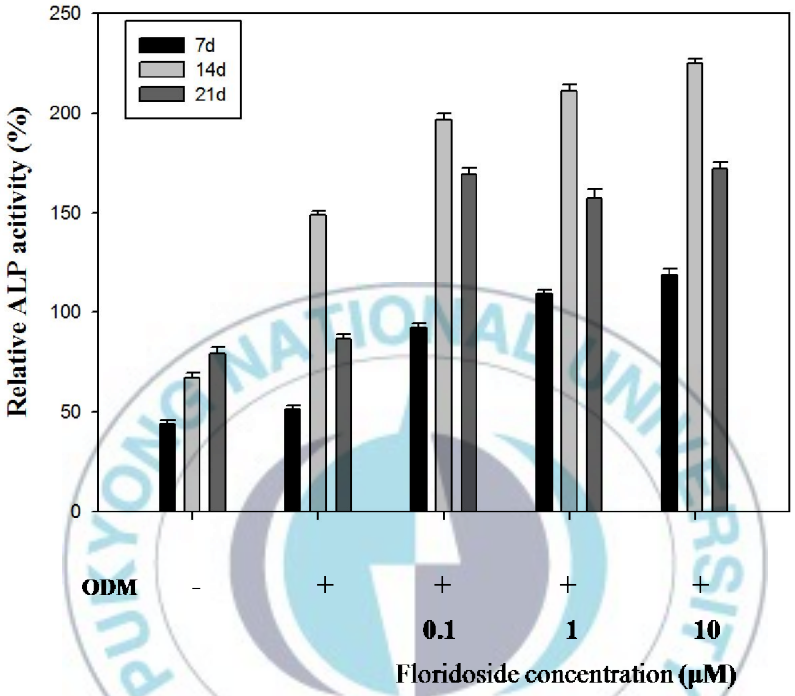


Figure 7: Effect of floridoside on the ALP activity of D1-cells, for testing ALP activity, floridoside treatments were for 1 and 2 weeks in ODM. The lysate cells were collected and ALP activity levels were determined as described previously. Each value is the mean ± S.D. of three independent experiments.

3.5. Effect of floridoside on the formation of mineralized matrix nodules

In studies using osteogenic cultures, mineralization is considered a functional *in vitro* endpoint reflecting advanced cell differentiation. Alizarin red staining is commonly used to detect and quantify calcium. Confluent osteogenic cultures, D1-cells, follow a two-stage developmental process including at 1–2-week initiation phase during which cells slowly proliferate, express ALP activity and other bone specific genes, and produce and assemble a collagen matrix.

The biological mineralization is controlled by the cell and involves a fine balance between stimulatory and inhibitory factors. To initiate the mineralization process, the cell must be ‘‘mineralization competent.” For example, only mature osteoblasts and terminally differentiated growth plate chondrocytes undergo mineralization events (Kirsch, 2007). The mineralization is initiated inside vesicles, either after secretion like matrix vesicles (MVs) or apoptotic bodies or before their secretion, inside the cell. Cells undergoing pathological mineralization are reminiscent of osteoblast or chondrocyte–phenotype, expressing several proteins, necessary for mineralization. Many secreted matrix proteins are involved in the initiation and directional growth of the mineral phase. Calcium and phosphate in metastable equilibrium can induce mineralization (Van de Lest *et al*., 2007). Calcification occurs at nucleation sites known as matrix vesicles present in the lacunae of mineralizing cartilage. They are believed to accumulate Ca^{2+} and inorganic phosphate which serve as nucleating agents for the formation of hydroxyapatite ($Ca_{10}(PO_4)_6(OH_2)_2$), the main inorganic component of bone (Wu *et al*., 1995).

After cultured for 2- 3 weeks, osteoblast nodes formed and reached peak quantity when osteoblasts started to mineralize (Stein *et al*., 1993). For initial studies to determine whether cultured D1-cells could differentiate to osteoblasts, we cultured D1-cells and exposed the cells to osteogenic media for up to 21 days. For negative control, culture DI-cells were treated in parallel alone with conventional media. The deposition of calcium by fibrocytes undergoing differentiation

to osteoblasts in osteogenic media was further evaluated using ARS staining. ARS staining assay provide evidence for the mineralization via appearances of stained photographs. D1-cells that had undergone differentiation to osteoblasts in osteogenic media for 21 days demonstrated positive ARS staining which represented deposition of calcium (Fig. 8a). Neither of D1-cells which is exposed to control media for 21 days showed any detectable calcium deposition by alizarin red S staining. These results suggest that D1-cells exposed to osteogenic media differentiated into cells that induce calcium deposition. According to the alizarine red-S staining data (Fig. 8b), floridoside (0.1, 1 and 10 μM) increased the amounts of hydroxyapatite in the cell. The amount of mineral intercellular level was higher in a concentration-dependent manner.

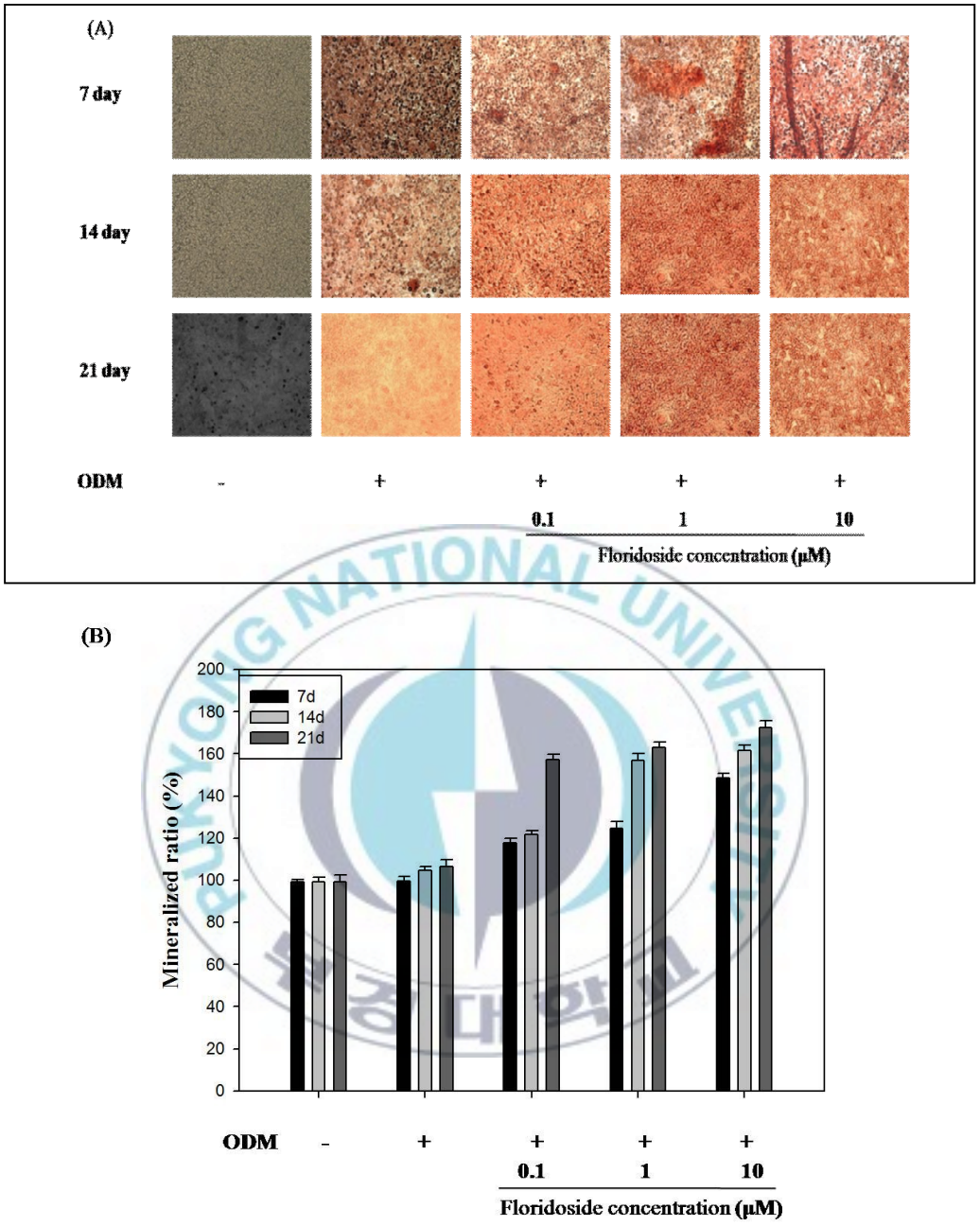


Figure 8. Effects of floridoside on mineralization of D1-cells. Cultures in the 12-well plate were treated with floridoside (0.1, 1 and 10 μM) for 7, 14 and 21 days. Firstly, cells were fixed with 70% ethanol for 1h and then stained with 40mM Alizarin Red S solution for 15 min. After moving solution cells were washed PBS three times abd theb take a picture. (A) Photographs of alizarin red S staining in D1 cells treated with floridoside. (B) Graph is that represent the area of dying zone using 10

cetylpyridium chloride.

3.6. Levels of soluble collagen secreted by D1-cells differentiated into osteoblats

D1-cells were treated with floridoside in the concentration range 0.1 – 10 μM during subsequent experiments. To investigate whether the floridoside has ability to increase the collagen synthesis based on the selective precipitation of [3H]-prolin labeled collagen. Collagen makes up 90% of bone matrix, one expects the osteoblastic production rate of collagen to be closely correlated to the formation of bone matrix (Bellows, Reimers & Heersche, 1999). An important postion of bone formation is the synthesis and deposition of collagen, which constitutes 90% of the total organic extracellular matrix in mature bone, by preosteoblasts or early undifferentiated osteoblasts cells (Reffitt *et al.*, 2003)

The effect of floridoside on D1-cell osteoblastic differentiation was further assessed by measuring the changes in levels of secreted factors representing primary osteoblast functions of extracellular matrix production (secreted collagen). The addition of floridoside with various concentration to culture media results in reduction of hydroxyproline recovered from the media, both during the 7, 14 and 21 days periods of culture. This can be due to inhibition of collagen breakdown and/or decreased collagen synthesis. However, the total amounts of hydroxyproline in the culture were not affected by the floridoside and therefore the reduced amounts of hydroxyproline found in the media from floridoside –treated cells, are most likely an effect of reduced bone formation (Fig. 9).

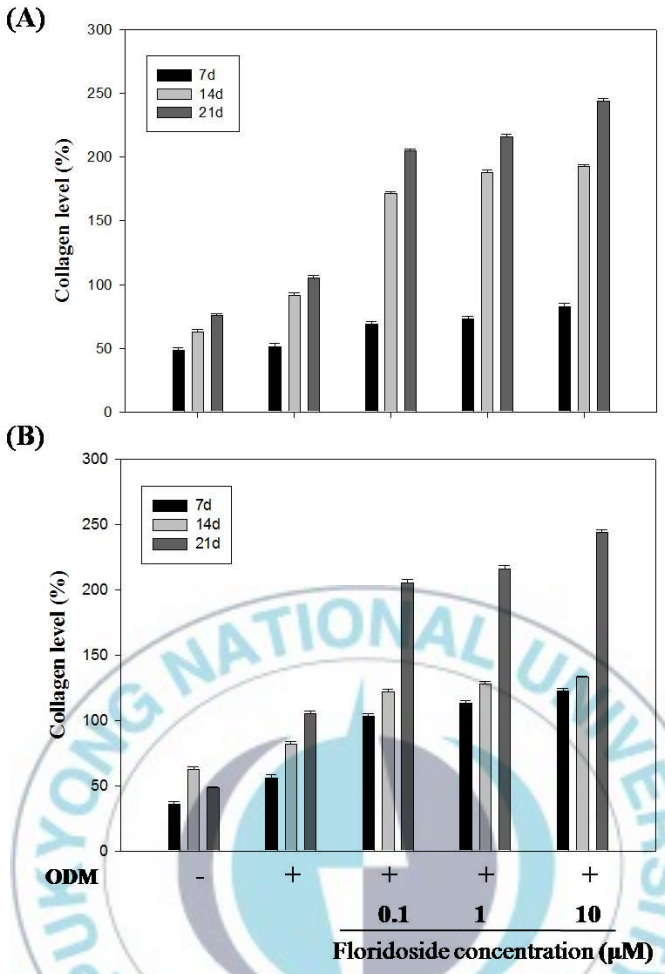


Figure 9: Effect of floridoside on collagen soluble in D-1 cells differentiated into osteoblats were grown in ODM for 7,14 and 21days. Floridoside with various concentration (0.1, 1, and 10 μM) were treated during 21 days of culturing. Media was changed every 2 days. (A) Collagen level result during 7, 14 and 21 days inculture of medium, (B) collagen level result during 7,14,and 21 days in lysate-cells. D1-cells with no added floridoside served as controls. Levels of soluble collagen secreted by osteoblasts into the medium in the presence and absence of floridoside were quantified using Picro-Sirius Red Method.

3.7. Effect of floridoside on differentiation of osteoblats by the production of type I collagen synthesis

Type I collagen is the most abundant extracellular protein of bones and is essential for bone strength (Sengupta *et al*., 2005). Because type I procollagen carboxyl and amino terminal domains are proteolytically removed during collagen secretion, antibodies to these domains have been used to stain fibroblasts synthesising type I collagen. In order to investigate the regulation of type I collagen and its role in the differentiation of mesenchymal stem cells, we employed D1-cells and found that these cells responded to floridoside by increasing their ALP activity and reducing their growth rate (Figs.5 and 7), as reported previously (Franceschi and Young, 1990). Collagen type 1 synthesis increased in all cell lines tested following treatment with floridoside at 0.1–10 μM. Treatment of the D1-cells with floridoside at 0.1–10 μM increased the amount of collagen type 1 in the culture medium by a factor of 1.75 ($P < 0.001$ vs untreated cells) (Fig. 10) in dose-dependent manner after 7 and 14 days of treatment . Floridoside did not alter collagen type 1 gene expression but our results suggest that it may modulate prolyl hydroxylase activity. Further studies are required to confirm this. Collagen synthesis and osteoblastic differentiation are tightly correlated, because the inhibition of collagen synthesis suppresses osteoblastic differentiation (Franceschi *et al*., 1992).

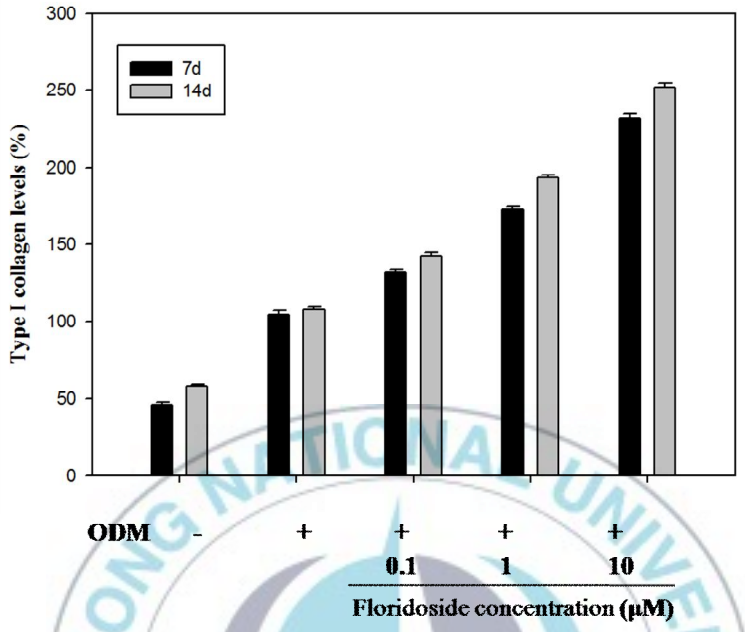


Figure 10: Effect of floridoside on increasing the osteoblastic activity, type I collagen synthesis in D1-cells. Cells were grown in ODM and treated with various concentrations of floridoside. Medium was collected and tested every 7 and 14 days. The production of type I collagen was assayed by Procollagen Type I C-Peptide (PIP) EIA kit. Each value is the mean ± S.D of three independent experiments.

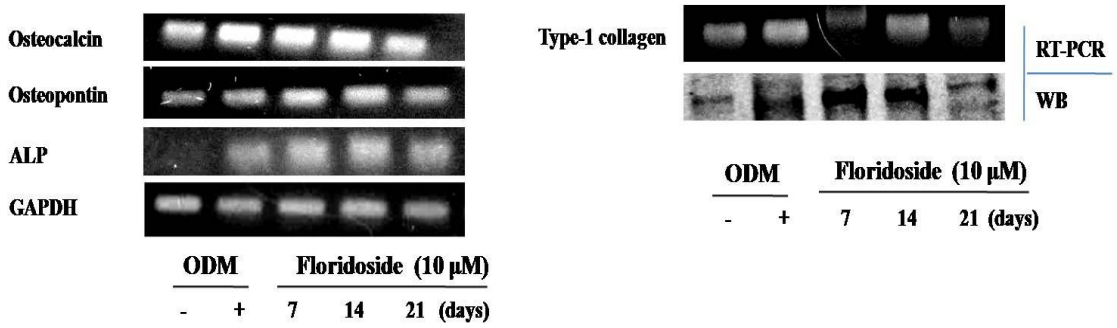
3.8. Effect of floridoside in D1-cells undergoing osteogenesis express genes for osteogenic marker

In order to confirm whether the D1-cells exposed to osteogenic media were differentiated into osteoblasts, we next evaluated whether these cells expressed specific osteogenic gene markers during osteogenesis. Osteocalcin (OCN) is a specific cell marker protein for the terminal cell differentiation of osteoblast which is selected into cell culture medium by the osteoblast (Abdallah *et al.*, 2004). Osteopontin (OPN) is expressed in developing bone cells during early stages of osteogenesis prior to mineralization (Mark *et al.*, 1987). Marker genes, including osteopontin and osteocalcin were markedly expressed increased in their expression during the osteogenic differentiation of MSCs. In the present study, to identify beneficial effects of floridoside on bone health and mineralization, we examined whether floridoside extracted from *Laurencia undulata* can increased activity of osteocalcin, osteopontin, alkaline phosphatase and type I collagen, phenotypic markers for early stage and terminally differentiation, respectively. Osteogenic differentiation of progenitor cells *in vitro* is characterized by formation of mineralized bone-like nodules containing ALP, osteopontin, and osteocalcin and type I collagen (Frank *et al.*, 2002). The four mRNA expressions of floridoside treated D1-cells were also compared to those of cells cultured in conventional media and ODM only and was assessed at 7, 14 and 21 days (Fig.11). On this basis, osteocalcin, osteopontin, ALP, and type I collagen mRNA were chosen as gene markers for evidence of osteogenesis as assessed by RT-PCR.

As shown in Fig.11, all four osteogenic genes were upregulated in both D1-cells exposed to osteogenic media as early as day 7. Each osteogenic gene marker showed a slightly different expression profile during the time course of osteogenic differentiation of D1-cells. D1-cells exposed to osteogenic media showed that osteocalcin, mRNA was highly expressed as early as day 7, with persistence of osteocalcin expression at days 14 and 21. In contrast, D1-cells undergoing osteogenic differentiation showed that osteopontin mRNA expression was induced as early as day 7 with

persistence of maximal expression at days 14 and 21 similar to osteocalcin. ALP gene expression in D1-cells undergoing osteogenic differentiation was maximally expressed by day 7, but was minimally by day 21. Type I collagen gene expression, a phenotypic markers for the later stage of osteoblat differentiation, was increased by floridoside treatment.

(A)



(B)

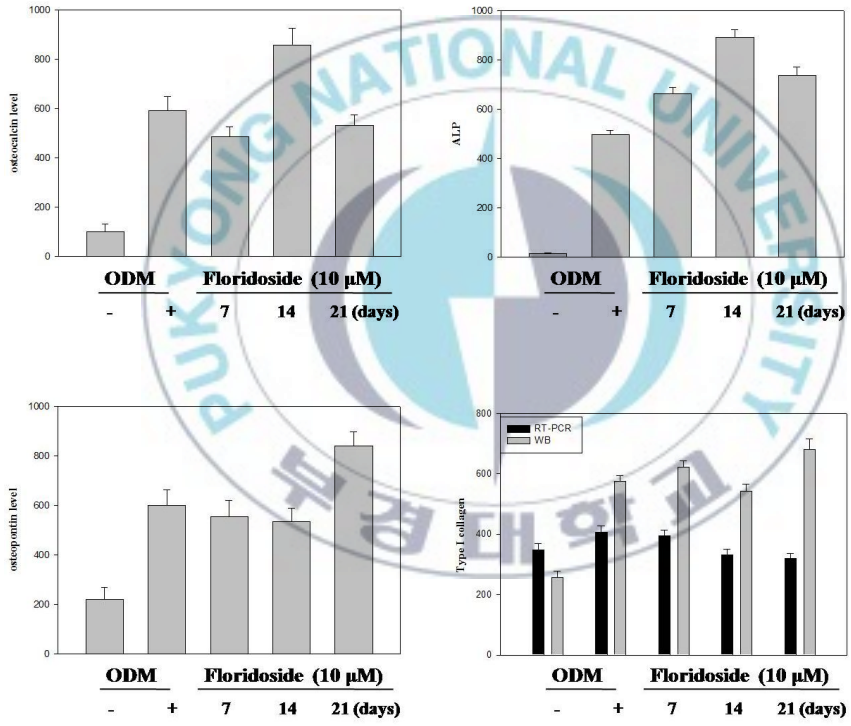
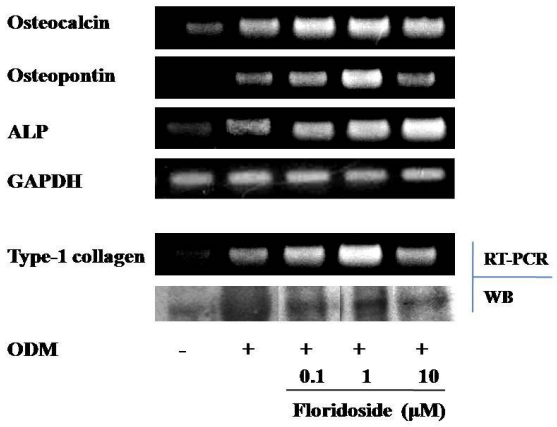


Figure 11: D1-cells undergoing osteogenesis express genes for osteogenic markers after treated with Floridoside for 7, 14 and 21 days. Time course of gene expression of osteocalcin, osteopontin, alkaline phosphatase and type I collagen from D1-cells undergoing differentiation to osteoblast cells in the presence or absence of osteogenic media (ODM). (A). Floridoside increased the mRNA levels of osteocalcin, osteopontin, and ALP in concentration. (B) PCR band are calculated by program of

LAS3000 analysis tools.

Osteoblasts are specialized cells which synthesize bone proteins such as osteocalcin, osteopontin, alkaline phosphatase, type I collagen and secrete a bone matrix which will mineralize. The mRNA expressions of osteocalcin, osteopontin, alkaline phosphatase and type I collagen of the floroside-exposed cells were compared with the control. When D1-cells osteoblatic-differentiate were administered with floridoside at 0.1, 1, 10 μM, all four mRNA expressions were up-reagulated in dose-dependent manner at day 14. Therefore, 14 days was selected as the optimal osteoblastic-differentiation time of cultivation. Expression of osteogenic genes treated with Floridoside at 0.1, 1 and 10 μM was assessed at 14 days post-transduction by real-time RT-PCR. mRNA expression of all four osteogenic genes was increased in dose-dependent manner (Fig.12).

(A)



(B)

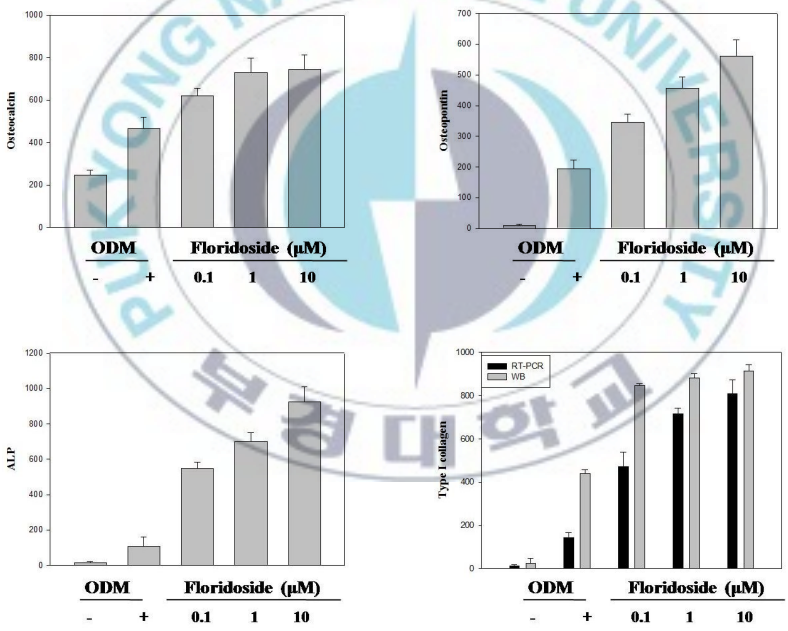


Figure 12: Increase of osteoblastic phenotype markers expression by floridoside in cultured D1-cells. D1-cells were treated with different concentrations of floridoside (0.1, 1 and 10 μM), and we find that 14 days as an optimal culture –time in osteoblast-differentiation by RT-PCR. (A) increased the mRNA level in a dose-dependent manner. (B) PCR band are calculated by program of LAS3000 anlysis tools.

3.9. Effects of floridoside on Bone morphogenetic proteins (BMPs) level by PCR and Western blotting

BMPs are members of the TGF-β1. BMPs play an important role in the process of bone formation and remodeling (Sykaras and Opperman, 2003). Its regulator such as BMP-2 and BMP-4 are known as stimulator in the osteoblastis differentiation of bone marrow-derived stromal cell lines (Yamaguchi *et al*., 1996). It has been well documented that stimulation of osteoblast differentiation is characterized mainly by increased expression of ALP, type I collagen, and osteocalcin (Xiao *et al*., 2004). The action of BMPs is mediated by heterotetrameric serine/threonine kinase receptors and the downstream transcription factors Smad protein and phosphorylated Smad proteins (pSmad 1/5/8). Our study indicates that the production of BMP-2 increases in floridoside-treated D1-cells. Also, phosphorylations of Smad1/5/8 are simultaneously enhanced in myricetin-treated osteoblasts. These results support the hypothesis that the BMP-2 signaling system plays an important role in floriside-mediated cell maturation and differentiation in osteoblasts. We next determined whether D1-cells undergoing osteogenic differentiation not only expressed mRNA for the osteogenic genes above, but also the relevant osteogenic protein. Total cellular protein was possessed from D1-cells undergoing osteogenic differentiation at days 7, 14 and 21 in response to osteogenic media, as compared to cells that were exposed to control media alone. mRNA-expression and western blot analysis demonstrated that D1-cells exposed to osteogenic media and treated with floridoside, displayed markedly elevated levels of BMP-2 as early as 7 days (Fig. 13).

In the present study, the two BMP-related regulators, Runx2 and Osx, were found closely related to the promoting effect of floridoside in osteogenesis. Ligation of BMP-2 to BMP receptor heteromeric complexes and subsequently activates Smads by phosphorylation. We first assessed activation (phosphorylation) of Smad proteins in floridoside-treated D1-cells. As shown in Fig.14, treatment with floridoside increase the amount of phospho-Smad 1/5/8 after 7 days exposure osteoblats to floridoside, with a progressive increase for up to 21 days. We find that, 14 days was

selected as the optimal osteoblastic-differentiation time of cultivation. The activation of Smads closely matched the appearance of BMP-2. Thus, BMP-2 signaling is necessary and sufficient to mediate the activation of Smad1/5/8 in floridoside-treated D1-cells.

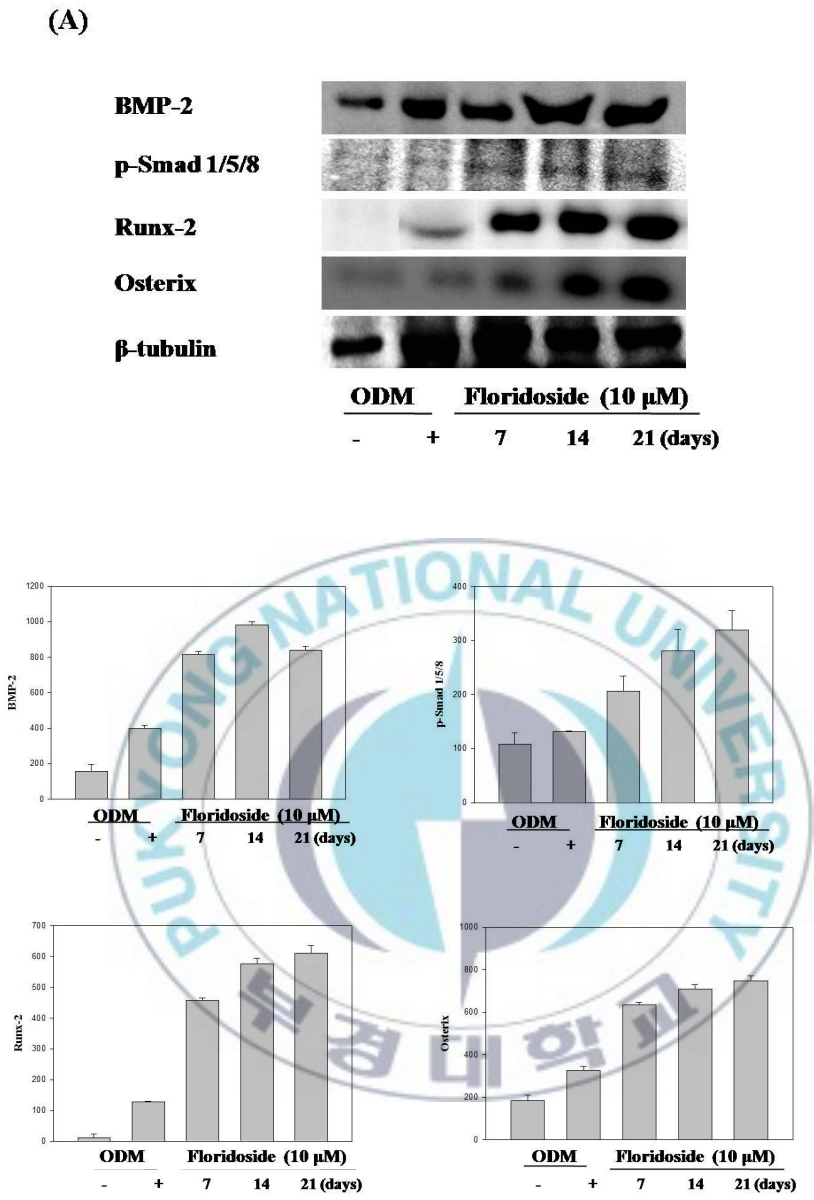


Figure 13: Effect of Floridoside on BMP-2 expression on protein expression in osteoblast cells, D1-cell line. Cells were treated with floridoside (10 μM). After incubation with ODM for 7,14 and 21 days, (A) cell lysate were collected and subjected to Western blots and SDS-PAGE using BMP-2, p-Smad 1/5/8, Runx-2 and Osterix antibodies. β-tubulin was used as an internal control. The antibody bindings were detected by enhanced chemiluminescence reagent using luminoimager. Western blot

band are calculated by program LAS3000 analysis tools.

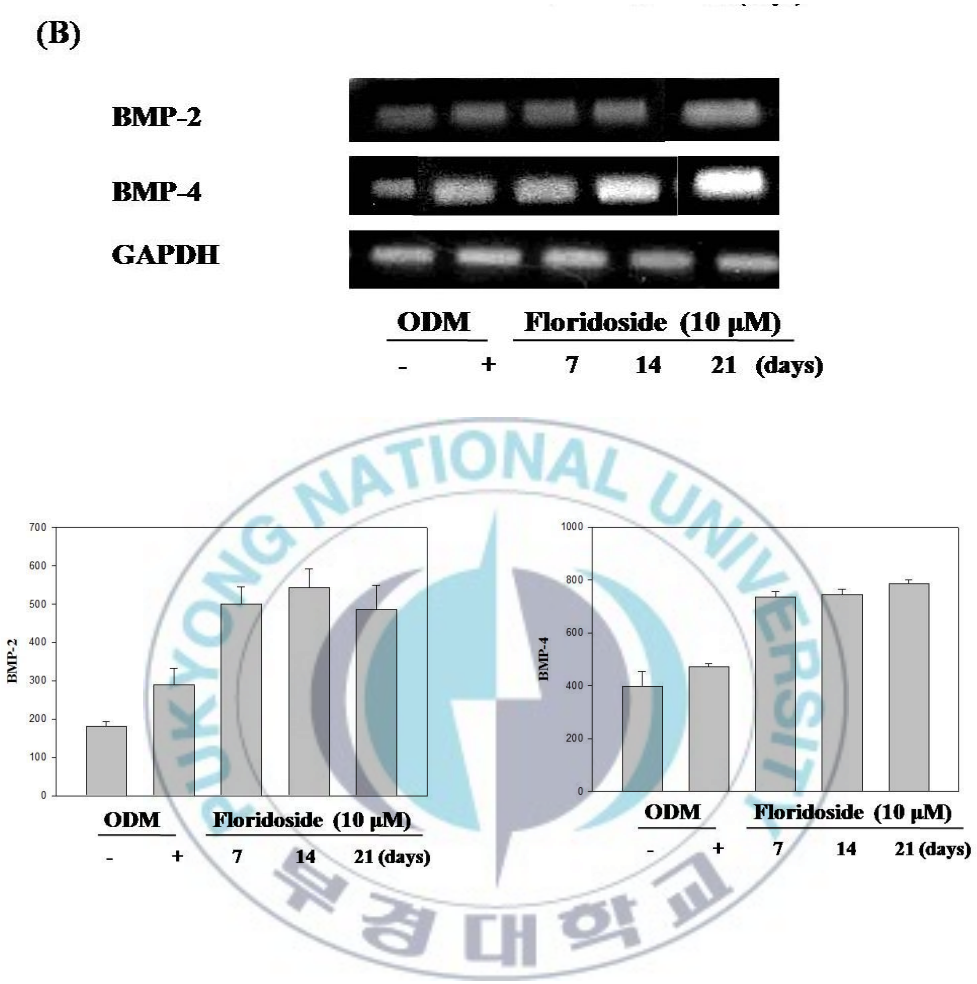


Figure 14: Effect of Floridoside on BMP-2 expression on mRNA expression in osteoblast cells, D1-cell line. Cells were treated with floridoside (10 μM). After incubation with ODM for 7,14 and 21 days, (B) The levels of BMP-2, BMP-4 mRNA were determined using RT-PCR analysis and GAPDH was used as an internal control. Western blot band are calculated by program Las3000 analysis tools.

The action of BMPs is mediated by heterotetrameric serine/threonine kinase receptors and the downstream transcription factors Smad1/5/8. Our study indicates that the production of BMP-2 increases in floridoside-treated D1-cells. Also, phosphorylations of Smad1/5/8 are simultaneously enhanced in floridoside-treated osteoblasts. Indeed, bone morphogenetic proteins. These results support the hypothesis that the BMP-2 signaling system plays an important role in floridoside-mediated cell maturation and differentiation in osteoblasts and also demonstrated that floridoside increased secretion of BMP-2 and are evidence that BMP-2 compare with blank (Fig.15 and 16).

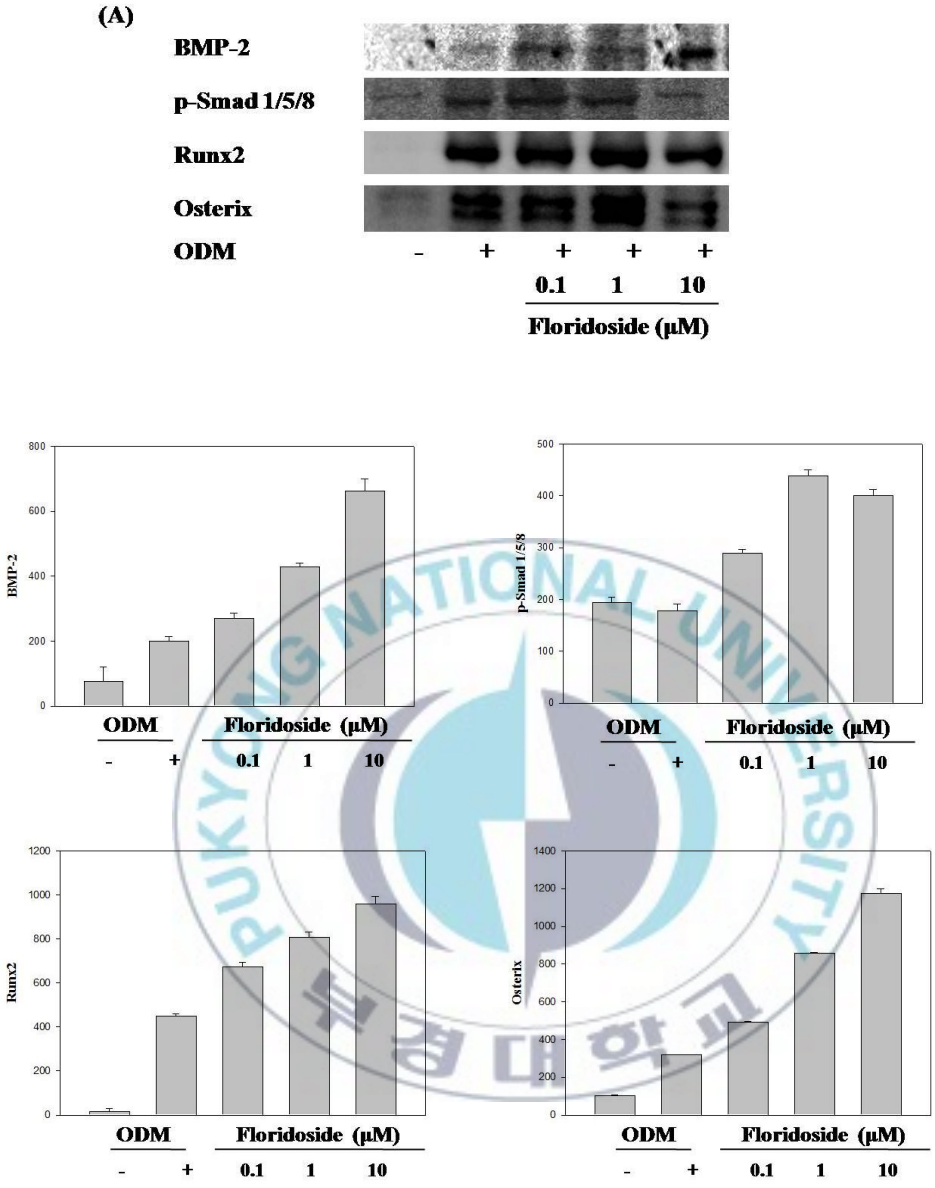


Figure 15: Effect of floridoside on BMP-2 expression on protein expression in osteoblast cells, D1-cell line. Cells were treated with various concentrations of floridoside (0.1, 1, 10 μM). After incubation with ODM for 14 days, as an optimal period (A) increased the protein levels of BMP-2, p-Smad 1/5/8, Runx-2 and Osterix in a dose-dependent manner. Western blot band area calculated by program of LAS3000 analysis tools.

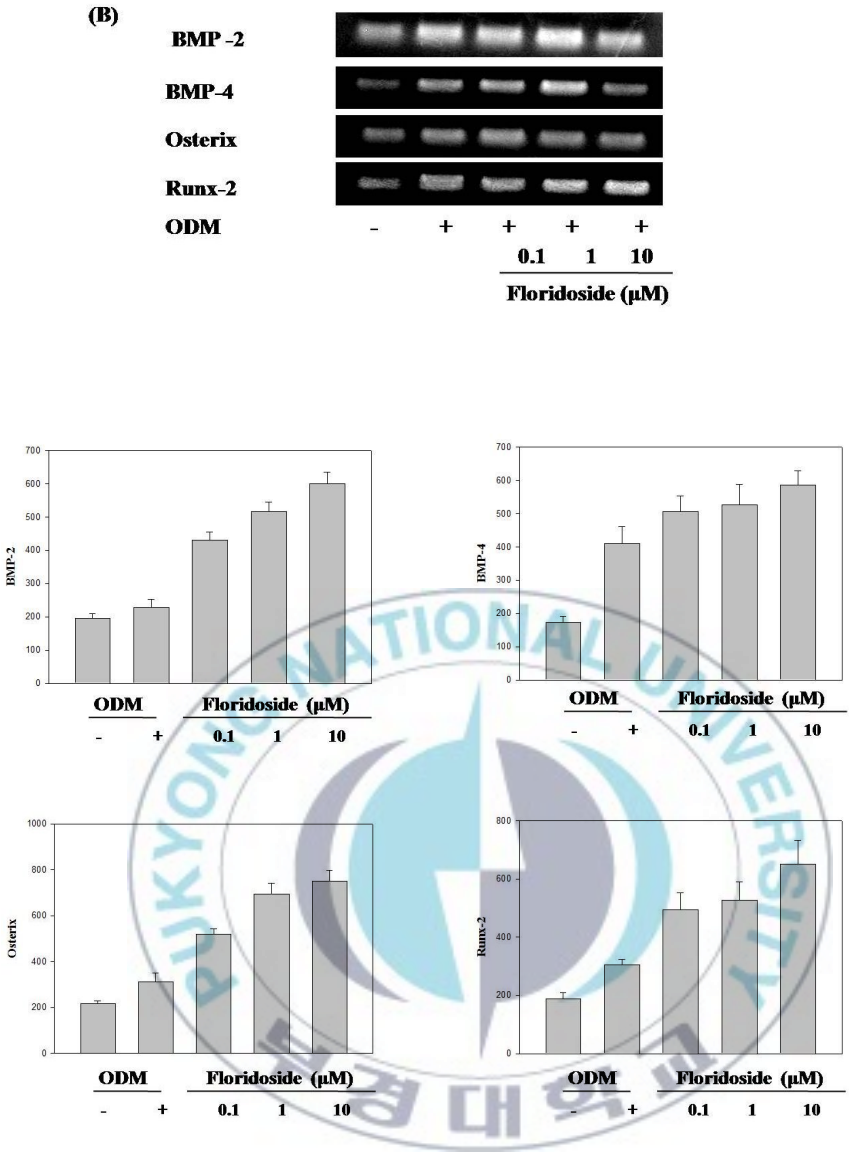


Figure 16: Effect of Floridoside on BMP-2 expression on mRNA expression in osteoblast cells, D1-cell line. Cells were treated with various concentrations of floridoside (0.1, 1, 10 μM). After incubation with ODM for 14 days, as an optimal period (B) The levels of BMP-2, BMP-4 mRNA were determined using RT-PCR analysis and GAPDH was used as an internal control. PCR band are calculated by program of LAS3000 anlysis tools.

4. Summary

During differentiation *in vitro*, osteoblast phenotypic markers appear in the following order: accumulation of collagenous matrix, expression of alkaline phosphate (ALP), secretion of osteocalcin, and finally, mineralization of bone nodules. Our results indicate that the presence of floridoside causes a significant increase in ALP activity, type I collagen synthesis and mineralization. As the appearance of ALP activity is an early phenotypic marker for mature osteoblasts, our results suggest that the presence of floridoside stimulates an early stage of osteoblast differentiation. The production of type I collagen, both phenotypic markers for the later stage of osteoblast differentiation, was increased by floridoside treatment. In addition, bone formation, as measured by mineralization, was also increased in osteoblastic differentiation D1-cells treated with floridoside. In this study we clearly show that the floridoside, investigated in the range 0.1 – 10 μM, increase the activity of ALP in osteoblastic differentiation D1-cells. The maximal stimulatory effect of ALP was observed after 14 days of treatment with various concentration of floridoside (0.1, 1, 10 μM). We therefore use this condition for futher studies.

BMPs play an important role in the process of bone formation and remodeling. Activation of BMP receptors initiates the BMP signaling pathway (mediated by Smad1, Smad5 and Smad8). Our study indicates that the production of BMP-2 increases in floridoside-treated D1-cells. Also, phosphorylations of Smad1/5/8 are simultaneously enhanced in floridoside-treated osteoblasts (ALP upreagulation and osteocalcin gene expression). In fact, BMP-2 induces the expression of Runx2, which then regulates the expression of Osx in osteoblastic differentiation. In the present study, the two BMP-related regulators, Runx2 and Osx, were found closely related to the promoting effect of floridoside in osteogenesis. These results clearly indicate an involvement of the BMP signal pathway in the floridoside-enhanced osteogenic differentiation. Our observation indicate that floridoside stimulates osteoblast differentiation at various stages in D1-cells. Floridoside's effect on cell maturation and differentiation is strongly associated with BMP-2/Smad 1/5/8 pathway. This

therefore suggests that floridoside may be beneficial in stimulating the osteoblastic activity resulting in bone formation.

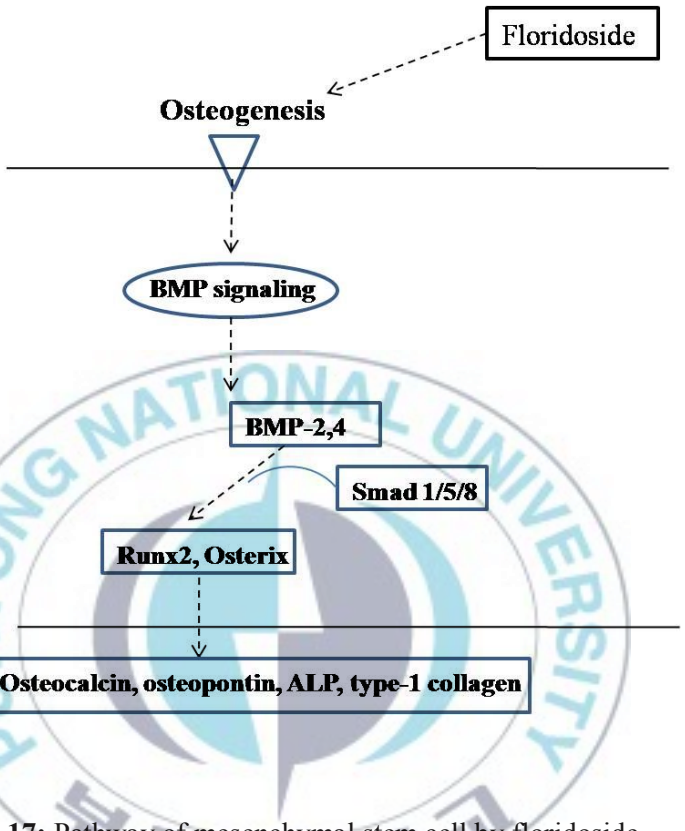


Figure 17: Pathway of mesenchymal stem cell by floridoside

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