โครงการ

ผลของลักษณะทางกายภาพของพื้นผิวไททาเนียมและซิมวาสเตตินต่อการเจริญเติบโตและพัฒนาเป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดมีเซนไคม์ของมนุษย์ในแบบจำลองภาวะกระดูกพรุน ในห้องปฏิบัติการ

โดย

นางสาวเปรมจิต อาภรณ์แม่กลอง และคณะ

กันยายน 2558

สัญญาเลขที่ **RSA5580016**

รายงานวิจัยฉบับสมบูรณ์

โครงการ

ผลของลักษณะทางกายภาพของพื้นผิวไททาเนียมและซิมวาสเตตินต่อการเจริญเติบโตและพัฒนาเป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดมีเซนไคม์ของมนุษย์ในแบบจำลองภาวะกระดูกพรุน ในห้องปฏิบัติการ

ผู้วิจัย นางสาวเปรมจิต อาภรณ์แม่กลอง

สังกัด ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และมหาวิทยาลัยสงขลานครินทร์

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณหน่วยงาน ผู้ทรงคุณวุฒิ เพื่อนร่วมงานและเจ้าหน้าที่ทุกท่านที่ได้ให้การสนับสนุนการดำเนินงานวิจัยนี้ทั้งในรูปของทุนการดำเนินการวิจัย การเอื้ออุปกรณ์วิทยาศาสตร์ ให้คำแนะนำที่เป็นประโยชน์ และช่วยเหลือ ใน การ ดำเนินงานต่าง ๆให้สำเร็จลุล่วงไปด้วยดีดังมีรายนามดังต่อไปนี้

1. สำนักงานกองทุนสนับสนุนการวิจัย
2. คณะกรรมการส่งเสริมการศึกษาในระดับอุดมศึกษา
3. มหาวิทยาลัยสงขลานครินทร์
4. สถานวิจัยวิศวกรรมเนื้อเยื่อแข็งเพื่อกะโหลกศีรษะใบหน้าและขากรรไกร คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์
5. เจ้าหน้าที่ประจำห้องปฏิบัติการกลาง คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์
6. บริษัท Institut Straumann AG, Basel, Switzerland ที่ให้การสนับสนุนแผ่นไททาเนียมเพื่อใช้ในการศึกษา

(ลายเซ็น).....

(นางสาวเปรมจิต อาภรณ์แม่กลอง)

หัวหน้าโครงการวิจัย

บทคัดย่อ

วัตถุประสงค์ การศึกษานี้มีเป้าหมายที่จะทำการศึกษาผลของลักษณะพื้นผิวไททาเนียมชนิดเรียบ (smooth) และไทเทเนียมที่ทำการปรับสภาพผิวด้วยการเป่าทรายและกรดกัด (sandblasted and acid-etched, SLA) และซิมวาสเตติน ต่อการเจริญเติบโต (growth) และการพัฒนาไปเป็นเซลล์สร้างกระดูก (osteogenic differentiation) ของเซลล์จากไขกระดูกในชั้นสตอร์มาลของมนุษย์ (human bone marrow stromal cells, hBMSCs) ในสภาวะคลาดแคลนเอสโตรเจน

วัสดุและวิธีการ นำเซลล์ hBMSCs มาวางลงบนถาดเลี้ยงเซลล์ ขนาด 24 หลุมต่อถาดและบนแผ่นไททาเนียม ชนิดผิวเรียบและหยาบ แบบ SLA (Straumann, Switzerland) ซึ่งแต่ละแผ่นได้ถูกวางไว้ในถาดเลี้ยงเซลล์ ขนาด 24 หลุมชนิดป้องกันการเกาะติดของเซลล์ หลังจากนั้นนำเซลล์ดังกล่าวมาเลี้ยงในน้ำเลี้ยงเซลล์เพื่อศึกษา การเจริญเติบโต (growth) และ การเหนี่ยวนำการพัฒนาไปเป็นเซลล์สร้างกระดูก (osteogenic media, OS) ชนิดปรกติ (FBS) ชนิดที่ขาดแคลนเอสโตรเจน (estrogen-deprived, ED) และ ชนิด ED ที่มี 100 นาโน โมลาร์ ซิมวาสเตติน (simvastatin) ผสมอยู่ด้วย (ED-SIM) เป็นเวลา 14 – 21 วัน แล้วทำการย้อมสีเพื่อตรวจสอบความมีชีวิต และการตายของเซลล์ (live/dead cell staining) ทำการวัดความมีชีวิตเพื่อตรวจสอบการเจริญเติบโตของเซลล์ (cell viability assay) ทำการตรวจสอบการเกาะติดและ รูปร่างของเซลล์ด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องกราด (scanning electron microscope, SEM) จากนั้นทำการศึกษาการพัฒนาไปเป็นเซลล์สร้างกระดูกโดยการตรวจวัดการแสดงออกของยีนส์ที่เกี่ยวข้องกับการพัฒนาไปเป็นเซลล์สร้างกระดูก อันได้แก่ runx2 และ bone sialoprotein (IBSP) และทำการวัด ระดับการทำงานของอัลคาไลค์ฟอสฟาเตส (alkaline phosphatase activity) ปริมาณของแคลเซียม (calcium contents) และระดับของออสติโอแคลซิน (osteocalcin) ส่วนระดับการแสดงออกของยีนส์ Bone morphogenetic protein-2 (BMP-2) ได้ถูกวัดเพื่อตรวจสอบผลการกระตุ้นเซลล์ของซิมวาสเตตินที่มีผลต่อการสร้างกระดูก และทำการย้อมสี

แคลซิน (calcein staining) เพื่อแสดงการสะสมของแคลเซียม ในโปรตีนเมตริกซ์นอกเซลล์ (extracellular matrix)

ผลการศึกษาและอภิปราย เซลล์จากไขกระดูกในชั้นสตอร์มาลของมนุษย์แสดงลักษณะการเกาะติดบนพื้นผิวและรูปร่างที่ต่างกันบนพื้นผิวไททาเนียมต่างชนิดกัน น้ำเลี้ยงเซลล์ชนิดขาดแคลนเอสโตรเจน (ED) มีผลลดการเกาะติดและลดการเจริญเติบโตของเซลล์โดยเฉพาะเซลล์ที่เลี้ยงบนผิวหยาบชนิด SLA แต่ถึงกระนั้นเซลล์สามารถเจริญเติบโตในน้ำเลี้ยงเซลล์ที่ขาดแคลนเอสโตรเจนซึ่งทำให้มีจำนวนเซลล์เพิ่มมากขึ้นจนมาบรรจบกัน (confluence) ในวันที่ 21 ของการเลี้ยงเซลล์ พื้นผิวไททาเนียมชนิด SLA สามารถส่งเสริมการพัฒนาไปเป็นเซลล์สร้างกระดูกของ hBMSCs ทั้งในน้ำเลี้ยงเซลล์ปรกติและในภาวะขาดแคลนเอสโตรเจน แต่ภาวะขาดแคลนเอสโตรเจนส่งผลลดความสามารถในการส่งเสริมการพัฒนาไปเป็นเซลล์สร้างกระดูกของพื้นผิวไททาเนียมชนิด SLA ลงไปอย่างมาก นอกจากนี้ยังพบว่าซิมวาสเตตินสามารถส่งเสริมการพัฒนาไปเป็นเซลล์สร้างกระดูกได้แม้ในภาวะขาดแคลนเอสโตรเจนโดยเฉพาะบนพื้นผิว SLA และผลการส่งเสริมนี้สอดคล้องกับการแสดงออกที่เพิ่มมากขึ้นของยีนส์ BMP-2 บนพื้นผิว SLA ในน้ำเลี้ยงเซลล์ที่ขาดแคลนเอสโตรเจน

สรุป แบบจำลองการเลี้ยงเซลล์ในภาวะขาดแคลนเอสโตรเจนที่นำเสนอในการศึกษานี้เป็นรูปแบบการศึกษาที่สามารถนำไปประยุกต์ใช้เพื่อศึกษาผลกระทบจากฮอร์โมนและสารเร่งการเจริญเติบโต (growth factors) ต่อปฏิสัมพัธ์ระหว่างเซลล์และพื้นผิวไททาเนียม การศึกษานี้พบว่าพื้นผิวไททาเนียมชนิด SLA ส่งเสริมฤทธิ์การกระตุ้นการพัฒนาไปเป็นเซลล์สร้างกระดูกของซิมวาสเตตินและผลการส่งเสริมร่วมกันนี้น่าที่จะสามารถนำมาประยุกต์ใช้ในทางคลินิก เพื่อส่งเสริม การยึดเกาะระหว่างรากฟันเทียมและกระดูกรองรับฟันต่อไป

คำหลัก ภาวะขาดแคลนเอสโตรเจน พื้นผิวไทเทเนียมที่ทำการปรับสภาพผิวด้วยการเป่าทรายและกรดกัด ซิมวาสเตติน การพัฒนาเป็นเซลล์สร้างกระดูก เซลล์จากไขกระดูกในชั้นสตอร์มาลของมนุษย์

Abstract

Purposes-The current study aimed to investigate effects of titanium surface topography and simvastatin on growth and osteogenic differentiation of human bone marrow stromal cells (hBMSCs) in estrogen-deprived (ED) cell culture.

Materials and Methods-Human BMSCs were seeded on cell culture plates and titanium (Ti) disks, smooth and sandblasted and acid etched (SLA) Ti surfaces (Straumann, Switzerland), and subsequently cultured in regular (FBS), estrogen-deprived (ED) and ED-with 100 nM simvastatin (ED-SIM) growth or osteogenic (OS) media for 14 – 21 days. Live/dead cell staining, scanning electron microscope (SEM) examination and cell viability assay were performed to determine cell attachment, morphology and growth. Expression levels of osteoblast-associated genes, Runx2 and bone sialoprotein (IBSP) and levels of alkaline phosphatase (ALP) activity, calcium contents and osteocalcin in culture media were measured to determine osteoblastic differentiation potenital. Expression levels of bone morphogenetic protein 2 (BMP-2) was investigated to examine stimulating effects of simvastatin. *In vitro* mineralization was verified by calcein staining.

Results-Human BMSCs exhibited different cell attachment and shapes on smooth and SLA titanium surfaces. Estrogen-deprived cell culture decreased cell attachment and growth, particularly on SLA titanium surface, but cells were able to grow to reach confluence on day 21 in ED-OS culture medium. Sandblasted and acid etch titanium surface promoted osteogenic differentiation in FBS- and ED-OS, but the promoting effects of SLA titanium surface in ED-OS were significantly decreased. Simvastatin significantly increased osteogenic differentiation of hBMSCs on SLA titanium surface in ED-OS medium and the promoting effects of simvastatin corresponded with the increasing of BMP-2 gene expression on SLA titanium surface in ED-OS-SIM culture medium.

Conclusions-Estrogen-deprived cell culture model provided a well-defined platform for investigating effects of hormone and growth factors on cells and titanium surface interaction. Titanium surface microtopography of SLA surface and simvastatin synergistically promoted osteoblastic differentiation of hBMSCs in ED condition and they might be applied to promote osteointegration in osteoporotic bone.

Keywords: Estrogen-deprived, titanium surfaces, sand blasted and acid etched, simvastatin, human bone marrow stromal cells, osteogenic differentiation

Executive Summary

Executive Summary

สัญญาเลขที่ RSA5580016

โครงการ ผลของลักษณะทางกายภาพของพื้นผิวไททาเนียมและซิมวาสเตตินต่อการเจริญเติบโตและพัฒนาเป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดมีเซนไคม์ของมนุษย์ในแบบจำลองภาวะกระดูกพรุนในห้องปฏิบัติการ (Effects of Titanium Surface Microtopography and Simvastatin on Growth and Osteogenic Differentiation of Human Mesenchymal Stem Cells in Estrogen-deprived Cell Culture)

สรุปข้อเสนอโครงการ

ผู้เสนอ : นางสาวเปรมจิต อาภรณ์แม่กลอง

ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ระยะเวลาดำเนินการ : 3 ปี (16 กรกฎาคม 2555 ถึง 31 กรกฎาคม 2558)

[ดำเนินการล่าช้ากว่ากำหนดเดิม 1 ปี (16 กรกฎาคม 2555 ถึง 15 กรกฎาคม 2557)]

งบประมาณ : 800,000 บาท (แปดแสนบาทถ้วน)

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1. ความสำคัญและที่มาของปัญหา

Osteoporosis, one of the public health problems associated with aging, might be considered a risk in implant therapy. A decreasing of estrogen level in senile osteoporosis decreases bone formation, promotes bone resorption and enhances adipogenic differentiation of mesenchymal stem cells (MSCs) (1). Unbalanced bone remodeling and deteriorating microarchitecture of osteoporotic bone create negative effects on osteointegration and implant stability in ovarectomised rabbits, rats and sheep (2-4). Bone mineral density (BMD) in bone marrow region of ovarectomized rats are significantly 30-40% lower than non-osteoporosis group (4), and removal torque of implants in osteoporotic tibia of ovarectomized rabbits is significantly decreased (3).

Various methods have been applied to increase BMD surrounding implant and improve bone implant contact in osteoporotic bone including implant surface modifications(5) and systemic and locally applied simvastatin (6, 7). Surface microtopography of titanium surface has been shown to be a major factor regulating cell response to biomaterials (8, 9). It is reported that rough titanium surface promotes osteogenic differentiation of hBMSCs (10, 11) and increase bone implant contact (12, 13). Surface roughness of Sand blasted and acid etched (SLA) titanium surface promotes early differentiation, bone formation, implant integration and reduce healing time of the implants (14). Superior effects of the SLA titanium surface on osteogenic differentiation have been well established (15, 16).

Simvastatin may synergistically enhance osteogenic differentiation of estrogen-deprived MSCs on titanium surface. Statin is primarily used in the treatment of hypercholesterolemia and has been reported to possess anabolic effects on bone (Song et al 2008). Simvastatin is reported to decrease fracture risk, increase bone mineral density (BMD),

enhance BMP2 expression and stimulate osteoblast differentiation *in vitro* (17). Enhancing effects of simvastatin on expression levels of BMP-2, a strong osteoinductive gene may be able to enhance osteogenic differentiation of estrogen-deprived hBMSCs on SLA titanium surface.

The current study aimed to establish estrogen-deprived cell culture model and investigate effects of titanium surface microtopography, smooth and SLA titanium surfaces, and simvastatin on growth and osteogenic differentiation of hBMSCs in estrogen-deprived cell culture. It was hypothesized that SLA titanium surface would be able to promote growth and osteogenic differentiation of estrogen-deprived human bone marrow stromal cells (ED-hBMSCs) and the promoting effects would be further enhanced by simvastatin supplementation.

2. วัตถุประสงค์

The current study aimed to

1. Establish estrogen-deprived cell culture model and
2. Investigate effects of *titanium surface microtopography*, smooth and SLA titanium surfaces, and *simvastatin* on growth and osteogenic differentiation of hBMSCs in estrogen-deprived cell culture.

3. ระเบียบวิธีวิจัย (Figure 1 and Tables 1 and 2)

Human bone marrow stromal cells (hBMSCs) at passages 3-5 in growth medium (FBS-growth medium) were seeded on 24-well cell culture plate and smooth and sandblasted and acid-etched titanium disks in non-treated 24-well cell culture plates. Cells were cultured in minimal growth medium (300 µl) for 3 hr. and 1 ml of culture medium / well for 24 hr. Then culture medium was changed to either regular with fetal bovine serum (FBS), estrogen-deprived

with charcoal strip FBS (ED) or ED with simvastatin (ED-SIM) culture media for 14 – 21 days according to groups of study (Figure 1 and Table 1).

To investigate cell adhesion, morphology and growth, live/dead cell (CellTracker^{TM} Green / Propidium iodide) staining and cell viability assay were performed. Quantitative real-time polymerase chain reaction (qRT-PCR) and alkaline phosphatase (ALP) activity, calcium contents and osteocalcin assays were performed to examine osteoblastic differentiation potential. After that growth and osteogenic differentiation of cells in different culture media and cell culture surfaces were compared. Results were derived from 2-3 independent experiments. The investigations at each time point were performed in 4-5 consecutive samples (n= 4-5, MEAN±SD) (Figure 1 and Table 2).

Figure 1 Outline of the study

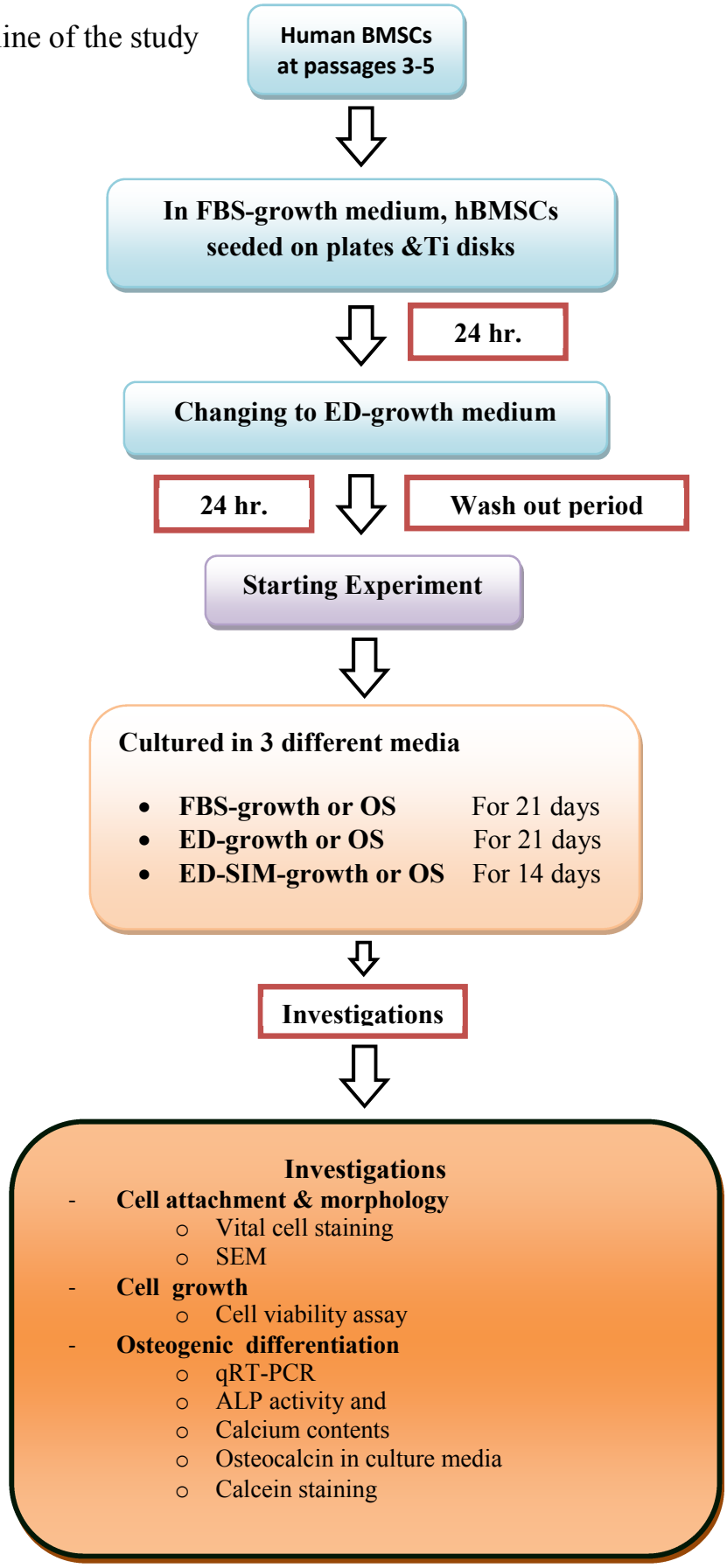

Table 1 Groups of study

Categories	Culture media	Groups	Description
I	Regular (FBS)	**A**	FBS- Plate
		B	FBS- SM
		C	FBS-SLA
II	Estrogen-deprived (ED)	**A**	ED-Plate
		B	ED-SM
		C	ED-SLA
III	ED-Simvastatin supplement (ED-SIM)	**A**	ED-SIM- Plate
		B	ED-SIM-SM
		C	ED-SIM- SLA

Note: FBS is an abbreviation for culture medium containing fetal bovine serum (FBS), ED-OS for estrogen deprived osteogenic medium containing charcoal stripped FBS, SIM for simvastatin, Plate, for cell culture plates and SM, smooth and SLA, sandblasted and acid etched titanium surfaces.

Table 2 Summary of the investigations

Investigations	Procedures	Investigating time
Cell attachment, spreading and morphology	Live/dead cell staining	At 3 hours after cell seeding
	Live/dead cell staining	At 24 hr. and on day 21
	SEM	On days 7 and 21
Cell growth	Cell viability assay	On days 2, 7, 14 and 21
Osteogenic differentiation	qRT-PCR analysis of osteoblast-associated genes	On days 7 and 21
	ALP activity analysis	On days 7 and 21
	Calcium content assay	On day 21
Expression of BMP-2	qRT-PCR analysis	On day 14

Note: ALP is an abbreviation for alkaline phosphatase, BMP-2, bone morphogenetic protein-2, qRT-PCR, quantitative Real-time polymerase chain reaction and SEM, scanning electron microscope

4. แผนการดำเนินการวิจัย (Table 3)

Table 3 Experimental time line

Activity		2555		2556				2557				2558		
		Jul-Sep	Oct - Dec	Jan - Mar	Apr - Jun	Jul-Sep	Oct - Dec	Jan - Mar	Apr - Jun	Jul-Sep	Oct - Dec	Jan - Mar	Apr - Jun	July
Preparation	Facility & chemical	←	→											
Experiment	Pilot study setting up estrogen-deprived cell culture		←	—	→									
	Pilot study on cell culture on titanium disks			←	→									
	Investigating cell growth and attachment on titanium surface				←	—	—	—	—	→				
	Performing qRT-PCR for gene expression						←	→						
	Measuring ALP activity and calcium contents						←	—	→					
	Analysis									↔				
	Pilot on simvastatin supplement									↔				
	Investigating effects of simvastatin on growth & differentiation									←	—	—	→	
Final report & Manuscript														↔

5. ชื่อเรื่องและชื่อวารสารที่คาดว่าจะตีพิมพ์ในวารสารวิชาการนานาชาติ

1. ชื่อเรื่อง Effects of titanium surface microtopography and simvastatin on growth and osteogenic differentiation of estrogen-deprived human mesenchymal stem cells, an *in vitro* study

ชื่อวารสาร International Journal of Oral and Maxillofacial Implant

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เนื้อหางานวิจัย

Effects of Titanium Surface Microtopography and Simvastatin on Growth and Osteogenic Differentiation of Human Mesenchymal Stem Cells in Estrogen-deprived Cell Culture

(ผลของลักษณะทางกายภาพของพื้นผิวไททาเนียมและซิมวาสเตตินต่อการเจริญเติบโตและพัฒนาเป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดมีเซนไคม์ของมนุษย์ในแบบจำลองภาวะกระดูก พรุนในห้องปฏิบัติการ)

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INTRODUCTION

Osteoporosis, one of the public health problems associated with aging, might be considered a risk in implant therapy. A decreasing of estrogen level in senile osteoporosis decreases bone formation, promotes bone resorption and enhances adipogenic differentiation of mesenchymal stem cells (MSCs) (1). Unbalanced bone remodeling and deteriorating microarchitecture of osteoporotic bone create negative effects on osteointegration and implant stability in ovarectomised rabbits, rats and sheep (2-4). Bone mineral density (BMD) in bone marrow region of ovarectomized rats are significantly 30-40% lower than non-osteoporosis group (4), and removal torque of implants in osteoporotic tibia of ovarectomized rabbits is significantly decreased (3).

Various methods have been applied to increase BMD surrounding implant and improve bone implant contact in osteoporotic bone including implant surface modifications(5) and systemic and locally applied simvastatin(6, 7). Surface microtopography of titanium surface has been shown to be a major factor regulating cell response to biomaterials (8, 9). It is reported that rough titanium surface promotes osteogenic differentiation of hBMSCs (10, 11) and increase bone implant contact (12, 13). Surface roughness of Sand blasted and acid etched (SLA) titanium surface promotes early differentiation, bone formation, implant integration and reduce healing time of the implants (14). Superior effects of the SLA titanium surface on osteogenic differentiation have been well established (15, 16).

Simvastatin may synergistically enhance osteogenic differentiation of estrogen-deprived MSCs on titanium surface. Statin is primarily used in the treatment of hypercholesterolemia and has been reported to possess anabolic effects on bone (Song et al 2008). Simvastatin is reported to decrease fracture risk, increase bone mineral density (BMD),

enhances BMP2 expression and stimulates osteoblast differentiation *in vitro* (17). Enhancing effects of simvastatin on expression levels of BMP-2, a strong osteoinductive gene may be able to enhance osteogenic differentiation of estrogen-deprived hBMSCs on SLA titanium surface.

The current study aimed to establish estrogen-deprived cell culture model and investigate effects of titanium surface microtopography, smooth and SLA titanium surfaces, and simvastatin on growth and osteogenic differentiation of hBMSCs in estrogen-deprived cell culture. It was hypothesized that SLA titanium surface would be able to promote growth and osteogenic differentiation of estrogen-deprived human bone marrow stromal cells (ED-hBMSCs) and the promoting effects would be further enhanced by simvastatin supplementation.

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MATERIALS AND METHODS

Outline of the study

Human bone marrow stromal cells (hBMSCs) at passages 3-5 in growth medium (FBS-growth medium) were seeded on 24-well cell culture plate and smooth and sandblasted and acid-etched titanium disks in non-treated 24-well cell culture plates. Cells were cultured in minimal growth medium for 3 hr. and 1 ml of culture medium / well for 24 hr. Then culture medium was changed to either regular, estrogen-deprived (ED) and ED with simvastatin culture media for 14 – 21 days according to groups of study (Fig. 1 and Table 1).

To investigate cell adhesion, morphology and growth, live/dead cell (CellTracker^{TM} Green / Propidium iodide staining) and cell viability assay were performed. Quantitative real-time polymerase chain reaction (qRT-PCR) and ALP activity, osteocalcin in culture medium and calcium contents in extra cellular matrix assays were performed to examine osteoblastic differentiation potential. After that growth and osteogenic differentiation of cells in different culture media and cell culture surfaces were compared. Results were derived from 2-3 independent experiments. The investigations at each time point were performed in 4-5 consecutive samples (n=4-5, MEAN±SD) (Fig. 1 and Table 2).

Groups of Study

The study was categorized into 3 categories and 3 groups, according to types of culture media and cell culture surfaces, respectively, Category I-Regular (FBS), II-Estrogen-deprived (ED) and III-ED-Simvastatin supplement culture media. In each category, cells were seeded on 3 different surfaces, Groups A: cell culture plates and B: smooth and C: sandblasted and acid etched titanium surfaces (Table 1).

Table 1 Groups of study

Categories	Culture media	Groups	Description
I	Regular (FBS)	**A**	FBS- Plate
		B	FBS- SM
		C	FBS-SLA
II	Estrogen-deprived (ED)	**A**	ED-Plate
		B	ED-SM
		C	ED-SLA
III	ED-Simvastatin supplement (ED-SIM)	**A**	ED-SIM- Plate
		B	ED-SIM-SM
		C	ED-SIM- SLA

Note: FBS is an abbreviation for culture medium containing fetal bovine serum (FBS), ED-OS for estrogen deprived osteogenic medium containing charcoal stripped FBS, SIM for simvastatin, Plate, for cell culture plates and SM, smooth and SLA, sandblasted and acid etched titanium surfaces.

Figure 1 Outline of the study

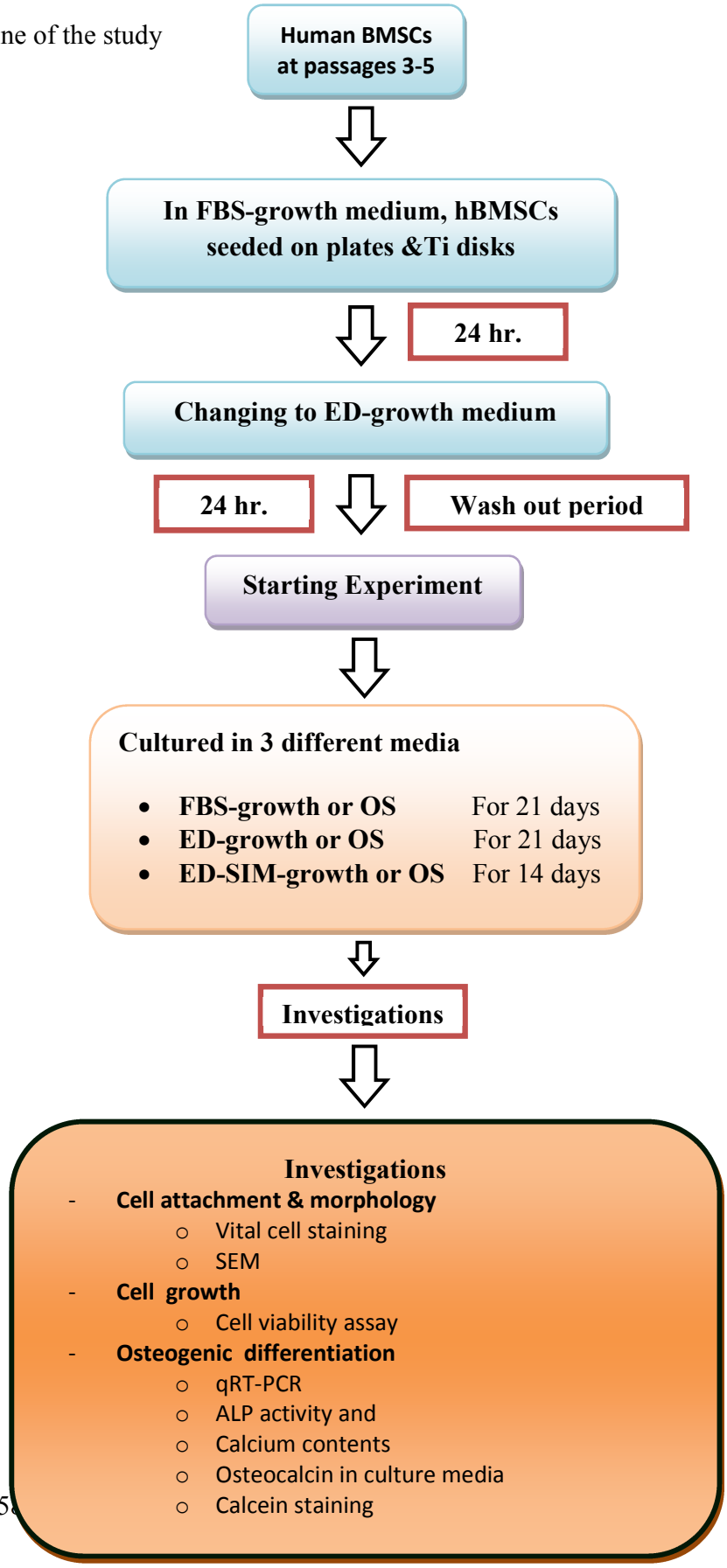


Table 2 Summary of the investigations

Investigations	Procedures	Investigating time
Cell attachment, spreading and morphology	Live/dead cell staining	At 3 hours after cell seeding
	Live/dead cell staining	At 24 hr. and on day 21
	SEM	On days 7 and 21
Cell growth	Cell viability assay	On days 2, 7, 14 and 21
Osteogenic differentiation	qRT-PCR analysis of osteoblast-associated genes	On days 7 and 21
	ALP activity analysis	On days 7 and 21
	Calcium content assay	On day 21
Expression of BMP-2	qRT-PCR analysis	On day 14

Note: ALP is an abbreviation for alkaline phosphatase, BMP-2, bone morphogenetic protein-2, qRT-PCR, quantitative Real-time polymerase chain reaction and SEM, scanning electron microscope

Human Bone Marrow Cell Culture

Obtaining a permission and approval from the Ethical Committee of Faculty of Medicine, Prince of Songkla University (Permission number EC-54-286-19-1-2) and patient written informed consent, human bone marrow stromal cells (hBMSCs) were harvested from healthy adult patients (age 19-45 years) undergoing orthopedic surgery at Prince of Songklanagarind hospital. Human BMSCs were harvested and expanded as described previously (18). Human MSCs at passages 4-5 were used in the analyses (Figures 2 & 3).

Human BMSCs were cultured in regular (Fetal bovine serum, FBS), estrogen-deprived (ED) and estrogen-deprived with simvastatin (ED-SIM) culture media. *Regular growth medium* (FBS-growth) comprised of DMEM-F12 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 0.5% fungizone (all from Gibco BRL/Life technologies, Rockville, MD, USA) (19). For *estrogen-deprived growth medium (ED-growth)*, phenol red free DMEM-F12 was supplemented with 10% charcoal stripped FBS (all from Gibco BRL/Life technologies) and 0.5% ITS+3 Liquid Media Supplement (100×) (Sigma Chemical Co., St. Louis, MO, USA), 1% penicillin/streptomycin and 0.5% fungizone (all from Gibco BRL/Life technologies). For osteogenic differentiation (OS) medium (FBS-OS and ED-OS), FBS and ED growth media were supplemented with 50 mM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone (all from Sigma Chemical Co.) (19). For ED-OS medium with 100 nM simvastatin supplement (ED-OS-SIM), 100 μM simvastation in dimethyl sulfoxide (DMSO) was supplemented just before used in a 1:1000 ratio of DMSO to culture medium. In a control group for ED-OS-SIM cell culture, ED-OS medium was also supplemented with DMSO in a ratio of 1:1000. The amount of DMSO was limited to 0.1% (all from Sigma Chemical Co.).

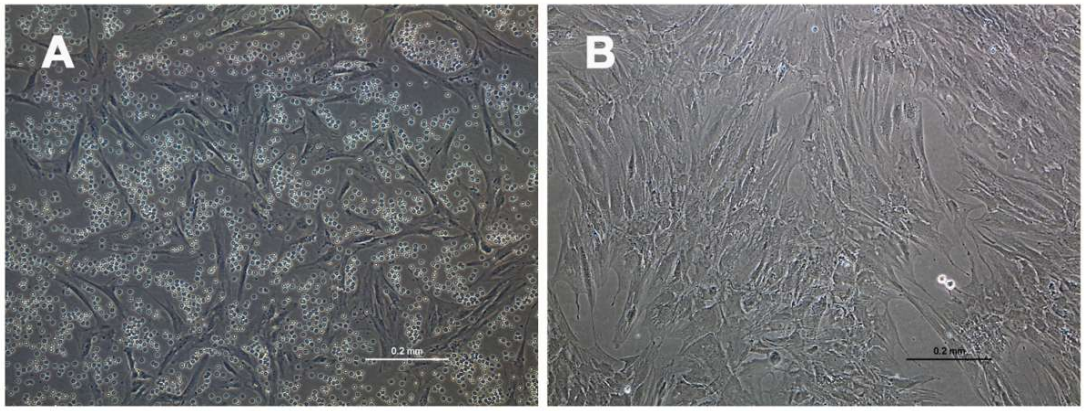


Figure 2 Images of hBMSCs at primary and third passages under inverted microscope, (A) Demonstrating small individual spindle-shaped cells attaching on cell culture plate and floating blood cells on day 5 after bone marrow cell seeding and (B) Exhibiting uniform fibroblast-like cells of hBMSCs at passage 3 at 80% confluence and being ready for cell seeding.

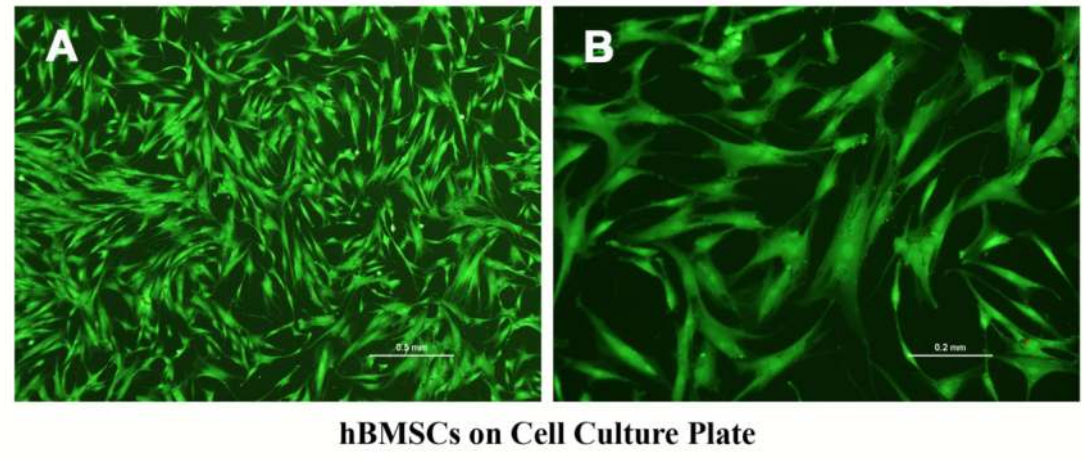


Figure 3 Confocal laser scanning microscope images of vital cell staining (CellTracker^{TM} Green, Molecular Probes, USA) of hBMSCs at passage 4 on cell culture plate in growth medium on day 7. Images demonstrated viability of spindle-shape and fibroblast-like cells creating intercellular contact at 70% confluence which were ready for cell seeding.

Cell Seeding and Cell Culture Scheme

Titanium disks with smooth and sandblasted and acid-etched titanium surfaces, 15 mm in diameter with 1 mm thickness, were kindly provided by Straumann (Institut Straumann AG, Basel, Switzerland) (Figure 5). Disks were placed in non-treated 24-well cell culture plate (Costar, Pittsburgh, PA, USA), one well for one disk for cell seeding and culture.

Human BMSCs were seeded on 24-well cell culture plate (Costar) and titanium disks, smooth and SLA titanium surfaces. Human BMSCs were seeded at $1x10^4$ cells/cm^2 or $2x10^4$ cells/disks for cell growth and attachment studies, and $2x10^4$ cells/cm^2 or $4x10^4$ cells/disk for osteogenic differentiation study. Cells, $2x10^4$ or $4x10^4$ cells, were suspended in 300 µl of growth medium and seeded on each well of 24-well cell culture plates and titanium disk. Then seeded cells were cultured in minimal FBS-growth medium for 3 hr. and then 24 hr. in 1 ml culture medium in a humidified incubator with 5% CO_2 at 37ºC. After that FBS growth medium was replaced with 1 ml ED-growth medium for 24 hr. wash out period (20). Subsequently, cells were cultured either in FBS-or ED-growth or osteogenic media for 21 day, and ED-OS-SIM for 14 days according to groups of the study for investigations (Figure 5 & Table 2).

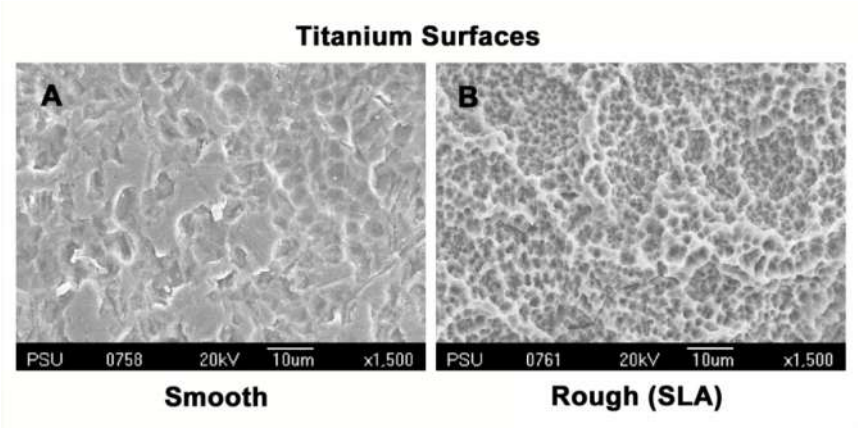
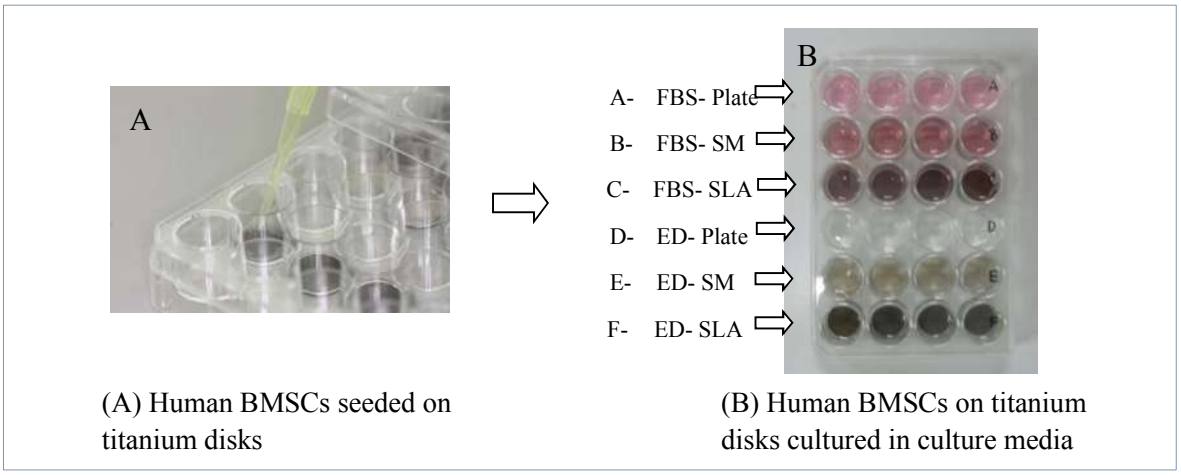


Figure 4 Scanning electron microscope images of titanium surfaces, (A) smooth and (B) rough, sandblasted and acid-etched (SLA) titanium surfaces



(A) Human BMSCs seeded on titanium disks

(B) Human BMSCs on titanium disks cultured in culture media

Figure 5 Demonstrating cell seeding on Ti disks and cell culture in different culture media according to groups of study

Live and Dead Cell Staining

To examine cell attachment, spreading, viability and cell dead, cells were incubated in a mixture of 5 μM CellTracker^{TM} Green (Molecular Probes/Invitrogen, Carlsbad, CA, USA) and 0.5 mg/ml propidium iodide (Sigma Chemical Co.) in ED growth medium for 30 minutes in a humidified incubator with 5% CO_2 at 37ºC. After that the disks were rinsed twice with phosphate buffer solution (PBS), fixed in 10% buffered formaldehyde and examined under fluorescence microscope (Ti-S100, Nikon, Japan) or confocal laser scanning microscope (CLSM) (FV300, Olympus, Japan). The staining was performed at 24 hr. after cell seeding in growth medium and then on days 7 and 21 in osteogenic medium (n=3) (Table 2) (21).

Scanning Electron Microscope

Cell attachment and morphology on titanium disks were assessed optically by scanning electron microscope (SEM) (5800LV, JEOL, Japan). At each investigation time, cells were fixed in 4% glutaraldehyde, dehydrated in ethanol series of 30-100%, dried, gold sputter-coated (SPI Module^{TM} Sputter Coater, SPI, USA) and examined under scanning electron

microscope (SEM, 5800LV, JEOL)(22). The examination was performed on days 7 and 21 (n=3) (Table 2) (21).

Cell Viability Assay

Growth curve was established using cell viability assay. Cell viability was measured as an indicator of cell growth and CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used following manufacturer's instructions. The formazan dye was quantified at 440 nm absorbance in duplicate using a microplate reader (Multiskan GO, Thermo Scientific, Finland). Then optical density values were extrapolated with a standard curve of cell numbers. Cell viability was determined on days 1, 7, 14 and 21 in growth media (n=4, Mean±SD) (Table 2) (21).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR was performed to determine expression levels of early and late osteoblastic differentiation associated genes, Runx2 and IBSP (23) and bone morphogenetic protein-2 (BMP-2) gene. Total RNA was extracted using Trizol (Invitrogen) and 1μg of RNA was reverse transcribed into cDNA using cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Equal amount of cDNA was amplified by PCR using the TaqMan Gene Expression Master Mix (Applied Biosystems) and 20×Target primers and Probe (Applied Biosystems). Genes and primers used are as followed, Runx2 (Hs01047973_m1), IBSP (Hs00173720_m1) and BMP-2 (Hs00154192_m1) (Applied Biosystems). The expression of the genes was measured by qRT-PCR on the Rotor Gene Q Detection System (Qiagen, Hilden, Germany). Levels of the target genes were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs02758991_g1) (Applied Biosystems) as an endogenous reference. Subsequently, the expression levels of investigated genes were normalized to the

expression levels of hBMSCs on cell culture plate in FBS-OS medium and reported as fold changes. Data presented for were averaged from 3 independent cultures (n=3, MEAN±SD) (24).

Alkaline Phosphatase (ALP) Activity Analysis

At each investigation time, hBMSCs on cell culture plates and titanium disks were lyzed in 1% Triton X-100 (Sigma Chemical Co.) to obtain total protein lysate and cell pellets. Amount of total protein contents and levels of alkaline phosphatase (ALP) activity in the protein lysis solution were measured, and pellets from the same samples were kept for calcium content assay (22).

The quantification of protein amount in cell lysate was performed using Bio-rad® DC™ Protein assay kit (Bio-Rad, Hercules, California, USA) following manufacturer's instruction. The reactions were read at 650 nm absorbance in duplicate using a microplate reader (Multiskan GO). Then ALP activity in cell lysate was measured. Procedures in brief: 100 µl protein extract solution was added in 400 µl of 2 mg/ml p-Nitrophenylphosphate in 0.75 mM 2-Amino-2-methyl-1-propanol, mixed well and incubated at 37ºC for 1 hr. Then 500 µl of 50 mM Sodium hydroxide was added to stop the reaction (all chemicals were from Sigma Chemical Co.) Color intensity was read at 405 nm absorbance in duplicate using a microplate reader (Multiskan GO). Optical density (OD) was extrapolated with a standard curve of serial dilutions of p-nitrophenol (Sigma). Then ALP activity was normalized by the amount of total protein contents of the same sample and reported as nano-Molar per milligram protein (nM/mg protein) (n=4, Mean±SD) (22).

Measuring Levels of Calcium Content in Extracellular Matrix

Following protein lysate procedures, cell pellets were demineralized at RT in 50 µl of 0.5 M HCL in PBS overnight on a horizontal shaker (HS260B, IKA® Werke, Germany), then centrifuged (Labofuge 400R) at 12000 rpm for 10 minutes. Subsequently, amount of calcium contents in the supernatant was measured using Calcium Colorimetric Assay kit (Biovision Inc. Milpitas, California, USA) following a manufacturer's instruction. The reactions were read at 575 nm absorbance in duplicate using a microplate reader (Multiskan GO). Then calcium content levels were normalized by the amount of total protein contents of the same samples and reported as nano-gram calcium per milligram protein (ng/mg protein) (n=4, Mean±SD) (22).

Measuring Osteocalcin Level in Culture Medium

On culture-day 20 in osteogenic medium, confluence cells were washed twice with PBS and cells were incubated in phenol red free DMEM-F12 culture medium overnight. After that culture medium was collected and centrifuged at 12000 rpm for 5 min. The supernatant was measure for amount of osteocalcin in culture medium using Takara Bio Osteocalcin ELISA Kit (TAKARA Bio Inc., Kyoto, Japan) following manufacturer's instruction. Optical density was measured at 450 nm in duplicate using a microplate reader (Multiskan GO), and extrapolated with standard curves to determine amount of osteocalin. Osteocalcin was reported in nano gram / mg protein contents of the same samples as ALP activity analysis. (n=4, Mean±SD) (21).

***In vitro* Mineralization Staining**

Live cell calcein staining was performed on culture-day 20. Cells were incubated in ED-growth medium with 2 mg/ml calcein (Sigma Chemical Co.) overnight. Then culture

medium was removed. After that cells were washed with PBS, fixed in 4% paraformaldehyde (Sigma Chemical Co.) and examined under fluorescence microscope (n=3) (25). Positive calcein staining was calibrated with von Kossa staining on the same cell culture disks of hBMSCs in FBS-OS culture medium on cell culture plate on day 20. After cells were incubated with calcein and examined under fluorescence microscope as stated above, cells were sequentially fixed in 4% Paraformaldehyde, incubated in 1% Silver nitrate in water (Sigma Chemical Co.) under UV light for 1 hr., incubated in 5% Sodium thiosulfate (Sigma Chemical Co.) for 5 min and examined under light microscope. Then positive green and black staining areas were compared to verify positive staining of calcein on cell culture plates. Subsequently, calcein staining was performed on titanium surfaces (26).

Statistical Analysis

The data were tested for normal distribution and homogeneity of variances then differences among groups at each time point were analyzed using one-way analysis of variance (ANOVA). When there were statistically differences, a multiple comparison test was then performed with either the Tukey HSD or Dunnette T3 methods as appropriate. If the data distribution was not normal, the Kruskal-Wallis analysis was used. Then if a difference was statistically significant, a MANN-Whitney test was performed. Significant differences were set at $p<0.05$. Data were derived from 3 independent experiments (n=4-5) and reported as Mean±SD.

RESULTS

Influences of titanium surface microtopography and estrogen-deprived cell culture on cell attachment, shape and growth on titanium surfaces

Live and dead cell staining

Live and dead cell staining demonstrated different cell attachment and shapes on smooth and SLA titanium surfaces. At 3 hr. after cell seeding in FBS growth medium, on smooth titanium surface cells spread out cell cytoplasm creating large cell-surface contact area, but on SLA titanium surface cells extended small cytoplasmic processes to attach on the rough surface creating multiple small contact points (Figure 6). At 24 hr. after cell seeding, it could be noticed that shapes of cells on smooth titanium surface and cell culture plates were similar, that cell body was elongated with protruding cytoplasmic process in the opposite directions forming spindle-shaped cells with a higher cell spreading on cell culture plate than titanium surface. On SLA titanium surface cells exhibited star-like shape with small cell body and multiple small cytoplasmic processes attaching on the rough surface. In addition, homogenous cell distribution in a high density at 80 – 90% coverage on cell culture surfaces could be observed before cells were cultured in ED-growth medium for 24 hr. wash out period (Figure 7). Human BMSCs grew and stayed vital in ED-OS medium till day 21. On day 21, in regular (FBS-OS) and estrogen-deprived osteogenic (ED-OS) media, cells grew in confluence and form multi-layer cell sheet while different cell morphologies on cell culture plate smooth and SLA titanium surfaces could be observed. Brighter green staining of cells in FBS-OS than ED-OS suggested lower cell viability in ED-OS than FBS-OS media. Red staining of propidium iodide of dead cells could be observed within the vital cell sheets (Figure 8).

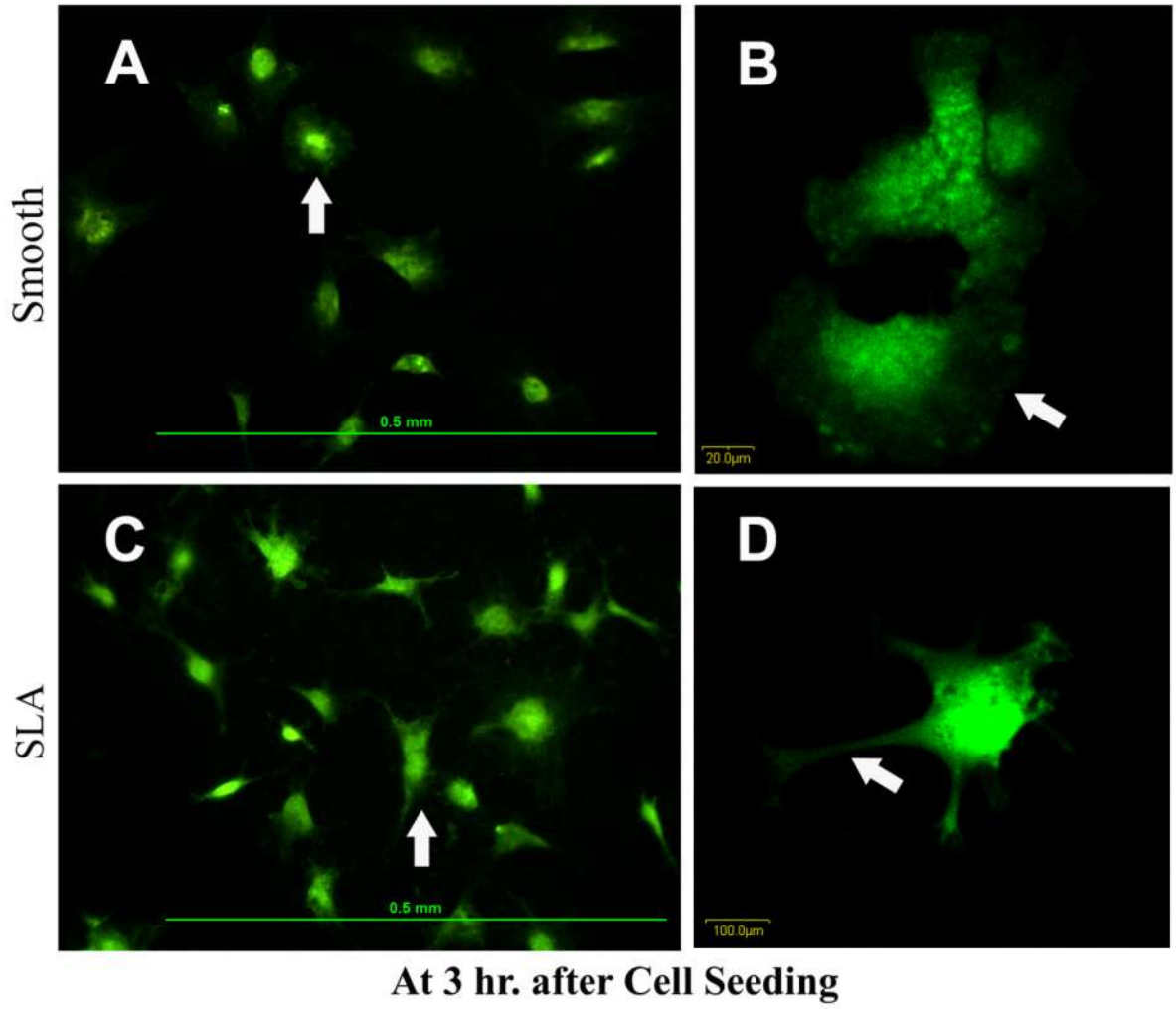


Figure 6 Green fluorescence vital cell staining (CellTracker^{TM} Green) of human bone marrow stromal cells (hBMSCs) at 3 hr. after cell seeding examined under (A & C) fluorescence microscope and (B & D) confocal laser scanning microscope; (A & B) on smooth and (C & D) sandblasted and acid etched (SLA) titanium surfaces. (A & B) Images demonstrate cell flattening cell body on the smooth surface (arrows) forming round shaped cells and (C & D) cell extending small multiple cytoplasmic processes (arrows) to attach on rough surface of SLA titanium surface forming start-like shaped cells.

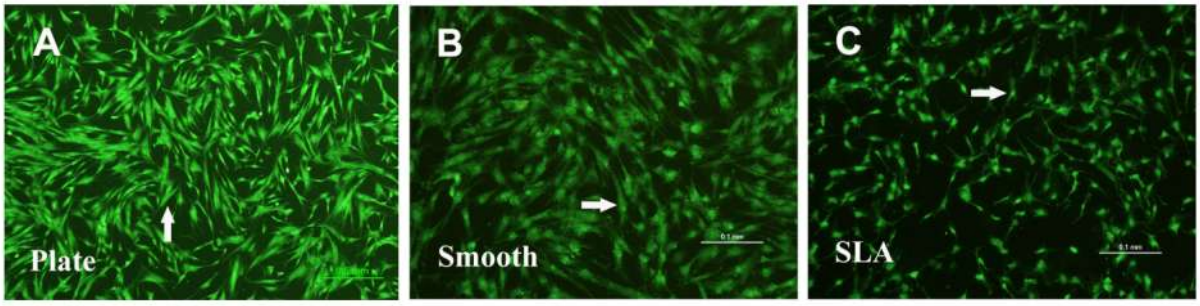


Figure 7 Green fluorescence vital cell staining (CellTracker^{TM} Green) of human bone marrow stromal cells (hBMSCs) at 24 hr. after cell seeding for an osteogenic differentiation study, (A) examined under confocal laser scanning microscope and (B & C) fluorescence microscope; (A) cell culture plate (Plate) and (B) smooth(Smooth) and (C) sandblasted and acid etched (SLA) titanium surfaces. Images exhibit high cell density and homogenous distribution of (A) spindle cell shaped on cell culture plate and (B) smooth titanium surface and (C) start-like shaped cells on the SLA titanium surface (arrows).

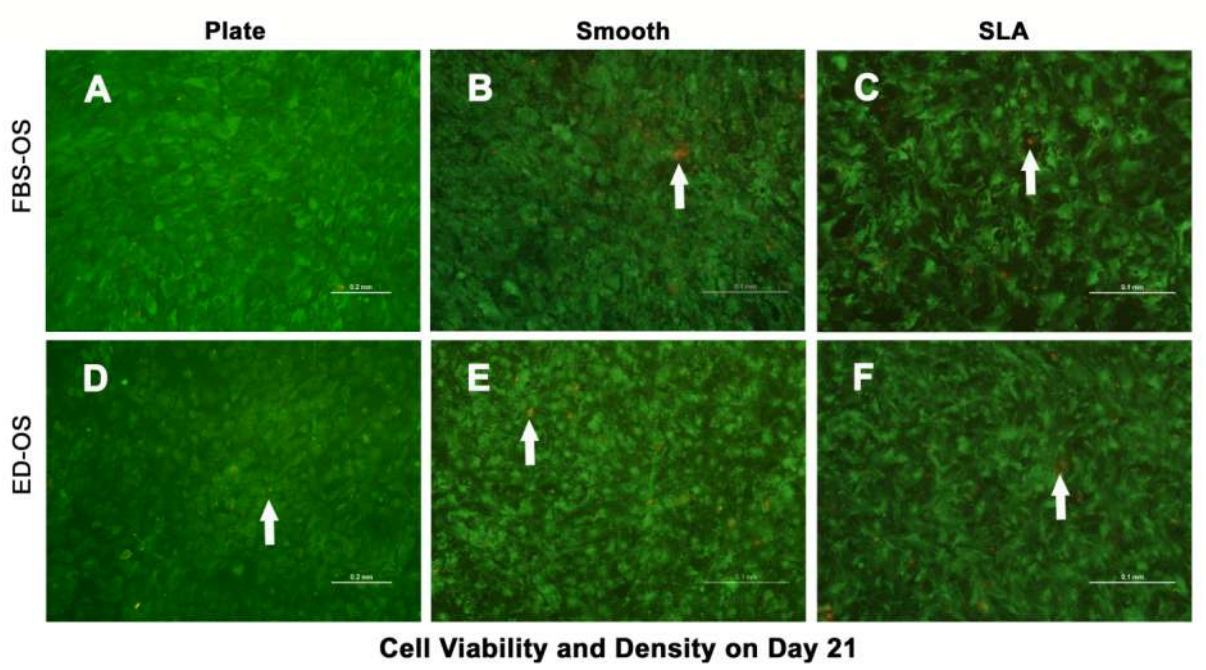


Figure 8 Fluorescence microscope images of green and red fluorescence live/dead cell staining (CellTracker^{TM} Green/propidium iodide (PI)) of human bone marrow stromal cells (hBMSCs) on day 21 in (A-C) regular (FBS-OS) and (D-E) estrogen-deprived osteogenic (ED-OS) media, (A, D) on cell culture plate (Plate), and (B, E) smooth (Smooth) and (C, F) sandblasted and acid etched (SLA) titanium surfaces. Green staining exhibited high level of cell viability and confluence on all surfaces. Red staining of PI (arrows) identified few dead cells scattering within confluence green viable cells on titanium surfaces. Brighter and greener staining in (A-C) FBS-OS suggested higher cell viability in FBS-OS than (D-F) ED-OS cell culture.

Scanning electron microscope

Scanning electron microscope images revealed that cell attachment, spreading and growth on smooth and SLA titanium surfaces were markedly different. On smooth titanium surface, cells were flattening out on the smooth surface, formed cell sheet and large cell-surface contact area. On SLA titanium surface, cells extended cytoplasmic processes to anchor on the rough surface and connect with other cells forming intercellular network (Figure 9). Multiple small contact points were created on the rough surface (Figure 10).

A decreasing of cell growth and spreading on titanium surfaces, particularly on SLA titanium surface might contribute to a lower cell density and smaller cell size in ED-OS than FBS-OS media, particularly on SLA titanium surface (Figure 10 . Density of cell sheet in FBS-OS appeared to be higher than ED-OS media. On SLA titanium surface, only cells in FBS-OS medium were able to formed cell sheet on culture-day 21 and SLA surface in ED-OS medium was covered with loose intercellular network (Figure 9).

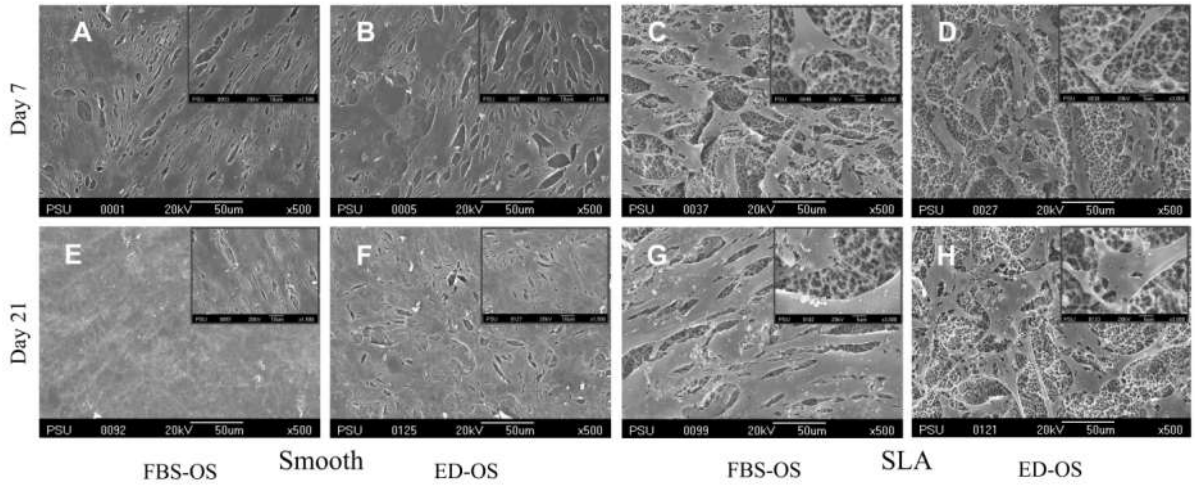


Figure 9 Scanning electron microscope (SEM) images of human bone marrow stromal cells (hBMSCs) on (A, B & E, F) smooth and (C, D & G, H) sandblasted and acid etched titanium surfaces in (A, E & C, G) regular (FBS-OS) and (B, F & D, H) estrogen-deprived osteogenic media (ED-OS) on (A-D) culture-days 7 (Day 7) and (E-H) 21 (Day 21). Images demonstrated different cell shapes and growth on smooth and SLA titanium surfaces in FBS-OS and ED-OS media. Cells formed cell sheet on smooth titanium surface (Smooth) but extended cytoplasmic process to attach on rough surface forming cells with multiple cytoplasmic process and intercellular network. Different cell spreading and growth in FBS-OS and ED-OS media were clearly shown on SLA titanium surface (SLA). Size of cells and cell density appeared to be smaller and lower in ED-OS (D & H) than FBS-OS culture media (C & G). On SLA titanium surface, only cells in FBS-OS could grow to form loose cell sheet on day 21 (G). High magnification images in the inlets magnified cell-surface contacts

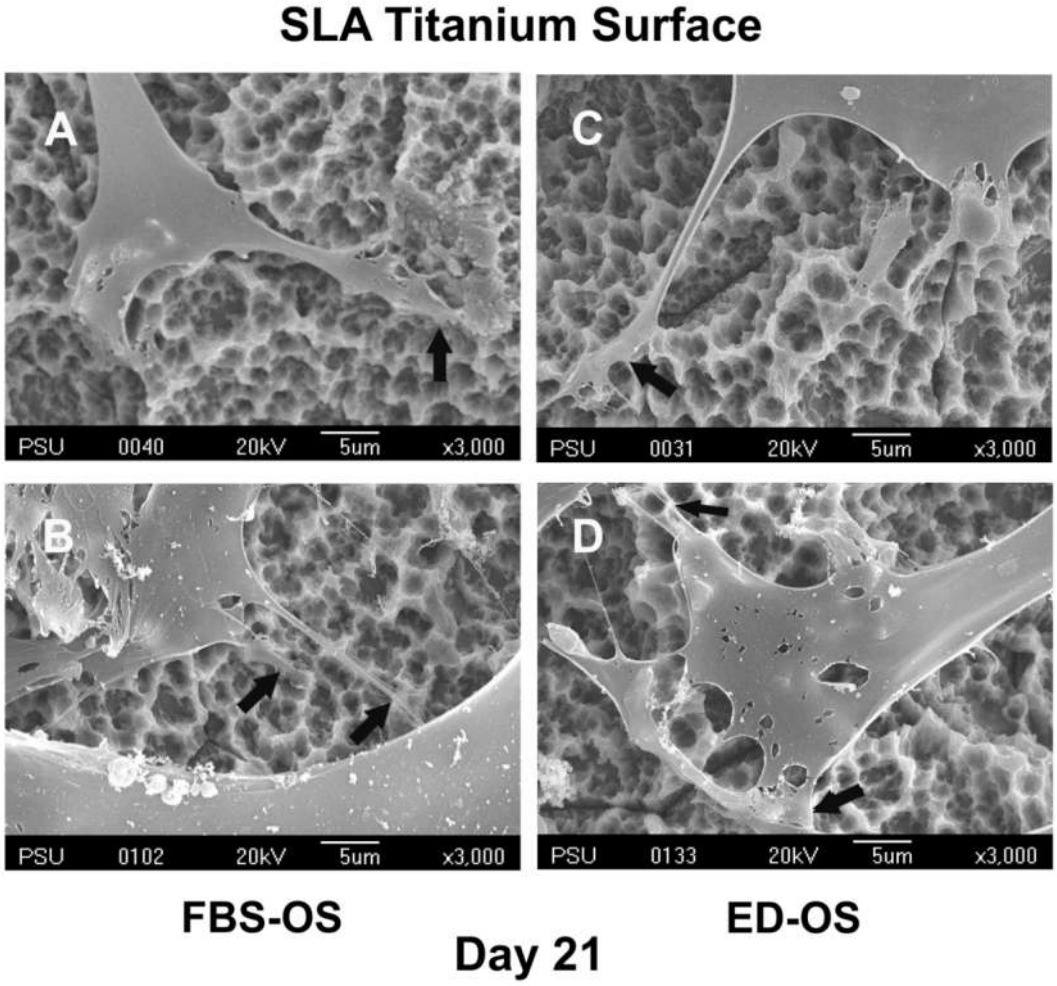


Figure 10 Scanning electron microscope images demonstrating intercellular-surface contact of hBMSCs on *rough, sandblasted and acid etched (SLA) titanium surface* in conventional (FBS-OS) and estrogen-deprived osteogenic mediums (ED-OS) on day 21, (A&B) in FBS-OS and (C&D) ED-OS media. Human BMSCs established focal contact points on the rough surface (arrows) by extending cell body across macro pores while attaching cytoplasmic process on micro pores of the SLA surface. Arrows indicate cell intercellular-surface contact of cytoplasmic processes on micro-pores of the SLA titanium surface.

Cell viability assay

Cell viability assay represented influences of estrogen-deprived cell culture and titanium surfaces on cell growth. Growth of cells in regular (FBS) was higher than estrogen-deprived (ED) growth media. In each cell culture medium, the highest level of cell growth was found on cell culture plate followed by smooth and SLA titanium surfaces, respectively. As a control group, hBMSCs on cell culture plate in FBS medium exhibited the highest levels of cell growth ($p<0.05$), while the lowest cell growth was on SLA titanium surface in ED culture medium ($p<0.05$). In FBS-medium, numbers of cells on every surface were continuously increased and reached the highest level on day 21, but in ED-culture medium, growth of cells were relatively stable on days 7 – 14 ($p>0.05$) and significantly decreased on day 21 ($p<0.05$). On day 21, Growth of cells on smooth and SLA titanium surfaces in FBS-OS medium was similar to growth of cells on cell culture plate in ED growth medium, which were significantly higher than growth of cells on smooth and SLA titanium surfaces in ED-growth medium ($p<0.05$) (Figure 11).

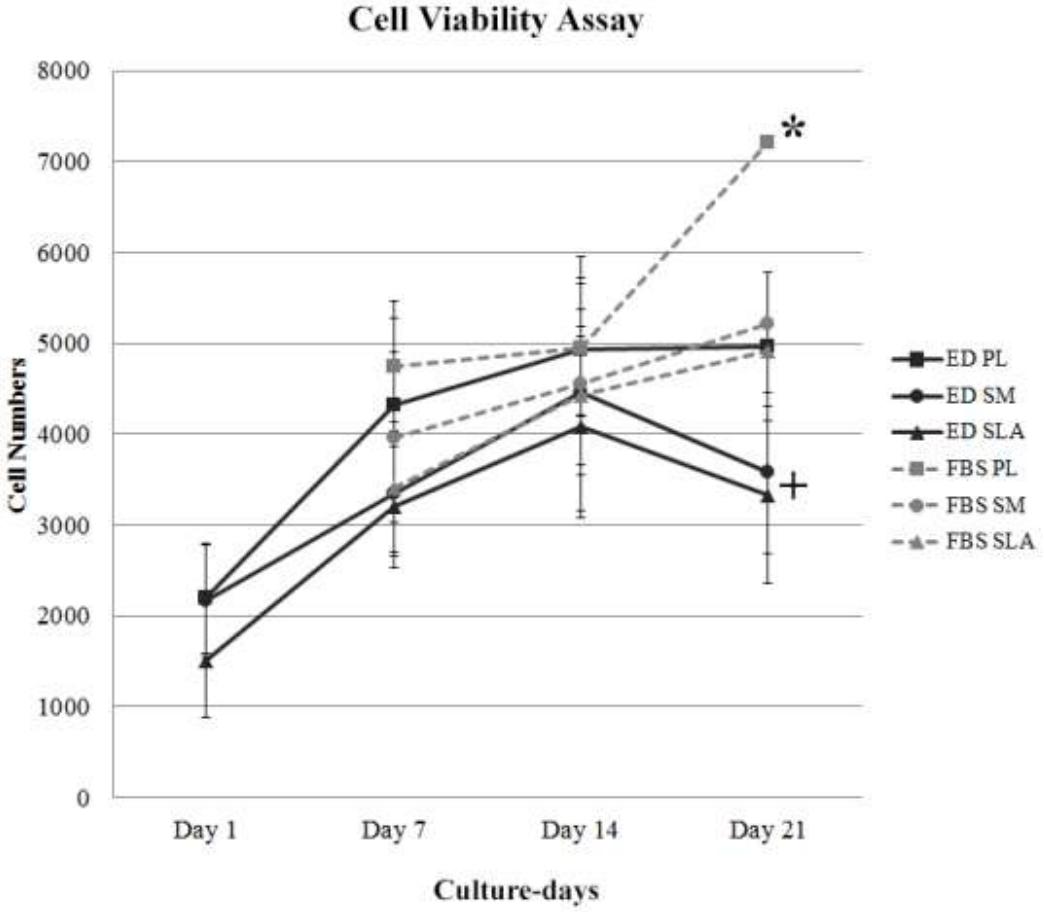


Figure 11 Cell viability assay demonstrates growth of human bone marrow stromal cells (hBMSCs) in regular (FBS) (dot lines) and estrogen-deprived (ED) growth media (solid lines) on cell culture plate (PL) and smooth (SM) and sandblasted and acid etched (SLA) titanium surfaces. On Day 1, numbers of cells on SLA titanium surface tended to be lower than cell culture plate and smooth titanium surface ($p>0.05$). Subsequently on days 7 – 21, numbers of cells on SLA tended to be lower than SM ($p>0.05$). On days 7 and 14 growth of cells was relatively stable and growth of cells on titanium surfaces in FBS-OS and ED-OS were not significantly different ($p>0.05$). Growth of cells was significantly different on day 21, when growth of cells on titanium surfaces in ED-OS was significantly lower than cell culture plate in ED and titanium surfaces in FBS, and cell culture plate in FBS, respectively (+, $p<0.05$). Cells on cell culture plate in FBS medium on day 21 showed the highest level of growth (*, $p<0.05$). The symbol * represents significantly higher than other groups and +, lower than other groups at $p<0.05$ a (n=4, MEAN±SD).

Effects of Titanium Surfaces and Estrogen-deprived Cell Culture on Osteogenic Differentiation of Human Bone Marrow Stromal cells (hBMSCs)

Quantitative real-time polymerase chain reaction (qRT-PCR)

ED-OS cell culture inhibited osteogenic differentiation of hBMSCs into mature osteoblasts and minimized promoting effects of SLA titanium surface on osteogenic differentiation of hBMSCs. Expression levels of Runx2, a marker of early osteoblastic differentiation (23), in ED-OS medium on day 21 were significantly higher than ED-OS on day 7 and FBS-OS on days 7 and 21 ($p<0.05$) (Fig 12A). On the contrary, expression levels of IBSP, a marker of late osteoblastic differentiation (23), on all surfaces in ED-OS medium were markedly lower than FBS-OS on days 7 and 21 ($p<0.05$). In FBS-OS medium, expression levels of IBSP on SLA titanium surface was significantly higher than smooth titanium surface and cell culture plates, respectively ($p<0.05$) (Fig 12B).

Alkaline phosphatase (ALP) activity and calcium content levels

Estrogen-deprived cell culture decreased ALP activity and *in vitro* mineralization (calcium contents), markers of early and late osteoblastic differentiation, respectively. Levels of ALP activity and calcium contents of hBMSCs on all surfaces in ED-OS were significantly lower than FBS-OS media ($p<0.05$). ALP activity on day 7 and calcium content levels on day 21 of hBMSCs on SLA in FBS-OS medium were significantly higher than cell culture plate and smooth titanium surface ($p<0.05$) (Figs. 12C & D).

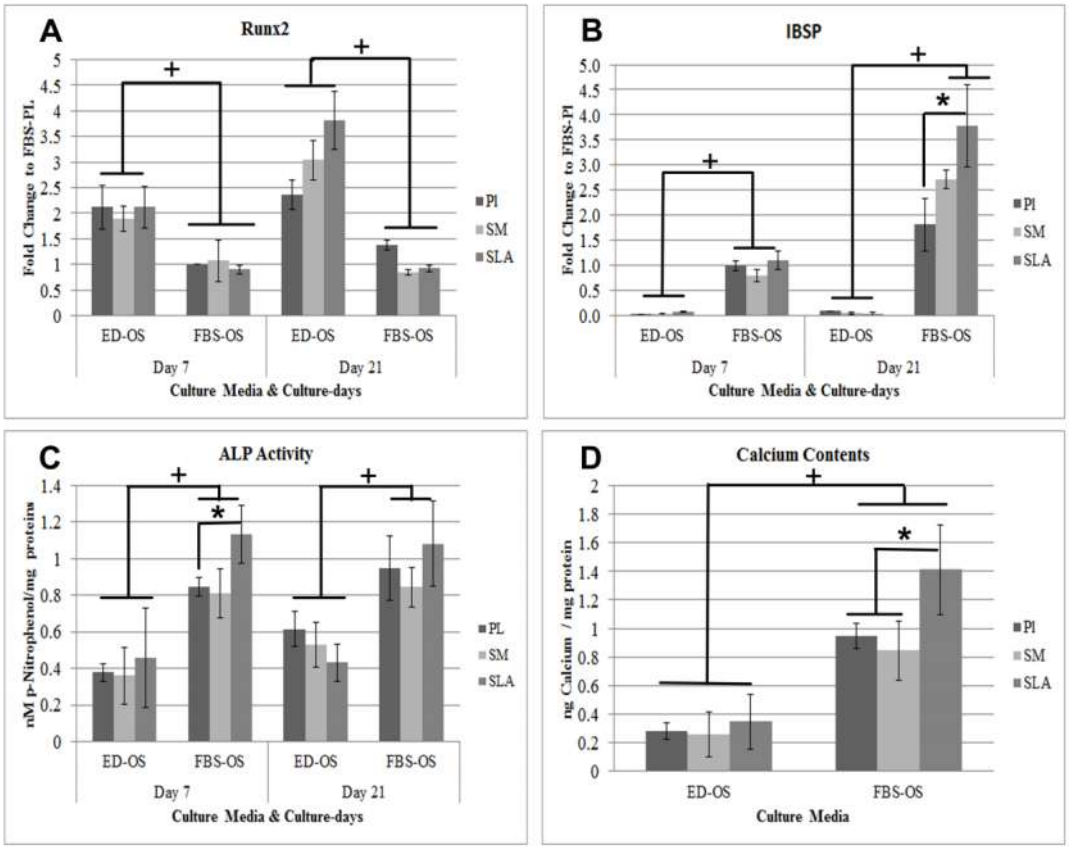


Figure 12 Demonstrating osteogenic differentiation potential of human bone marrow stromal cells (hBMSCs) in regular (FBS-OS) and estrogen-deprived osteogenic (ED-OS) media on cell culture plate (PL) and smooth (SM) and sandblasted and acid etched (SLA) titanium surfaces on culture-days 7 (Days 7) and 21 (Day 21), (A) quantitative real-time polymerase chain reaction (qRT-PCR) exhibits expression of Runx2 and (B) bone sialoprotein (IBSP) genes, (C) alkaline phosphatase activity and (D) calcium content levels. Estrogen-deprived-OS medium significantly increased expression levels of Runx2, but decreased IBSP expression, ALP activity and calcium content levels (+, $p<0.05$). On day 21, (B) levels of IBSP expression and (D) calcium contents on SLA titanium surface in FBS-OS were significantly higher than cell culture plate and smooth titanium surface (*, $p<0.05$). (C) Levels of ALP activity in each culture medium on days 7 and 21 were not significantly different ($p>0.05$). Symbols * represents significant difference among surfaces in the same group and +, differences between media at $p<0.05$. Data were from 2 independent experiment (n=4, MEAN±SD).

Effects of Simvastatin on Osteogenic Differentiation of hBMSCs on Titanium Surface in Estrogen-deprived Cell Culture

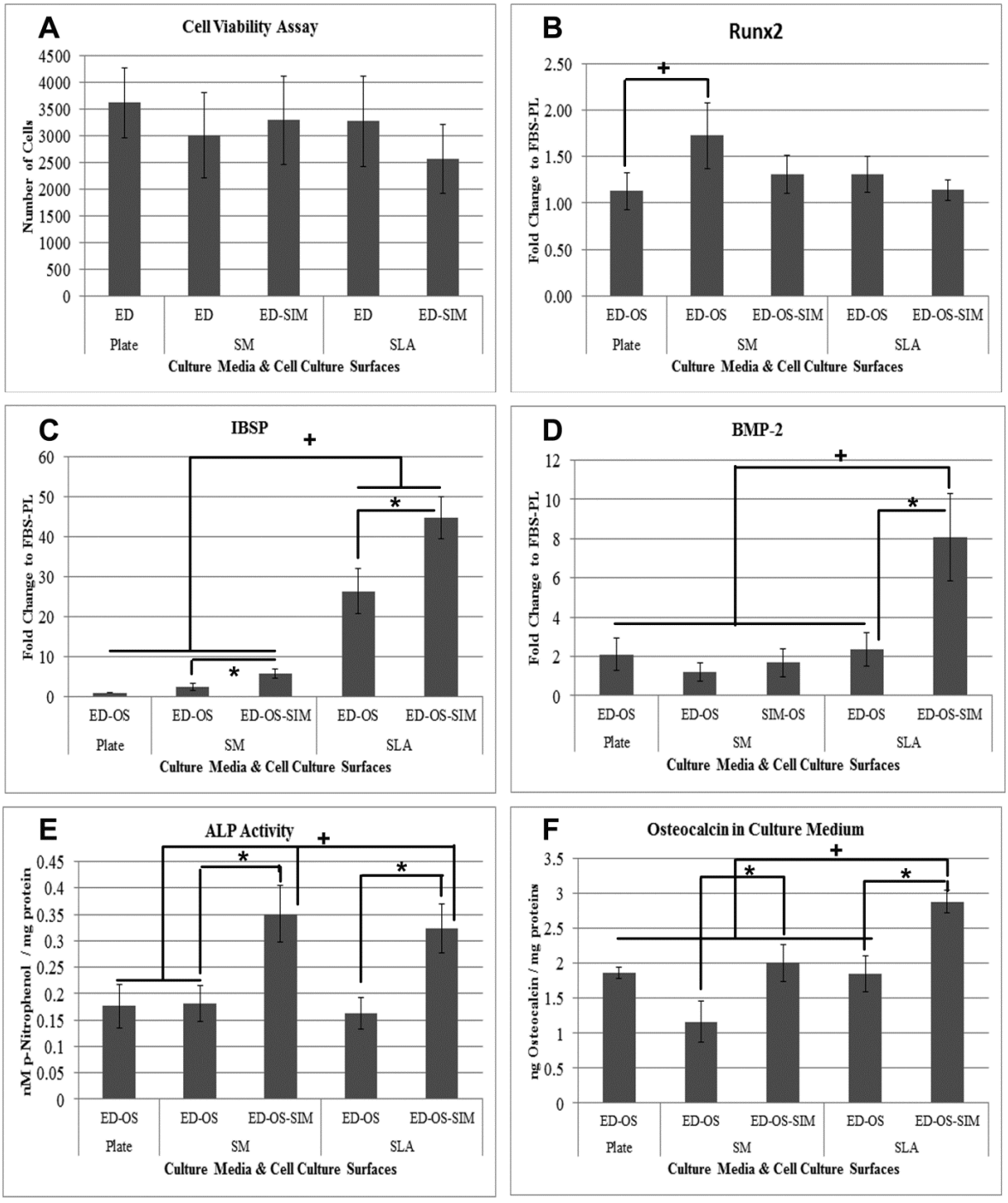
When simvastatin was supplemented in ED-growth medium for 14 days, simvastatin tended to decrease cell growth on SLA titanium surface but the differences were not significant ($p>0.05$) (Figure 13A). Simvastatin promoted late osteoblastic differentiation on SLA titanium surface. Simvastatin tended to decrease expression levels of Runx2, but significantly increase IBSP expression levels on titanium surfaces ($p>0.05$) (Figure 13B). It was clearly shown that in ED-OS medium the expression level of IBSP on SLA titanium surface was significantly higher than smooth titanium surface and cell culture plate ($p<0.05$) and simvastatin markedly enhanced IBSP levels on SLA titanium surface ($p<0.05$). The highest level of IBSP expression was on SLA titanium surface in ED-OS with simvastatin followed by SLA in ED-OS only, smooth titanium surface in ED-OS-SIM and smooth titanium surface in ED-OS media, respectively ($p<0.05$) (Figure 13C). Simvastatin markedly enhanced expression of BMP-2 on SLA titanium surface. An expression level of BMP-2 on SLA titanium surface was significantly higher than other groups ($p<0.05$) and the differences among other groups were not significant ($p>0.05$) (Figure 13D).

Simvastatin promoted osteogenic differentiation in ED-cell culture. Simvastatin increased levels of ALP activity and osteocalcin in culture medium. Levels of ALP activity on smooth and SLA titanium surfaces in ED-OS medium with simvastatin were significantly higher than cell culture plate and smooth and SLA titanium surfaces in ED-OS alone ($p<0.05$). The activity levels on cell culture plate and smooth and SLA titanium surfaces in ED-OS alone were not significantly different ($p>0.05$) (Figure 13E).

Simvastatin increased osteocalcin levels on titanium surfaces in ED-OS cell culture. Levels of osteocalcin on titanium surfaces in ED-OS-Sim were significantly higher than

ED-OS alone ($p<0.05$). The highest level was on SLA in ED-OS-SIM, followed by SLA in ED-OS alone and SM in ED-OS-SIM and cell culture plate in ED-OS alone, and smooth titanium surface in ED-OS alone, repectively, $p<0.05$). The levels of SLA in ED-OS alone and SM in ED-OS-SIM and cell culture plate in ED-OS alone were not significantly different ($p>0.05$) (Figure 13F).

In vitro calcein staining verified *in vitro* mineralization on titanium surfaces by exhibiting varying levels of green staining of calcium deposition on extracellular matrix of hBMSCs in FBS-OS, ED-OS and ED-OS supplemented with simvastatin. Calcein staining on cell culture plate was similar to von Kossa staining, a positive control stain (Figure 15 A&B) Bright green staining was clearly shown on titanium surfaces in simvastatin supplemented groups, particularly on SLA titanium surfaces (Figure 14C-I).


A
Cell Viability Assay
Number of Cells
4500
4000
3500
3000
2500
2000
1500
1000
500
0
ED
ED
ED-SIM
ED
ED-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
B
Runx2
Fold Change to FBS-PL
2.50
2.00
1.50
1.00
0.50
0.00
+
ED-OS
ED-OS
ED-OS-SIM
ED-OS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
C
IBSP
Fold Change to FBS-PL
60
50
40
30
20
10
0
+
*
*
ED-OS
ED-OS
ED-OS-SIM
ED-OS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
D
BMP-2
Fold Change to FBS-PL
12
10
8
6
4
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ED-OS
ED-OS
SIM-OS
ED-OS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
E
ALP Activity
nM p-Nitrophenol / mg protein
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
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ED-OS
ED-OS
ED-OS-SIM
ED-OS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
F
Osteocalcin in Culture Medium
ng Osteocalcin / mg proteins
3.5
3
2.5
2
1.5
1
0.5
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ED-OS
ED-OS
ED-OS-SIM
ED-OS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces

Figure 13 Demonstrating effects of simvastatin on growth, osteogenic differentiation potential and expression of bone morphogenetic protein 2 (BMP-2) of human bone marrow stromal cells (hBMSCs) in estrogen-deprived (ED) cell culture. Human BMSCs, seeded on cell culture plate (PL) and smooth (SM) and sandblasted and acid etched (SLA) titanium surfaces, were cultured in regular (FBS-OS), estrogen-deprived osteogenic (ED-OS) and ED-OS with 100 nM simvastatin (ED-OS-SIM) culture media for 14 days. Investigated parameters were (A) cell growth (cell viability assay), (B & C, E & F) osteogenic differentiation markers, (B) qRT-PCR of Runx2, (C) bone sialoprotein (IBSP), (E) alkaline phosphatase (ALP) activity and (F) osteocalcin in culture media, and (D) qRT-PCR of bone morphogenetic protein-2 (BMP-2). Simvastatin tended to decrease (A) cell growth on SLA and (B) expression levels of Runx2 on SM ($p>0.05$). (B) Runx 2 expression levels on SM in ED-OS was significantly higher than PL in ED-OS (*, $p<0.05$) and tended to be higher than other groups ($p>0.05$). (C) ED-OS-Sim significantly increased expression levels of IBSP and (D) BMP-2 and (F) levels of osteocalcin on SLA, and (E) enhanced ALP activity on SM and SLA titanium surfaces (*, $p<0.05$). In ED-OS alone expression (C) levels of IBSP and (F) osteocalcin on SLA were significantly higher than SM titanium surfaces (+, $p<0.05$) and the expressions on SLA titanium surface were significantly increased in ED-OS-Sim medium (*, $p<0.05$). Symbols * represents significant difference among surfaces in the same culture medium at $p<0.05$, and +, differences between groups of culture medium at $p<0.05$. Data were from 2 independent experiment (n=4, MEAN±SD).

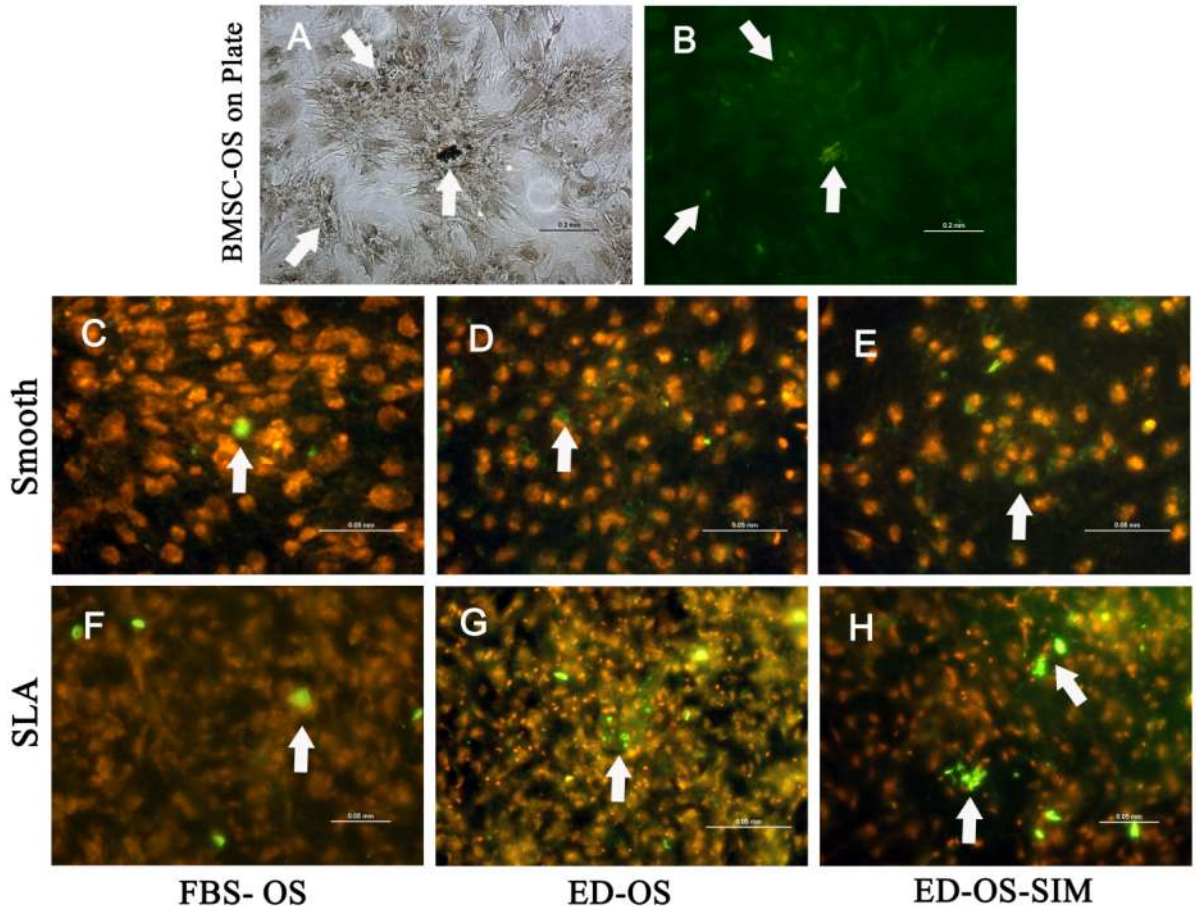

Figure 14 Demonstrating calcein staining of *in vitro* mineralization of human bone marrow stromal cells on (A & B) cell culture plate and (C-E) smooth and (F-H) sandblasted and acid etched (SLA) titanium surfaces in (C & F) regular (FBS-OS), (D & G) estrogen-deprived (ED-OS) and (E & H) ED-OS with 100 nM simvastatin (ED-OS-SIM) culture media. (A) Von Kossa staining exhibited black staining on mineralized nodules in ECM (arrows) (positive control) and (B) green calcein staining (arrows) on cell culture plate which corresponded with black staining in (A). (C-H) Exhibiting varying levels of green calcein staining on mineralized nodules (arrows).

DISCUSSION

In an effort to improve osteoblastic differentiation in osteoporotic bone, the current study investigated effects of titanium surface microtopography, smooth and SLA titanium surfaces, and simvastatin on growth and osteogenic differentiation of hBMSCs in estrogen-deprived cell culture (ED-hBMSCs).

Estrogen-deprived cell culture

A long term estrogen-deprived (ED) cell culture model was established to mimic estrogen deprived condition of hBMSCs in menopause osteoporotic cases. Estrogen-deprived condition was created by utilizing phenol red free culture medium and charcoal stripped bovine serum (27, 28). Estrogen-deprived cell culture with low levels of growth factors and lipophilic materials was a harsh condition for cell growth and differentiation, particularly for 21 day cell culture. As previously published, it was found that growth factor deprivation decreased cell attachment, cytoplasmic spreading and cell growth (29). Therefore to minimize adverse effects of complex hormone and growth factor deprivation on cell growth and functions in ED-cell culture, a liquid media supplement was supplemented in culture media and cells were seeded in a high cell density. Liquid media supplementation added essential factors for cell growth and function, which are insulin-transferrin-sodium selenite and linoleic;oleic-BSA and has been used as a supplement in serum free cell culture (Sigma Chemical Co) (30). At the same time high cell density promotes intercellular communication and paracrine and autocrine functions of cells (31). Thus medium supplement and inter-cellular communication might have supported ED-hBMSCs to sustain low levels of growth and osteoblastic differentiation of ED-hBMSCs throughout cell culture. As a result, cells in ED-deprived culture media were able to reach confluence and grow in multilayer and mineralize ECM on culture-day 21. In the current study,

hBMSCs were cultured in ED-growth medium for 24 hr. wash-out period before starting the experiment to ensure a removal of serum residual effects (20). In summary, a well define experimental model for hormone and growth factor cell response was established.

Effects of titanium surfaces and estrogen-deprived-cell culture on growth and osteogenic differentiation

Different cell growth and differentiation on smooth and SLA titanium surfaces might relate to different cell attachment and shapes on smooth and SLA titanium surfaces. The results agree with previous studies that cell attachment and proliferation of hBMSCs are decreased, but osteoblastic differentiation is supported on SLA titanium surface (32-34). Sandblasted and acid etched titanium surface increased expression levels of late osteoblastic differentiation markers IBSP, alkaline phosphatase (ALP) activity and osteocalcin (OCN) production (15, 35, 36). Scanning electron microscope (SEM) images of hBMSC on SLA titanium surface suggested that surface microtopography of SLA titanium surface supported attachment of cells by promoting multiple contact points of cell cytoplasm and cytoplasmic processes on the macro and micro pores of the surface (12, 16). Promoting effects of SLA titanium surface on osteogenic differentiation could be results of morphological change during cell attachment on different substrate architectures that stimulate focal adhesion signal transduction and adhesion molecules (37-39). In the current study, surface microtopography must have influenced cell functions since initial cell seeding, as different cell shapes on smooth and rough surfaces had been shown since 3 hr. after cell seeding and continued throughout 21-day cell culture.

Effects of surface microtopography on growth and osteogenic differentiation were influenced by hormones and growth factors in local environment, as promoting effects of SLA titanium surface on osteoblastic differentiation was significantly decreased in ED-OS medium.

Decreasing of osteogenic differentiation of hBMSCs on titanium surfaces in ED-OS medium could be a result of a reduction of ECM synthesis in estrogen-deprived cell culture. Because estrogen promotes extracellular matrix (ECM) synthesis (40, 41) and ECM provides external signal regulating growth and survival of contact dependent cells (42), a reduction of ECM in ED-cell culture might attribute to a decreasing of cell growth, attachment and osteogenic differentiation of ED-hBMSCs, particularly on SLA titanium surface. As a result, growth and osteogenic differentiaion on SLA titanium surface were severely affected by ED-condition and promoting effects of SLA surface was markedly decreased in ED cell culture.

Effects of simvastain on growth and osteogenic differentiation

Simvastatin was able to promote osteogenic differentiation on titanium surface in ED-OS medium and promoting effects of simvastatin was increased on SLA titanium surface. Significant increase of IBSP expression and levels of ALP activity and osteocalcin on SLA titanium surface with simvastatin supplement corresponded with a markedly increase of BMP2 expression levels on SLA titanium surface in ED-OS medium with simvastatin supplement. The findings suggested that enhancing effects of simvastatin on osteogenic differentiation on SLA titanium surface was at least partially mediated by inducing BMP-2 (43). Promoting effects of simvastatin supports a previous study finding a correlation between increasing of bone formation markers and levels of simvastatin in serum (44) and underlines promoting effects of simvastatin on bone healing and osteointegration in animal and clinical studies (45, 46).

The association between the findings and *in vivo* and clinical studies

In contrast, the results are contradicted to clinical reports on implant survival that survival of dental implant is not effected by osteoporosis and up to now, osteoporosis is not a contra indication for dental implant placement (47). This might be because impaired

osteogenesis in osteoporotic bone prolongs secondary stability buildup time and increases dipping levels of primary strength during 2-4 week after implant insertion (16). At the same time, these defects could be minimized by applying good clinical care such as surgical techniques, longer non-loading time and good dental implant prosthesis (48). Thus effects of osteoporosis on osteointegration could be subtle and manageable and might not affect implant survival. However, based on previous reports (2, 4) and the current study, implant placement in osteoporotic bone requires close attention to ensure osteointegration and function of dental implant. Careful surgical technique, proper clinical management and implant dental prosthesis designs are recommended to accommodate compromised osteogenesis in an osteoporotic bone.

Limitations of the study design

Limiting of cell growth and attachment on titanium surface in ED-cell culture could be considered as a limitation of ED-cell culture model that could not completely simulate clinical situation in skeletal defects. In clinical environment, titanium surface inserted in the osteoporotic bone will be covered with blood clot and body fluid that would be able to enhance cell attachment and growth on the titanium surfaces and alleviated direct effects of estrogen-deficiency on growth and differentiation on titanium surface and osteointegration (49, 50). Thus, the effects of estrogen-deficiency on osteointegation might be delay or obscured in animal and clinical studies (2-4).

CONCLUSION

In conclusion, the current cell culture model provided a well control experimental model for studying effects of hormones and growth factors on growth and differentiation of cells on titanium surfaces *in vitro*. It was clearly shown that SLA titanium surface microtopography and simvastatin synergistically promoted osteoblastic differentiation of ED-hBMSCs. The

findings underscore hypotheses that estrogen deficiency in postmenopausal osteoporosis cases could compromise osteointegration of the dental implants, and simvastatin supplement would enhance osteointegration on SLA titanium surface in osteoporotic bone.

LIMITATIONS OF THE STUDY

Estrogen deprived cell culture

Estrogen-deprived cell culture model was changed from using Fulvestrant to block function of estrogen receptor alpha generating estrogen-receptor alpha deficient hBMSCs to be cell culture in charcoal strip and phenol red free culture medium. This was because an inconsistent result of fulvestrant blocking and the proposed culture model did not support a long term estrogen-deprived cell culture of 21 day. Thus author changed a culture model, as stated in previous progress reports.

Changing scope of the study

Scope of the current study was narrower than the one stated in a proposal. Because technical, financial difficulties and unexpected loss of sample, study model was modified and the second objective of the study, examining regulating roles of Wnt10b and mechanotransduction genes and signals, generated by cell surface contact on titanium surfaces, on osteogenic differentiation of ERα-def-hMSCs on titanium surfaces, was not performed. Difficulties were difficulty in determining working concentration and condition of fulvestrant and estrogen supplements, technical difficulty for western blotting, requiring large numbers of titanium disks, very high cost of qRT-PCR and insufficient funding of the project.

Requiring large numbers of disks and cells

Large numbers of titanium disks and hBMSCs were required for the experiments and analysis. Low levels of ALP levels and cell growth in ED-OS and a necessity to use high

concentration protein lysis compelled a combining of 4 titanium disks for one sample or 16 disks per group for n=4 at each investigation time, and also an increasing of cell seeding density to $4x10^4$ cells/disk. This resulted in a handle of large numbers of titanium disks and a demand for large numbers of cells at each round of the experiment. Titanium disks should have a larger diameter to fit in 6 well-cell cell culture plates instead of 24 well-cell culture plates as used in the current study.

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Output ที่ได้จากโครงการ

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว และมหาวิทยาลัยสงขลานครินทร์

1. การเสนอผลงานในที่ประชุมวิชการ

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2. **ใช้ประกอบในการ**บรรยาย

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Title

Effects of Titanium Surface Microtopography and Simvastatin on Growth and Osteogenic Differentiation of Human Mesenchymal Stem cells in Estrogen-deprived Cell Culture

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Abstract

Purposes-The current study aimed to investigate effects of titanium surface topography and simvastatin on growth and osteogenic differentiation of human bone marrow stromal cells (hBMSCs) in estrogen-deprived (ED) cell culture.

Materials and Methods-Human BMSCs were seeded on cell culture plates, smooth surface titanium (Ti) disks, and sandblasted with large grits and acid etched (SLA) surface Ti disks; and subsequently cultured in regular (FBS), estrogen-deprived (ED) and ED-with 100 nM simvastatin (ED-SIM) osteogenic (OS) media for 14 – 21 days. Live/dead cell staining, scanning electron microscope examination and cell viability assay were performed to determine cell attachment, morphology and growth. Expression levels of osteoblast-associated genes, Runx2 and bone sialoprotein and levels of alkaline phosphatase (ALP) activity, calcium contents and osteocalcin in culture media were measured to determine osteoblastic differentiation. Expression levels of bone morphogenetic protein 2 was investigated to examine stimulating effects of simvastatin (n=4-5, Mean±SD). *In vitro* mineralization was verified by calcein staining.

Results-Human BMSCs exhibited different attachment and shapes on smooth and SLA titanium surfaces. Estrogen-deprived cell culture decreased cell attachment and growth, particularly on the SLA titanium surface, but cells were able to grow to reach confluence on day 21 in the ED-OS culture medium. Promoting effects of the SLA titanium surface in ED-OS were significantly decreased. Simvastatin significantly increased osteogenic differentiation of hBMSCs on the SLA titanium surface in ED-OS medium and the promoting effects of simvastatin corresponded with the increasing of BMP-2 gene expression on the SLA titanium surface in ED-OS-SIM culture medium.

Conclusions-Estrogen-deprived cell culture model provided a well-define platform for investigating effects of hormone and growth factors on cells and titanium surface interaction. Titanium, the SLA surface and simvastatin synergistically promoted osteoblastic differentiation of hBMSCs in ED condition and might be useful to promote osteointegration in osteoporotic bone.

Keywords

Estrogen-deprived, osteogenic differentiation, sand blasted and acid etched, simvastatin, titanium surfaces

INTRODUCTION

Osteoporosis, one of the public health problems associated with aging, might be considered a risk in implant therapy. A decreasing of estrogen level in senile osteoporosis decreases bone formation, promotes bone resorption and enhances adipogenic differentiation of mesenchymal stem cells (MSCs) (1). Unbalanced bone remodeling and deteriorating microarchitecture of osteoporotic bone create negative effects on osteointegration and implant stability in ovarectomised rabbits, rats and sheep (2-4). Bone mineral density (BMD) in bone marrow region of ovarectomized rats are significantly 30-40% lower than non-osteoporosis group (4), and removal torque of implants in osteoporotic tibia of ovarectomized rabbits is significantly decreased (3).

Various methods have been applied to increase BMD surrounding an implant and improve bone implant contact in osteoporotic bone including implant surface modifications(5) and systemic and locally applied simvastatin(6, 7). Surface microtopography of titanium surface has been shown to be a major factor regulating cell response to biomaterials (8, 9). It is reported that rough titanium surface promotes osteogenic differentiation of hBMSCs (10, 11) and increase bone implant contact (12, 13). Surface roughness of sand blasted with large grits and acid etched (SLA) titanium surface promotes early differentiation, bone formation, implant integration and reduce healing time of implants (14). Superior effects of the SLA titanium surface on osteogenic differentiation have been well established (15, 16).

Simvastatin may synergistically enhance osteogenic differentiation of estrogen-deprived MSCs on titanium surface. Statin is primarily used in the treatment of hypercholesterolemia and has been reported to possess anabolic effects on bone (17). Simvastatin is reported to decrease fracture risk, increase bone mineral density (BMD), enhances

BMP2 expression and stimulates osteoblast differentiation *in vitro* (18). Enhancing effects of simvastatin on expression levels of BMP-2, a strong osteoinductive gene may be able to enhance osteogenic differentiation of estrogen-deprived hBMSCs on SLA titanium surface.

The current study aimed to establish estrogen-deprived cell culture model and investigate effects of titanium surface microtopography, smooth and SLA titanium surfaces, and simvastatin on growth and osteogenic differentiation of hBMSCs in estrogen-deprived cell culture. It was hypothesized that SLA titanium surface would be able to promote growth and osteogenic differentiation of estrogen-deprived human bone marrow stromal cells (ED-hBMSCs) and the promoting effects would be further enhanced by simvastatin supplementation. .

MATERIALS AND METHODS

Human Bone Marrow Cell Culture

Obtaining a permission and approval from the Ethical Committee of Faculty of Medicine, Prince of Songkla University and a patient written informed consent, human bone marrow stromal cells (hBMSCs) were harvested from 4 healthy adult male patients (age 19-30 years) undergoing orthopedic surgery at Songklanagarind hospital, Prince of Songkla University. Human BMSCs were harvested and expanded as described previously (19). Human MSCs at passages 4-5 were used in the analyses.

Human BMSCs were cultured in regular (Fetal bovine serum, FBS), estrogen-deprived (ED) and estrogen-deprived with simvastatin (ED-SIM) culture media. *Regular growth medium* (FBS-growth) comprised of DMEM-F12 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 0.5% fungizone (all from Gibco BRL/Life technologies, Rockville, MD, USA) (20). For *estrogen-deprived growth medium (ED-growth)*, phenol red free DMEM-F12 was supplemented with 10% charcoal stripped FBS (all from Gibco BRL/Life technologies) and 0.5% ITS+3 Liquid Media Supplement (100×) (Sigma Chemical Co., St. Louis, MO, USA), 1% penicillin/streptomycin and 0.5% fungizone. For osteogenic differentiation (OS) medium (FBS-OS and ED-OS), FBS and ED growth media were supplemented with 50 mM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone (all from Sigma Chemical Co.) (20). For ED-OS medium with 100 nM simvastatin supplement (ED-OS-SIM), 100 μM simvastation in dimethyl sulfoxide (DMSO) was supplemented just before used in a 1:1000 ratio of DMSO to culture medium. In a control group for ED-OS-SIM cell culture, ED-OS medium was also supplemented with DMSO in a ratio of 1:1000. The amount of DMSO was limited to 0.1% (all from Sigma Chemical Co.).

Groups of Study

The study was categorized into 3 categories and 3 groups, according to types of culture media and cell culture surfaces, respectively, Category I-Regular (FBS), II-Estrogen-deprived (ED) and III-ED-Simvastatin supplement culture media. In each category, cells were seeded on 3 different surfaces, Groups A: cell culture plates and B: smooth and C: sandblasted and acid etched titanium surfaces (Table 1).

Cell Seeding and Cell Culture Scheme

Smooth Titanium disks and sandblasted with large grits and acid-etched surface (SLA) titanium disks, 15 mm in diameter with 1-mm thick, were kindly provided by Straumann (Institut Straumann AG, Basel , Switzerland) (Fig 1). Disks were placed in non-treated 24-well cell culture plate (Costar, Pittsburgh, PA, USA), one well for one disk for cell seeding and culture.

Human BMSCs were seeded on 24-well cell culture plate and titanium disks, smooth and SLA titanium surfaces. Human BMSCs were seeded at $1x10^4$ cells/cm^2 or $2x10^4$ cells/disks for cell growth and attachment studies, and $2x10^4$ cells/cm^2 or $4x10^4$ cells/disk for osteogenic differentiation study. Cells, $2x10^4$ or $4x10^4$ cells, were suspended in 300 µl of growth medium and seeded in each well of 24-well cell culture plates and titanium disk. Then seeded cells were cultured in minimal FBS-growth medium for 3 hr. and then 24 hr. in 1 ml culture medium in a humidified incubator with 5% CO_2 at 37ºC. After that FBS growth medium was replaced with 1 ml ED-growth medium for 24 hr. wash out period (21). Subsequently, cells were cultured either in FBS-or ED-growth or osteogenic media for 21 day, and ED-OS-SIM for 14 days according to groups of the study for investigations (Table 2).

Live and Dead Cell Staining

To examine cell attachment, spreading, viability and cell dead, cells were incubated in a mixture of 5 μM CellTracker™ Green (Molecular Probes/Invitrogen, Carlsbad, CA, USA) and 0.5 mg/ml propidium iodide (Sigma Chemical Co.) in ED growth medium for 30 minutes in a humidified incubator with 5% CO_2 at 37ºC. After that the disks were rinsed twice with phosphate buffer solution (PBS), fixed in 10% buffered formaldehyde and examined under a fluorescence microscope (Ti-S100, Nikon, Japan) or confocal laser scanning microscope (CLSM) (FV300, Olympus, Japan). The staining was performed at 24 hr. after cell seeding in growth medium and then on days 7 and 21 in osteogenic medium (n=3) (Table 2) (22).

Scanning Electron Microscope

Cell attachment and morphology on titanium disks were assessed optically by a scanning electron microscope (SEM) (5800LV, JEOL, Japan). At each investigation time, cells were fixed in 4% glutaraldehyde, dehydrated in ethanol series of 30-100%, dried, gold sputter-coated (SPI Module™ Sputter Coater, SPI, USA) and examined under scanning electron microscope (SEM, 5800LV, JEOL)(23). The examination was performed on days 7 and 21 (n=3) (Table 2) (22).

Cell Viability Assay

Growth curve was established using cell viability assay. Cell viability was measured as an indicator of cell numbers and CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used following manufacturer's instructions. The formazan dye was quantified at 440 nm absorbance in duplicate using a microplate reader (Multiskan GO, Thermo Scientific, Finland). Then optical density values were

extrapolated with a standard curve of cell numbers. Cell viability was determined on days 1, 7, 14 and 21 in growth media (n=4, Mean±SD) (Table 2) (22).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR was performed to determine expression levels of early and late osteoblastic differentiation associated genes (24) and bone morphogenetic protein-2 (BMP-2) gene. Total RNA was extracted using Trizol (Invitrogen) and 1μg of RNA was reverse transcribed into cDNA using cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Equal amount of cDNA was amplified by PCR using the TaqMan Gene Expression Master Mix (Applied Biosystems) and 20×Target primers and Probe (Applied Biosystems). Genes and primers used are as followed, Runx2 (Hs01047973_m1), IBSP (Hs00173720_m1) and BMP-2 (Hs00154192_m1) (Applied Biosystems). The expression of the genes was measured by qRT-PCR on the Rotor Gene Q Detection System (Qiagen, Hilden, Germany). Levels of the target genes were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs02758991_g1) (Applied Biosystems) as an endogenous reference. Subsequently, the expression levels of investigated genes were normalized to the expression levels of hBMSCs on cell culture plate in FBS-OS medium and reported as fold changes. Data presented for were averaged from 3 independent cultures (n=3, MEAN±SD) (25).

Alkaline Phosphatase (ALP) Activity Analysis

At each investigation time, hBMSCs on cell culture plates and titanium disks were lyzed in 1% Triton X-100 (Sigma Chemical Co.) to obtain total protein lysate and cell pellets. Amount of total protein contents and levels of alkaline phosphatase (ALP) activity in the protein lysis solution were measured, and pellets from the same samples were kept for calcium content assay (23).

The quantification of protein amount in cell lysate was performed using Bio-rad® DC™ Protein assay kit (Bio-Rad, Hercules, California, USA) following manufacturer's instruction. The reactions were read at 650 nm absorbance in duplicate using a microplate reader (Multiskan GO). Then ALP activity in cell lysate was measured. Procedures in brief: 100 µl protein extract solution was added in 400 µl of 2 mg/ml p-Nitrophenylphosphate in 0.75 mM 2-Amino-2-methyl-1-propanol, mixed well and incubated at 37ºC for 1 hr. Then 500 µl of 50 mM Sodium hydroxide was added to stop the reaction (all chemicals were from Sigma Chemical Co.) Color intensity was read at 405 nm absorbance in duplicate using a microplate reader (Multiskan GO). Optical density (OD) was extrapolated with a standard curve of serial dilutions of p-nitrophenol (Sigma). Then ALP activity was normalized by the amount of total protein contents of the same sample and reported as nano-Molar per milligram protein (nM/mg protein) (n=4, Mean±SD) (23).

Measuring Levels of Calcium Content in Extracellular Matrix

Following protein lysate procedures, cell pellets were demineralized at RT in 50 µl of 0.5 M HCL in PBS overnight on a horizontal shaker (HS260B, IKA® Werke, Germany), then centrifuged (Labofuge 400R) at 12000 rpm for 10 minutes. Subsequently, the amount of calcium contents in the supernatant was measured using Calcium Colorimetric Assay kit (Biovision Inc. Milpitas, California, USA) following manufacturer's instructions. The reactions were read at 575 nm absorbance in duplicate using a microplate reader (Multiskan GO). Then calcium content levels were normalized by the amount of total protein contents of the same samples and reported as nano-gram calcium per milligram protein (ng/mg protein) (n=4, Mean±SD) (23).

Measuring Osteocalcin Level in Culture Medium

On culture-day 20 in osteogenic medium, confluence cells were washed twice with PBS and cells were incubated in phenol red free DMEM-F12 culture medium overnight. After that culture medium was collected and centrifuged at 12000 rpm for 5 min. The supernatant was measure for the amount of osteocalcin in culture medium using Takara Bio Osteocalcin ELISA Kit (TAKARA Bio Inc., Kyoto, Japan) following manufacturer's instructions. Optical density was measured at 450 nm in duplicate using a microplate reader (Multiskan GO), and extrapolated with standard curves to determine amount of osteocalin. Osteocalcin was reported in nano gram / mg protein contents of the same samples as ALP activity analysis. (n=4, Mean±SD) (22).

In vitro Mineralization Staining

Live cell calcein staining was performed on culture-day 20. Cells were incubated in ED-growth medium with 2 mg/ml calcein (Sigma Chemical Co.) overnight. Then culture medium was removed. After that cells were washed with PBS, fixed in 4% paraformaldehyde (Sigma Chemical Co.) and examined under fluorescence microscope (n=3) (26). Positive calcein staining was calibrated with von Kossa staining on the same cell culture disks of hBMSCs in FBS-OS culture medium on cell culture plate on day 20. After cells were incubated with calcein and examined under fluorescence microscope as stated above, cells were sequentially fixed in 4% Paraformaldehyde, incubated in 1% Silver nitrate in water (Sigma Chemical Co.) under UV light for 1 hr., incubated in 5% Sodium thiosulfate (Sigma Chemical Co.) for 5 min and examined under light microscope. Then positive green and black staining areas were compared to verify positive staining of calcein on cell culture plates. Subsequently, calcein staining was performed on titanium surfaces.

Statistical Analysis

The data were tested for normal distribution and homogeneity of variances. The differences among groups of normally distributed data were analyzed using one-way analysis of variance (ANOVA) and either the Tukey HSD or Dunnette T3 methods as appropriate. When the data distribution was not normal, the Kruskal-Wallis analysis and a MANN-Whitney test was performed were performed. Significant differences were set at $p<0.05$. Data were derived from 3 independent experiments (n=4-5) and reported as Mean±SD.

RESULTS

Influences of titanium surface microtopography and estrogen-deprived cell culture on cell attachment, shape and growth on titanium surfaces

Live and dead cell staining demonstrated different cell attachment and shapes on smooth and SLA titanium surfaces. At 3 hr. after cell seeding in FBS growth medium, on the smooth titanium surface, cells spread out cell cytoplasm creating large cell-surface contact area, but on the SLA titanium surface cells extended small cytoplasmic processes to attach on the rough surface creating multiple small contact points (Fig 2). At 24 hr. after cell seeding, it could be noticed that shapes of cells on smooth titanium surface and cell culture plates were similar, that cell body was elongated with protruding cytoplasmic process in the opposite directions forming spindle-shaped cells with a higher cell spreading on cell culture plate than titanium surface. On the SLA titanium surface cells exhibited star-like shape with small cell body and multiple small cytoplasmic processes attaching on the rough surface. In addition, homogenous cell distribution in a high density at 80 – 90% coverage on cell culture surfaces could be observed before cells were cultured in ED-growth medium for 24 hr. wash out period (Fig 3). Human BMSCs were grown and stayed vital in ED-OS medium till day 21. On day 21, in regular (FBS-OS) and estrogen-deprived osteogenic (ED-OS) media, cells grew to confluence and form multi-layer cell sheet while different cell morphologies on cell culture plate smooth and SLA titanium surfaces could be observed. Brighter green staining of cells in FBS-OS than ED-OS suggested lower cell viability in ED-OS than FBS-OS media. Red staining of propidium iodide of dead cells could be observed within the vital cell sheets (Fig 4).

Scanning electron microscope images revealed that cell attachment, spreading and growth on smooth and SLA titanium surfaces were markedly different. On the smooth titanium surface, cells were flattening out on the smooth surface, formed cell sheet and large cell-surface

contact area. On the SLA titanium surface, cells extended cytoplasmic processes to anchor on the rough surface and connect with other cells forming intercellular network. Multiple small contact points were created on the rough surface (Fig 5).

A decreasing of cell growth and spreading on titanium surfaces, particularly on SLA titanium surface might contribute to a lower cell density and smaller cell size in ED-OS than FBS-OS media, particularly on the SLA titanium surface. Density of cell sheet in FBS-OS appeared to be higher than ED-OS media. On the SLA titanium surface, only cells in FBS-OS medium were able to formed cell sheet on culture-day 21 and the SLA surface in ED-OS medium was covered with loose intercellular network (Fig 5).

Cell viability assay reflected influences of estrogen-deprived cell culture and titanium surfaces on cell growth. The growth of cells in regular (FBS) was higher than estrogen-deprived (ED) growth media. In each cell culture medium, the highest level of cell growth was found on cell culture plate followed by smooth and SLA titanium surfaces, respectively. As a control group, hBMSCs on cell culture plate in FBS medium exhibited the highest levels of cell growth ($p<0.05$), while the lowest cell growth was on the SLA surface in ED culture medium ($p<0.05$). In FBS-medium, numbers of cells on every surface were continued to increase and reached the highest level on day 21, but in ED-culture medium, growth of cells were relatively stable on days 7 – 14 ($p>0.05$) and were significantly decreased on day 21 ($p<0.05$). On day 21, the growth of cells on smooth and SLA titanium surfaces in FBS-OS medium was similar to growth of cells on cell culture plate in ED growth medium, which were significantly higher than the growth of cells on smooth and SLA titanium surfaces in ED-growth medium ($p<0.05$) (Fig 6).

Effects of Titanium Surfaces and Estrogen-deprived Cell Culture on Osteogenic Differentiation of Human bone marrow stromal cells (hBMSCs)

ED-OS cell culture inhibited osteogenic differentiation of hBMSCs into mature osteoblasts and minimized promoting effects of the SLA titanium surface on osteogenic differentiation of hBMSCs. Expression levels of runx2, a marker of early osteoblastic differentiation (24), in ED-OS medium on day 21 were significantly higher than ED-OS on day 7 and FBS-OS on days 7 and 21 ($p<0.05$) (Fig 7A). On the contrary, expression levels of IBSP, a marker of late osteoblastic differentiation (24), on all surfaces in ED-OS medium were markedly lower than FBS-OS on days 7 and 21 ($p<0.05$). In FBS-OS medium, expression levels of IBSP on the SLA titanium surface was significantly higher than the smooth titanium surface and cell culture plates, respectively ($p<0.05$) (Fig 7B).

Estrogen-deprived cell culture decreased ALP activity and *in vitro* mineralization (calcium content), markers of early and late osteoblastic differentiation, respectively. Levels of ALP activity and calcium contents of hBMSCs on all surfaces in ED-OS medium were significantly lower than FBS-OS medium ($p<0.05$). ALP activity on day 7 and calcium content levels on day 21 of hBMSCs on the SLA surface in FBS-OS medium were significantly higher than cell culture plate and the smooth titanium surface ($p<0.05$) (Figs 7C and D).

Effects of Simvastatin on Osteogenic Differentiation of hBMSCs on Titanium Surface in Estrogen-deprived Cell Culture

When simvastatin was supplemented in ED-growth medium for 14 days, simvastatin tended to decrease cell growth on the SLA titanium surface but the differences were not significant ($p>0.05$) (Fig 8A). Simvastatin promoted late osteoblastic differentiation on the SLA titanium surface. Simvastatin tended to decrease expression levels of Runx2, but significantly increase IBSP expression levels on titanium surfaces ($p>0.05$) (Fig 8B). It was

clearly shown that in ED-OS medium the expression level of IBSP on the SLA was significantly higher than the smooth titanium surface and cell culture plate ($p<0.05$) and simvastatin markedly enhanced IBSP levels on the SLA titanium surface ($p<0.05$). The highest level of IBSP expression was on the SLA titanium surface in ED-OS with simvastatin followed by the SLA in ED-OS only, the smooth titanium surface in ED-OS-SIM and the smooth titanium surface in ED-OS media ($p<0.05$) (Fig 8C). Simvastatin markedly enhanced expression of BMP-2 on the SLA titanium surface. An expression level of BMP-2 on the SLA titanium surface was significantly higher than other groups ($p<0.05$) and the differences among those groups were not significant ($p>0.05$) (Fig 8D).

Simvastatin promoted osteogenic differentiation in ED-cell culture. Simvastatin increased levels of ALP activity and osteocalcin in culture medium. Levels of ALP activity on titanium surfaces both smooth and SLA in ED-OS medium with simvastatin were significantly higher than all groups in ED-OS only ($p<0.05$). However the activity levels among each groups in ED-OS only was not significantly different ($p>0.05$) (Fig 8E).

Simvastatin increased osteocalcin levels on titanium surfaces in ED-OS cell culture, particularly SLA titanium surface. Levels of osteocalcin in culture media on SLA titanium surface and cell culture plate were significantly higher than the smooth titanium surface ($p<0.05$). The highest level of osteocalcin was on the SLA titanium surface in ED-OS with simvastatin (ED-OS-SIM), followed by the SLA titanium surface in ED-OS and the smooth titanium in ED-OS-SIM, cell culture plate and the smooth titanium surface in ED-OS only ($p<0.05$) (Fig 8F).

In vitro calcein staining verified *in vitro* mineralization on titanium surfaces by exhibiting varying levels of green staining of calcium deposition on extracellular matrix of

hBMSCs in FBS-OS, ED-OS and ED-OS supplemented with simvastatin. Calcein staining on a cell culture plate was similar to a positive control stain of von Kossa staining (Fig 9A and B). Bright green staining was clearly shown on titanium surfaces in simvastatin supplemented groups, particularly on SLA titanium surfaces (Fig 9).

DISCUSSION

In an effort to improve osteoblastic differentiation in osteoporotic bone, the current study investigated effects of titanium surface microtopography, smooth and SLA titanium surfaces, and simvastatin on growth and osteogenic differentiation of hBMSCs in estrogen-deprived cell culture (ED-hBMSCs).

A long term estrogen-deprived (ED) cell culture model was established to mimic estrogen deprived condition of hBMSCs in menopause osteoporotic cases. The estrogen-deprived condition was created by utilizing phenol red free culture medium and charcoal stripped bovine serum (27, 28). The ED-culture with low levels of growth factors and lipophilic materials was a harsh condition for cell growth and differentiation, particularly for 21 day cell culture. As previously published, it was found that growth factor deprivation decreased cell attachment, cytoplasmic spreading and cell growth (29). Therefore to minimize adverse effects of complex hormone and growth factor deprivation on cell growth and functions in ED-cell culture, a liquid media supplement was supplemented in culture media and cells were seeded in a high cell density. Liquid media supplementation added essential factors for cell growth and function, which are insulin-transferrin-sodium selenite and linoleic;oleic-BSA and has been used as a supplement in serum free cell culture (Sigma Chemical Co) (30). At the same time high cell density promotes intercellular communication and paracrine and autocrine functions of cells (31). Thus medium supplement and inter-cellular communication might have supported ED-hBMSCs to sustain low levels of growth and osteoblastic differentiation of ED-hBMSCs throughout cell culture. As a result, cells in ED-deprived culture media were able to reach confluence and grow in multilayer and mineralize ECM on culture-day 21. In the current study, hBMSCs were cultured in ED-growth medium for 24 hr. wash-out period before starting the

experiment to ensure elimination of serum residual effects (21). In summary, a well define experimental model for hormone and growth factor cell response was established.

Different cell growth and differentiation on smooth and SLA titanium surfaces might relate to different cell attachment and shapes on smooth and SLA titanium surfaces.The results agree with previous studies that cell attachment and proliferation of hBMSCs are decreased, but osteoblastic differentiation is supported on the SLA titanium surface (32-34). The sandblasted with large grits and acid etched titanium surface increased expression levels of late osteoblastic differentiation markers IBSP, alkaline phosphatase (ALP) activity and osteocalcin (OCN) production (15, 35, 36). Scanning electron microscope (SEM) images of hBMSC on the SLA titanium surface suggested that surface microtopography of the SLA titanium surface supported attachment of cells by promoting multiple contact points of cell cytoplasm and cytoplasmic processes on the macro and micro pores of the surface (12, 16). Promoting effects of SLA titanium surface on osteogenic differentiation could be results of morphological change during cell attachment on different substrate architectures that stimulate focal adhesion signal transduction and adhesion molecules (37-39). In the current study, surface microtopography has influenced on cell functions since initial cell seeding, as different cell shapes on smooth and rough surfaces had been shown since 3 hr. after cell seeding and continued throughout 21-day cell culture.

Effects of surface microtopography on growth and osteogenic differentiation were influenced by hormones and growth factors in local environment, as promoting effects of the SLA titanium surface on osteoblastic differentiation was significantly decreased in ED-OS medium. Decreasing of osteogenic differentiation of hBMSCs on titanium surfaces in ED-OS medium could be a result of a reduction of ECM synthesis in estrogen-deprived cell culture.

Because estrogen promotes extracellular matrix (ECM) synthesis (40, 41) and ECM provides external signal regulating growth and survival of contact dependent cells (42), a reduction of ECM in ED-cell culture might attribute to a decreasing of cell growth, attachment and osteogenic differentiation in ED-hBMSCs, particularly on the SLA titanium surface. As a result, growth and osteogenic differentiaion on the SLA titanium surface were severely affected by ED-condition and promoting effects of the SLA surface was markedly decreased in ED cell culture.

Limiting of cell growth and attachment on the titanium surface in ED-cell culture could be considered as a limitation of ED-cell culture model that could not completely simulate clinical situation in skeletal defects. In clinical environment, titanium surface inserted in the osteoporotic bone will be covered with blood clot and body fluid that would be able to enhance cell attachment and growth on the titanium surfaces and alleviated direct effects of estrogen-deficiency on growth and differentiation of osteogenic cells on the titanium surface and osteointegration (43, 44). Thus, the effects of estrogen-deficiency might delay or jeopardize osteointegation in animal and clinical studies (2-4).

Simvastatin was able to promote osteogenic differentiation on titanium surface in ED-OS medium and promoting effects of simvastatin was increased on the SLA titanium surface. Significant increase of IBSP expression and levels of ALP activity and osteocalcin on the SLA titanium surface with simvastatin supplement corresponded with a markedly increase of BMP2 expression levels on the SLA titanium surface in ED-OS medium with simvastatin supplement. The findings suggested that enhancing effects of simvastatin on osteogenic differentiation on the SLA titanium surface was at least partially mediated by inducing BMP-2 (17). Promoting effects of simvastatin supports a previous study that found a correlation between increasing of bone formation markers with levels of simvastatin in serum (45) which

underlined promoting effects of simvastatin on bone healing and osteointegration in animal and clinical studies (46, 47).

CONCLUSION

In conclusion, the current cell culture model provided a well control experimental model for studying effects of hormones and growth factors on growth and differentiation of cells on titanium surfaces *in vitro*. It was clearly shown that the SLA titanium surface microtopography and simvastatin synergistically promoted osteoblastic differentiation of ED-hBMSCs. The findings underscore hypotheses that estrogen deficiency in postmenopausal osteoporosis cases could compromise osteointegration of the dental implants, and simvastatin supplement would enhance osteointegration on the SLA titanium surface in osteoporotic bone.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig 1.

Scanning electron microscope images of titanium surfaces, (A) smooth and (B) sandblasted with large grits and acid etched (SLA) titanium surfaces.

Fig 2.

Green fluorescence vital cell staining (CellTracker™ Green) of human bone marrow stromal cells (hBMSCs) at 3 hr. after cell seeding examined under (A & C) Fluorescence microscope and (B & D) confocal laser scanning microscope; (A & B) on smooth and (C & D) sandblasted and acid etched (SLA) titanium surfaces. (A & B) Images demonstrate cell flattening cell body on the smooth surface (arrows) forming round shaped cells and (C & D) cell extending small multiple cytoplasmic processes (arrows) to attach on rough surface of SLA titanium surface forming start-like shaped cells.

Fig 3.

Green fluorescence vital cell staining (CellTracker™ Green) of human bone marrow stromal cells (hBMSCs) at 24 hr. after cell seeding for an osteogenic differentiation study, (A) examined under confocal laser scanning microscope and (B & C) Fluorescence microscope; (A) cell culture plate (Plate), (B) smooth(Smooth) titanium surface and (C) sandblasted with large grits and acid etched (SLA) Ti surface. Images exhibit high cell density and homogenous distribution of (A) spindle cell shaped on cell culture plate and (B) smooth Ti surface and (C) star-like shaped cells on the SLA Ti surface (arrows).

Fig 4.

Fluorescence microscope images of green and red fluorescence live/dead cell staining (CellTrackerTM Green/propidium iodide (PI)) of human bone marrow stromal cells (hBMSCs) on day 21 in (A-C) regular (FBS-OS) and (D-E) estrogen-deprived osteogenic (ED-OS) media, (A, D) on cell culture plate (Plate), (B, E) smooth titanium (Ti) surface (Smooth) and (C, F) sandblasted with large grits and acid etched (SLA) Ti surface. Green staining exhibited high level of cell viability and confluence on all surfaces. Red staining of PI (arrows) identified few dead cells that could be seen scattering within confluence green viable cells on titanium surfaces. Brighter and greener staining in (A-C) FBS-OS suggested higher cell viability in FBS-OS than (D-F) ED-OS cell culture.

Fig 5.

Scanning electron microscope (SEM) images of human bone marrow stromal cells (hBMSCs) on (A, B & E, F) smooth titanium (Ti) surface and (C, D & G, H) sandblasted with large grits and acid etched (SLA) Ti surface in (A, E & C, G) regular (FBS-OS) and (B, F & D, H) estrogen-deprived osteogenic media (ED-OS) on (A-D) culture-days 7 (Day 7) and (E-H) 21 (Day 21). Images demonstrated different cell shapes and growth on smooth and SLA titanium surfaces in FBS-OS and ED-OS media. Cells formed cell sheet on smooth titanium surface (Smooth) but extended cytoplasmic process to attach on rough surface forming cells with multiple cytoplasmic process and intercellular network. Different cell spreading and growth in FBS-OS and ED-OS media were clearly shown on SLA titanium surface (SLA). Size of cells and cell density appeared to be smaller and lower in ED-OS (D & H) than FBS-OS culture media (C & G). On

SLA titanium surface, only cells in FBS-OS could grow to form loose cell sheet on day 21 (G). High magnification images in the inlets magnified cell-surface contacts.

Fig 6.

Cell viability assay demonstrates growth of human bone marrow stromal cells (hBMSCs) in regular (FBS) (dot lines) and estrogen-deprived (ED) growth media (solid lines) on cell culture plate (PL), smooth (SM) titanium surface and sandblasted with large grits and acid etched (SLA) Ti surface. On Day 1, numbers of cells on SLA titanium surface tended to be lower than cell culture plate and smooth titanium surface ($p>0.05$). Subsequently on days 7 – 21, numbers of cells on SLA tended to be lower than SM ($p>0.05$). On days 7 and 14 growth of cells was relatively stable and growth of cells on titanium surfaces in FBS-OS and ED-OS were not significantly different ($p>0.05$). Growth of cells was significantly different on day 21, when growth of cells on titanium surfaces in ED-OS was significantly lower than cell culture plate in ED and titanium surfaces in FBS, and cell culture plate in FBS, respectively (+, $p<0.05$). Cells on cell culture plate in FBS medium on day 21 showed the highest level of growth (*, $p<0.05$). The symbol * represents significantly higher than other groups and +, lower than other groups at $p<0.05$ a (n=4, MEAN±SD).

Fig 7.

Demonstrating osteogenic differentiation potential of human bone marrow stromal cells (hBMSCs) in regular (FBS-OS) and estrogen-deprived osteogenic (ED-OS) media on cell culture plate (PL) and smooth (SM) titanium (Ti) surface and sandblasted and acid etched (SLA) Ti surfaces on culture-days 7 (Days 7) and 21 (Day 21), (A) quantitative real-time polymerase chain reaction (qRT-PCR) exhibits expression of Runx2 and (B) bone sialoprotein (IBSP) genes, (C)

alkaline phosphatase activity and (D) calcium content levels. Estrogen-deprived-OS medium significantly increased expression levels of Runx2, but decreased IBSP expression, ALP activity and calcium content levels (+, $p<0.05$). On day 21, (B) levels of IBSP expression and (D) calcium contents on SLA titanium surface in FBS-OS were significantly higher than cell culture plate and smooth Ti surface (*, $p<0.05$). (C) Levels of ALP activity in each culture medium on days 7 and 21 were not significantly different ($p>0.05$). Symbols * represents significant difference among surfaces in the same group and +, differences between media at $p<0.05$. Data were from 2 independent experiment (n=4, MEAN±SD).

Fig 8.

Demonstrating effects of simvastatin on growth, osteogenic differentiation potential and expression of bone morphogenetic protein 2 (BMP-2) of human bone marrow stromal cells (hBMSCs) in estrogen-deprived (ED) cell culture. Human BMSCs, seeded on cell culture plate (PL), smooth (SM) titanium (Ti) surface and sandblasted and acid etched (SLA) Ti surface were cultured in regular (FBS-OS), estrogen-deprived osteogenic (ED-OS) and ED-OS with 100 nM simvastatin (ED-OS-SIM) culture media for 14 days. Investigated parameters were (A) cell growth (cell viability assay), (B & C, E & F) osteogenic differentiation markers, (B) qRT-PCR of Runx2, (C) bone sialoprotein (IBSP) (E) alkaline phosphatase (ALP) activity and (F) osteocalcin in culture media, and (D) qRT-PCR of bone morphogenetic protein-2 (BMP-2). Simvastatin tended to decrease (A) cell growth on SLA and (B) expression levels of Runx2 on SM ($p>0.05$). (B) Runx 2 expression levels on SM in ED-OS was significantly higher than PL in ED-OS (*, $p<0.05$) and tended to be higher than other groups ($p>0.05$). (C) ED-OS-Sim significantly increased expression levels of IBSP and (D) BMP-2 and (F) levels of osteocalcin on SLA, and (E) enhanced ALP activity on SM and SLA (*, $p<0.05$). In ED-OS, (C) expression

levels of IBSP and (F) osteocalcin on SLA were significantly higher than SM (+, $p<0.05$) and the expressions on SLA Ti surface were significantly increased in ED-OS-Sim medium (*, $p<0.05$). Symbols * represents significant difference among surfaces in the same culture medium at $p<0.05$, and +, differences between groups of culture medium at $p<0.05$. Data were from 2 independent experiment (n=4, MEAN±SD).

Fig 9.

Demonstrating calcein staining of *in vitro* mineralization of human bone marrow stromal cells on (A & B) cell culture plate and (C-E) smooth titanium (Ti) surface and (F-H) sandblasted with large grits and acid etched (SLA) Ti surface in (C & F) regular (FBS-OS), (D & G) estrogen-deprived (ED-OS) and (E & H) ED-OS with 100 nM simvastatin (ED-OS-SIM) culture media. (A) Von Kossa staining exhibited black staining on mineralized nodules in ECM (arrows) (positive control) and (B) corresponding green calcein staining (arrows) on cell culture plate to (A). (C-H) Exhibiting varying levels of green calcein staining on mineralized nodules (arrows)

Table 1

Groups of study

Categories	Culture media	Groups	Description
I	Regular (FBS)	**A**	FBS- Plate
		B	FBS- SM
		C	FBS-SLA
II	Estrogen-deprived (ED)	**A**	ED-Plate
		B	ED-SM
		C	ED-SLA
III	ED-Simvastatin supplement (ED-SIM)	**A**	ED-SIM- Plate
		B	ED-SIM-SM
		C	ED-SIM- SLA

Note: FBS is an abbreviation for culture medium containing fetal bovine serum (FBS), ED-OS for estrogen deprived osteogenic medium containing charcoal stripped FBS, SIM for simvastatin, Plate, for cell culture plates and SM, smooth and SLA, sandblasted with large grits and acid etched titanium surfaces.

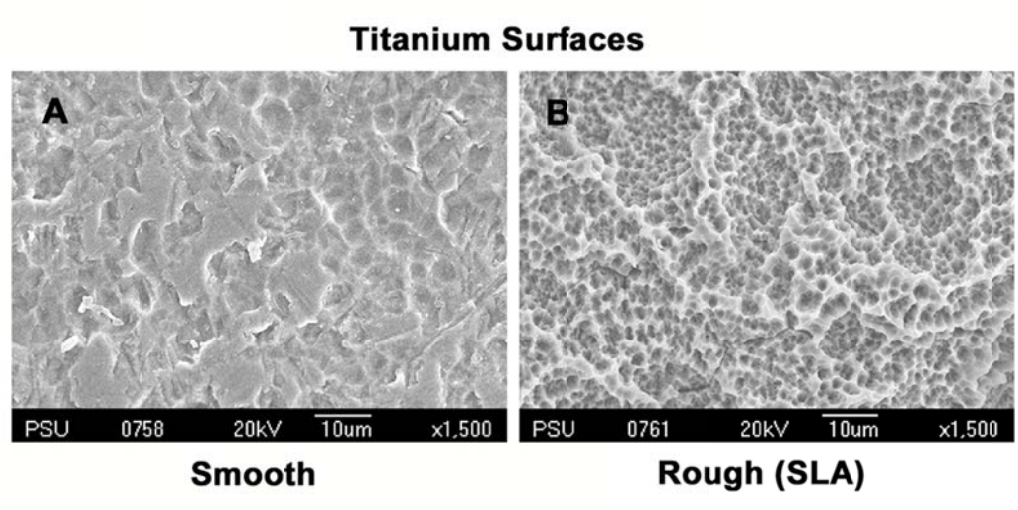
Table 2

Summary of the investigation procedures

Investigations	Procedures	Investigating time
Cell attachment, spreading and morphology	Live/dead cell staining	At 3 hours after cell seeding
	Live/dead cell staining	At 24 hr. and on day 21
	SEM	On days 7 and 21
Cell growth	Cell viability assay	On days 2, 7, 14 and 21
Osteogenic differentiation	qRT-PCR analysis of osteoblast-associated genes	On days 7 and 21
	ALP activity analysis	On days 7 and 21
	Calcium content assay	On day 21
Expression of BMP-2	qRT-PCR analysis	On day 14

Note: ALP is an abbreviation for alkaline phosphatase, BMP-2, bone morphogenetic protein-2, qRT-PCR, Quantitative real-time polymerase chain reaction and SEM, scanning electron microscope

"Titanium surface and simvastatin in estrogen-deprived cell culture"



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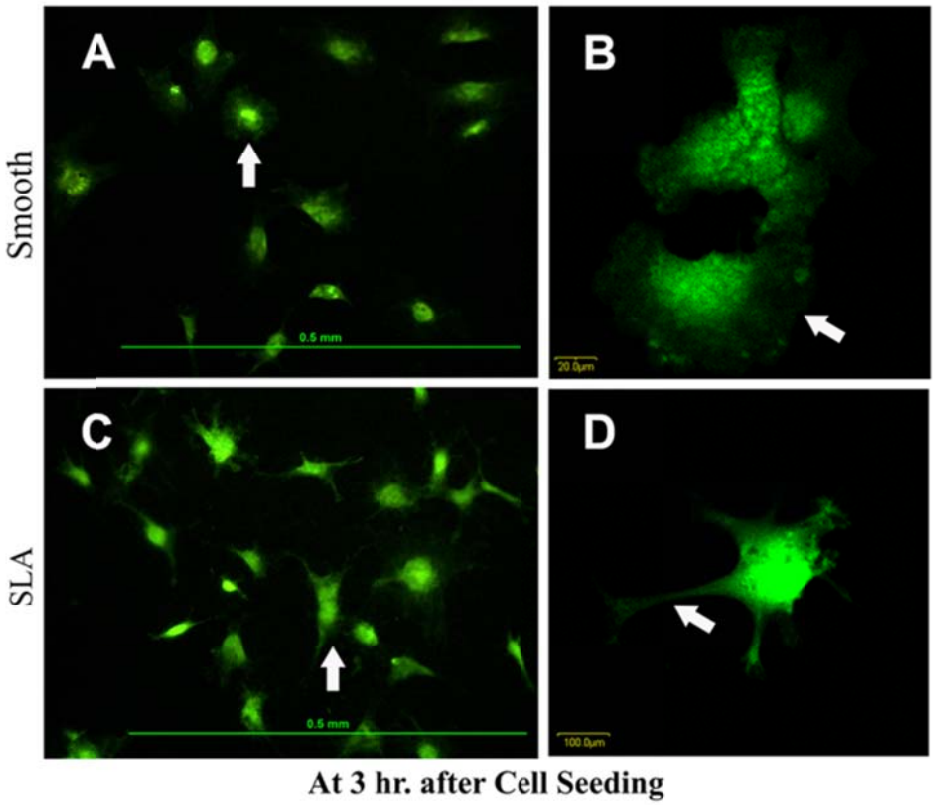

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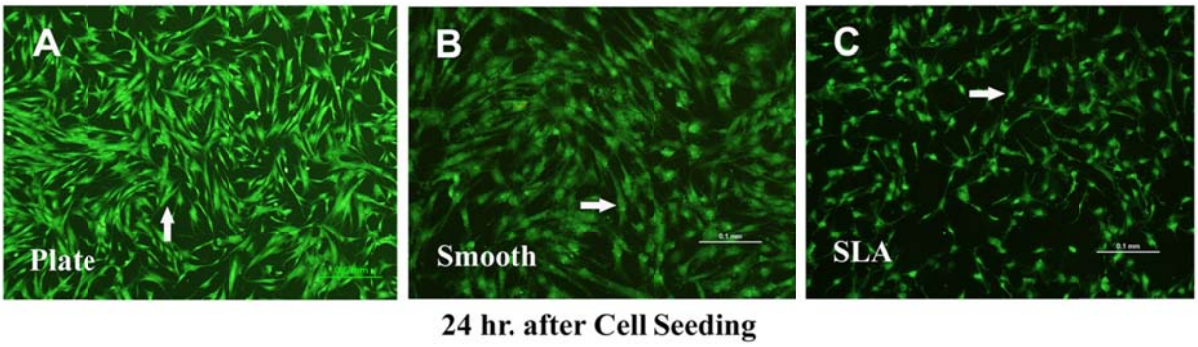

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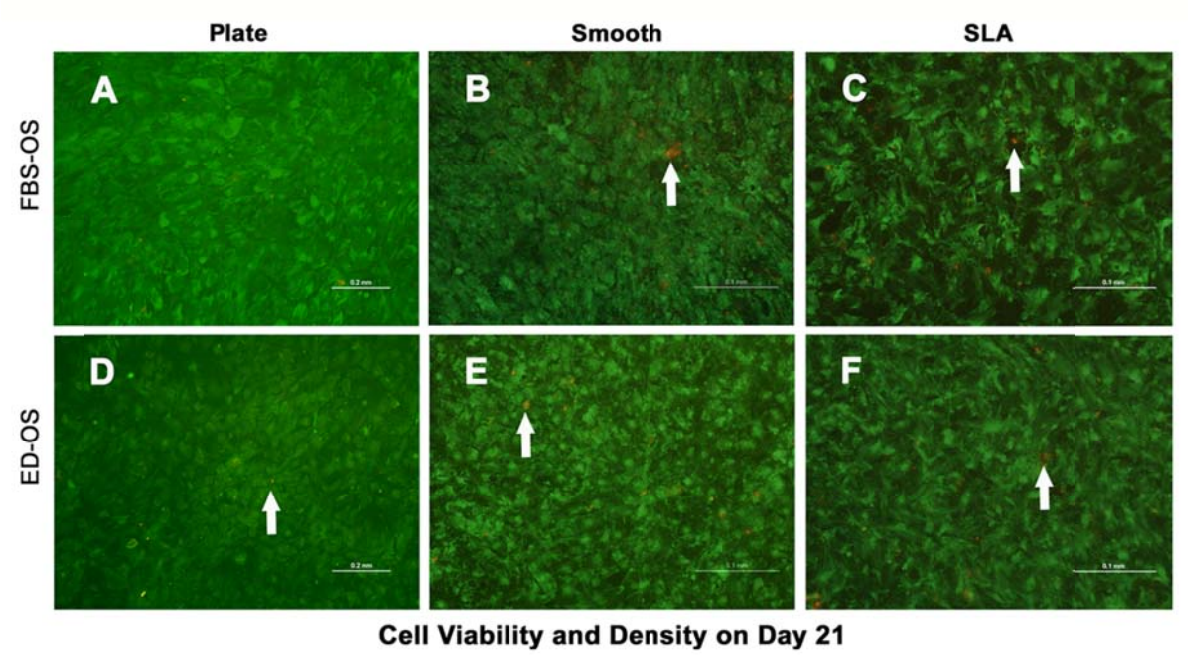

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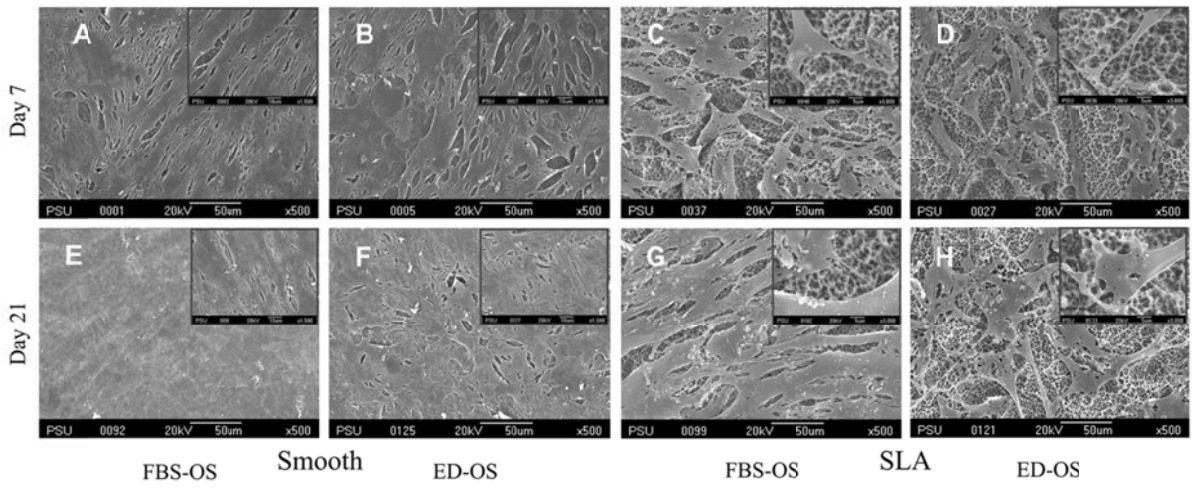
Fluorescence microscope images of green and red fluorescence live/dead cell staining (CellTrackerTM Green/propidium iodide (PI)) of human bone marrow stromal cells (hBMSCs) on day 21 in (A-C) regular (FBS-OS) and (D-E) estrogen-deprived osteogenic (ED-OS) media, (A, D) on cell culture plate (Plate), (B, E) smooth titanium (Ti) surface (Smooth) and (C, F) sandblasted with large grits and acid etched (SLA) Ti surface. Green staining exhibited high level of cell viability and confluence on all surfaces. Red staining of PI (arrows) identified few dead cells that could be seen scattering within confluence green viable cells on titanium surfaces. Brighter and greener staining in (A-C) FBS-OS suggested higher cell viability in FBS-OS than (D-F) ED-OS cell culture.



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Scanning electron microscope (SEM) images of human bone marrow stromal cells (hBMSCs) on (A, B & E, F) smooth titanium (Ti) surface and (C, D & G, H) sandblasted with large grits and acid etched (SLA) Ti surface in (A, E & C, G) regular (FBS-OS) and (B, F & D, H) estrogen-deprived osteogenic media (ED-OS) on (A-D) culture-days 7 (Day 7) and (E-H) 21 (Day 21). Images demonstrated different cell shapes and growth on smooth and SLA titanium surfaces in FBS-OS and ED-OS media. Cells formed cell sheet on smooth titanium surface (Smooth) but extended cytoplasmic process to attach on rough surface forming cells with multiple cytoplasmic process and intercellular network. Different cell spreading and growth in FBS-OS and ED-OS media were clearly shown on SLA titanium surface (SLA). Size of cells and cell density appeared to be smaller and lower in ED-OS (D & H) than FBS-OS culture media (C & G). On SLA titanium surface, only cells in FBS-OS could grow to form loose cell sheet on day 21 (G). High magnification images in the inlets magnified cell-surface contacts.

"Titanium surface and simvastatin in estrogen-deprived cell culture"

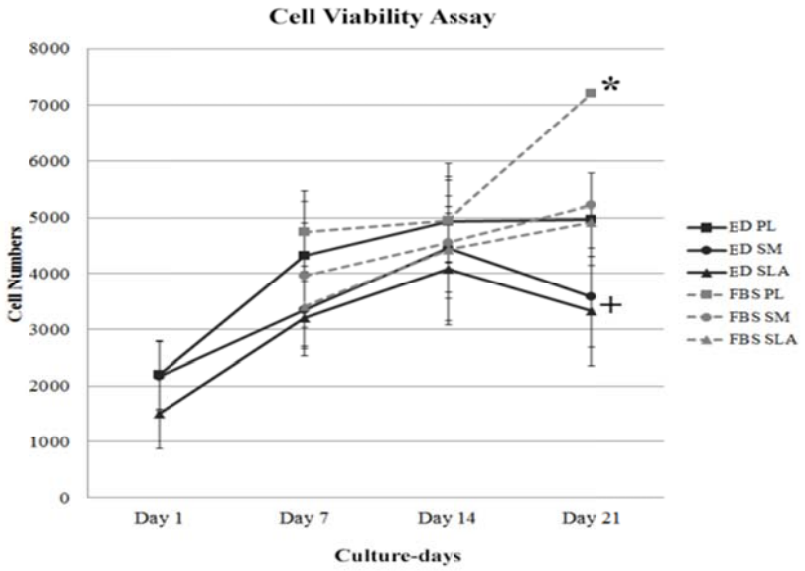


Fig 6.

Cell viability assay demonstrates growth of human bone marrow stromal cells (hBMSCs) in regular (FBS) (dot lines) and estrogen-deprived (ED) growth media (solid lines) on cell culture plate (PL), smooth (SM) titanium surface and sandblasted with large grits and acid etched (SLA) Ti surface. On Day 1, numbers of cells on SLA titanium surface tended to be lower than cell culture plate and smooth titanium surface ($p>0.05$). Subsequently on days 7 – 21, numbers of cells on SLA tended to be lower than SM ($p>0.05$). On days 7 and 14 growth of cells was relatively stable and growth of cells on titanium surfaces in FBS-OS and ED-OS were not significantly different ($p>0.05$). Growth of cells was significantly different on day 21, when growth of cells on titanium surfaces in ED-OS was significantly lower than cell culture plate in ED and titanium surfaces in FBS, and cell culture plate in FBS, respectively (+, $p<0.05$). Cells on cell culture plate in FBS medium on day 21 showed the highest level of growth (*, $p<0.05$). The symbol * represents significantly higher than other groups and +, lower than other groups at $p<0.05$ a (n=4, MEAN±SD).

"Titanium surface and simvastatin in estrogen-deprived cell culture"

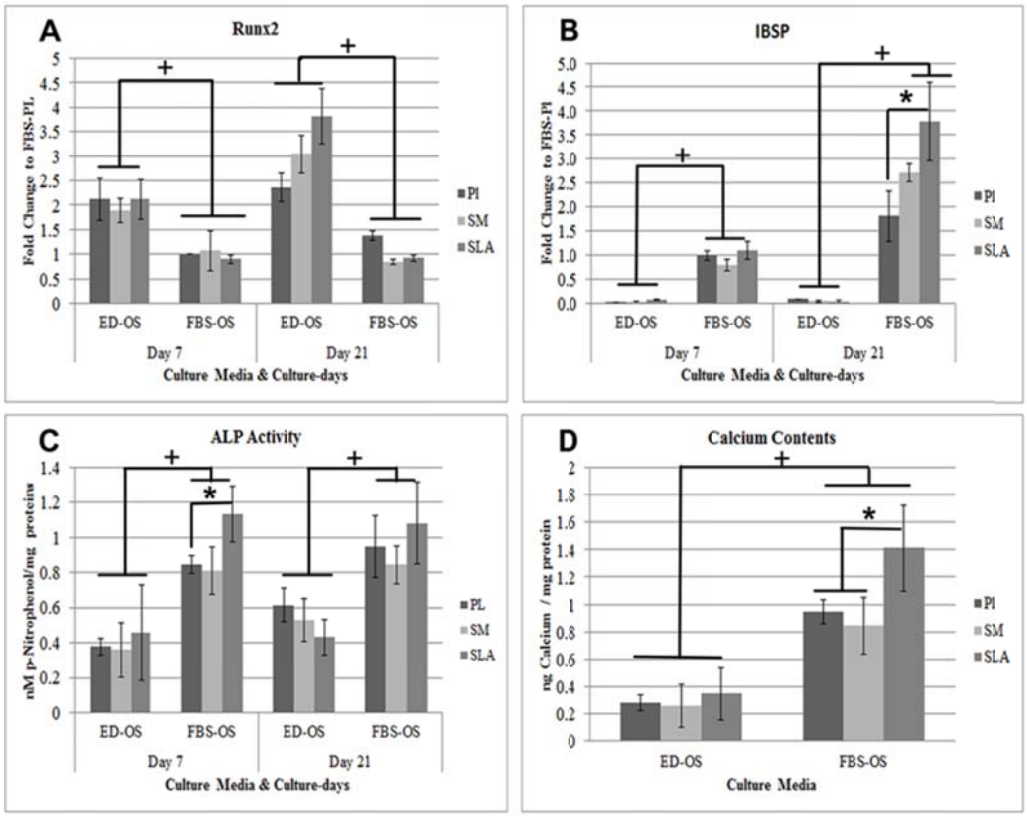
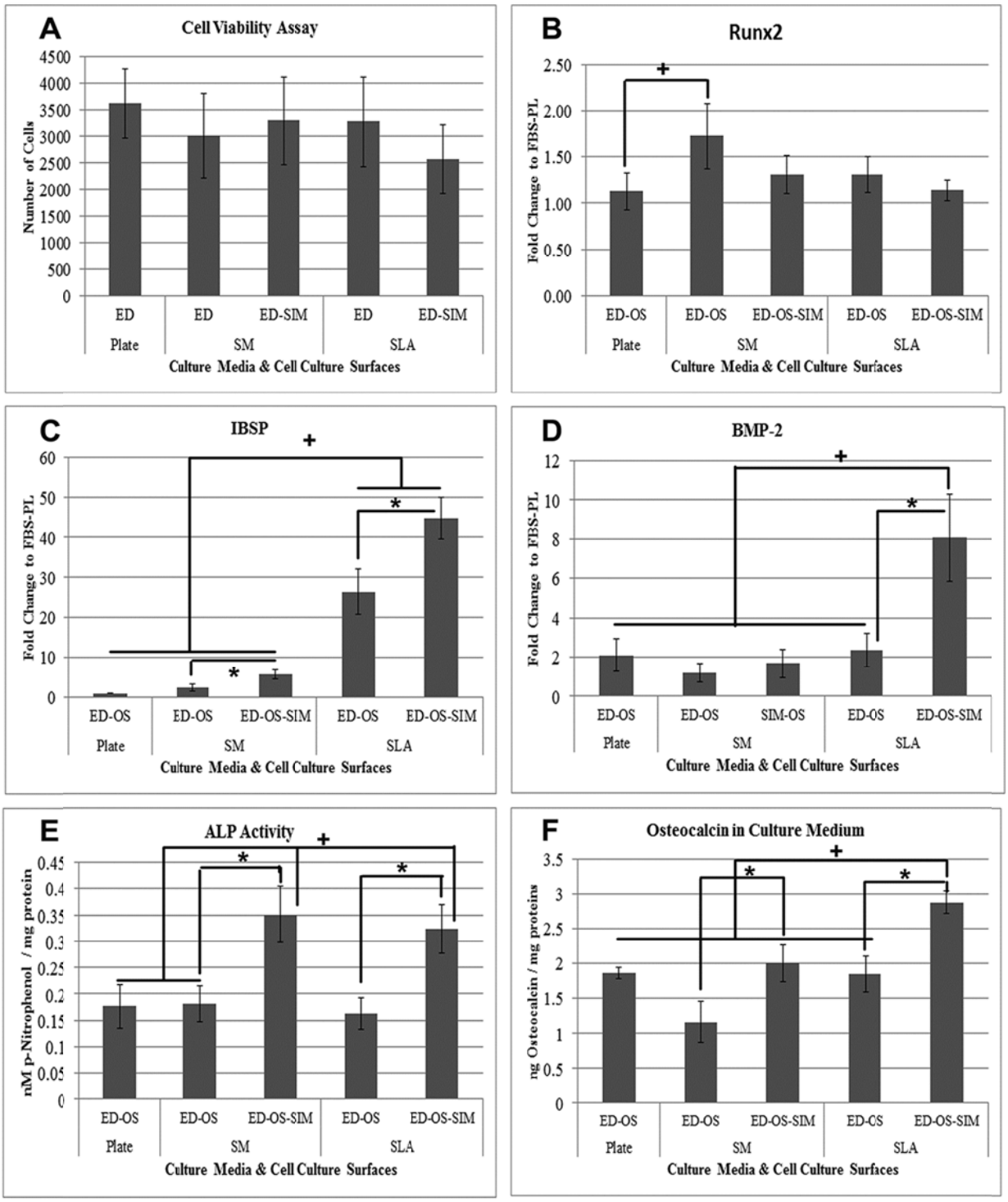


Fig 7.

Demonstrating osteogenic differentiation potential of human bone marrow stromal cells (hBMSCs) in regular (FBS-OS) and estrogen-deprived osteogenic (ED-OS) media on cell culture plate (PL) and smooth (SM) titanium (Ti) surface and sandblasted and acid etched (SLA) Ti surfaces on culture-days 7 (Days 7) and 21 (Day 21), (A) quantitative real-time polymerase chain reaction (qRT-PCR) exhibits expression of Runx2 and (B) bone sialoprotein (IBSP) genes, (C) alkaline phosphatase activity and (D) calcium content levels. Estrogen-deprived-OS medium significantly increased expression levels of Runx2, but decreased IBSP expression, ALP activity and calcium content levels (+, $p<0.05$). On day 21, (B) levels of IBSP expression and (D) calcium contents on SLA titanium surface in FBS-OS were significantly higher than cell culture plate and smooth Ti surface (*, $p<0.05$). (C) Levels of ALP activity in each culture medium on days 7 and 21 were not significantly different ($p>0.05$). Symbols * represents significant difference among surfaces in the same group and +, differences between media at $p<0.05$. Data were from 2 independent experiment (n=4, MEAN±SD).


A
Cell Viability Assay
Number of Cells
4500
4000
3500
3000
2500
2000
1500
1000
500
0
ED
ED
ED-SIM
ED
ED-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
B
Runx2
Fold Change to FBS-PL
2.50
2.00
1.50
1.00
0.50
0.00
+
ED-OS
ED-OS
ED-OS-SIM
ED-OS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
C
IBSP
Fold Change to FBS-PL
60
50
40
30
20
10
0
+
*
*
ED-OS
ED-OS
ED-OS-SIM
ED-OS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
D
BMP-2
Fold Change to FBS-PL
12
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8
6
4
2
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ED-OS
ED-OS
SIM-OS
ED-OS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
E
ALP Activity
nM p-Nitrophenol / mg protein
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
+
*
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ED-OS
ED-OS
ED-OS-SIM
ED-OS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces
F
Osteocalcin in Culture Medium
ng Osteocalcin / mg proteins
3.5
3
2.5
2
1.5
1
0.5
0
+
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ED-OS
ED-OS
ED-OS-SIM
ED-CS
ED-OS-SIM
Plate
SM
SLA
Culture Media & Cell Culture Surfaces

"Titanium surface and simvastatin in estrogen-deprived cell culture"

Fig 8.

Demonstrating effects of simvastatin on growth, osteogenic differentiation potential and expression of bone morphogenetic protein 2 (BMP-2) of human bone marrow stromal cells (hBMSCs) in estrogen-deprived (ED) cell culture. Human BMSCs, seeded on cell culture plate (PL), smooth (SM) titanium (Ti) surface and sandblasted and acid etched (SLA) Ti surface were cultured in regular (FBS-OS), estrogen-deprived osteogenic (ED-OS) and ED-OS with 100 nM simvastatin (ED-OS-SIM) culture media for 14 days. Investigated parameters were (A) cell growth (cell viability assay), (B & C, E & F) osteogenic differentiation markers, (B) qRT-PCR of Runx2, (C) bone sialoprotein (IBSP) (E) alkaline phosphatase (ALP) activity and (F) osteocalcin in culture media, and (D) qRT-PCR of bone morphogenetic protein-2 (BMP-2). Simvastatin tended to decrease (A) cell growth on SLA and (B) expression levels of Runx2 on SM ($p>0.05$). (B) Runx 2 expression levels on SM in ED-OS was significantly higher than PL in ED-OS (*, $p<0.05$) and tended to be higher than other groups ($p>0.05$). (C) ED-OS-Sim significantly increased expression levels of IBSP and (D) BMP-2 and (F) levels of osteocalcin on SLA, and (E) enhanced ALP activity on SM and SLA (*, $p<0.05$). In ED-OS, (C) expression levels of IBSP and (F) osteocalcin on SLA were significantly higher than SM (+, $p<0.05$) and the expressions on SLA Ti surface were significantly increased in ED-OS-Sim medium (*, $p<0.05$). Symbols * represents significant difference among surfaces in the same culture medium at $p<0.05$, and +, differences between groups of culture medium at $p<0.05$. Data were from 2 independent experiment (n=4, MEAN±SD).

"Titanium surface and simvastatin in estrogen-deprived cell culture"

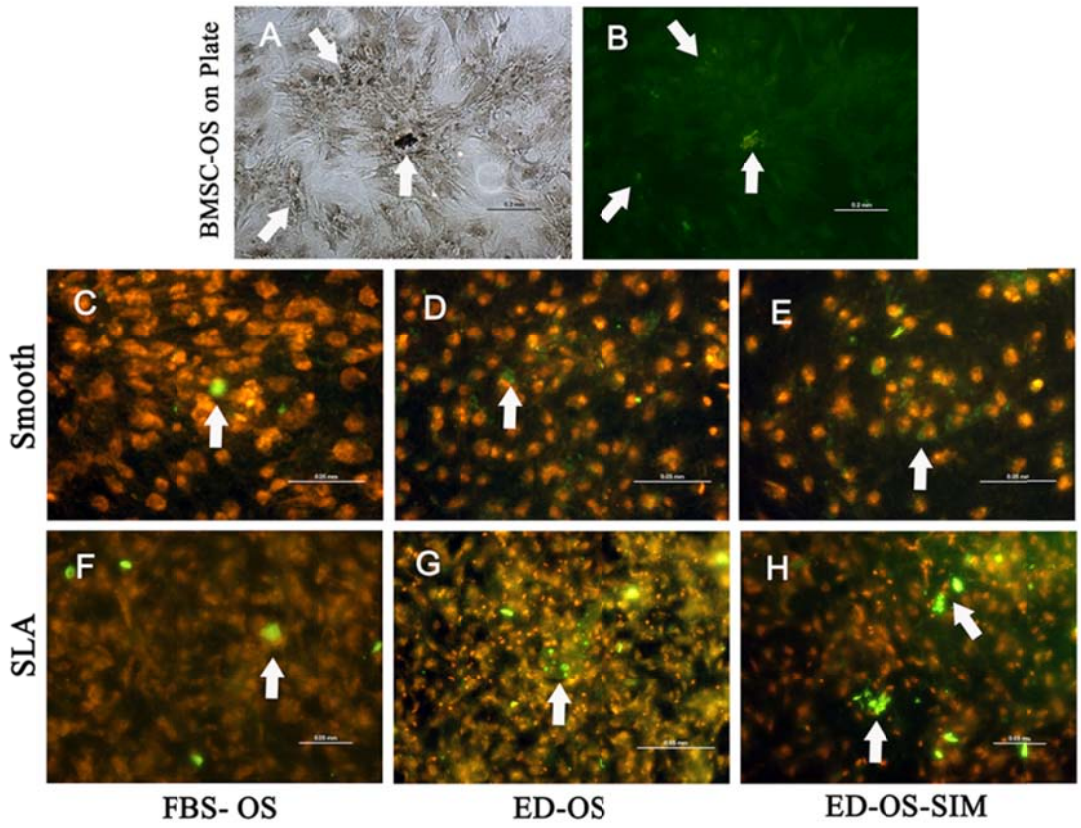

Fig 9.

Demonstrating calcein staining of *in vitro* mineralization of human bone marrow stromal cells on (A & B) cell culture plate and (C-E) smooth titanium (Ti) surface and (F-H) sandblasted with large grits and acid etched (SLA) Ti surface in (C & F) regular (FBS-OS), (D & G) estrogen-deprived (ED-OS) and (E & H) ED-OS with 100 nM simvastatin (ED-OS-SIM) culture media. (A) Von Kossa staining exhibited black staining on mineralized nodules in ECM (arrows) (positive control) and (B) corresponding green calcein staining (arrows) on cell culture plate to (A). (C-H) Exhibiting varying levels of green calcein staining on mineralized nodules (arrows)